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Original Article

The design and evaluation of the antimicrobial activity of a novel conjugated penta-ultrashort antimicrobial peptide in combination with conventional antibiotics against sensitive and resistant strains of *S. aureus* and *E. coli.*

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Abstract

Background and purpose: Antimicrobial resistance still constitutes a major health concern to the global human population. The development of new classes of antimicrobial agents is urgently needed to thwart the continuous emergence of highly resistant microbial pathogens.
Experimental approach: In this study, we have rationally designed a novel conjugated ultrashort antimicrobial peptide. The peptide named naprolyginine was challenged against representative strains of wild-type and multidrug-resistant bacteria individually or in combination with individual antibiotics by employing standard antimicrobial and checkerboard assays.
Findings / Results: Our results displayed that the peptide exhibits potent synergistic antimicrobial activity against resistant strains of gram-positive and gram-negative bacteria when combined with individual antibiotics. Additionally, the peptide was evaluated for its hemolytic activity against human red blood cells and displayed negligible toxicity.
Conclusion and implications: Naprolyginine could prove to be a promising candidate for antimicrobial drug development.

Keywords: Antimicrobial peptides; Bacteria; Drug design; Hemolysis; Naproxen.

INTRODUCTION

Antimicrobial resistance and the emergence of multidrug-resistant bacteria are considered as one of the major health threats facing the human population worldwide (1,2). This situation is made worse by the fact that antimicrobial agent development and discovery pipelines are dry and very few classes of antimicrobial agents have managed to reach the clinic in recent decades (3,4). Accordingly, the development of novel classes of antimicrobial agents is of utmost importance and should be a priority for policymakers responsible for setting the health and pharmaceutical priorities of all countries worldwide.

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Antimicrobial peptides (AMPs) represent a promising class of antimicrobial agents due to their wide-spectrum activity, potency, and unique mode of antimicrobial activity (5,6). Classical AMPs range from 12 to 50 amino acids in length, are amphipathic, and display an overall positive charge (7,8). These physicochemical properties play a major role in the antimicrobial activity of this class of molecules as they are responsible for the interaction with the negatively charged bacterial membranes while the amphipathic nature of the peptide is responsible for peptide-induced membrane lysis and consequently bacterial cell death (9,10).

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Several challenges have hampered the development of classical AMPs into effective therapeutics including the cost of manufacturing, lack of target selectivity, and consequently increased toxicity.

In this study and to overcome the obstacles faced by classical AMPs in regards to AMPs manufacturing costs and inherent cell toxicity, we have designed an ultrashort AMP (USAMP) composed of 5 alternating amino acids. The pentapeptide was conjugated to highly hydrophobic naproxen moiety and was named naprolyginine. This conjugated hydrophobic moiety will enhance the hydrophobic nature of the peptide and act as an anchor in regard to peptide-membrane insertion and permeability. The design strategy of creating USAMPs and consequently shortening the length of the peptides when compared with their classical counterparts will significantly reduce manufacturing costs. Additionally, this reduction in the peptide length and hydrophobic conjugation is expected to enhance the peptide's cytotoxic profile by reducing its hemolytic activity. The antimicrobial activity of the peptide was evaluated against sensitive and resistant strains of gram-positive and gram-negative bacteria. Additionally, the synergistic activity of naprolyginine when combined with conventional antibiotics was evaluated by checkerboard assays to assess the impact of peptide-antibiotic synergism on the reduction of the effective minimum inhibitory concentrations of naprolyginine and the antibiotics. Finally, the hemolytic activity of the peptide was evaluated using hemolytic assays.

MATERIALS AND METHODS

Peptide design and synthesis

Naprolyginine is a penta-USAMP that was rationally designed to include five alternating subunits of both arginine and biphenylalanine in conjugation with a hydrophobic moiety of naproxen (2-(6-methoxy-2-naphthyl) propionic acid). The designed peptides used in the present study were synthesized by (GL Biochem Ltd., Shanghai, China) using the solid-phase method and Fmoc chemistry was finally obtained as a lyophilized state. Reverse phase high-performance liquid chromatography (RP-HPLC) was used for purification of the peptide using a C18 internsil® ODS-SP column, the column was eluted with acetonitrile / H2O-TFA gradient at a flow rate of 1.0 mL/min. The purification and identification of the synthesized peptides were confirmed by electrospray ionization mass spectrometry (ESI-MS).

