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A3, a Scorpion Venom Derived Peptide Analogue with Potent Antimicrobial and Antibiofilm activity against Clinical Isolates of Multi-Drug Resistant Gram Positive Bacteria

Ammar Almaaytah[1*], Ahmad Farajallah[1], Ahmad Abualhaijaa[2], Qosay Al-Balas[3]

1 Department of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

2 Department of Applied Biological Sciences, Faculty of Science and Arts, Jordan University of Science and Technology, Irbid, Jordan

3 Department of Medicinal Chemistry, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

*Corresponding author: amalmaaytah@just.edu.jo; Tel: +962777658820; Fax: +962-2-7201075.

Abstract:

Current research in the field of antimicrobials is focused on the development of novel antibiotics and antimicrobial agents to counteract the huge dilemma that the human population is mainly facing in regards to the rise of bacterial resistance and biofilm infections. Host Defense peptides (HDPs) are a promising group of molecules for antimicrobial development as they share unique characteristics suitable for antimicrobial activity including their broad spectrum of activity and potency against bacteria. AamAP1 is a novel HDP that was identified through molecular cloning from the venom of the North African scorpion *Androctonus amoeruxi*. *In vitro* antimicrobial assays revealed that the peptide displays moderate activity against different strains of Gram-positive and Gram-negative bacteria. Additionally, the peptide proved to be highly hemolytic and displaying significantly high toxicity against mammalian cells. In our study, a novel synthetic peptide analogue named A3 was designed from the naturally occurring scorpion venom host defense peptide. The design strategy depended on modifying the amino acid sequence of the parent peptide in order to increase its net positive charge, percentage helicity and optimize other physico-chemical parameters involved theoretically in HDPs activity. Accordingly, A3 was evaluated for its in vitro antimicrobial and anti-biofilm activity individually and in combination with four different types of conventional antibiotics against clinical isolates of multi-drug resistant (MDR) Gram-positive bacteria. A3 was also evaluated for its cytotoxicity against mammalian cells. A3 displayed potent and selective *in vitro* antimicrobial activities against a wide range of MDR Gram-positive bacteria. Our results also showed that combining A3 with conventional antibiotics displayed a synergistic mode of action which resulted in decreasing the MIC value for A3 peptide as low as 0.125 μM. These effective concentrations were

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associated with negligible toxicities on mammalian cells. In conclusion, A3 exhibits enhanced activity and selectivity when compared with the parent natural scorpion venom peptide. The combination of A3 with conventional antibiotics may be pursued as a potential novel treatment strategy against MDR and biofilm forming bacteria.

1. Introduction

Bacterial infectious diseases represent a major health problem currently facing humanity and threatening the effective treatment of an ever-increasing range of diseases caused by pathogens that have acquired the ability to almost resist all types of conventional antibiotics currently found in the clinic [1]. According to a recent World Health Organization (WHO) report regarding the emergence of bacterial resistance, the most notorious forms of bacteria causing the major types of resistant nosocomial infections were attributed to three distinct pathogens and they include *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* [2].

A significant population of these microbial organisms are resistant to many of the known conventional antibiotics found in the clinic due to the wide-scale misuse of these antibiotics in the last decades [3]. The emergence of resistant strains of bacteria has been accompanied by a sharp decrease in the number of antibiotics being developed for clinical use and successfully reaching clinical trials [4].

One of the most notorious infectious causing bacteria includes Gram-positive bacteria such as the multidrug-resistant *Staphylococcus aureus* and the multidrug-resistant *Enterococcus spp*. These pathogens are found in the hospital environment, and they exhibit a multidrug resistance mode of survival that complicates antimicrobial therapy. Infections caused by multidrug-resistant Gram-positive bacteria represent a major global health burden, not only in terms of morbidity and mortality, but also in terms of increased expenditure on patient management and implementation of infection control measures [5].

The increasing resistance of bacteria to conventional antibiotics is creating an urgent need for developing novel classes of antimicrobial agents with different modes of action other than the conventional antimicrobial agents currently used in the clinic [6].

Host defense peptides (HDPs) are considered as a major component of the innate host immune system of several types of eukaryotic organisms including insects, amphibians, vertebrates, mammals and plants [7]. These peptides act as the first line of defense against a broad spectrum of pathogens including viruses, bacteria, protozoa and fungi [8]. HDPs can be defined generally as short sequences of peptides having less than 50 amino acid residues and carrying a net positive charge ranging from (+2 to +9) and displaying more than 30% hydrophobic residues with an amphipathic nature [9]. These structural determinants permit the peptides to fold into amphipathic or amphiphilic structures once they are in contact with plasma membrane and consequently damaging bacterial membranes and causing cell lysis and death [10].

The main structural determinants that are responsible for defining HDPs' membrane activity and are thought to influence the activity and toxicity of the peptides are conformation or helicity, charge, hydrophobicity, hydrophobic moment and amphipathicity. These factors cannot be assessed individually when trying to analyze the activity or toxicity of an antimicrobial peptide, as all factors are interactive and of significant importance [11, 12].

Due to their attractive properties regarding the broad-spectrum antimicrobial activity and rapid killing kinetics, HDPs can be considered as potentially promising candidates for development of novel therapeutics against multi-drug resistant bacteria. HDPs may offer several advantages as candidates for antimicrobial development over traditional antibiotics as their use may include defeating infections individually or in synergy with other antimicrobial agents for the purpose of the reducing the effective killing concentrations and consequently reducing cytotoxicity.

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AamAP1 is a natural cationic alpha-helical peptide that was identified through molecular cloning from the venom of the North African scorpion *Androctonus amoeruxi*, the peptide carries a net positive charge of (+1) and was found to display relatively mild antimicrobial activity against standard representatives of Gram-positive and Gram-negative bacteria [13].

