Novel Antimicrobial Peptides for enhanced antimicrobial activity against Methicillin Resistant *Staphylococcus aureus*: Design, Synthesis and Formulation

by

Mbuso Faya
(MSc Health Science – University of KwaZulu-Natal)

Submitted as fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmaceutics at the Discipline of Pharmaceutical Sciences of the School of Health Sciences at the University of KwaZulu-Natal

UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Supervisor: Professor Thirumala Govender
(PhD, University of Nottingham, United Kingdom)

Date submitted: December 6th 2018

"A candle loses nothing by lighting another candle''

- James Keller -

"***This thesis is dedicated to my family who have consistently pushed beyond boundaries and refused earthly definitions to press on to discover new realities".***

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Details of contribution to publications that form part and/or include research presented in this thesis:

The following publications were submitted to their respective reputable high impact factor peer reviewed journals.

Conjugates and nano-delivery of antimicrobial peptides for enhancing therapeutic activity

M. Faya, R.S. Kalhapure, H.M. Kumalo, A.Y. Waddad, C. Omolo, T. Govender, Conjugates and nano-delivery of antimicrobial peptides for enhancing therapeutic activity, Journal of Drug Delivery Science and Technology. 44 (2018) 153–171. doi:https://doi.org/10.1016/j.jddst.2017.12.010.

Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation

M. Faya, R.S. Kalhapure, D. Dhumal, N. Agrawal, C. Omolo, K.G. Akamanchi, T. Govender, Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation, Journal of Biomololecular Structure and Dynamics. (2018) 1–31.

Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against methicillin-resistant *Staphylococcus aureus* (MRSA)

Mbuso Faya, Calvin A. Omolo, Fernando Albericio, Beatriz G. de la torres, Heba A. Hazzah, Ruma Maji, Pavan Walwaker, Chunderika Mocktar, Bongani Nkambule, Thirumala Govender*, European Journal of Pharmaceutics and Biopharmaceutics (manuscript ID: EJPB_2018_1382)

The published, accepted and submitted first author manuscripts can be found in Chapters three to five of this thesis.

Mr. Mbuso Faya contributed to the conceptualization and design of the papers. In addition, he was responsible for analysis and data collection and wrote the papers and undertook all revisions. He also contributed to the synthesis, and characterization of the AMPs and formulation, and characterization of the nanostructured liposomal carriers in terms of particle size, polydispersity index, zeta potential, surface morphology, entrapment efficiency, *in vitro* and antimicrobial activity and analysis of Molecular dynamics (MD) simulations studies. Dr. R.S. Kalhapure assisted with the inception, overall design of the project. Mr. Dinesh Dhumal was responsible for proving the QSAR data. Mr. Calvin Omolo assisted with the conceptualization and minor revisions of the paper. Mr. Pavan Walwaker assisted in performing the cytotoxicity studies. Dr Ruma Maji assisted with the formulation of liposomes. Dr Hezekiel Kumalo and Dr Ayman Waydad assisted in the revisions of the review paper. Dr Nikhil Agrawal assisted in MD simulation studies. Dr Heba Hazzah assisted with synthesis and purification of the AMPs. Dr. Chunderika Mocktar supervised the *in vitro* antibacterial activity studies. Dr. Bongani Nkambule assisted with all flow cytometric work. Prof K.G Akamanchi, Prof Fernando Albericio and Prof Beatriz G. de la Torre served as collaborators on the project. Prof. Thirumala Govender served as supervisor and was responsible for project conceptualization, problem-solving, paper and abstract editing and general supervision of the study.

Research output from the dissertation

1. First authored Publications

The following research papers were published as results generated from specific objectives from this study and they include: -

- Faya, M., Kalhapure, R. S., Kumalo, H. M., Waddad, A. Y., Omolo, C., & Govender, T. (2017). Conjugates and nano-delivery of antimicrobial peptides for enhancing therapeutic activity. *Journal of Drug Delivery Science and Technology*. https://doi.org/10.1016/j.jddst.2017.12.010

- Faya, M., Kalhapure, R. S., Dhumal, D., Agrawal, N., Omolo, C., Akamanchi, K. G., & Govender, T. (2018). Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation. *Journal of Biomolecular Structure and Dynamics*, (just-accepted), 1-31.

Mbuso Faya[1], Heba A. Hazzah, Calvin A. Omolo[1], Ruma Maji[1], Pavan Walwaker[1], Chunderika Mocktar[1], Bongani Nkambule[2], Fernando Albericio, Beatriz G. de la Torre, Thirumala Govender[*,1]. Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against methicillin-resistant Staphylococcus aureus (MRSA). European Journal of Pharmaceutics and Biopharmaceutics (manuscript ID: EJPB_2018_1382)

2. Conference Presentations

Poster Presentations

The following conference presentations were produced from data generated during this study:

1. Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation. Mbuso Faya, Rahul S. Kalhapure, Dinesh Dhumal, Nikhil Agrawal, Calvin Omolo, Krishnacharya G. Akamanchi & Thirumala Govender. Journal of Biomolecular Structure and Dynamics. Nano Africa, 23-25 April 2018, Durban South Africa, (senior Author.

Oral presentations

2. Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation. Mbuso Faya, Rahul S. Kalhapure, Dinesh Dhumal, Nikhil Agrawal, Calvin Omolo, Krishnacharya G. Akamanchi & Thirumala Govender. Journal of Biomolecular Structure and Dynamics. 38th Annual Conference of the Academy of Pharmaceutical Sciences,06-08 July 2017, Johannesburg, South Africa.
3. Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against methicillin-resistant *Staphylococcus aureus* (MRSA). Mbuso Faya[1], Calvin A. Omolo [1], Fernando Albericio[1], Beatriz G. de la torres[1], Heba A. Hazzah[3], Ruma Maji[1], Pavan Walwaker[1], Chunderika Mocktar[1], Bongani Nkambule[2], Thirumala Govender[*,1] University of KwaZulu-Natal Nanotechnology Platform Workshop, 22nd November 2017.

The posters can be found in Appendix A.

Abstract

The control of infectious diseases is seriously threatened by the steady increase in the number of microorganisms that are resistant to antimicrobial agents. Some of the interventions to address the problem of resistance include the use of drug combinations and improvements in patient compliance with dosing. The discovery of antimicrobial peptides (AMPs) has given hope to the problem of drug resistance. Therefore, the aim of this study was to design, synthesize and evaluate novel AMPs for their bacterial membrane penetration and activity followed by their co-encapsulation with vancomycin (VCM) and oleic acid (OA) in a liposomal system to enhance their antimicrobial activity. In this study a QSAR model which can simultaneously estimate antimicrobial potential (MIC) and bacterial cell penetrating ability (TI) of antimicrobial cell penetrating peptides (aCPPs) against *S. aureus* was developed and novel AMPs were designed, synthesized and employed to decorate vancomycin and oleic acid containing liposomes to achieve pH responsiveness for enhanced antimicrobial activity. The QSAR study proved the viability of the therapeutic index of aCPPs in relation to their cell penetrating ability and antimicrobial potential by building a QSAR model which outlined specific descriptors responsible for their potency. The synthesized novel AMPs were found to be biosafe, exhibiting cell viability of above 85% in all the cell lines tested using an MTT assay. The membrane penetration studies using molecular dynamics and flow cytometry revealed that the AMPs were able to traverse the bacterial membrane. The formulated liposomal systems were characterized in terms of sizes, polydispersity indices (PDI), zeta potential (ZP), surface morphology, *in vitro* antibacterial activity. The liposomes formulated from the two best bioactive AMPs were formulated to produce AMP_2-Lipo-1 and AMP_3-Lipo-2. The size, PDI and ZP at pH 7.4 of the drug loaded liposomes (**AMP_2-Lipo-1 and AMP_3-Lipo-2**) were 102.6±1.81 nm, PDI of 0.157±0.01 and -9.81±1.69 mV and 146.4±1.90 nm, PDI of 0.412±0.05 and -4.27±1.25 mV for **AMP_2-Lipo-1 and AMP_3-Lipo-2** respectively. However, when the liposomes were placed in pH 6.0 it was observed that both liposomal formulations had an increase in size and decrease in negative charge. **AMP_2-Lipo-1** had a size of 387.4±51.11 nm and PDI of 0.81±0.03 and a zeta potential of - 2.19±0.57 mV whereas **AMP_3-Lipo-2** had a size of 229.4±13.8 nm and PDI of 0.74±0.01, with a zeta potential of 0.14±0.31 mV. Measurements were also made at pH 4.5 where **AMP_2-Lipo-1** had a size of 192.4±6.9 nm and PDI of 0.49±5.24, with a zeta potential of 1.50±0.31 mV. **AMP_3-Lipo-2** reflected similar changes at pH 4.5 where the size was 218.6±6.18 nm and PDI of 0.63±0.02, with a zeta potential of 1.80±2.21 mV. The observed differences (particle swelling and charge switch) at the three different pH is indicative of pH responsiveness of our formulation and therefore could function considerable well in drug delivery. *In vitro* antibacterial activity showed that the liposomal formulations had enhanced activity compared to bare AMPs against MRSA. In summary, the synthesized novel AMPs showed high biosafety profiles and enhanced activity compared to bare AMPs and further revealed the potential for application in clinical trials.

Acknowledgements

My first and sincere appreciation goes to my family for their support and guidance throughout the course of my PhD. I would like to express my appreciations towards my friends who encouraged me to further my studies. I would also like to thank my supervisor Prof. Thirumala Govender for her support and guidance and my fellow colleagues, Sfiso Makhathini, Calvin Omolo, Dr Lucky Kumalo, Nawras Abdolemien, Mohammed Abdeen, Daniel Hassan, Pavan Walvaker, as well as the postdoctoral researcher's Dr Ruma Maji, Dr Ayman Waydad, Dr Ramesh Ganimani. Finally, I would like to acknowledge Dr Chundereka Moktar for availing her research facilities towards my research work, Dr Bongani Nkambule for availing his flow cytometry instrumentation, Dr Nikhil Agrawal for providing guidance with regards to computational studies and Prof Fernando Albericio, Prof Beatriz de la Torres and Heba Hazza for assisting with our synthetic work. I would also like to acknowledge the National Research Foundation (NRF) and talent acceleration scholarship program (TAES) for their financial assistance towards my research and also a special thanks to the University of KwaZulu-Natal for giving me the opportunity to carry out my research work and add to the on-going knowledge development sphere.

TABLE OF CONTENTS

List of Abbreviations

AMPs	Antimicrobial peptides
SLNs	Solid lipid nanoparticles
VCM	vancomycin
OA	Oleic acid
QSAR	Quantitative structure activity relationship
MRSA	Methilicin resistant *Staphylococcus aureus*
PC	Phospatidyl choline
DLS	Dynamic light scattering
aCPPs	Antimicrobial cell penetrating peptides
TI	Therapeutic index
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
S. aureus	*Staphylococcus aureus*
HPLC	High performance liquid chromatography
LCMS	liquid chromatography mass spectrometry
HR-TEM	High resolution transmission electron microscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
A	hydrogen bond acceptor
aCPP's	antimicrobial cell penetrating peptides
AMPs	antimicrobial peptides
ANN	artificial neural network
AntiBP2	antibacterial peptide prediction
APD2	antimicrobial peptide database
COM	centre of mass
CPPs	cell penetrating peptides
F value	Fisher test
H	hydrophobic group
IC_{50}	half maximal inhibitory concentration
LA	lipid A
LINCS	linear constraint solver
LPS	lipopolysaccharides
Lys	lysine
MD	molecular dynamics
MIC	minimum inhibitory concentration
NPT	isothermal-isobaric ensemble
NVT	canonical ensemble
PLS	partial least squares
PME	Particle mesh Ewald
PO_4	Phosphate group
Q^2	cross-validation coefficient
R	aromatic ring
R^2	correlation coefficient
RMSE	Root-mean squared error
SD	Standard deviation
TI	therapeutic index
Trp	tryptophan

vdW	van der Waals
TEM	Transmission electron microscopy
PDI	Polydispersity index
VCM-HCl	Vancomycin hydrocloride
RBCs	Red blood cells
MBHA	paramethylbenzhydrylamine
DIC	N,N′-Diisopropylcarbodiimide
SPPS	Solid phase peptide synthesis
DMF	Dimethyl formamide
DCM	dicloromethane
PBS	Phosphate buffer saline
A 549	Adeno carcinomic alveolar basal epithelial cells
HEK-293	Human embryonic kidney
HeLa	Henrietta Lacks
ZP	Zeta potential
ABS	Absorbance
RMSE	Root mean square error
MHB broth	Muller Hinton broth
CFU	Colony forming units
MHA	Muller Hinton Agar
DMEM	Dulbecco's Modified Eagle's Medium
MOI	Multiplicity of infection
PI	Propidium iodide
dH_2O	Deionized water
MDT	Mean dissolution time
E.coli	*Escherichia Coli* (bacteria)
FIC	fractional inhibitory concentration
IV administration	Intravenous administration
°C	Degrees Celsius
MIC	Minimum inhibitory concentration
min	Minutes
UV-VIS	Ultraviolet-visible spectroscopy

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CHAPTER 1
INTRODUCTION

1.1 Introduction

The focus of this chapter is to briefly give a background and overview of drug resistance which has rendered many therapeutic interventions ineffective. Furthermore, it provides an alternative route with enhanced therapeutic outputs compared to known antibiotics which has resulted in the proposed aims and objectives of the study, thus highlighting the novelty and significance of the study.