Determination of the minimum inhibitory concentration and minimum bactericidal concentration of naprolyginine and the individual antibiotics

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of both naprolyginine and the eight individual antibiotics (levofloxacin, chloramphenicol, rifampicin, amoxicillin, clarithromycin, doxycycline, vancomycin, and cefixime) representing a variety of modes of antimicrobial activity were determined using the microbroth dilution method as outlined by the Clinical and Laboratory Standards Institute and as described previously (11,12). Briefly, bacterial strains were grown in Muller Hinton broth (MHB) medium and diluted to 10^{60} CFU/mL in the same medium before use. Naprolyginine and the different antibiotics were prepared in different concentrations and aliquoted with the bacteria in 96-well plates and incubated for 18-24 h at 37 °C. The cell growth and the MICs were determined by reading the plates on an ELISA reader at OD λ = 570. The MBC was determined by transferring 10 µL from the negative well onto agar plates and incubating for 24 h at 37 °C. The MBC was determined as the concentration that caused the eradication of 99.9% of viable cells. All experiments were performed in triplicate.

Determination of the synergistic activity of naprolyginine in combination with individual antibiotics

The synergistic activity and the MIC values of naprolyginine, when combined with antibiotics, were determined using the microbroth checkerboard assay as described previously (13,14). The antimicrobial assays

were performed as described in the previous section with the modification of adding an individual antibiotic in combination with naprolyginine to determine the MIC. All experiments were performed in triplicate.

Determination of the fractional inhibitory concentration

The fractional inhibitory concentration (FIC) for naprolyginine was determined using a standard antimicrobial checkerboard assay as described previously (15). The FIC is the summation of the inhibitory concentration values of each antimicrobial component in the antimicrobial combination divided by the inhibitory concentration of the individual antimicrobial agent. An FIC index < 0.5 was considered synergistic; an FIC index of 0.5-1 was considered an additive while an FIC value above 1 was considered antagonistic.

Hemolytic activity of naprolyginine

The ability of naprolyginine to damage the membrane of normal mammalian cells was determined using the erythrocyte hemolytic assays as described previously (16,17). All experiments were performed in triplicate.

RESULTS

Design and synthesis of naprolyginine

Naprolyginine was designed as an ultrashort conjugated pentapeptide consisting of alternating subunits of both the amino acids arginine and lysine (RBRBR) that were conjugated to 2-(6-methoxy-2-naphthyl) propionic acid (Fig. 1). The design strategy depended on providing the peptide with minimal cationicity employing both the two previously mentioned positively charged amino acids needed to allow the peptide to bind to the negatively charged membranes of bacterial cells through electrostatic interaction. Additionally, and to confer the hydrophobicity needed to allow the peptide to permeabilize the target membranes, the pentapeptide was conjugated to naproxen which is a highly hydrophobic moiety that is expected to act as an anchor for peptide-membrane hydrophobic interaction. Naprolyginine displays a net positive charge of +3 and a molecular weight of 1144.34 Da. The peptide's characterization profile including its identity and purity was confirmed using HPLC and ESI-MS, respectively (Figs. 2 and 3).