In this study, we have analyzed the physiochemical properties of AamAP1 in order to modify these parameters for rational design of a new modified synthetic peptide analogue with enhanced antimicrobial activity and reduced toxicity against normal mammalian cells. The design strategy employed in this study depended on modifying the net positive charge of the natural peptide while keeping other physiochemical parameters within normal ranges. The aim of the design strategy focused on producing a modified peptide with enhanced and potent activity against clinical isolates of multidrug-resistant Gram-positive bacteria of the *Staphylococcus aureus* and *Enterococcus spp*. Additionally, and in order to reduce the minimum effective antibacterial concentrations of the modified peptide and consequently reduce its toxicity on mammalian cells, we combined the modified peptide named A3 with four conventional antibiotics to enhance the activity of each individual antimicrobial agent through a synergistic mode of action. Additionally, the anti-biofilm activity of A3 was evaluated against biofilm-forming Gram-positive bacteria. Finally, the *in vitro* cytotoxicity of A3 against normal mammalian cell lines in addition to human erythrocytes was evaluated in order to assess the outcome of the design strategy and the synergistic mode of action in the reduction of the toxicity of HDPs.

2. Materials

2.1 Bacterial Strains

In the present study, the Gram-positive bacterial strains used for the determination and testing of the antimicrobial activity of A3, the antibiotics, and A3-antibiotic combinations were acquired from the American type tissue culture collection (ATCC) and these include: *Staphylococcus aureus* (ATCC

29213), *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecalis* (ATCC 19433), *Staphylococcus epidermidis* (ATCC 12228) which were used as control strains. Additionally, the multi-drug resistant bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), (ATCC 43300) and (ATCC BAA-41), and the multi-drug resistant Enterococcus *faecalis* (ATCC BAA-2356) and *Enterococcus faecium* (ATCC BAA-2316) were also employed in our tests. The bacteria was cultured on Mueller Hinton Agar obtained from (Scharlap, S.L, Spain).

2.2 Antimicrobial Substances

Different antimicrobial substances were employed in the study, including AamAP1, A3 and four kinds of conventional antibiotics that include levofloxacin, chloramphenicol, rifampicin and erythromycin.

AamAP1 and its modified analogue (A3) were synthesized by solid-phase method and Fmoc chemistry, and purified by reverse phase high performance liquid chromatography using an acetonitirile / H2O-TFA gradient. The identity of the peptide was confirmed by ESI-MS mass spectrometry (GL Biochem Ltd., Shanghai, China).

All antibiotics were obtained from (Sigma-Aldrich, China) except erythromycin which was obtained from (Sigma-Aldrich, USA). Stock solutions were prepared according to the manufacturer's recommendation and stored at optimum temperature for each antibiotic.

The media used to dissolve the peptide and the antibiotics and also to prepare bacterial suspensions was Mueller Hinton Broth obtained from (Oxoid LTD., England).

2.3 Cell lines

The cell lines employed for the toxicity assays were the Human Embryonic Kidney 293 cells line (HEK 293) and Vero cell line. The culture media employed for cell propagation include RPMI media with 1% streptomycin and ampicillin and 10% fetal Bovine serum (Sigma-Aldrich, China).

3. Methods

3.1 Bioinformatics Analysis and Design of the Modified Peptide

The Network Protein Sequence Analysis SOPM secondary structure prediction software (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopm.pl) was used for estimation of the α-helical content of AamAP1 and the modified peptide A3 in order to control the helicity parameter of the modified peptide within optimum range [14]. The mean hydrophobicity (hydrophobicity <H>, hydrophobic moment <μH>), was calculated using the HydroMCalc software for AamAP1 and A3 (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py) while Innovagen's peptide calculator was used for calculating the water solubility, net charge at neutral pH, molecular weight and Isoelectrical point of both peptides (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp).

3.2 Molecular modeling and *In silico* analysis of AamAP1 and its modified peptide analogue A3

Computer simulation was employed for the evaluation of the physicochemical parameters for AamAP1's structural analogue A3. Identification and structure prediction for the best template for homology modeling of A3 was performed using the HHpred (HHsearch 2.0) software by HMM–HMM comparison [15]. The RAMPAGE: Assessment of the Ramachandran Plot software was employed for three dimensional structure validations [16]. The final model was visualized using Accelrys® Discovery studio software (http://toolkit.tuebingen.mpg.de/modeller). Where Confirmation of the model reliability was performed using the I-TASSER software [17].

3.3 Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of A3 against the bacterial strains were determined by adapting the microbroth dilution

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method outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines [18, 19]. Briefly, bacterial cells were cultured overnight in Muller Hinton Broth (MHB) and diluted to 10^6 CFU/ml in the same medium prior to use. Different concentrations of A3 peptide were prepared by using the serial dilution method in culture medium. 50 µl of each peptide concentration and 50 µl of diluted bacterial suspension were added to each well in sterile flat- bottomed 96-well microtiter cell culture plates. Plates were incubated for 18-24 hours, at 37°C, in a humidified atmosphere. Following this, the growth of bacteria was determined by means of measuring optical density (OD) at λ = 600 nm by an ELISA plate reader (BioTek Epoch). For the minimum bactericidal concentration (MBC), 10 µl aliquots were taken from wells of each peptide concentration, then transferred into a pre-sterilized labeled agar plates and incubated over night at 37°C for overnight according to CLSI guidelines. The MBC was determined as the lowest concentration that resulted in <0.1% survival of the subculture.

3.4 MIC and MBC Determination of Individual Antibiotics and Checkerboard Assay

The (MICs) and (MBCs) of antibiotics were tested and determined against bacterial strains as described in the previous section by preparing eight different concentrations of each antibiotic. The (MICs) and (MBCs) of combinations of A3 and the different antibiotics were tested and determined against bacterial strains as described in section 3.3, in addition to the broth microdilution checkerboard technique [20]. A mixture of the peptide and one of the antibiotics in different concentrations were added to each microtiter well, where 25 µl of each peptide concentration and 25 µl of each antibiotic concentration were added to six wells of a sterile flat- bottomed 96 well-plate containing 50 µl of diluted bacterial suspension. All MIC and MBC determinations were made in triplicate.