1.2 Background to this study

Pathogenic microorganisms have been a global threat for decades resulting in the numerous diseases that are rising exponentially over the years[1]. These pathogens such as bacteria, viruses, parasites or fungi, have the potential to spread disease directly or indirectly and from one person to another[2]. Globally, the rate of infections has increased in both nosocomial and community settings, therefore creating a major need for newer effective therapeutic interventions in both developed and developing countries[3][4]. This incremental rise of infectious diseases has also posed a threat to global trade, population growth and further escalated the disease burden in developing countries (**Figure 1**)[5][6]. Besides the cause of mortality, infectious diseases also contribute greatly as opportunistic infections in immune compromised people, which further increases mortality rates[7][8]. Based on the broad overview of the spread of infectious diseases, various novel routes are required to circumvent the spread of infectious diseases.

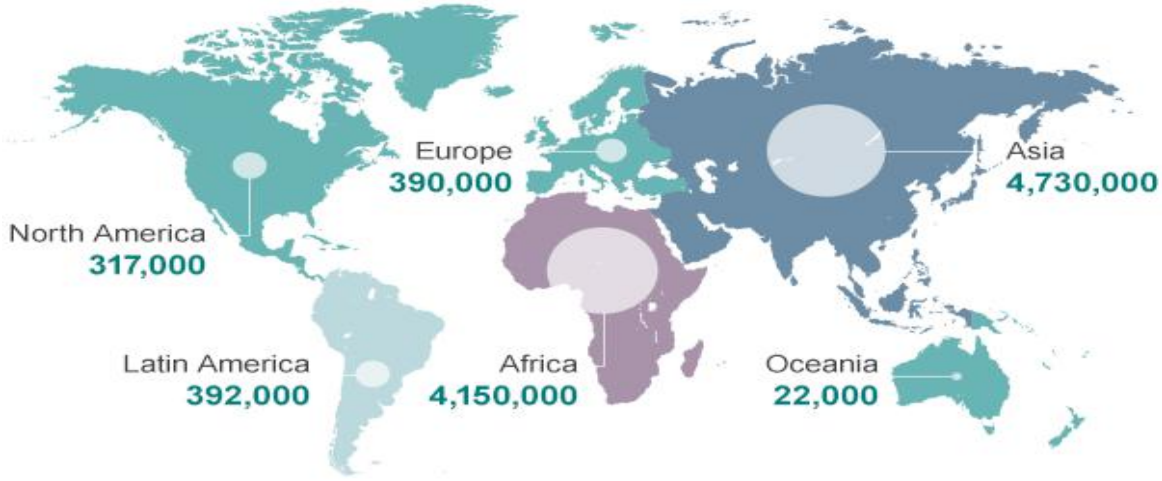

Figure 1: Antimicrobial resistance deaths prediction by 2050[9]

One of the very first attempts to control infectious diseases was through the introduction of salvarsan, an anti-syphilis therapeutic agent which was synthesized by Ehrlich in 1910[10]. Two decades after, sulphonamides were introduced into the antibiotic market by Domagk together with other scientists[11]. These early synthetic compounds had lowe bio-safety and efficacy levels (**Figure 2**). In 1928, penicillin was discovered by Alexander Fleming, where he found that the growth of *Staphylococcus aureus* was inhibited in a zone surrounding a contaminated blue mold (a fungus from the Penicillium genus) in culture dishes, which led to the finding that a microorganism would produce substances that could inhibit the growth of other microorganisms[12]. Streptomycin (obtained from *Streptomyces griseus*) was later discovered in 1944 and thereafter, chloramphenicol, tetracycline, macrolide, and glycopeptides (e.g., vancomycin) were discovered from soil bacteria[13].

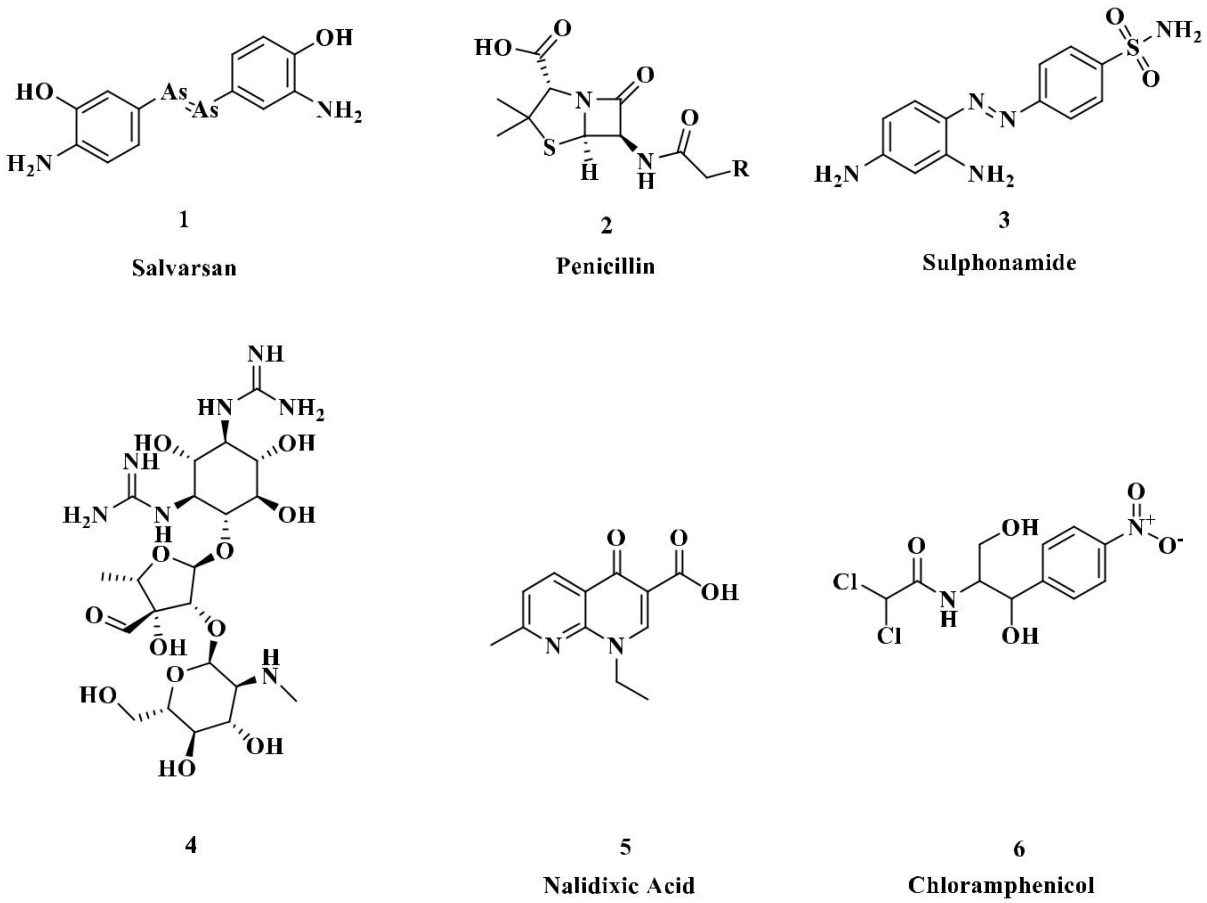

Figure 2: Structures of early antimicrobial agents

The goal of these antimicrobial agents was to have highly selective toxicity towards pathogens with minimal and preferably no toxicity in humans[14]. This selectivity was thought to occur through the inhibition of specific bacterial-intracellular targeting or through

the inhibition of biochemical pathways which are vital to the bacterial survival[15][16][17]. Some of the well-studied targeted pathways by different drugs include inhibition of cell wall synthesis (β-lactams, Penicillins, cephalosporin, vancomycin, bacitracin, cycloserin), inhibition of cell membrane function (Polymyxin, amphotericin B, imidazole, Daptomycin), inhibition of protein synthesis (chloramphenicol, erythromycin, clindamycin), and inhibition of nucleic acid synthesis (ciprofloxacin, flucytosine, fluoroquinolones, rifamycin)[18] (**Figure 3**).

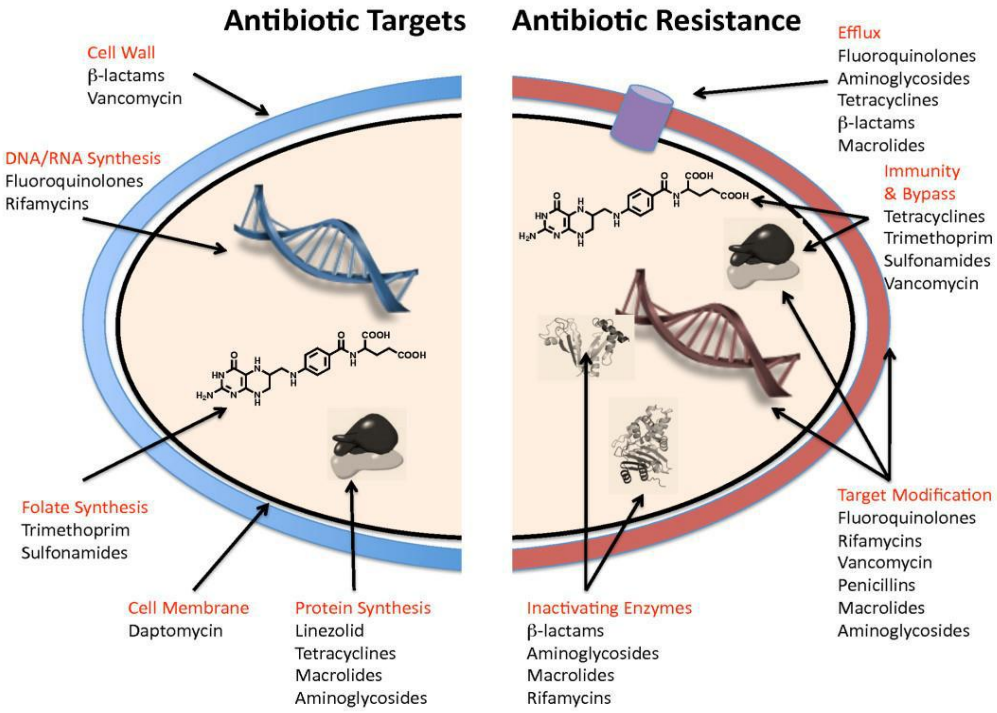

Figure 3: Antibiotic targets and resistance mechanisms[19]

Even with the plausible prospects of antibiotics such as reduction of morbidity and mortality rates, numerous limitations have attenuated their function, thus rendering them ineffective against various bacterial strains[20]. Some of these limitations include bacterial resistance brought on by improper usage, transposable genetic elements and mutations[21][22]. Others include enzymatic degradation of antibiotics in the gastro-intestinal tract and biocompatibility together with short circulation in the bloodstream[23].

Bacteria use different resistance mechanisms to repel and protect themselves from the function of antibiotics. Firstly, antibiotic modification where the resistant bacteria retains the

same sensitive target, but the antibiotic is prevented from reaching it[24]. Secondly, augmented efflux via transport pumps is another mechanism of resistance where antibiotics are actively pumped out of the bacteria[17][25]. Thirdly, alterations in the primary site of action where the antibiotic penetrates the cell and reaches the target site but is unable to inhibit the activity of the target because of structural changes in the molecule[26]. The fourth mechanism is carried out through the application of enzymes, where the bacteria may protect themselves from antibiotics by the production of an alternative target (an enzyme) that is resistant to inhibition by the antibiotic while continuing with the production of the original sensitive target[27].