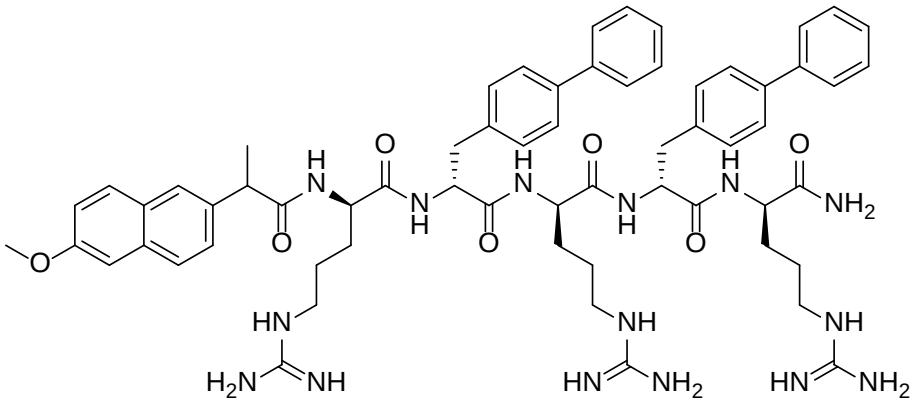

Fig. 1. Chemical structure of naprolyginine

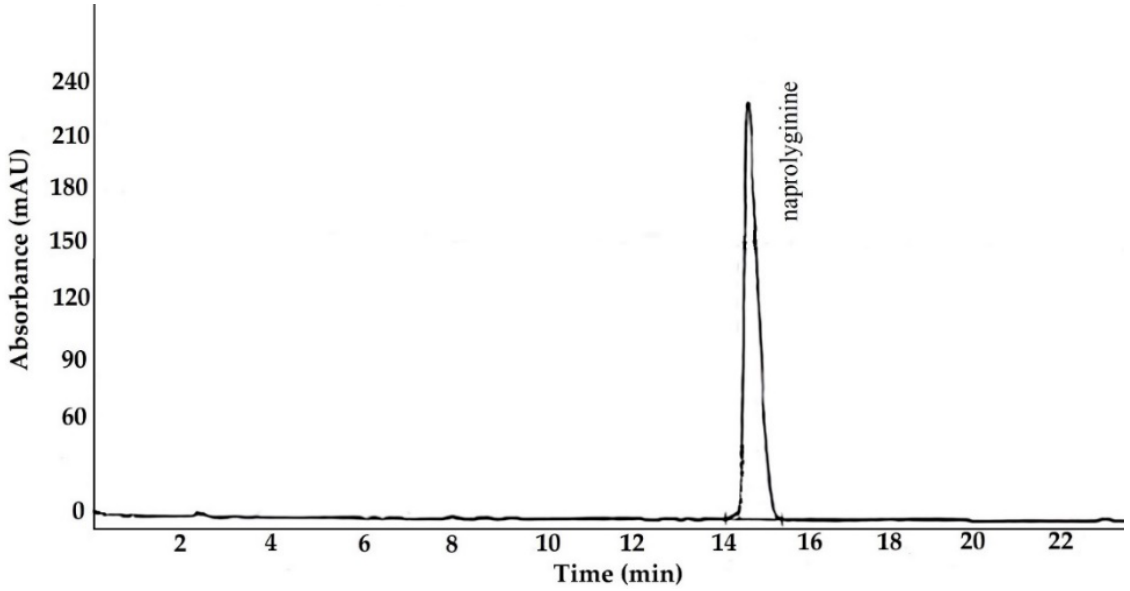

Fig. 2. Reverse phase high-performance liquid chromatography (HPLC) chromatogram of the naprolyginine indicating 99% purity of the synthesized peptide. The absorbance was at λ = 214 nm.