3.5 Determination of the Fractional Inhibitory Concentration (FIC)

The fractional inhibitory concentration index (FIC) and the antimicrobial activities of A3-antibiotic combinations were assessed using the broth microdilution checkerboard technique [20]. The FIC is

defined as the inhibitory concentration of the antimicrobial combination divided by that of the single antimicrobial component. The FIC index for the combination of different two antimicrobial agents is calculated according to the following equation:

FIC index= (MIC of drug X in combination) / (MIC of drug X alone) + (MIC of drug Y in combination) / (MIC of drug Y alone). FIC indices were interpreted as follows:

≤ 0.5: synergistic activity, 0.5-1: additive activity, 1-4: indifference, > 4: antagonism [108].

A Synergistic activity means that each individual component in the combination is supplementing the other in increasing its potency. Additive activity represents an increase in the potency of only one component in the combination, while indifference means there is no change in the activity between individual and combination treatments. Finally, antagonistic activity means that one or even both components in the combination are working against each other.

3.6 Antibiofilm Activity

The Anti-biofilm activity for modified AamAP1, A3 and the antibiotics were performed according to Luca et al. [21]. Briefly, Biofilm formation was performed by employing the Calgary biofilm device (Innovotech, Canada). The biofilm forming Gram positive *S. aureus* (29213) and *S. aureus* (BAA-41) bacterial strains were incubated in MHB for twenty hours at 37°C, and cultures were diluted in the same medium to achieve a concentration of 10^7 CFU/ml. 150 µl of this bacterial culture were added to the sterile 96 pegs-lids on which biofilm cells can build up. Negative control lanes were prepared by adding 150 µl MHB to six wells. And then the pegs were incubated for 20 h under a rotation of 125 rpm at 35°C to allow biofilm formation on the purpose-designed pegs. The minimum biofilm eradication concentration (MBEC) is defined as the lowest concentration of the antimicrobial agent that prevents bacterial re-growth from a treated biofilm, within the exposure time of the antimicrobial agent. Once the biofilms were allowed to form, the pegs were rinsed twice with phosphate buffered saline (PBS) to remove planktonic cells as a washing step. Each peg-lid was then

transferred into a "challenge 96-well microtiter plate" containing two hundred microlitres of different peptide concentrations and the peg lids containing the biofilms were incubated for two hours at 37°C. After biofilm treatment with the challenge plate, the biofilms were transferred into a recovery plate and incubated for eight hours. The MBEC of the each antibiotic was determined as described previously and by preparing eight antibiotic solutions with different concentrations.

For viable cell determination, 5 µl from each well of peptide concentrations was transferred to MHB and then transferred into a pre-sterilized labeled agar plates and incubated over night at 37°C. The following day, the number of bacterial colonies was counted. The minimum bactericidal concentration for biofilm (MBCb) was defined as the lowest peptide concentration that showed no growth (99.9% killing).

3.7 Mammalian Cytotoxicity Assays

The MTT assay was employed for the cytotoxicity assays, the adherent kidney cell lines (VERO and HEK) were seeded in 5 x 10^3 cells per well in flat- bottomed 96 -well plate, and the plate were incubated for 18-24 hours at 37°C under 5% CO_2. This was followed by preparing different concentrations of A3 and the plates were incubated under 5% CO_2 and 37°C for 24 hours. After 24 hours of treatment, 30 µl MTT solution were added to all wells and the plates were incubated under the same condition for 4-6 hours. After this incubation period, the MTT/Peptide solution was removed and 100 µl of DMSO was added to each well and mixed thoroughly by pipetting to dissolve the formazan crystals at the bottom of the wells until a clear purple color was achieved. The plates were then placed on an ELx808™ Absorbance Microplate Reader (BioTek, USA) and the absorbance measured at λ600 nm.

3.8 Erythrocyte Hemolytic Assay

For the determination of the ability of A3 to induce hemolysis to human erythrocytes, the hemolytic activity was performed by centrifuging 10 ml of human heparinized blood in at 3000 x g for 5 min,

after that plasma was removed and the cells were re-suspended in 25 ml PBS to wash them and then re-centrifuged again as mentioned above; this step was repeated two times. Finally, 2 ml of cells were re-suspended in a pre-sterile tube containing 48 ml PBS to reach a final concentration of 4% RBC. Seven peptide solutions with different concentrations were prepared, and then 2 ml of each peptide concentration was added to 2 ml of erythrocyte suspension.

Positive controls were prepared by adding 5 µ l of 0.1% (Triton X) to 2 ml of 4% RBC suspension (Positive control) and negative controls were prepared by adding 2 ml of 4% RBC suspension only (Negative control), the suspension was incubated with the peptides for 60 min at 37°C. After the incubation step, the tubes were gently vortexed and 1 ml of each sample was removed and placed into a pre-sterilized Eppendorf tubes and then centrifuged for 5 min at 3000 x g and 200 µ l of each supernatant was placed into a well of a 96 well plate. Absorbance was measured at λ= 450 nm with the aid of an ELISA reader. The percentage hemolysis was calculated according to the following equation:

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

Where:

A: is Optical Density (OD) 450 with the peptide solution,

A0: is OD 450 of negative control (0.9% NaCl),

and AX: is OD 450 of positive control (0.1% Triton 10X).