Based on the ineffective application of antibiotics to curb new infection rates due to drug resistance, there is a clear need for the development of novel therapeutic alternatives to effectively bypass the antibiotic shortfalls. One of the current therapeutic options researched is antimicrobial peptides (AMPs). These peptides are composed of different amino acid arrangement with lengths ranging from 15-50 amino acids residues, have been seen to possess a broad spectrum of activity against most pathogenic microorganisms[28]. Their antimicrobial activity is attributed to their net positive charge, hydrophobicity as well as structural conformation[29][30]. Resistance to these compounds have not been well researched, suggesting that AMPs have the potential to be developed for therapeutic application. The development of antimicrobial peptide antibiotics is increasingly gaining much attraction as their roles in physio-pathological processes are being unravelled[31]. However, due to lack of delivery at an appropriate dosage, time, specificity of the target cells, they can be rendered unusable[32]. There are numerous methods for obtaining AMP delivery systems. AMPs could be immobilized via a linker molecule into a variety of materials or adsorption onto a variety of surfaces and still retain their ability to bind and kill bacteria[33]. One of the methodologies currently used is nanoencapsulation. This is achieved through encapsulation or adsorption on nanocarriers by various methods such as emulsion, polymerization, solvent evaporation, combination of sonication and layer by layer technology and solvent displacement/solvent diffusion[34].

Due to the inability of current dosage forms to curb the rise of drug resistance, researchers have explored various strategies to combat this occurrence. Some strategies involve the use of molecular modelling for the design of potent and selective AMPs for optimal membrane targeting. Other reported strategies involve the co-delivery of current drugs such as vancomycin with fatty acids (e.g. oleic acid) and the encapsulation of bioactive antimicrobial

peptides in a nano system. Vancomycin is known as a good antimicrobial agent that inhibits bacterial cell wall synthesis by complexation with peptidoglycan precursors[35]. In a study by Sande et al. (2012), it was reported that liposomal vancomycin improved MRSA killing in comparison to bare vancomycin[36]. The application of oleic acid in targeting pathogenic microorganisms have been also been evaluated. However, its encapsulation in a liposomal system has been shown to possess minimum bactericidal concentrations (MBC), that is 12 times lower than free oleic acid against MRSA[37][38].

Currently, nanotechnology has been seen to be possess the ability to address the antibiotic shortfalls due to drug resistance[39]. In the context of drug delivery, various nanobased structures have been used to delivery drugs to their specific target site. Some of these nanoparticles include micelles, solid lipid nanoparticles (SLNs), liposomes, dendrimers, hydrogels which are further discussed in the following chapter[40]. The advantages that these nanostructures offer include, enhanced drug solubility, sustained and controlled drug release at infection site, biocompatibility, reduced toxicity[41]. Therefore, in order to address the rise in drug resistance and limitations associated with conventional dosage forms of antibiotics, novel nano-antibiotic approaches are warranted. One such approach involves the application of liposomes which have attracted much attention due to their biocompatibility and biodegradability, which makes them very attractive for biomedical investigation[42]. They have been seen to possess the capability to solubilize and encapsulate hydrophilic and hydrophobic materials by nature, making them ideal nano structures for the delivery of amphiphilic compounds.

Considering the rise in drug resistance and the slow development of antibacterial drugs, there is a huge scope in the development of novel therapeutic interventions to curb the rate of infection and to halt the propagation of pathogenic microorganisms. In this study we have explored firstly the design and synthesis of novel antimicrobial peptides to specifically target the bacterial membrane. Secondly, we have explored the non-covalent co-delivery of three antibacterial agents (AMPs, OA, VCM) in a liposome to target MRSA infections. This non-covalent strategy is referred to as a supramolecular lipidation which involves two or more larger molecules in a system without the application of chemical bonding. This strategy offers an enhanced multi-directional approach in targeting infection sites, where the inner contents can act on the bacterial membrane whilst other agents are eluted to intracellular targets.

There are several reports on the liposomal encapsulation of AMPs or the co-delivery of drugs with other antimicrobial agents in nano systems. However no study has reported on the non-covalent encapsulation of novel AMPs with other agents to target MRSA infections.

Currently, nano systems such as liposomes have been designed to respond to a change in pH. This pH responsiveness has the potential to offer plausible therapeutic advantages such as effectiveness in reaching the target site, where the infection site acidosis triggers a release of the nano system contents on that target area. Therefore, the design of pH responsive liposomal delivery systems comprising of novel AMPs and other antimicrobial agents known to target the bacterial membrane and intracellular contents could prove to be a valuable strategy in targeting resistant strains.

Therefore, the aim of this study was to design, synthesize and evaluate novel AMPs for their bacterial membrane penetration and activity followed by their co-encapsulation with VCM and OA in a liposomal system to enhance their antimicrobial activity. Therefore, chapters 3 and 4 represent our first efforts to develop novel AMPs followed by their non-covalent incorporation with two other antimicrobial agents (OA and VCM) in a liposome to target MRSA infections. We envisaged that through this supramolecular nano composite, a system comprising of the membrane penetrating power of AMPs in conjunction with the known antimicrobial activity of oleic acid and VCM would produce a plausible system that would effectively halt MRSA infections and offer a sustained release of the nanoparticle at the target site.

1.3 Problem statement

Drug resistance has significantly threatened the control of infectious diseases. Moreover, it has rendered many antibiotics ineffective towards specific bacterial strains. This incumbent has led to a rise in mortality and morbidity rates especially in developing countries. Current dosage forms have been seen to possess several limitations such as inadequate drug concentration at infection/target sites, fast degradation and short circulation in the bloodstream, severe side effects and poor patient compliance. All these factors stated above are indicative of the huge gap in the development of novel therapeutic compounds to curb the rise of drug resistance. This also points to the need for molecular modelling approaches to design optimal AMPs with bacterial membrane specificity. The application of nanotechnology in the development on nano-based drug delivery systems has proven to possess the ability to significantly address the antibiotic shortfalls brought on by drug resistance. The design and development of AMPs which target the bacterial cell membrane due to their cationicity followed by their co-encapsulation with OA and VCM in a liposomal system is expected to enhance antibacterial activity and thus contributing toward curbing drug resistance. With this in mind, molecular modelling approaches, followed by the

development and application of nano-based drug delivery systems are paramount to effectively understand the mechanism of bacterial pathway inhibition with increased antimicrobial activity, whilst lowering infections rates globally.

1.4 Aims and objectives of this study

The broad aim of this study was to design antimicrobial peptides that have bacterial membrane penetrating ability and explore their co-encapsulation with a drug and a non-drug antimicrobial agents in a delivery system to target MRSA infections. The specific research aims of the novel antimicrobial peptide and their co-encapsulation to enhance their antimicrobial activity are further discussed with respective objectives in the following chapters.

Aim 1

The aim of this study was to develop a QSAR model which can simultaneously estimate antimicrobial potential and bacterial cell penetrating ability of antimicrobial CPPs (aCPPs) against *S. aureus*.

In order to achieve this aim, the objectives of the study were to:

1. To divide the 28 aCPPs into training and test set compounds and use PHASE 3.0 suite to build and determine the most appropriate pharmacophore model (AAHRR.114).
2. Select the AAHRR.114 pharmacophore model to determine a QSAR pharmacophoric alignment of the training and test set compounds to find the best fit aCPP.
3. Perform molecular dynamics simulation using the best fit compound on a POPC model membrane to ascertain its membrane penetrating ability.

Aim 2

The aim of this study was to design and synthesize novel AMPs for the decoration of vancomycin and oleic acid containing liposomes to achieve pH responsiveness for enhanced antimicrobial activity.

In order to achieve this aim, the objectives of the study were to:

1. Design nine novel AMPs using a data filtering technology, CellPPD in the antimicrobial database (APD).

2. Synthesize and characterize the novel AMPs and assess their antimicrobial activity against MRSA and *S. aureus*.
3. Select the AMPs with the best antimicrobial activity and evaluate their membrane penetrating ability using molecular dynamics and flow cytometry.
4. Determine their cytotoxicity and haemolysis to confirm their biosafety profiles.
5. Formulate AMP, VCM and OA loaded pH responsive liposomes and characterize them in terms of particle size, polydispersity index, ZP, surface morphology, entrapment efficiency, *in vitro* antimicrobial activity
6. Determine the liposomal clearance of intracellular MRSA in HEK 293 cells.

1.5 Novelty of the study

The novelty of the work is presented in two experimental studies.

Aim 1

The novelty of this research as depicted in chapter 3 is discussed herein below;

- This study will focus on the development of a QSAR model which can estimate the antimicrobial potential (MIC) of a set of antimicrobial cell penetrating peptides (aCPPs) based on their therapeutic index (TI). TI is the ratio of eukaryotic cell viability to the minimum inhibitory concentration values of a CPP. Furthermore, molecular dynamics simulation will be performed to confirm the membrane insertion ability of the the most active aCPP obtained from the QSAR study with the POPC membrane. The aCPPs form when small sequence modifications of CPPs as well as AMPs take place. While their mechanism of action has not been fully explored, it is thought that they primarily target the bacterial cell membrane.

- The novelty of this study is based on the activity of these aCPPs by correlating its TI to bacterial cell penetrating potential and further confirming its membrane insertion by molecular dynamics simulation. To date, there has been no QSAR study conducted to predict the cell penetrating-antimicrobial potential of aCPPs based on their TI. This study aimed at the development of a QSAR model to validate the cell penetrating ability of aCPPs based on their TI.
- A total 28 aCPPs were divided into training and test set compounds and PHASE 3.0 suite was used to build a pharmacophore. It was found that the most appropriate pharmacophore model (AAHRR.114) to predict aCPP activity had a

five-point hypothesis that consisted of two hydrogen bond acceptor (A), one hydrophobic group (H) and two aromatic ring features (R). AAHRR.114 was selected for QSAR model development and the 3D-QSAR was evaluated by cross-validation coefficient (Q2), Fisher test (F), correlation coefficient (R^2) and Pearson-R. Standard deviation (SD) and Root-mean squared error (RMSE).

- The results obtained showed that the higher the TI, the higher the PHASE predicted activity which is consistent with our hypothesis. The findings that will be achieved are important for future research in peptide design and will allow researchers to focus more on optimizing the TI of aCPPs for bacterial cell specificity.
- This study reports for the first time the application of the TI of aCPPs in relation to their cell penetration ability and antimicrobial potential by building a QSAR model which will outline the specific descriptors of aCPPs responsible for their potency.

Aim 2

The novelty of this research as depicted in chapter 4 is discussed herein below;

- Supramolecular lipidation of AMPs using different nano systems have been widely reported, however no work has reported on the supramolecular strategy employing the co-encapsulation of AMPs with a drug and a non-drug antimicrobial agent in a liposomal system for enhanced antimicrobial activity.
- Co-delivery of antibiotics such as vancomycin with agents such as linolenic acid has improved activity against bacteria such as MRSA and *S. aureus*. However, there is no report on the co-delivery of AMPs with antibiotics drugs and other non-drug antimicrobial agents in a single delivery system to target MRSA.
- This study reports for the first time the application of this supramolecular liposomal assembly in improving antimicrobial activity of AMPs against MRSA.

1.6 Significance of the study

Infectious diseases continue to be plague humanity globally and therapeutic efforts to curb this phenomenon have been greatly attenuated. This is due to re-emergance and proliferation of multidrug-resistant strains which has led to the search for effective therapeutic agents. Antimicrobial peptides (AMPs) therefore represent a new class of potential drug candidates

and are currently being explored for conjugation to other antibiotics as well as for delivery via novel advanced nano-delivery systems. The significance of this study is highlighted below:

New pharmaceutical products: It is envisaged that the proposed novel AMPs and the supramolecular assembly comprising of the AMPs, VCM and OA encapsulated in a liposomal system as a single entity will be new pharmaceutical products that have not been reported. These agents can serve as new drug entities for effective therapeutic outputs, contributing to the production of low-cost drug entities in the pharmaceutical industry.

Improved patient therapy and disease treatment: The supramolecular assembly can improve patient therapy and disease treatment caused by drug resistant bacterial infections by augmenting the antimicrobial activity of the encapsulated agents for effective targeting, allowing for minimal doses to be used for improving patience compliance, improved treatment outcomes, saving lives of patients.