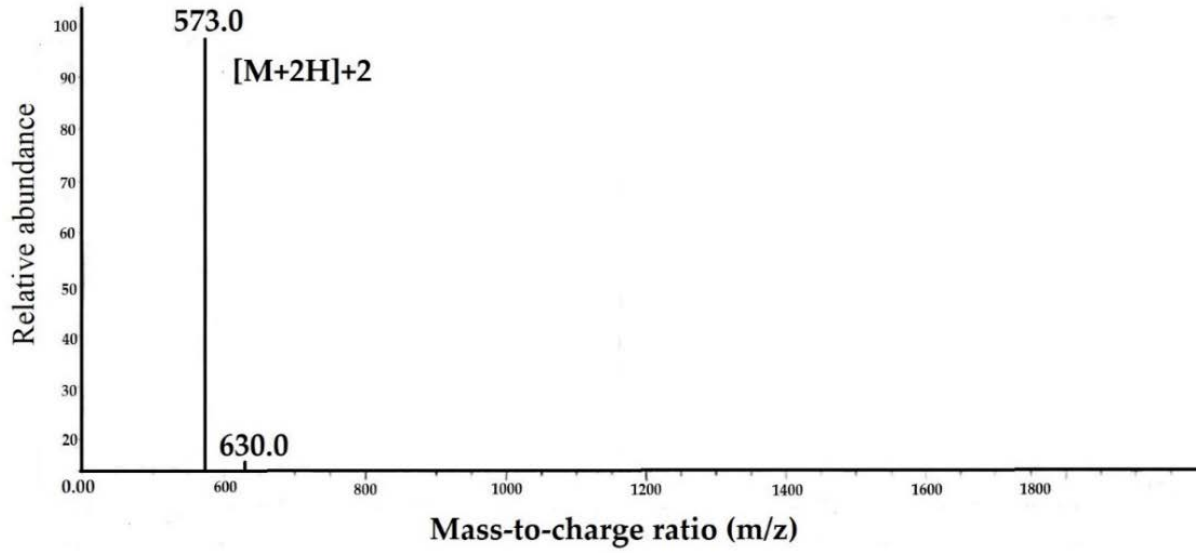

Fig. 3. Positive electrospray ionization mass spectrometric (ESI-MS) analysis of the naprolyginine. The peptide shows major peak in the +2 state of 573 Da.

In vitro antimicrobial activity of naprolyginine and the individual antibiotics

The antimicrobial activity of naprolyginine and the eight different antibiotics employed in this study action (levofloxacin, chloramphenicol, rifampicin, amoxicillin, clarithromycin, doxycycline, vancomycin, and cefixime) were evaluated against gram-positive bacteria represented by the reference *Staphylococcus aureus* (ATCC 29215) and methicillin-resistant *S. aureus* (MRSA; ATCC BAA-41). Additionally, the peptide was also challenged against reference and resistant strains of gram-negative bacteria represented by *Escherichia coli* (ATCC 25922) and extended-spectrum beta-lactamases

(ESBL) *E. coli* (ATCC BAA-3054), respectively. Table 1 details the MIC and MBC values for naprolyginine in addition to the individual antibiotics. The rationale for the types of antibiotics employed in this study was based on selecting antibiotics that represent different classes of antibiotics that cover a wide spectrum of antimicrobial mechanisms of action including the inhibition of cell wall synthesis, protein synthesis, and RNA synthesis. Naprolyginine managed to inhibit the growth of gram-positive reference *S. aureus* and MRSA at MIC values of 8 and 15 µM, respectively. When assessed for its antimicrobial activity against gram-negative bacteria, naprolyginine managed to inhibit gram-negative *E. coli* (ATCC 25922) and ESBL *E. coli* (ATCC BAA-3054) with MIC values of 18 µM and 25 µM, respectively. The MBC values were equal to the MIC values indicating a bactericidal antimicrobial behavior. As displayed in Table 1, four antibiotics exerted a bactericidal antimicrobial behavior and these include levofloxacin, rifampicin, cefixime, and amoxicillin while chloramphenicol, clarithromycin, vancomycin, and doxycycline displayed bacteriostatic activity.

Synergistic activity of naprolyginine

The synergistic activity of naprolyginine in combination with eight different antibiotics was evaluated by employing the checkerboard technique. Synergistic values for naprolyginine in combination with different antibiotics were identified using the FIC index and as represented in Table 2. Six peptide-antibiotic combinations displayed synergistic activities with the most potent combination attributed to naprolyginine in combination with levofloxacin with an FIC index of 0.12 and 0.26 that was reported against *S. aureus* (ATCC 29215) and *E. coli* (ATCC 25922), respectively. For the resistant strains of both gram-positive and gram-negative bacteria, naprolyginine displayed synergistic activity against MRSA in combination with vancomycin and ESBL *E. coli* (ATCC BAA-3054) in combination with chloramphenicol.

Hemolytic assay

Naprolyginine did not cause any hemolytic activity against human erythrocytes up to a concentration of 100 µL. The results of the hemolytic assay display that the peptide displays selective membrane destabilizing activity against microbial cells (Table 3).

Table 1. MIC and MBC values of naprolyginine and the individual antibiotics against gram-positive and gram-negative bacteria.