4. Results

4.1 Bioinformatics Analysis of AamAP1 and the Design of A3

AamAP1 was used as a platform for the development of novel synthetic peptide analogue named A3 with enhanced antimicrobial activity and lowered mammalian cell toxicity. Initially, the NPS SOPM analysis software was used for the prediction and calculation of the percentage helicity of the parent peptide. The rationale used for designing A3 was to increase the net positive charge and percentage helicity in regards to the parent peptide by substituting specific amino acids with lysine and arginine amino acids while relatively optimizing other physico-chemical parameters of the parent peptide that are known to affect the peptide's activity such as hydrophobicity and hydrophobic moment. As charge and helicity are considered crucial structural determinants of antimicrobial activity as they are believed to be responsible for the initial interaction of cationic HDPs with the negatively charged bacterial membranes, an increase in the net positive charge of the parent peptide is expected to enhance its antimicrobial activity and minimize its toxicity. Additionally, increasing the overall helicity of the peptide plays a major role in decreasing the toxicity of the peptides towards mammalian cells and consequently enhances the selectivity of the peptide. As shown in Table 1, A3 was designed to display an extra two charges by substituting the proline with arginine and histidine with lysine on positions 7 and 8 respectively.

The results shown in (Table 1) display that parent peptide AamAP1 exhibits 55.56% helicity, while the structural modification performed on the parent peptide increased the percentage helicity of A3 to 100%. The hydrophobicity range generated for AmaAP1 was reported to be (0.904) while for A3, it was reported to be 0.746. The hydrophobic moment was also simulated for each peptide with a reported value of (0.435) for the AamAP1 and a value of 0.517 for A3.

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Table 1: The amino acid sequences and properties of the peptides employed in this study.

Peptide	Sequence	Hydrophobicity <H>	Hydrophobic moment <μH>	% Helicity	Net Charge z
AamAP1	FLFSLIPHAIGGLISAFK	0.904	0.435	55.56 %	+1
A3	FLFSLIRKAIGGLISAFK	0.746	0.517	100 %	+3

The HHpred (HHsearch 2.0) software was employed for the evaluation of the physicochemical parameters of A3 and for structure prediction and identification of the best template that could be employed for homology modeling. The homology prediction reported that A3 displays good alignment with Pardaxin (an antimicrobial and anticancer peptide identified previously from Pacific Peacock sole). The homology score reported for A3 was 21.8, which is considered to be of high quality. Three-dimensional structural modeling of A3 revealed the peptide is exhibiting a continuous uninterrupted alphe helix confirmation in accordance with the theoretical calculations performed previously (Figure 1)

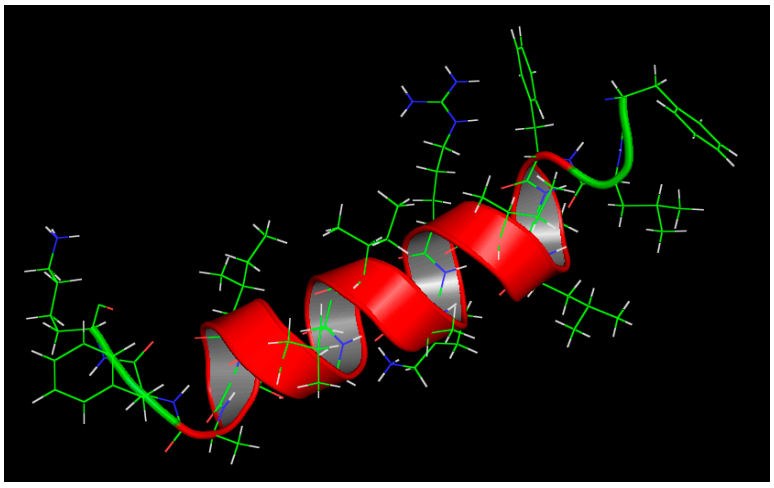

Figure 1: Three-dimensional structural modeling of A3 peptide. Red regions correspond to helical structures within the peptide.

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4.2 Peptide Synthesis and Purification

A3 was synthesized according to the solid-phase method and standard Fmoc chemistry. The peptide purity was assessed by high performance liquid chromatography (HPLC) (Figure 1, supplementary material). The peptide Identity was confirmed by performing positive electrospray ionization (ESI) mass spectrometric (MS) analysis as the synthetic replicate of modified AamAP1 peptide showing major peaks in the +2 and +3 charge state of 991.64 Da and 661.37 Da respectively (Figure 2, supplementary material).

4.3 Bacterial Susceptibility Assay

A3 proved to be a highly active antimicrobial agent against all the studied bacterial strains. It was able to inhibit the growth of control strains of Gram-positive bacteria within a range of 2.5-12.5 µM. Additionally, it inhibited the growth of clinically isolated resistant Gram-positive strains within a range of 5-15 µM. *S. epidermidis* (12228) was the most sensitive strain with a MIC value of 2.5 µM.

The bactericidal activity of A3 peptide was assessed by measuring the minimal bactericidal concentration (MBC) for each tested bacterial strain. The MBC values reported for A3 peptide against all studied bacterial strains were equal to the MIC values which indicate that the peptide is exhibiting a bactericidal antimicrobial nature. The MIC and MBC values of A3 against the bacterial strains are summarized in Table 2.

The antimicrobial activities of the antibiotics employed in the current study were determined against wild type and multi-drug resistant bacterial strains. The MIC values of all antibiotics against the bacterial strains are summarized in Table 3. Four different types of antibiotics were included in the current study including levofloxacin, chloramphenicol, rifampicin and erythromycin. Five strains of bacteria were selected for the determination of the MICs in regards to the previously mentioned antibiotics. One from the control group, represented by *S. aureus* (29213) and four strains

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representing the MDR group and these include *S. aureus* (33591*), S. aureus* (BAA-41), *E. faecalis* (BAA-2356) and *E. faecium* (BAA-2316).

Table 2: A summary of the MIC and MBC values of A3 against all tested bacterial strains. (Results represent triplicates)

Control Gram-positive strains	**ATCC**	**MIC value (µM)**	**MBC value (µM)**
Staphylococcus aureus	29213	5	5
Enterococcus faecalis	29212	10	10
Enterococcus faecalis	19433	12.5	12.5
Staphylococcus epidermis	12228	2.5	2.5
MDR Gram-positive strains	**ATCC**	**MIC value (µM)**	**MBC value (µM)**
Staphylococcus aureus	43300	5	5
Staphylococcus aureus	BAA-41	5	5
Staphylococcus aureus	33591	5	5
Enterococcus faecalis	BAA-2356	5	5
Enterococcus faecium	BAA-2316	15	15

Table 3: Minimum inhibitory concentrations (MICs) of antibiotics against the tested bacterial strains. The MIC values are displayed in (µM) concentration. (Results represent triplicates).