Addition to the knowledge base of drug discovery:

It is envisaged that the proposed studies can lead to the addition and generation of new knowledge in drug discovery and delivery. It can include the following:

- Design of optimal novel materials and compounds for lead optimization in drug-candidate discovery.
- Synthesis of novel entities and their evaluation *in vitro* and *in silico* for bacterial membrane penetration can add to the conception of new knowledge.
- Co-delivery of these compounds with drug and non-drug agents will also contribute to new scientific knowledge.
- The extensive evaluation of these novel systems can provide knowledge for application in clinical trials.

Stimulation of new research:

AMPs possess great potential to change antimicrobial therapy and their non-covalent co-delivery with other antibacterial agents will allow them to effectively treat diseases associated with bacterial infections. The proposed research holds great prospects in combating drug resistance for the following reasons:

- The findings are important for future research in peptide design and will allow researchers to focus on optimizing the TI of aCPPs for bacterial cell specificity. It will also facilitate the synthesis of novel aCPPs for the design and development of aCPP based drug delivery systems. A good TI also implies that the hemolytic index is low. A low hemolytic index suggests that the designed aCPP can be applied in clinical trials.
- The proposed supramolecular liposomal assembly can further stimulate the research area of drug, non-drug and peptide encapsulation for enhanced membrane penetration and activity. Furthermore, this research area can be evaluated for enhanced drug delivery and peptide intracellular targeting for combatting drug resistance.

1.7 Overview of thesis

- The research work performed is presented in this thesis in the publication format according to University of Kwa-Zulu Natal, College of Health Sciences guidelines. It specifies the inclusion of brief introductory chapter, published papers and a final chapter on the conclusions. A PhD study is expected to generate at least 3 first authored papers, 2 of which must be experimental.

CHAPTER 1. INTRODUCTION:

- This chapter gives a brief contextual to the study and provides details on the status of infectious diseases, drug resistance and therapeutic interventions that have been conducted thus far. Furthermore, details on innovative solutions to curb drug resistance and improve antibiotic therapy are described, resulting in aims and objectives, novelty and significance of the study pursued.

CHAPTER 2. REVIEW PAPER:

- This chapter focuses mainly on the diversity and broad spectrum antimicrobial activity of AMPs and its conjugates, computational studies depicting AMP-antibiotic conjugate action and mechanisms of membrane penetration as well as Nano delivery of AMPs. The novelty of this review is elucidated in the ability of the AMPs to be conjugated to other compounds and further used in drug delivery and it identifies a considerable gap in AMP conjugates exploitation as potential therapeutic agents and in drug delivery. This review paper is a first authored publication (Drug Delivery

Science and Technology - impact impactor = 2.297) and has vastly contributed to the literature on peptide science and their utilisation in combating infectious diseases. Lastly, this review also highlights the direction of future research of peptides, highlighting their potential for conjugation strategies involving other compounds such as natural products, nano delivery of AMP-vaccine conjugates for enhanced immunity, encapsulation of AMP-gene conjugates in nano systems for targeted gene therapy. This would greatly enhance the applications of AMPs and broaden their scope in finding therapeutic agents.

CHAPTER 3. EXPERIMENTAL PAPER 1:

- This chapter addresses Aim 1, Objectives 1- 3 and is a first authored experimental paper published in the Journal of Biomolecular Structure and Dynamics, impact factor = 2.15. This research focussed on the development of a QSAR model which can estimate the antimicrobial potential (MIC) of a set of aCPPs based on their therapeutic index (TI), which is the ratio of eukaryotic cell viability to the minimum inhibitory concentration values of a CPP. Furthermore, molecular dynamics simulation was performed to confirm the membrane insertion ability of the most active aCPP obtained from the QSAR study with the POPC membrane. The novelty of this paper is based on the activity of these aCPPs by correlating its TI to its bacterial cell penetrating potential and further confirming its membrane insertion by molecular dynamics simulation.

CHAPTER 4. EXPERIMENTAL PAPER 2:

- This chapter addresses Aim 2, Objectives 1 – 6 and is a first authored experimental article that has been completed and submitted to European Journal Pharmaceutics and Biopharmaceutics (impact factor 4.491) an ISI international journal (manuscript ID: EJPB_2018_1382). This paper reports on the supramolecular lipidation of novel antimicrobial peptides for enhanced antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). This research revealed that the co-delivery of novel AMPs, VCM and OA in a liposomal system can potentially be used to enhance activity and penetration of AMPs, as well as offering synergism between the encapsulated materials thereby improving the treatment of bacterial infections.

CHAPTER 5. CONCLUSION:

- This chapter describes the overall conclusions from the research findings in the different research undertaken. It further provides information on recommendations for future research in peptide science for their utilisation to curb drug resistance.

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CHAPTER 2, Review Paper

2.1. Introduction

This chapter is a first authored review paper titled, conjugates and nano-delivery of antimicrobial peptides for enhancing therapeutic activity and had been published in the Journal of Drug Delivery Science and Technology. This review focuses mainly on the diversity and broad-spectrum antimicrobial activity of AMPs and its conjugates, computational studies depicting AMP-antibiotic conjugate action and mechanisms of membrane penetration as well as Nano delivery of AMPs. The novelty of this review is elucidated in the ability of the AMPs to be conjugated to other compounds and further used in drug delivery. The review paper includes the design and synthesis of novel antimicrobial peptides for delivery in nano systems for enhanced antimicrobial activity which is part of the main objective this study.

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Conjugates and nano-delivery of antimicrobial peptides for enhancing therapeutic activity

Mbuso Faya[a], Rahul S. Kalhapure[a], Hezekiel M. Kumalo[b], Ayman Y. Waddad[a], Calvin Omolo[a], Thirumala Govender[a,*]

[a] Department of Pharmaceutical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa
[b] School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Medical School, Durban 4000, South Africa

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ABSTRACT

The current global crisis of antibiotic drug resistance is driving the search for novel treatment approaches. Antimicrobial peptides (AMPs) are small molecular weight proteins with varying number of amino acids found in both eukaryotes and prokaryotes. They have recently been targeted as novel antimicrobial agents with the potential to treat multiple-drug resistant infections. Their conjugation with various classes of materials such as antibiotics, polymers, DNA, salts, phenolic derivatives and their delivery via nano carrier systems are strategies being used to enhance their therapeutic efficacy. An update and understanding of their applicability as conjugates and nano delivery are essential to optimise their development and activity. This review focuses on computational studies depicting their permeation through model membranes and identification of physicochemical descriptors for activity. It also highlights the potential of AMPs and their conjugates and encapsulation into nano delivery systems for improving activity. Further, research to realise their potential as conjugates and delivery via nano carrier systems are also identified. To our knowledge, this current review presents the first account that comprehensively highlights AMPs targeting various microorganisms, and their conjugation to different compounds to showcase the potential for nano delivery alone or in their respective conjugates for enhanced activity.

1. Introduction

Infectious diseases are one of the leading cause of mortality globally, despite modern technological advances of the 21st century on new drugs and diagnostic equipment used to improve healthcare [1,2]. Over the past 10 years in particular, re-emerging infectious diseases have challenged researchers and the public health systems in their efforts to curb the rise of pathogenicity [3–12]. Bacteria possess numerous drug target sites, with the number of exploited sites being relatively small [13]. This gap in the exploitation of bacterial intracellular targets allows for the synthesis and design of newer antimicrobial agents. Antimicrobial drugs have various modes of action, and depend on factors such as their structural conformation and affinity to certain target sites [14]. The most effective antibiotics act as inhibitors of cell wall synthesis (e.g. penicillins, cephalosporins, bacitracin and vancomycin) [15], cell membrane function (e.g. polymixin B and colistin) [16], protein synthesis (e.g. aminoglycosides, macrolides, lincosamides, streptogramins, chloramphenicol, tetracyclines), nucleic acid synthesis (e.g. quinolones, metronidazole, and rifampin) and other metabolic processes (e.g. sulfonamides and trimethoprim) [17]. Despite the development of numerous potent antibiotics, infections continue to be a challenge to treat, with the bacteria developing strategies to circumvent their action [18–21].

While antibiotics have revolutionised the therapy of infections, several disadvantages with current dosage forms have been observed. These include inadequate concentration at target infection sites, poor penetration of the antibiotics, side effects and poor adherence [22–24]. These limitations have contributed to antibiotic resistance by microorganisms, causing infections on a global scale [25]. The World health organization (WHO) also identified other causes of drug resistance that include the inappropriate use of antibiotics, lack of quality medicines, animal husbandry practices, poor infection control, weak surveillance systems and a lack of progress in developing new vaccines to combat drug resistance [26]. The reduction in effectiveness of a drug [27] is mainly used in the context of pathogenesis, and occurs through a number of mechanisms, such as: (a) drug modifications by enzymes, such as β-lactamases, (b) target site alterations, (c) metabolic pathways alterations, and (d) reduced drug accumulation due to efflux pump

* Corresponding author.
E-mail address: govenderth@ukzn.ac.za (T. Govender).

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Table 1
Examples of bacteria resistant to antibiotics.

Bacteria	Type	Drugs resistant to	Ref
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Gram (+) cocci	Vancomycin, Linezolid, Daptomycin, Eicoplanin	[214]
Vancomycin resistant *Staphylococcus aureus* (VRSA)	Gram (+) cocci	Erythromycin, Vancomycin	[215]
S. pneumoniae	Gram (+) diplo-coccus	Doxycycline, Erythromycin, Penicillin G	[216]
E. faecium, VRE	Gram (+) cocci	Vancomycin, Streptomycin, Gentamicin, Penicillin, Ampicillin	[217]
E. coli	Gram (−) rods	Ciprofloxacin, Levofloxacin	[218]
P. aeruginosa	Gram (−) rod	Imipenem, Meropenem, non-antipseudo-monal Penicillins	[219]
K.pneumoniae	Gram (−) rods	Colistin,	[220]
A. baumanii	Gram (−) rod	Imipenem, Meropenem	[221]

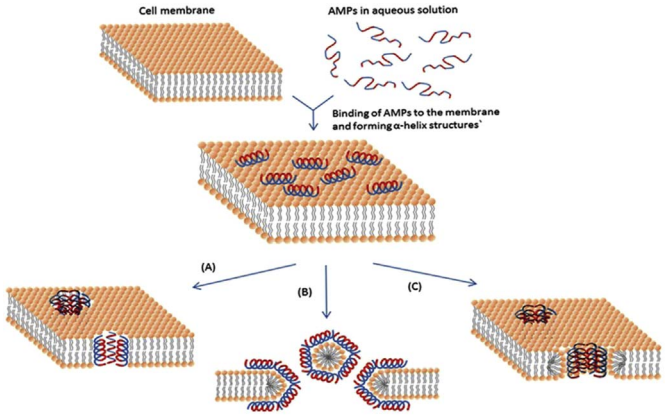

Fig. 1. The various AMPs mechanisms. A represents the Barrel-Stave model (AMPs penetrate the membrane in a perpendicular fashion). B represents the carpet Model (sections of the membrane are coated with AMPs). C represents the Toroidal-pore model (AMPs are in a constant interaction with the membrane phospholipid head groups). AMP Hydrophobic and hydrophilic parts are represented by the colour blue and red respectively. Permission granted [69]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activity [28,29]. Drug resistance has led to the inadequacy of current dosage forms and has significantly hindered the efficacy of antibiotics [30]. This includes resistance to bacteria, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) (resistant to beta-lactams), *E. faecium* (resistant to streptomycin), *K. pneumonia* (resistant to 2nd and 3rd generation cephalosporins) and *A. baumanii* (Table 1) [31]. The proliferation of multidrug-resistant strains has led to the search for effective therapeutic agents, and has ignited research into the design and synthesis of novel antimicrobial molecules [32,33]. The development of alternative therapeutic agents remains one of the major challenges to circumventing the problem of drug resistance [34]. Antimicrobial peptides (AMPs) represent a new class of potential drug candidate and are proteins of smaller molecular weight (2–8-kDa) that are broad spectrum in their activity against pathogenic bacteria, viruses and fungi [35,36]. They are also known as host defence proteins (HDPs), and are part of the innate immune system found in all classes of life [37]. The discovery of AMPs dates back to 1939, with gramicidins being discovered first and isolated from *B. brevis* [38]. Gramicidins have been used to treat infected wounds on the skin of guinea-pigs [39], which led to their consideration for clinical use, after which they were commercially synthesized as antibiotics. The number of AMPs discovered and/or synthesized to date is above 5000 [40].