Antimicrobial agents	***Staphylococcus aureus* (ATCC 29215)**	**MRSA (ATCC BAA-41)**	***Escherichia coli* (ATCC 25922)**	**ESBL *Escherichia coli* (BAA-3054)**
	MIC/MBC (µM)	**MIC/MBC (µM)**	**MIC/MBC (µM)**	**MIC/MBC (µM)**
Naprolyginine	8/8	15/15	18/18	25/25
Levofloxacin	0.5/0.5	10/10	2/2	12/12
Chloramphenicol	20/30	25/40	80/100	150/200
Rifampicin	0.025/0.025	0.005/0.005	15/15	50/20
Amoxicillin	5/5	40/40	25/25	200/250
Clarithromycin	0.5/1.5	125/150	125/150	125/200
Doxycycline	2/10	10/20	1.5/15	16/25
Vancomycin	0.5/0.5	2/2	200/200	250/200
Cefixime	4/4	30/30	6/6	80/80

MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration; ESBL, extended-spectrum beta-lactamases; MRSA, methicillin-resistant *Staphylococcus aureus.*

Table 2. Combinatorial antimicrobial activity of naprolyginine and antibiotics including the FIC indices for the antimicrobial combinations against tested bacterial species.

Bacterial strains	Antibiotic	Antibiotic individual MIC (µM)	Antibiotic synergistic MIC (µM)	Naprolyginine individual MIC (µM)	Naprolyginine synergistic MIC (µM)	FIC* Index
Staphylococcus aureus (ATCC 29215)	Levofloxacin	0.5	0.05	8	0.125	0.12*
	Chloramphenicol	30	10	8	2	0.58
	Rifampicin	0.025	0.015	8	0.5	0.66
	Amoxicillin	5	2.5	8	6	1.25
	Clarithromycin	1.5	0.5	8	4	0.83
	Doxycycline	10	2	8	4	0.7
	Vancomycin	0.5	0.025	8	2	0.3*
	Cefixime	4	1	8	4	0.75
Methicillin-resistant *Staphylococcus aureus* (ATCC BAA-41)	Levofloxacin	10	8	15	6	1.2
	Chloramphenicol	40	20	15	6	0.9
	Rifampicin	0.005	0.0025	15	10	1.17
	Amoxicillin	40	25	15	10	1.3
	Clarithromycin	200	80	15	15	1.4
	Doxycycline	20	10	15	6	0.9
	Vancomycin	2	0.5	15	4	0.5*
	Cefixime	30	15	15	8	1.03
Escherichia coli (ATCC 25922)	Levofloxacin	2	0.25	18	2.5	0.26*
	Chloramphenicol	100	25	18	10	0.81
	Rifampicin	15	0.5	18	6	0.37*
	Amoxicillin	25	8	18	8	0.76
	Clarithromycin	150	100	18	12	1.33
	Doxycycline	15	6	18	8	0.84
	Vancomycin	150	100	18	10	1.22
	Cefixime	6	2	18	4	0.56
ESBL *Escherichia coli* (BAA-3054)	Levofloxacin	12	10	25	15	1.43
	Chloramphenicol	200	15	25	7.5	0.38*
	Rifampicin	50	5	25	15	1.1
	Amoxicillin	250	150	25	10	1
	Clarithromycin	200	125	25	15	1.23
	Doxycycline	25	10	25	10	0.8
	Vancomycin	200	150	25	15	1.35
	Cefixime	80	20	25	15	0.85

*The synergistic FIC values; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; ESBL, extended-spectrum beta-lactamases; FIC, fractional inhibitory concentration.

Table 3. Hemolytic activity of naprolyginine against human erythrocytes.