Antibiotic	***S. aureus* (29213)**	***S. aureus* (33591)**	***S. aureus* (BAA-41)**	***E. faecalis* (BAA-2356)**	***E. faecium* (BAA-2316)**
Levofloxacin	0.5	10	10	27.5	12.5
Chloramphenicol	20	130	25	30	20
Rifampicin	0.025	0.04	0.005	0.03	7.5
Erythromycin	0.5	8	350	35	40

4.4 Synergistic Activity of A3 and the Antibiotics in Combination

The MIC values for A3 in combination with the antibiotics in most combination groups decreased dramatically. Eight combinations out of the overall sixteen antimicrobial combinations displayed synergistic effects against planktonic cells of the tested bacterial strains. For *S. aureus* (ATCC: 29213), A3-levofloxacin, A3-chloramphenicol, A3-rifampicin and A3-erythromycin displayed potent synergistic activities as the MIC of A3 in the first two combinations was reduced by 60% and in the last two combinations, the reduction in MIC was 97.5%. Levofloxacin's MIC in the combination was reduced by 93.6%, Chloramphenicol's MIC by 93.8%, rifampicin's MIC by 60%, and finally erythromycin by 87.5% when compared to the MICs of these antibiotics individually. For the MRSA strain of *S. aureus* (ATCC 33591), A3-levofloxacin and A3-rifampicin combinations displayed significant synergistic effects. The MIC of A3 in these combinations was reduced by 70% and 60% respectively, when compared to its individual MIC. The MIC values of levofloxacin and rifampicin in the combination groups were reduced by 92.5% and 96.9% respectively compared to the MICs of these antibiotics individually (Table 4).

For the clinically isolated MRSA strain of *S. aureus* (ATCC BAA-41), only the A3-chloramphenicol combination displayed a synergistic effect. The MICs of A3 and chloramphenicol in the combination were reduced by 60% and 90% respectively compared to their individual MICs. For the clinically isolated multi-drug resistant *E. faecium* (BAA-2316), the A3-levofloxacin combination displayed a synergistic effect. The MICs of A3 and Levofloxacin in the combination were reduced to 86.7% and 90% respectively compared to their individual MICs.

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Table 4: Minimum inhibitory concentrations (MICs) of A3 and the antibiotics in combination against Gram-positive bacteria. (Results represent triplicates).

MIC in combination against Gram-positive bacteria **MIC in combination/(Individual MIC)**			
Bacterial strain	**Antibiotics (µM)**		**A3 (µM)**
S. aureus (*29213*)	**Levofloxacin**	0.03215/(0.5)	2/(5)
	Chloramphenicol	1.25/(20)	2/(5)
	Rifampicin	0.01/(0.025)	0.125/(5)
	Erythromycin	0.0625/(0.5)	0.125/(5)
S. aureus (*33591*)	**Levofloxacin**	0.75/(10)	1.5/(5)
	Chloramphenicol	30/(130)	2.5/(5)
	Rifampicin	0.00125/(0.04)	2/(5)
	Erythromycin	3/(8)	2/(5)
S. aureus (*BAA-41*)	**Levofloxacin**	0.7/ (10)	3/(5)
	Chloramphenicol	2.5/(25)	2/(5)
	Rifampicin	0.0002/(0.005)	3/(5)
	Erythromycin	50/(350)	2/(5)
E. faecium (*BAA-2316*)	**Levofloxacin**	1.25/(12.5)	2/(15)
	Chloramphenicol	10/(20)	2/(15)
	Rifampicin	6.25/(7.5)	2.5/(15)
	Erythromycin	30/(40)	1/(15)

4.5 The FIC Index

The FIC index results indicated that several A3-antibiotic combination groups exhibited potent synergistic effects with (FIC ≤ 0.5) against target bacteria. The combination of A3-erythromycin

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against *S. aureus* (29213), A3-levofloxacin against *E. faecium* (BAA-2316) and A3-levofloxacin against *S. aureus* (33591) exhibited the highest synergistic activity among all combinations with FIC indices reaching 0.15, 0.23 and 0.38 respectively. The combinations of A3-levofloxacin, A3-chloramphenicol and A3-rifampicin against *S. aureus* (29213) exhibited synergistic activity with the FIC indices reaching 0.46, 0.46 and 0.43 respectively. The combination of A3-Rifampicin against *S. aureus* (33591) displayed a synergistic activity with the FIC index reaching 0.43. The combination of A3-chloramphenicol against *S. aureus* (BAA-41) exhibit synergistic activity with the FIC indices reaching 0.5. On the other hand, the other combinations exhibited only additive effects with FICs in the range of ($0.5 < FIC < 1$) . Table 5 displays the entire FIC indices of all peptide-antibiotic combinations.

Table 5: The fractional inhibitory concentration (FIC) indices of the antimicrobial combinations. (Results represent triplicates).

	FIC Index			
Antimicrobial combinations	***S. aureus* (29213)**	***S. aureus* (33591)**	***S. aureus* (BAA-41)**	***E. faecium* (BAA-2316)**
A3-Levofloxacin	**0.46**	**0.38**	0.68	0.23
A3-Chloramphenicol	**0.46**	0.73	**0.5**	**0.63**
A3-Rifampicin	**0.43**	**0.43**	0.64	**1.0**
A3-Erythromycin	**0.15**	0.78	0.54	**0.82**

4.6 Antibiofilm Activity

The antibiofilm activities of A3 and AamAP1 were assessed by two independent methods using the Calgary biofilm device. The first method depends on visual observation of bacterial growth and the determination of the Minimal Biofilm Eradication Concentration (MBEC), while the second method depends on viable bacterial cell counts after the treatment using the colony count method. The

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minimum A3 concentration that was able to inhibit the re-growth of bacteria from peptide treated of *S. aureus* (29213) and *S. aureus* (BAA-41) biofilms (MBEC) was found to be 25 and 30 µM respectively. There was no biofilm activity seen for the AamAP1, as shown in Table 6.