AMPs are either natural based obtained from prokaryotes and eukaryotes [41], or synthetic based. They are divided into four structural groups' viz. (a) β-sheet; (b) α-helical; (c) loop and (d) extended peptides with broad spectrum activity [42], with α-helix and β-sheets specifically being more common [43]. As AMPs are constructed by coupling amino acids, it is easy to modify their structure [44], which is an advantage in designing various combinations. This ability also allows for the possibility to change the AMP targets and improve their stability against the degradative effects of proteases [45]. AMP activity occurs mainly by disrupting the integrity of the membrane protein, inhibiting DNA and RNA synthesis, or disrupting intracellular targets [46]. AMP action is dependent on their cationic charge, which allows them to be attracted to the anionic membrane of its targets and leads to the destruction of the cell membrane [47,48]. Fig. 1 shows the different mechanisms AMPs used to traverse the bacterial membrane [49]. The AMPs membrane penetrating ability is a major advantage over conventional antibiotics, which may find it difficult to cross bacterial cell membrane and make their way into intracellular targets [50]. Several review papers have highlighted the applicability of AMPs as antibacterial agents for enhancing activity against various organisms, such as LL-37, melittin and magainin-II, which are active against *P. aeruginosa, L. monocytogens*, and MRSA respectively [51–53]. A number of AMPs, such as pexiganan acetate, Omiganan, IMX924, Arenicin, Semaglutide and Dulaglutide, were found to be active against Gram positive and negative bacteria, and are now in clinical trials (Table 2). Their intended use was for diabetic ulcers, catheter infection prevention and Type 2 diabetes mellitus [54,55]. Despite their beneficial potent antibiotic activity, their inherent drawbacks, including poor physicochemical stability, a short circulating plasma half-life and a high haemolytic effect [56] have the potential to render AMPs un-usable [57–59]. Various strategies are therefore being used to overcome these limitations. To potentiate their activity, AMPs are also being increasingly explored for conjugation to several classes of materials. The conjugation strategy of AMPs to other compounds amplifies their potential to overcome the current drug resistance crisis [60] as it offers in combination multiple benefits as opposed to the AMP alone. These AMP conjugates can lead to multiple mechanisms of action against bacteria, facilitate self-assembly of AMPs into nanostructures for delivery, achieve intracellular targeting and prolong circulation life [61–64]. Administration of AMPs or its conjugates will eventually require its incorporation into a dosage form for patient administration. The use of current conventional dosage forms will limit the potential of AMPs as they lead to inadequate delivery to the infection site, may not offer protection against degradation by proteases and other degradative enzymes [23,65]. Although it has been noted that the mechanisms of bacterial resistance to AMPs are still not well understood, and their occurrence very unlikely, physico-chemical modifications in the bacterial cell membrane seems to be the first step to developing resistance [66]. Once the bacteria changes the AMP target to make it less susceptible to the action of AMPs, fluidity and bacterial cell permeability decrease due to alterations in the architecture of the outer and inner membranes. Bacterial membrane surface modifications, which can lead to reduced levels of specific membrane proteins and ions, as well as changes in the membrane lipid composition, can promote resistance, which alters the activity of the AMP at its site of action [67,68]. To circumvent this occurrence, the encapsulation or association of these AMPs into nanosized carriers as delivery systems is being explored to achieve targeted delivery to the infection site and reduce resistance [69]. This would provide an added advantage since these nano carriers provide adequate delivery with selective targeting to the infection site as well protection from enzymatic degradation. Also the nano carriers will provide high stability, high carrier capacity, feasibility of incorporation of both hydrophilic and hydrophobic substances, and

Table 2
AMPs under clinical trials and development.

Product	Description	Indication	Phase	Company (location)
Magainin peptide/ pexiganan acetate	22-amino-acid linear antimicrobial peptide, isolated from the skin of the African clawed frog (*Xenopus laevis*)	Diabetic foot ulcers	3	Dipexium Pharma (White Plains, New York)/Macro Chem/Genaera
Omiganan	Synthetic cationic peptide derived from Indolicidin	Rosacea	2	BioWest Therapeutics/Maruho (Vancouver)
OP-145	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Chronic bacterial middle-ear infection	2	OctoPlus (Leiden, The Netherlands)
Novexatin	Cyclic cationic peptide, 1093 daltons	Fungal infections of the toenail	1/2	NovaBiotics (Aberdeen, UK)
Lytixar (LTX-109)	Synthetic, membrane-degrading peptide	Nasally colonized MRSA	1/2	Lytix Biopharma (Oslo)
NVB302	Class B lantibiotic	*C. difficile*	1	Novacta (Welwyn Garden City, UK)
MU1140	Lantibiotic	Gram-positive bacteria (MRSA, *C. difficile*)	Preclinical	Oragenics (Tampa, Florida)
Arenicin	21 amino acids; rich in arginine and hydrophobic amino acids	Multiresistant Gram-positive bacteria	Preclinical	Adenium Biotech Copenhagen
Avidocin and purocin	Modified R-type bacteriocins from *Pseudomonas aeruginosa*	Narrow spectrum antibiotic for human health and food safety	Preclinical	AvidBiotics (S. San Francisco, California)
IMX924	Synthetic 5-amino-acid peptide innate defense regulator	Gram- negative and positive bacteria (improves survival and reduces tissue damage)	Preclinical	Iminex (Coquitlam, British Columbia, Canada)

Adapted from Fox et al., 2013 [222].

feasibility of variable routes of administration and allows controlled drug release from the matrix [70]. Several types of nanoparticles, which are described in this review, such as liposomes, micelles, nanofibers, metallic nanoparticles (silver and gold nanoparticles) and hydrogel nanoparticles, also possess various mechanisms to bypass resistance. Once the AMP is incorporated into these nano systems, it would promote the formation of reactive oxygen species, improve the delivery of the bioactive AMPs by functioning as circulating micro-reservoirs for sustained release at the infection site, and provide resistance to corrosion and oxidation in the case of metallic nano particles [71]. These nano-based drug delivery systems have distinguished themselves as the best approach to mitigate the development of drug resistance by decreased uptake and increased efflux of drug from the microbial cell, biofilm formation and intracellular bacteria. Finally, nanoparticles can target antimicrobial agents to the infection site, which leads to higher drug doses accumulating at the infected site while keeping the total dose of drug administered low. The strategy of having a high local dose at the infection site promotes bacterial killing before resistance could develop, while the lower total dose decreases the possibility that bacteria outside of the nanoparticle site of action will develop drug resistance [72].

Molecular modelling of AMPs is being increasingly reported to understand their mechanisms of action and how they traverse the bacterial cell membrane [73–75]. Studies to identify structural and physicochemical descriptors for AMP activity are also being undertaken. An understanding of such studies is critical to facilitate the design and optimisation of future new conjugates and delivery systems of AMPs.

Reviews thus far on AMPs have focussed on their sources, applications, structural make-up, activity against various classes of bacteria and design [38,76–80]. To date, there is no review paper focusing on AMP-conjugates in combination with the nano delivery of AMPs and molecular modelling approaches as strategies to potentiate the applicability and activity of AMPs. An overview of molecular modelling and quantitative structure activity relationship (QSAR) investigations of AMP activity on the bacterial cell membrane specifically is also lacking. This review paper provides an overview of available computational studies depicting AMP-membrane penetration, as well as their quantitative structure activity relationships (QSAR) that identify the characteristic descriptors responsible for their cell membrane permeation. It focuses mainly on the diversity and broad spectrum antimicrobial activity of AMPs and their conjugates, as well as on the formulation and evaluation of AMPs into various nano delivery systems. Future research to realise the potential of AMP-conjugates and AMP delivery via nano drug delivery are also identified. This review paper therefore highlights the AMPs ability to target various microorganisms, their conjugation to different antibiotics, polymers and other conjugates, such as phenolic compounds, DNA and salts, which have the potential to enhance AMP activity. This review also highlights the molecular modelling approaches that could structurally elucidate the mechanism of membrane penetration, and showcases the potential for nano delivery AMPs alone and in their respective conjugates for enhanced activity.

2. *In silico* studies of AMPs in membranes

Since the advancement of computational drug designing and machine learning, *In silico* simulations have become a complementary counterpart to experiments to understand the molecular mechanism of macromolecules and develop novel drug candidates [81]. Molecular modelling approaches, including molecular dynamics and QSAR, have become an integral part of modern drug discovery [82]. An understanding of bacterial membrane penetration by AMPs is key to its utilisation to circumvent drug resistance [83]. The mechanism of membrane penetration by AMPs is not well understood [84], however, *In silico* studies have emerged that utilised complex computational tools to study receptor-ligand interactions and their binding affinities [85]. In the literature, molecular studies, such as molecular dynamics (MD) and quantitative structure-activity relationship (QSAR), have been utilised to understand the mechanical behavior of biomolecules e.g. AMP_S [86–88]. As this review focuses on AMP-conjugates and AMP nano delivery, a mechanistic understanding of the actions of AMPs is important to elucidate their membrane penetration ability. This section will focus on a brief overview of molecular dynamic (MD) simulations of AMPs through model membranes. Quantitative structure–activity relationship (QSAR) is also discussed, which provides a deeper understanding of the structural descriptors that could be attributed to membrane penetration.

2.1. Molecular dynamics (MD) simulations of AMPs in model membranes

Molecular dynamics is an *In silico* method that provides structural insights, binding affinities and stabilities of proteins by complex calculations of time dependent behavior of a molecular system [89]. Its main aim is to provide a simulation that studies the conformational rearrangements of molecules, the interaction and motion of atoms and

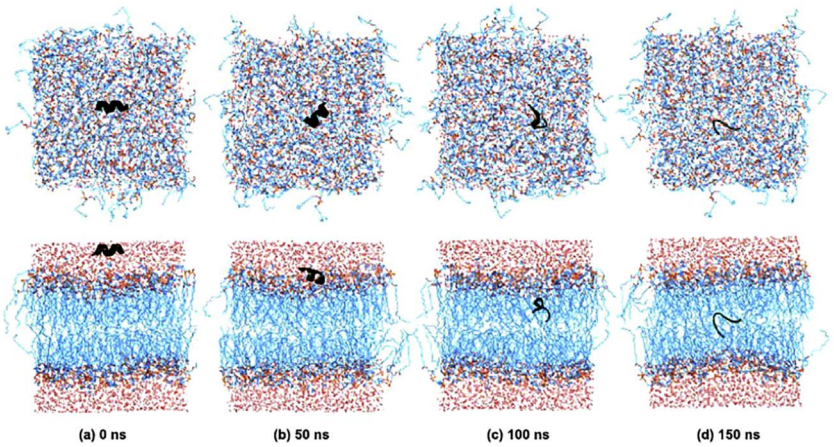

Fig. 2. MD simulation depicting the interaction of the amphipathic helical AMP F[Nle]W[Hag]RWWV[Orn]L with an artificial lipid bilayer. Permission granted [78].

molecules according to Newtons equation of motion [90]. MD utilises force fields (e.g. CHARMM) to estimate the forces between interacting atoms and to calculate the overall energy of system [91]. MD simulations, the integration of Newton's laws of motions generates successive configurations of the evolving system, providing trajectories that specify positions and velocities of the particles over time. From these MD trajectories, a variety of properties can be calculated, including free energy, kinetics measures, and other macroscopic quantities, which can be compared with experimental observables [90]. Khandelia et al. (2008) reviewed the impact of peptides in lipid membranes, and observed that such MD simulations on model membranes are essential for understanding the structural factors that account for membrane permeation, such as peptide amphiphilic character, conformation and electrostatic effects [92]. MD simulations of AMPs in model membranes allow for the behavioral and structural insights of how they are inserted into the lipid bilayers, as well as the outlining factors that influence lipid-peptide interactions, such as membrane thickness, lipid acyl-chain order and dynamics, membrane elasticity, lipid-domain and annulus formation to be described [93,94]. In this review, we focused on the different MD simulations studies as examples to elucidate the AMP-membrane interaction, with the hope that this will give an indication of AMP factors that could attenuate or enhance their membrane penetration potential. Sengupta et al. (2008) conducted a series of simulations with the AMP, Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) interacting with dipalmitoylphosphatidylcholine (DPPC) bilayers using the GROMACS software package for MD simulations. The GROMOS force field 43a2 was used to interpret the peptide-bilayer interactions. The simulation revealed that two AMPs were inserted into the DPPC bilayer pore, while the remainder were found to line the mouth of the pore [95]. The authors indicated that the AMPs lining the mouth of the pore are expected to give an initial burst effect and then a controlled release behavior will be followed. We believe that this study not only explains the interaction of AMPs with the membrane, but can also indicate the AMPs' release behavior from future liposomal formulations due to the similarity of the components between the liposomes and the membrane bilayer. In another study, Dittmer et al., (2009) conducted a study on incorporating AMPs (alamethicin) into membranes employing liquid-State NMR and MD, with the C monomer from the X-ray crystal structure of alamethicin being used for the system set up. The MD simulations revealed the fluidity of the membrane environment, where the AMP was dissolved rather than incorporated [96]. Dan et al. (2011) reviewed the interaction of the AMP protegrin on lipid bilayer membranes. An interesting observation from this review was that these AMPs interact with the components of the outer membrane, and have an increased bilayer disruption with increased AMP concentration [97].