Concentration (µM)	Hemolysis (%)
0	0
5	0
10	0
20	0
40	0
60	0
80	0
100	1

DISCUSSION

The escalating challenge of antimicrobial resistance is raising alarm among global health authorities and governments worldwide (18). Several recent reports are warning about the impending threat of antimicrobial resistance and its devastating consequences on global human health if not addressed urgently (19). Antibiotics which have been the backbone of anti-infective therapy and have saved millions of human lives over the past century could be rendered ineffective and would ultimately usher human transition to the post-antibiotic era (20). Accordingly, there is an urgent need to develop novel classes of antimicrobial agents to combat this imminent escalating threat. AMPs represent an attractive class of molecules for antimicrobial drug development due to their intrinsic wide-spectrum antimicrobial activity. Several efforts have been undertaken to move AMPs into the clinic as effective therapeutics with little success due to the toxicity issues associated with this class of molecules and their high manufacturing costs (21). To overcome these obstacles, recent efforts focused on alleviating these issues by designing USAMPs that can be attractive candidates for drug development as they would allow large-scale production with economic feasibility and provides a significant reduction in manufacturing costs due to simplicity in their structure and short amino acid sequence. Additionally, USAMPs can be designed to exert minimal cell cytotoxicity and consequently accelerate the introduction of AMPs into the clinic (22). The design of USAMPs requires careful selection of the amino acids that constitute the primary structure of the peptide to achieve the needed physicochemical properties that allow AMPs to exert their antimicrobial mode of action. This is translated into producing a short peptide that exhibits sufficient cationic potential to bind the negatively-charged bacterial membranes while maintaining the minimal threshold of hydrophobicity needed to allow the peptide to traverse through bacterial membranes and create membrane transient pores that consequently lead to membrane leakage and cell death (23). In this study, we have designed a novel conjugated USAMP (naprolyginine) based on the previously-mentioned structural parameters needed to achieve the AMP's antimicrobial efficacy and minimal cytotoxicity. Naprolyginine is a pentapeptide composed of alternating subunits of arginine and biphenylalanine and consequently displays a net positive charge of +3, the charge is in alignment with the recommended cationicity needed for antimicrobial activity of AMPs which is within the (+3-+6) range. Biphenylalanine was incorporated into the primary structure of the peptide to act as an anchor for the conjugated naproxen which represents the hydrophobic part of the peptide that is responsible for membrane perturbation and eventually pore formation. As displayed in our results, the design strategy proved to be successful in generating a USAMP with potent activity against reference and resistant strains of gram-positive and gram-negative bacteria capable of destroying bacterial cells individually with concentrations as low as 8 µM. The peptide also proved to be very efficient in destroying bacterial cells when combined with conventional antibiotics. Naprolyginine managed to inhibit bacterial cells when combined with antibiotics such as levofloxacin with concentrations as low as 0.125 µM which is equivalent to around a 600-fold decrease in the effective antimicrobial concentration of the native peptide. This pattern of enhanced synergistic antimicrobial activity has been reported previously and could prove to be a very successful strategy in advancing USAMPs into effective therapeutics. Naprolyginine also caused negligible hemolysis indicating a selective antimicrobial activity. The low-hemolytic activity of naprolyginine is attributed to the nature of mammalian cell membranes which are zwitterionic and neutral concerning their charge potential. Additionally, mammalian membranes contain a significant amount of cholesterol which could reduce the ability of USAMPs to bind to membranes and induce pore formation (24). However, the main limitations of our study are related to the inability to provide a full cytotoxicity profile of naprolyginine and this issue has to be further elucidated in future studies. Naprolyginine's cytotoxicity should be evaluated against mammalian cells *in vitro* in addition to *in vivo*

studies to generate evidence regarding the success of the conjugation strategy in reducing AMPs toxicity. In conclusion, naprolyginine could serve as a potential candidate for antimicrobial drug development.

CONCLUSION

The design and antimicrobial characterization of a novel conjugated ultrashort antimicrobial peptide with potent activities against clinically resistant isolates of gram-positive and gram-negative bacteria and negligible hemolytic activities are presented in this manuscript. When combined with conventional antibiotics, the peptide (naprolyginine) demonstrated a number of synergistic activities and may prove to be an important candidate for future antimicrobial research.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

A. Almaaytah and R. Darwish conceptualized the study; R. Darwish supervised the study; A. Salama performed the experimental parts of the study; A. Almaaytah, R. Darwish, and A. Salama analyzed the data; A. Almaaytah and R. Darwish wrote the manuscript. The final version of the manuscript was approved by all authors.

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