Table 6: The MBEC values of A3 and AamAP1 against biofilm-forming bacterial strain of *S. aureus.* MBEC values are displayed in (µM) concentration. (Results represent triplicates)

Gram-positive strains	A3	Parent peptide
S. aureus (29213)	25	No activity
S. aureus (BAA-41)	30	No activity

The minimum concentration of A3 needed to reduce the number of viable bacterial cells of *S. aureus* (29213) and *S. aureus* (BAA-41) biofilms to almost zero (99.9% killing) (MBCb) was calculated and the results of the minimum bactericidal concentration (MBCb) assay are summarized in Table 7. A3 managed to reduce the number of viable bacterial cells to almost zero and achieve the minimum bactericidal concentration (MBCb) at 60 µM which confirms the potent activity of the peptide and further increases the spectrum of activity of A3 to include bacterial biofilms in addition to the clinically isolated resistant strains of Gram-positive bacteria.

Table 7: The percentage reduction of viable bacterial cells of *S. aureus* biofilms including the minimum biofilm bactericidal concentration (MBCb) for A3 against tested bacterial strains. The shaded column represents the MBCb for both bacterial strains (Results represent triplicates)

Peptide Conc. (µM)	**80**	**60**	**40**	**35**	**30**	**25**	**20**	**15**
S. *aureus* (29213)	0.028 %	0.083 %	0.13%	0.24%	0.46%	5.7%	11.6%	25.3%
S. *aureus* (BAA-41)	0.013 %	0.098 %	1.2%	2.6%	4.7%	13.2%	27.4%	35.2%

4.7 MTT Cell Proliferation Assay

The cell proliferation assay was performed for the purpose of measuring the antiproliferative activity of A3 against two types of mammalian cell lines (Vero and HEK 293) in order to measure the peptide selectivity and cytotoxicity. A3 managed to inhibit the proliferation of Vero and HEK cell lines at IC_{50} values of 26.1 and 33.2 µM respectively (Table 8).

Table 8: IC_{50} values for A3 on HEK and Vero mammalian cell lines. (Results represent triplicates)

A3	**Mammalian IC_{50} (µM)**	
	HEK	**Vero**
	33.2	26.1

4.8 Hemolysis Assay

The Hemolytic activity of A3 against human erythrocytes (RBC) was determined as measure of the peptide's toxicity toward normal mammalian cells. At the MIC & MBC concentrations for A3 in combination with the antibiotics that were reported to be in the range of (0.025 - 3 µM) in addition to the individual MIC concentrations of A3 against most bacterial strains evaluated previously. A3 caused almost zero (negligible) hemolysis after 60 min of incubation with human erythrocytes while significant hemolysis to human erythrocytes was reported with concentrations above 20 µM, which are significantly higher than the concentrations needed to inhibit bacterial growth individually, or with the antibiotic combinations (Table 9).

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Table 9: Hemolytic effect A3 on human erythrocytes after 60 min of incubation.
(Results represent triplicates)

Peptide concentration (μM)	Hemolysis (%)
1	0
5	0
10	5.1
20	16.8
40	36.1
60	47.9
80	49.4

Discussion

Host defense peptides (HDPs) represent an attractive group of molecules for development as novel therapeutic agents to be used in the treatment of antimicrobial infections, especially the ones caused by the antibiotic multi-resistant bacteria [22]. One of the most significant obstacles for the clinical development of HDPs in the treatment of microbial infections is related to their toxicity against normal cells due to their lack of microbial target selectivity as in the case with conventional antibiotics. Several HDPs display potent antimicrobial activity but suffer from severe toxicity and hemolytic activity against normal cells. To overcome this dilemma many studies focused on advising new methods to limit the cytotoxicity of HDPs either through structural modification or novel formulation technologies [23, 25].

Scorpion venoms represent a cocktail of biologically active molecules that are classified into two groups and these include the disulfide bridged peptides that target membrane bound ion channels and the recently discovered non-disulfide bridged peptides which display a diversity of biological activities including antimicrobial peptides [25, 26]. One of the major drawbacks of scorpion

antimicrobial HDPs is the lack of distinct cell selectivity against target cells as the peptides induce severe hemolytic effects against mammalian cells and human erythrocytes [27]. AamAP1, a novel HDP that was identified through molecular cloning from the venom derived cDNA library of the North African scorpion *Androctonus amoeruxi*, was found to display moderate broad spectrum antimicrobial activities against representative strains of Gram-positive, Gram-negative bacteria and yeast in the range of 20–150μM. The peptide also displayed significant hemolytic activity against sheep erythrocytes at concentrations that were employed in the antimicrobial studies and showed no selectivity against mammalian cells [13].

In the present study, AamAP1 was used as platform for computer aided rational design in order to develop a modified peptide with improved antimicrobial activity combined with improved cell selectivity and decreased toxicity against mammalian cells. The design strategy was mainly based on substitution of different amino acid in order to increase the positive net charge and the overall percentage helicity while taking into account the optimization of all the other physicochemical parameters responsible for HDPs activity. The main parameter that was initially employed for modification of the parent peptide focused on increasing the positive net charge and generation of a continuous helical amino acid sequence. The resultant modified peptide named A3 was later screened for all the structural parameters involved in HDPs activity such as hydrohobicity, hydrophobic moment, charge, percentage and helicity

The overall net charge of HDPs is considered an essential parameter regarding HDPs activity. Charge is responsible for the initial electrostatic attraction of cationic HDPs with the negatively charged bacterial membranes. The increase in A3's net positive charge while not drastically altering other physicochemical parameters is expected to enhance the peptide's antimicrobial activity and minimize its cytotoxicity by enhancing its selectivity against bacterial membranes. An overall net charge range of ($+2 < Q < +9$) has been determined in literature as an optimal range for HDPs activity

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[28]. A3 displayed a positive cationic charge of (+3). When compared to the parent peptide AamAP1 that originally displayed a net charge of (+1).