Using another model lipid bilayer membrane of phosphatidylglycerol (POPG) and phosphatidylcholine (POPC), Wang et al. (2012) conducted a MD simulation of the AMP CM15 at 100 ns. The AMP was reported to penetrate the model membranes with no hemolytic activity. The study concluded that the initial conformation of the peptide played an important role in inserting the peptide into the model membranes [70]. From this study, we expect that the hydrophobicity of the AMPs could determine their distribution between the lipid bilayer of the membrane and the aqueous core, which is essential in formulating similar structured nanocarriers. In a study conducted by Li et al. (2012), molecular simulations were conducted to propose how a branched AMP disrupts a bacterial membrane, with MD simulations being performed for each model membrane using the CHARMM36 force field. The concentration dependent effects of the branched AMP were studied by performing simulations with varying peptide–lipid ratios: 1:128, 2:128 and 3:128. These ratios corresponded to different concentrations of the AMP, and all the simulations were run for 200 ns using the GROMACS package 4.5. The results indicated that the activity of the branched AMP (B2088) is concentration dependent, and that at higher concentrations (using 3 B2088 molecules with 128 lipid molecules), significant membrane perturbation may occur [87]. This study can be considered significant because it can be used to predict the conjugation of the AMPs with different ratios of polymers, and to interpret the interaction behavior based on an analysis of the entropic energy values, which could affirm the continuity or discontinuity of the interaction. Wang et al. (2016) analyzed the susceptibility of AMPs that incorporated unnatural amino acids against microbial infections. The MD simulation revealed the amphipathic-helix conformation of the designed peptides, as depicted in Fig. 2, which were also seen to unfold when they traversed the simulated lipid bilayer that mimics the bacterial membrane. *In vitro* susceptibility was conducted against *P aeruginosa, S. aureus*, and *E coli,* with one AMP (F[Nle]W[Hag]RWWV[Orn]L) showing very potent antimicrobial activity, having MIC's of 5.6, 18.9 and 11.2 μg/ml for *S. aureus, P. aeruginosa* and *E. coli* respectively [98]. We conclude that the importance of the Wang et al. (2016) study is that a correlation between amino acid sequences and bacterial phospholipids could be established to create the foundation for further experimental studies using techniques that investigate the interaction between the AMPs. Balatti et al. (2017) conducted a study on the differential interaction of amphiphilic AMPs (aurein 1.2 and maculatin) with POPC lipid structures using coarse-grained MD simulations. The AMP-lipid simulation was conducted in three initial configurations: (a) peptides in water in the presence of a pre-equilibrated lipid bilayer; (b) peptides inside the hydrophobic core of the membrane; and (c) random configurations that allow self-assembled molecular structures. The results showed that both AMPs were capable of forming membrane aggregation, however, the aurein 1.2 were seen to form pore-like structures, whereas the maculatin formed clusters and induced curvature at low peptide–lipid ratios [99].

The above reports have been useful in demonstrating that the nature of the AMP dictates how it will interact with the bacterial membrane,

and whether it will be incorporated or disintegrate as it interacts with it. These molecular dynamic studies of AMP-membrane interaction allowed for the concise evaluation of the AMPs mechanism of action as they traverse the lipid bilayer. This is important for guiding future research that incorporates peptide conjugates and their delivery in nano systems. It should be noted though that a challenging factor is the experimental validation of computer modelled simulated results, as most MD studies having not been evaluated experimentally [100]. This suggests the need to combine both computational and experimental approaches in AMP design and application. In contrast to the above research, where the authors focused on a single AMP (Protegrin), this review focuses on various AMPs interacting with model membranes, and further postulates that these AMP activities could be enhanced by conjugation strategies.

The remainder of the above studies focused on molecular modelling with AMPs on model membranes. The review of the current literature therefore indicates that there is a considerable gap in molecular dynamic simulations of AMP-antibiotic conjugates and their delivery in nano systems. Research on simulations of AMP-conjugates with model membranes have potentially great prospects, specifically as some target intracellular organelles. An MD study of these conjugates as they penetrate the bacterial membrane, as well as when they interact with intracellular targets, would offer a better understanding of their mechanism and how they could be improved. Only one study so far has reported the modelling on AMP-nanoparticles. This study by Liu, Xu et al. (2009) showed the self-assembly of cationic peptide nanoparticles as an efficient antimicrobial agent. Those nanoparticles showed efficient penetration through the blood brain barrier (BBB) in *S. aureus*-infected meningitis rabbits for the treatment of brain infections [101]. Molecular dynamic studies on the various nano carrier systems that can be used for AMP delivery is therefore highly warranted.

2.2. Quantitative structure activity relationship (QSAR)

QSAR is another well-known computational method used for studying AMPs, and relies on identifying a set of structural or physiochemical descriptors to describe their activity [102,103]. This computational tool provides the added advantage of AMP structure-membrane activity. While most studies employing QSAR modelling to study peptides use this tool to predict structural descriptors attributed to peptide activity, haemolysis and cytotoxicity [104], there are no QSAR model studies that have related the structure of AMPs to their ability to penetrate bacterial membranes. This review provides insights into AMP structural descriptors in relation to their ability to penetrate bacterial membranes. Frecer et al. (2006) developed a QSAR model to analyse the haemolytic effects and antimicrobial activity of cyclic cationic AMPs obtained from protegrin-1. The study utilised the genetic function approximation algorithm to relate antibacterial activity to the AMPs net charge and amphipathicity and the haemolytic activity paralleled with the lipophilicity of the residues from the nonpolar surface of the β-hairpin. However, the results reflected that the protegrins, together with their analogs containing a single or double disulfide bridge, demonstrated greater antimicrobial potency compared to their counterparts, with no disulfide bridges [105]. Jianbo Tong et al. (2008) reported on a novel descriptor of amino acids and its use in designing peptides with the principal component analysis (PCA) method, where 99 molecular indexes of amino acids were examined. For each amino acid, nine principal component scores were selected and applied as new vectors of descriptors. Vector of principal component scores (VSW) were derived from the principal component analysis of the invariant molecular indices of the amino acids. The observed and calculated activities of the AMP sequences were compared, and were found to be very similar [106].

Wang et al. (2012) built a QSAR model of cationic AMPs, basing it on the structural properties of amino acids. The amino acid index database was used to select 89 indices that depicted three classes of AMPs in the model (Surface-tethered cationic peptides, 101 synthetic cationic polypeptides and novispirin AMPs) in order to calculate the contribution of the amino acids to the activity of the AMPs. The high performance STR-MLR model enabled the prediction of antimicrobial activity and identified the most suitable amino acids in the sequence to be used for designing novel AMPs [107]. The importance of amino acid sequence was highlighted later in another study by Mariya et al. (2015). They built a QSAR model, for which the Monte Carlo method was used, of the activity of AMPs (mastoparan analogs) as a mathematical function of a sequence of amino acids. The results obtained were reasonably good, with the pMIC values being better than those of the experimental pMIC values.

From the above reports, it is clear that the use of QSAR studies is important to establish the most potent descriptors that will allow for optimal AMP activity and will subsequently facilitate the design of optimal AMPs with good antimicrobial activity and less haemolytic effects [108]. Importantly, we believe that these descriptors identified could also be crucial in elucidating the most probable candidates that are important in bacterial cell membrane penetration and nanoparticle formation. As most AMPs main target is the cell membrane, we contend that the descriptors outlined in the above studies not only account for antimicrobial activity but membrane penetration as well. In conjunction with QSAR modelling, more in-depth computational approaches are needed to further describe the mechanism of action of these descriptors on the cell membrane components leading to membrane disruption. Zelezetsky et al. (2006) equates the activity of AMPs to their structural conformation, where α-helical structuring permits optimal spatial arrangement of aliphatic side chains for membrane insertion, and their hydrophobicity allows for deeper insertion into the whole lipid bilayer [109]. We also contend that since the formulation of nano delivery systems requires certain physicochemical conditions, such as pH, hydrophobicity, charge, solubility, entrapment and release profiles [101], QSAR studies could assist in elucidating those descriptors required for nanoparticle formulation, and how these AMPs would behave in those nano systems.

A possible limitation of current QSAR reports on AMPs is that they have mainly focused on the elucidation of possible physicochemical descriptors responsible for activity and those that attenuate activity [103,110]. Studies on membrane destabilization, AMP activity within the bacterial cell wall and how it disrupts intracellular pathways are lacking. Future reports on QSAR studies with AMPs can be strengthened by the inclusion of experimental studies such as DNA based testing for determining lysis of the microbial cell wall and microscale thermophoresis (MST) for determining the intracellular interactions causing the disruption of the intracellular pathways to provide a greater in-depth understanding of how specific descriptors enhance membrane permeation and intracellular interactions [111].

3. AMP-antibiotic conjugates

One approach to enhance the performance of AMPs is the application of conjugation strategies [112]. AMPs can be conjugated to various classes of materials forming AMP-conjugates, and are designed solely for the purpose of combining the antimicrobial power of AMPs with the desired conjugates to effectively kill microorganisms [45,60]. The AMP-antibiotic conjugates offer better biological activities than AMPs alone, this being confirmed by studies focused on using them as vectors to deliver their respective conjugates [113–115].

Conjugation requires knowledge of both the type of conjugates to be used and a thorough understanding of the microorganism being targeted to ensure optimum conjugate activity without attenuating the antibacterial action of the agent intended to be conjugated to the AMP. Novel synthetic routes and various conjugation approaches using antibiotics, polymers, salts, DNA and phenoloic derivatives have been reported thus far and are reviewed here under.

Table 3
AMP-Antibiotic conjugates synthesized for activity against microorganisms.

Peptide	Peptide sequence	Antibiotic	Microorganism	Main findings	Ref
UBI_{29-41}	GRAKRRMQYNRR	Chlorampenicol (CAP)	*S. aureus, E. coli, P. aeruginosa*	MIC for CAP-UBI_{29-41} was found to be 3.8 ± 0.9 μg/mL for *E. coli* and 15.0 ± 2.6 μg/mL for *S. aureus*	[113]
Tridecaptin (H-TriA1)	WDGSTSDDXGVYS	Rifampicin, Vancomycin, Erythromycin	*K. pneumoniae*	H-TriA1-Rifampicin did not increase the *in vitro* activity of Rifamicin. Moderate activity was observed with H-TriA1-VMC and H-TriA1-Ery.	[128]
Indolicidin	ILPWKWPWWPWRR	Levofloxacin	*S. aureus, E. coli, P. aeruginsa, B. subitilis*	The conjugate showed moderate activity against the tested strains.	[223]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	Vancomycin	*enterococci*	The MIC of Magainin 2-VMC conjugate was 2 and 4 μg/mL against MRSA and VSE respectively.	[127]
Nisin	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	Vancomycin	VSE, VRE, *Moraxella catarrhalis*	Derivative 5 of the conjugate was found to have good activity with an MIC value of 0.6 μg/mL against VSE (15A797)	[224]
Anoplin	GLLKRIKTLL	Vancomycin		DOPC/DOPG vesicles were used for leakage assay where conjugation of temporin L to VMC enhanced its activity.	[225]
temporin L	FVQWFSKFLGRIL			Anoplin-VMC had lower membrane disruption activity.	
Peptide-resin conjugate	FVVKKKKKVF	Vancomycin	*S. aureus*	The MIC of the peptide-resin conjugate was 0.0156 μg/mL in the presence of VMC.	[123]
CRAMP	KIGEKLKKIGQKIKNFFQKLVPQPEQ	Vancomycin	*S. aureus,*	CRAMP-VMC conjugate did not inhibit bacterial growth better than its separate compounds.	[226]

3.1. AMP-antibiotic conjugates targeting gram positive bacteria

AMP-antibiotic conjugation offers a promising new class of therapeutic agents and great prospects in reviving drugs that were rendered ineffective by microorganisms' resistance strategies. Table 3 reflects some of the AMP and their respective conjugates together with their biological activity towards specific Gram positive and negative bacterial strains. AMPs have the ability to traverse the bacterial cell membrane and disrupt intracellular targets [116], which makes them ideal for conjugation with antibiotics that have difficulty crossing the membrane barrier [117]. In this regard, they would have a dual activity: i) transporting antibiotics across bacterial membranes, and ii) providing their own potent antibacterial activity. This dual role has led researchers to explore conjugation strategies to enhance the activity of antibiotics, as well as to take advantage of the activity of AMPs on bacterial membranes.