The α-helical content and the percentage helicity of A3 was evaluated in order to confirm that the substitution design generated a continuous uninterrupted α-helical peptide. The results displayed that the percentage helicity of A3 was 100% with a significant increase over the parent peptide that exhibited 50.56% percentage helicity. As helicity is considered crucial for HDPs activity, this increase in helicity in the modified peptide is expected to generate modified peptide with significant selectivity against target cells.

In order to confirm the initial helicity analytical results, the HHpred (HHsearch 2.0) software was used for the evaluation of the physicochemical parameters of A3 and for the structure prediction and identification of the best template that could be employed for homology modelling of the modified peptide. The homology results displays significant alignment with Pardaxin, a previously identified group of HDPs with potent antimicrobial and anticancer activities and an uninterrupted helical structure that is in accordance with initial helicity findings that were generated from the SOPM secondary structure prediction software .

The antimicrobial studies displayed that A3 was active against the wild type and the multi-drug resistant clinical isolates of Gram-positive bacteria that were employed in this study. A3 was able to inhibit the growth of control strains of Gram-positive bacteria within a range of 2.5-12.5 μM. Additionally, it inhibited the growth of the multi-drug resistant and clinical isolates of Gram-positive strains within a range of 5-15 μM. The synergistic effects of combing A3 with conventional antibiotics was also evaluated in order to further reduce the effective antimicrobial concentration needed for bacterial elimination. The results form the synergistic studies display that the MIC values for a significant number of antibiotics in combination with A3 decreased dramatically. Out of the overall sixteen antimicrobial combinations, 50% were shown to be synergistic and 50% are additive against

the planktonic cells of the tested bacterial strains while non-shown any indifference or antagonistic effect according to FIC index. For the control strain of *S. aureus* (ATCC: 29213), A3-levofloxacin, A3-chloramphenicol, A3- Rifampicin and A3-erythromycin combinations displayed synergistic effects. The most notable reduction was related to levofloxacin's MIC reduction when combined with A3 as its MIC value was reduced by 93.6%. Additionally, chloramphenicol's MIC value was reduced by 93.8%, while erythromycin's MIC was reduced by 87.5% compared to the MICs of these antibiotics individually.

For the MRSA strain of *S. aureus* (ATCC 33591), A3-levofloxacin and A3-rifampicin combinations displayed synergistic effects. The MIC values of Levofloxacin and rifampicin in the combination groups were reduced by 92.5% and 96.9% respectively compared to the MICs of these antibiotics individually. For the clinical isolated MRSA strain of *S. aureus* (ATCC BAA-41), only the A3-chloramphenicol combination displayed a synergistic effect. The MIC of chloramphenicol in the combination was reduced to 90% compared to its individual alone. For the clinical isolated multi-drug resistant *E. faecium* (BAA-2316), A3-levofloxacin combination displayed a synergistic effect and the MIC of levofloxacin in the combination was reduced to 90% compared to its MIC alone.

It is well documented that antibiotics can kill or inhibit the bacteria through different mechanisms of action such as inhibiting proteins synthesis, interfering with nucleic acid synthesis, and blocking cell wall synthesis [29]. In the present study, although the tested antibiotics exhibited different antibacterial mechanisms, many of the test combinations displayed synergistic outcome or at least an additive effect, which suggested that the A3's mechanism of action is different from all mechanisms of the investigated antibiotics. However, the mechanisms of the synergistic effects of the HDP-antibiotic combinations were still unclear yet. Several studies showed that many HDPs had the ability to lyse the biological membrane and generate pores by different mechanisms such as toroidal pore and barrel stave models which consequently allow the antibiotics to bypass the bacterial cell wall in large numbers and destroy the bacteria [30].

Bacterial membranes are rich in the acidic phospholipids peptidoglycans (PG), phospholipids (PS), and Cardiolipins (CL), these biomolecules contribute collectively in conferring the overall negative charge on bacterial membranes. Moreover, LPS and teichoic or teichuronic acids of Gram-positive bacteria impart additional negative charge to the surfaces of these respective organisms [12].

One of the proposed mechanisms for the synergistic effect was that the HDPs cause destruction of the peptidoglycan layer and cause permeabilization of the membrane and consequently allowing the rapid entry of antibiotics [31]. From our data, we propose that this mechanism is the most probable mechanism for A3 and the tested antibiotics combinations. This mechanism explains our results where all combinations displayed either a synergistic or additive effects against all tested bacterial strains. The targets of all antibiotics employed in this study (levofloxacin, chloramphenicol, rifampicin, and erythromycin) are located inside the bacterial cell, which means this mechanism augmented the antibiotics in reaching their targets and accomplish their molecular function.

The structural modification not only enhanced the antimicrobial activity of the parent peptide but also supplemented it with potent antibiofilm activity. The minimum A3 concentration that was able to inhibit the re-growth of the clinically isolated multi-drug resistant Gram-positive bacteria of *S. aureus* (ATCC: 29213 and BAA-41) from peptide treated biofilm was found to be 25 µM and 30 µM respectively. For the four conventional antibiotics used in our study levofloxacin, chloramphenicol, rifampicin and erythromycin, the reported data for biofilm eradication was above 500 µM for each antibiotic (Data not shown). This data suggests that A3 is highly potent agent in biofilm inhibition unlike conventional antibiotics.