Levofloxacin, is a broad spectrum drug that belongs to the fluoroquinolone antibiotic class [118]. Recently, microorganism's resistant against levofloxacin have been discovered in bacteria such as *E. coli, P. aeruginosa* and *S. aureus* [119], which has attenuated the activity of this drug. In an early study, to restore the potency of levofloxacin and obtain synergistic activity, this antibiotic was conjugated to indolicidin (ILPWKWPWWPWRR), a linear cationic AMP rich in tryptopan (Trp) and proline (Pro), reported to be active against both Gram positive and negative bacteria, fungi, and protozoa. The MIC values ranged from 0.03 to 0.1 μg/mL against *P. aeruginosa* and *S. aureus* and *E. coli.* The conjugate activity was better than the activity of levofloxacin and Indolicidin alone indicating the success of the conjugation in restoring the potency of Levofloxacin. It was hypothesized that the combination of levofloxacin with indolicidin, a highly hydrophobic peptide, may improve delivery of the antibiotic through the outer membrane of the bacteria. In addition, the cationic antimicrobial peptide, Indolicidin is known to further enhance antibiotic delivery by altering the membrane integrity [47]. In the same study, the authors conjugated Tat (GRKKRRQRRRPQ) to Levofloxacin via an amide bond or ester linkage, and evaluated it against a number of strains, including *E. coli, P. aeruginosa* and *S. aureus*. The MIC values of the conjugates against the tested strains ranged between 0.08 and 0.12 μg/mL [120]. The conjugate had better activity than TAT alone against all the tested strains and the conjugate also showed better potency compared to Levofloxacin alone against *S. aureus.*

Vancomycin is an antibiotic which interrupts cell wall synthesis by complexation with peptidoglycan precursors and this makes it an ideal antibiotic for conjugation with AMPs for membrane perturbation [121]. It has also been used as a last resort drug to treat serious infections caused by penicillin resistant bacteria [122]. Instead of directly conjugating the antibiotic to the AMP as in the studies above, Cho et al. (2007) investigated another strategy which involved firstly the synthesis of antibacterial peptide-resin conjugates and then its subsequent conjugation to vancomycin. The cationic antimicrobial peptide (CAP)-vancomycin conjugate displayed potent activity against *S. aureus* and *M. luteus,* with MICs of 1.56 μg/mL and 3.12 μg/mL respectively, which revealed that the activity of vancomycin was amplified by the peptide-resin conjugate [123]. Another study by Nigam et al. (2015) evaluating the synthesis of cathelicidin-related antimicrobial peptides (CRAMP)-vancomycin conjugates using different linkers, the results indicating that the conjugate with the short and hydrophobic linkers bearing an aromatic group had better activity compared to those with longer chain linkers without an aromatic group [124]. Other AMPs that have been conjugated to vancomycin include Nisin, Anoplin and temporin L [115]. All these AMPs were seen to have good to moderate antimicrobial activity against Gram-positive bacteria. Using another antibiotic, Schmidt et al. (2014) synthesized a peptide-tobramycin conjugate and tested it against *S. aureus*. The results reflected that the conjugate (Pentobra) was able to destabilize the bacterial membrane and inhibit protein synthesis and also had significant bactericidal

activity towards the Gram positive bacteria [125].

Contrary to the above-mentioned studies where activity was amplified, there is indeed the possibility that activity is decreased. This was shown in a study by Arnusch et al. (2012), where ultra-short peptide bioconjugates were synthesized and evaluated for antimicrobial activity. The conjugates had no activity on all Gram positive strains and were seen to be selective towards fungi [126]. Arnusch et al. (2012) also synthesized vancomycin-Magainin II peptide derivatives via click chemistry and tested them against MRSA, vancomycin susceptible enterococci (VSE) and vancomycin resistant enterococci (VRE). The MIC values of the most promising conjugate was found to be 2–16 μg/mL against MRSA, VSE and VRE respectively [127]. The conjugate had no higher activity on the microorganisms than vancomycin alone where the MIC values were 0.4 μg/mL, 0.5 μg/mL and 128 μg/mL against MRSA, VSE and VRE respectively. Although it would have been useful, these authors did not provide possible reasons for this unexpected finding. We think that factors in the conjugate that could have resulted in the undesirable effect were the choice of the linker, steric hindrance mediated by the vancomycin on the AMP, amino acid sequence and/or electron withdrawing effects in the peptide-linker-vancomycin sequence. With the above knowledge concerning AMP-antibiotic conjugation, the design of these conjugates requires further studies on their activity, such as suitable linkers, how they confer such potent activity with regards to their surface charge and stability to optimise their activity. Using another antibiotic, Schmidt et al. (2014) synthesized a peptide-tobramycin conjugate and tested it against Gram Positive *S. aureus*. The results reflected that the conjugate (Pentobra) was able to destabilize the bacterial membrane and inhibit protein synthesis and also had significant bactericidal activity towards *S. aureus*.

3.2. AMP-antibiotic conjugates targeting gram negative bacteria

In addition to Gram positive bacteria, studies have also focused on targeting Gram negative bacteria. Cochrane et al. (2015) investigated the synthesis of Tridecaptin – antibiotic conjugates, which have activity against Gram-negative bacteria. Tricadeptin was conjugated to rifampicin, vancomycin and erythromycin, the latter combination providing better activity against *K. pneumoniae* infections, with an MIC value of 0.4 μg/mL [128]. The conjugation however did not increase the *in vitro* activity of rifampicin, the lowest MIC being 25 μg/ml against *E. coli* and *A. baumannii*. The authors explained this occurrence as the possibility of the peptide and antibiotic not arriving at the target site together due to possible cleavage by proteolytic enzymes. However, linking the AMP derivative H-TriA_1 to vancomycin resulted in a 16-fold increase in activity against *E. coli*, and an 8-fold increase against multidrug resistant *K. pneumoniae* and *A. baumannii.* Targeting *E. Coli* was the focus also of a study reported by Chen et al. (2015) who synthesized and conjugated chloramphenicol (CAP) to the antimicrobial peptide $UBI_{29\text{-}41}$, with the *in vitro* studies revealing an enhanced antibacterial activity against *E. coli*. The activity of CAP alone on *E. coli* was 6.2 ± 1.7 μmol/L, whereas that of CAP-$UBI_{29\text{-}41}$ conjugate on *E. coli was* 3.8 ± 0.9 μmol/L. The toxicity of the conjugate on normal cells decreased significantly compared to CAP and most importantly CAP-$UBI_{29\text{-}41}$ conjugate exhibited more favourable antibacterial efficacy than CAP alone. In addition, the toxicity of CAP-UBI_{29-41} on ordinary cells was reduced noticeably in contrast with CAP alone. Schmidt et al. (2014) also synthesized a peptide-tobramycin conjugate and tested it against *E. coli*. The conjugate (Pentobra) was able to destabilize the bacterial membrane and inhibit protein synthesis and also had significant bactericidal activity towards *E. coli*. These results confirm the importance of AMP-antibiotic conjugation as a synergistic approach in targeting pathogenic microorganisms. The difference in the activity of AMP conjugates against both Gram positive and negative bacteria as shown in these studies indicate the existence of various possibilities causing either increased potency or reduced potency of the AMP-antibiotic conjugates. These could include surface charge of the AMP-antibiotic conjugate, aggregation before the AMP-antibiotic conjugate enters the cell membrane, site of conjugation as well as the choice of linker used which will effectively decrease or increase the accessibility of the antibiotics. The conjugation of AMPs to antibiotics offers a promising approach in enhancing the therapeutic ability of antibiotics and their targeted delivery to specific intracellular organelles. This approach allows AMPs to be used as delivery vectors as well as antimicrobial agents themselves.

4. Cell culture and *in vivo* models evaluating AMP efficacy

The concept of AMP cell selectivity creates the necessities for evaluation tools to determine the biosafety and efficacy on both *in vitro* and *in vivo* levels. One of the major advantage of the AMPs and AMP conjugates over the conventional antibiotics is their selectivity towards the bacterial cells rather than the host cells. In addition, as the lipid composition of the cell surface determines the selectivity of the AMPs and AMPs conjugates, cell culture studies could consider a good source of information to understand such selectivity, biosafety and efficacy [129]. The antimicrobial peptide Magainin II was tested for its cytotoxicity on tumour cells MCF-7 and normal cells HSF. The cells were stained with FITC-Annexin V and propidium iodide and then observed under fluorescence microscope for their apoptotic or necrotic state, respectively. Quantitatively, FACS analysis was used to determine the number of apoptotic and necrotic cells. The cell viability was also measured using CCK-8 (cell counting kit-8), XTT and MTT [130–132]. The MICs (minimum inhibitory concertation) of cationic helical peptide was investigated using broth microdilution method in which the AMPs or AMPs conjugate were dissolved in broth medium with different range of dilution using 96-well plates and then the absorbance measured spectrophotometrically. Field emission-scanning electron microscopy and confocal microscopy could also be used to determine the antibacterial mechanisms by direct monitoring of bacterial membrane structure as well as pore formation of the bacterial membrane respectively [133]. Bacterial Killing assay to determine MBC (minimum bactericidal concentration) was used by *Salomone* et al. (2016) when they tested the bactericidal activity of a novel cell penetrating peptide [134]. An alternative technique to MIC and MBC could be flow cytometry which is could have been useful in displaying the penetration potential of these peptides. The effect of Megainin II and Megainin II conjugate on MCF-7 and HSF cell lines were studied using flow cytometry method [131]. The effect of chimeric peptide with disruptive membrane properties when incubated with Hela cell line and the activity of AMPs in conjugation with silver nanoparticles on *E. coli* were studied using FACS (fluorescence activated cell sorting) a synonym for flow cytometry [132,134,135].

Ron-Doitch et al. (2016) determined the antiviral effect of liposomal indolicidin on Herps Simplex Virus-infected 3D epidermis model, the formation of the tissues was validated and stained with hematoxylin and eosin B stain for further evaluation [135]. Tridecaptin-Antibiotic conjugates was evaluated on C57BL/6L mice infected with *K. pneumoniae* then survival rate was determined using Kapaln-Meier plot. Although the activity of the peptide-antibiotic conjugates retained *in vivo*, variation in the effect depended on the type of AMP-antibiotic conjugates (Rifampicin, Erythromycin and Vancomycin) used. Particularly, Tridecaptin-Erythromycin conjugate exerted better activity than the antibiotic alone [128]. The $UBI_{29\text{-}41}$ AMP fragment was attached to chloramphenicol antibiotic and ICG02 (near infra-red dye) for targeting *E. coli* and *S. aureus* in ICR mice. The targeting capability was proved by detecting the presence of the ICG02 dye at the site of infection using *in vivo* imaging system [113].

From all of the above studies, it was observed that the AMPs or AMP-conjugates had a higher selectivity toward a wide range of microorganisms with enhanced antibacterial effects. Plausible cytotoxicity and binding affinity towards the cancer cell lines evaluated were also observed, suggesting that these AMPs could be considered for cancer

therapy. *In vivo* murine toxicity was not observed, indicating their biocompatibility and potential for further exploration.

5. AMP polymer conjugates

Polymers such as hyperbranched polygycerol (HPG), polyethylene glycol (PEG), poly- L-lysine (PLL), Chitosan, and poly Lactic-*co*-glycolic acid (PLGA) are generally used extensively in drug delivery, where they function as drug carriers across bacterial membrane, and when inside the cell membrane, they disintegrate and release the drug to its specific target site [136]. These polymers have also been used for AMP delivery, and function by protecting them from degradation and allowing them to be released effectively to the target site [63]. The main role of polymeric carriers in antibiotic or peptide delivery is to deliver the cargo to the disease site, to function as protector groups of the cargo being delivered, and to protect the cargo or peptide from degradation by proteases and efflux channels [59]. The following section describes conjugation approaches involving AMPs and polymers.