The increase in the cationicity and overall helicity of A3 not only enhanced its antimicrobial activity but also influenced its selectivity index. The increase in antimicrobial activity was also accompanied by a decrease in the hemolytic activity of the peptide against human erythrocytes. A3 caused negligible hemolysis at MIC concentrations needed to inhibit bacteria.

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The reduced cytotoxicity of the peptide studies were also confirmed using the MTT cytotoxicity assay against human HEK 293 and Vero cell lines, as the modified A3 peptide caused mild disruption of eukaryotic cells than the its parent AamAP1 peptide. This improvement in the toxicity profile and selectivity index could also be attributed to the increase in cationicity and improved binding to microbial membranes. The anti-proliferative concentrations of of A3 was reported to be five to six fold higher than the geometric average MIC values which confirm that the design strategy was successful in reducing the overall toxicity of the peptide. The results of the cytotoxicity studies confirm the results from the previous hemolytic assays of A3.

In this study, we report the design and functional characterization of the antimicrobial, antibiofilm and mammalian cytotoxic activity of a synthetic scorpion venom peptide analogue named A3. The antimicrobial, haemolytic and antiproliferative studies indicate that A3 displays potent selective activities against a wide range of Gram-positive bacteria including the clinical isolates of multi drug resistant Gram-positive bacteria. The results obtained from the hemolytic and MTT assay indicate that A3 is exerting moderate toxicity against eukaryotic cells at the antimicrobial concentrations and is displaying significant selectivity against microbial cells. The potency of A3 peptide combined with its mild cytotoxic profile indicate that the peptide has a great potential as an effective antimicrobial agent and combining A3 with conventional antibiotics may be pursued as a potential novel treatment strategy against MDR and biofilm forming bacteria.

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Author Contributions: Ammar Almaaytah designed the experiments and prepared the manuscript; Ahmad Farjallah and Ahmad Abualhaijaa performed the experiments; Qosay Al-Bals performed the moelling experiments and contributed to check and revise the wording in the manuscript.

Conflicts of Interest: The authors declare no conflict of interest

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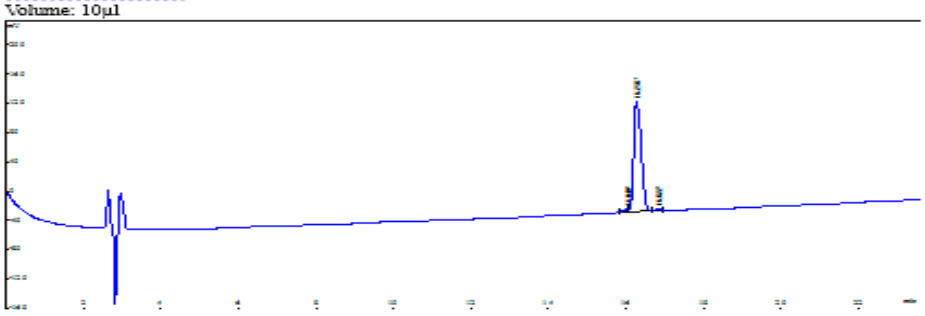
Supplementary Material:

HPLC REPORT

Product Name:FK-18
Instrument No: 0200194
Lot No :P150414-SL343091
Column :4.6*250mm, phenomenex C18-5
Solvent A :0.1%Trifluoroacetic in 100% Acetonitrile
Solvent B :0.1%Trifluoroacetic in 100% Water
Gradient :

	A	B
0.01min	48%	52%
25in	73%	27%
25.1min	100%	0%
30min	Stop	

Flow rate :1.0ml/min
Wavelength :220nm
Volume: 10μl



Rank	Time	Name	Conc.	Area	Height
1	16.049		1.618	29090	3700
2	16.258		97.55	1754252	150172
3	16.822		0.829	14907	1504
Total			100	1798249	155376

Figure 1: Analytical RP-HPLC chromatogram of A3

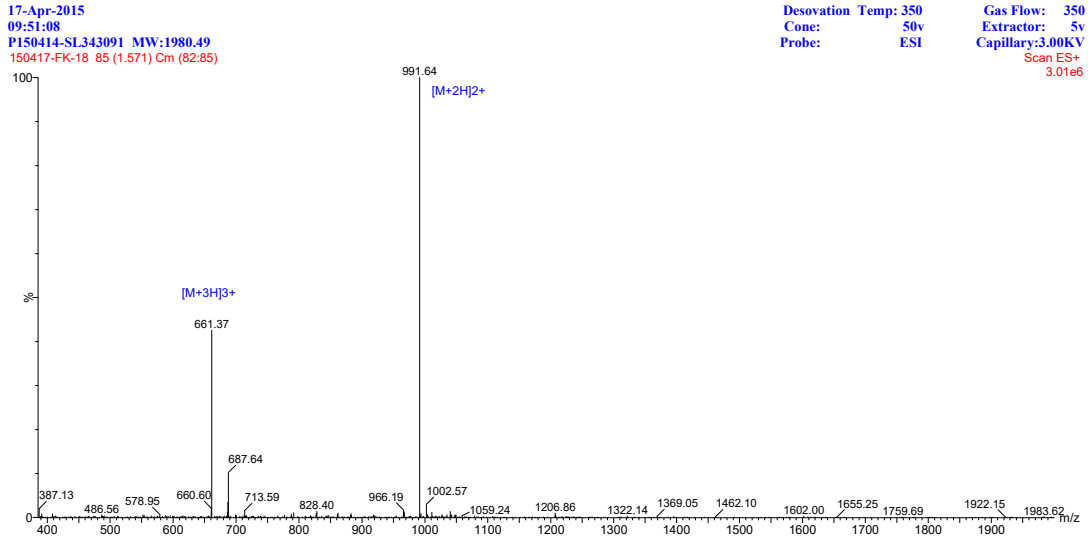


Figure 2: Positive electrospray ionization (ESI) mass spectrometric (MS) analysis of the A3. The peptide showing major peaks in the +2 and+3 charge state of 991.64 Da and 661.37 Da respectively