5.1. AMP- hyperbranched polygycerol (HPG) conjugates

HPGs are dendritic macromolecules with random branch-on-branch topology, which has numerous advantages [137]. Firstly, HPGs are more hydrophilic than PEG, and secondly, the hyper-branched assembly allows the HPG to efficiently cover the surface more than PEG. Thirdly, HPGs have a number of hydroxyl groups that allow for the attachment of several ligands on the HPG [138]. In a study conducted by Kumar et al. (2015), Aurein 2.2 was conjugated to a hyperbranched polygycerol (HPG), with the MIC values for the conjugate being determined, and *S. aureus* and S. *epidermidis* being used as test strains. The MICs of the peptide against *S. aureus* and *S. epidermidis* were 16 and 32 μg/mL respectively, whereas the MICs for the conjugates were 110 μg/mL and 120 μg/mL respectively [139]. The authors postulated that the possible reasons for the decreased activity could be due to peptide substitution where a higher peptide density within the conjugate would result in a higher antimicrobial activity. Despite the decrease in activity, the HPGylated peptides were also non-toxic to human umbilical vein endothelial cells (HUVECs) and fibroblasts indicating biocompatibility.

5.2. AMP- polyethylene glycol (PEG) conjugates

PEG is a non-toxic, non-immunogenic and FDA approved polymer used to enhance the biocompatibility of many compounds [140]. PEGylation of peptide drugs has been shown to enhance biocompatibility of the peptide in question [139,141,142]. Conjugation of AMPs with PEG have been undertaken with the aim of prevention of recognition and degradation by proteolytic enzymes and increases the size of the AMP, thus reducing the renal filtration and altering bio-distribution [143]. Guiotto et al. (2003) Pegylated the AMP nisin, and determined their MIC values for a number of bacterial strains. The results showed the nisin-PEG conjugate to be less effective than the original AMP alone, with MIC values of the conjugate ranging from 250 μM for *S aureus* and > 500 μM for *P aeruginosa* [144]. In a study by Morris et al. (2012), the AMP CaLL was Pegylated, forming a PEG-CaLL conjugate, with the *in vitro* activity revealing that the CaLL was more active that the conjugate [145]. Benincasa et al. (2015) also studied the PEGylation of the peptide Bac7 and tested the conjugate against *S. typhimurium,* with the MIC values of the BacE-PEG ranging between 4 and 8 μM when assayed in MH broth. The MIC values were also determined in the presence of human serum and plasma, and were 1 μM and 0.25 μM in plasma for the BacE-PEG conjugate in the presence of human serum [146]. The above studies which all showed a decreased activity of the Pegylated AMP as compared to AMP alone, indicate that even with prospects of PEG protecting the AMPs from degradative enzymes and increasing their biocompatibility, there is also a possibility of activity being compromised. As PEG coats both the hydrophobic and hydrophilic parts of the AMP, this could result in the AMP not being freely available for interaction with the bacterial membrane, thus decreasing its cell penetrative activity. PEG also has a high molecular weight, which confers steric hindrance [147], which could be the reason for the attenuated activity of the AMPs. This phenomenon is well described by Lee et al. (2014), who conducted a molecular simulation of PEGylated peptides. It was seen that the PEG chains wrap around the AMPs and weaken their binding interactions with the lipid bilayers. From the reviewed PEGylated AMPs, it was observed that decreased activity was higher in β-sheets than in α-helical AMPs, indicating a structural influence on PEGylation [148]. The AMPs reported so far for Pegylation were all hydrophilic. Pegylation could be advantageous for highly hydrophobic AMPs as PEG is thought to play a crucial role in increasing their solubility and reducing their antigenicity [55], and as such, can play a crucial role in enhancing the AMP activity.

5.3. AMP-chitosan conjugates

Chitosan is a non-toxic cationic polysaccharide natural polymer with a wide range of biomedical applications [149]. It is also easily absorbable at low pH, and has antacid and antiulcer activities that prevent and weaken drug irritation in the gastrointestinal tract [149,150]. These properties make it an ideal candidate for controlled drug release formulations that would provide an added advantage in AMP-chitosan conjugate formulations. Batista et al. (2009) reported on the novel synthesis of chitosan-pexiganan conjugate through the Sulfo-EMCS Cross-Linker for treating infected skin lesions Fig. 3 [151]. In this study, only the successful conjugation was reported, and no antibacterial studies were performed. But based on the known activities of both chitosan and the antimicrobial peptide pexiganan, the authors indicated that this conjugate is very likely to undergo clinical trials for topical uses [152,153]. Their assertion is supported by the following two studies on chitosan and pexiganan. Flamm et al. (2015) tested a non-conjugated pexiganan against a selected number of resistant strains which included MRSA, *S. aureus, E. faecium, E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii*. The MIC values from these pathogens derived from diabetic foot infections ranged between 16 and 32 μg/mL [154]. In Costa et al. (2014) described the antibacterial activity of chitosan derivatives on *C. albicans* and the MIC values as being 1 μg/mL for high molecular weight (HMW) chitosan and 3 μg/mL for low molecular weight (LMW) chitosan [155]. Therefore, based on the antibacterial activity of these materials individually against the Gram-positive microorganisms, a conjugate would be expected to show enhanced activity towards biofilm disruption. It has been reported that *C. albicans* mycofilms actively enhance *S. aureus* colonization and their interaction in a biofilm mode promotes staphylococcal infections [156]. To date, that is the only study involving the conjugation of pexiganan to chitosan or other polymers and additional experimental research involving pexiganan and chitosan conjugates is required. The above studies involving pexiganan and chitosan activities indicate the potential of this polymer and peptide conjugation strategy to yield good results that can lead to clinical trials. Sahariah et al. (2015) reported on the antimicrobial activity of Anoplin-chitosan conjugates, which were synthesized using Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) chemistry. The conjugates showed promising activity compared to their parent peptide, with the lowest MIC observed against E. coli (4 μg/ml) [157]. Future research and reviews should explore the chemical composition of chitosan using computational approaches and how it can be used to enhance biocompatibility of AMPs, this component making it ideal for conjugation studies with AMPs and to enhance the delivery of AMPs.

Polymers have been used broadly as drug delivery vectors, and in the formulation of polymeric nanoparticles due to their improved bioavailability, enhanced encapsulation, controlled drug release and attenuated toxicity demonstrated [158,159]. Their utilisation, in

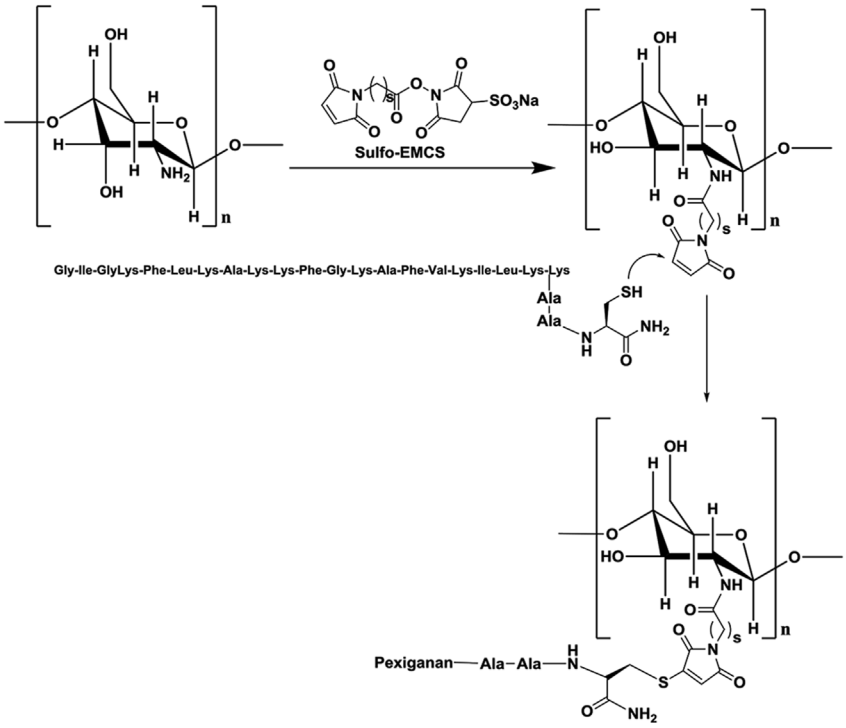

Fig. 3. Pexiganan-chitosan conjugate. Permission granted [125].

conjunction with AMPs, would also offer an enhanced nanoparticle system that contains cell penetrating power due to the cationic properties of AMPs [63]. These types of nano systems would allow AMPs to have a bi-directional approach of enhancing the cell penetrating ability of the nanoparticle as well as interacting with the intracellular organelles.

6. Miscellaneous AMP conjugates

The following section discusses other various AMP conjugates that have been reported.

6.1. AMP- phenolic conjugates

Phenolic groups are found in most drug molecules and have been used in pro-drug modifications to overcome pharmacological barriers that would attenuate drug action [160]. One of the strategies used by phenols is to cover polar groups, and this allows a molecule to be more lipophilic thus promoting membrane permeability [161]. This strategy would be ideal in AMP-phenolic conjugates to effectively deliver the AMP to its target sites through bacterial membrane penetration. Findlay et al. (2012) reported on neomycin–phenolic conjugates with broad-spectrum antibacterial activity. The conjugates had good activity against neomycin sulfate resistant bacteria with low activity towards neomycin susceptible strains. Several conjugates had activity towards MRSA that was also similar to *S. aureus*, while activity against *P. aeruginosa* was slightly increased (64 μm/mL). Therefore, these conjugates displayed improved activity towards Gram positive and negative bacteria. These conjugates had triclosan and clofoctol linkers, which could be responsible for their activity [162]. The phenolic group in antibacterial agents can be used in structural modifications to overcome various properties that could be barriers in the application of the compounds [160]. With the above promising data, future research should explore conjugating phenolic groups to already known AMPs to enhance their bioavailability [163]. There is considerable scope in nano encapsulation of AMP-phenolic conjugates, with an absence of experimental or review studies to elucidate the activity of AMP-phenols in nano systems. As phenols show improved bioavailability and stability, it is thought that their conjugation with AMPs would provide stability to the formulation and protect them from degradation.

6.2. AMP-DNA conjugates

The strategy of AMP conjugation has been extended to DNA as this offers the potential of to deliver AMPs to nucleic acids.

Ghosal et al. (2012) conducted research on the conjugation of Peptide–Peptide Nucleic Acid (PNA) against *P. aeruginosa*, these being nucleobase oligomers that are regarded as DNA, with a neutral peptide backbone that is stable and resistant to hydrolytic cleavage [164]. These conjugates were synthesized by continuous solid phase synthesis using Boc-chemistry and purified by HPLC. The conjugates had MIC values ranging from 1 to 20 μM, indicating their potential to be used as antibacterial agents and their activity was significantly higher than that of the non-conjugated peptide [165]. Williams et al. (2012) synthesized peptide-oligonucleotide conjugates via solid phase synthesis and its formation was confirmed by reverse phase HPLC, and Maldi-Tof. Antimicrobial activity was not carried out as the authors focussed only on demonstrating the viability of this conjugation strategy, as this is an emerging field. It is envisaged that this field of peptide-DNA nanotechnology will be very useful to direct individual peptides to specific locations on the surface of a DNA nanostructures [166]. Conjugation of AMPs to these nucleic acids would allow AMPs to be utilised as delivery vehicle to pass through the membrane to deliver the nucleic acids into the bacterial nucleus. This would allow the conjugate to inhibit bacterial replication, and to possibly attenuate genetic material that bring about resistance. There is still a considerable gap in research focusing on the nano delivery of AMPs with peptide nucleic acids and the encapsulation of these conjugates would allow effective targeted delivery and a reduction in the dosing frequency.

6.3. AMP-salt conjugates

The strategy of conjugating AMPs to Imidazolium salt is that these salts exhibit biological activity when part of ionic liquids and can form hydrogen bonds with drugs and proteins.

Reinhardt et al. (2014) synthesized Imidazolium salt − peptide

conjugates and screened them for their biological activity. Recent observations suggest that imidazolium cations, when part of ionic liquids, exhibit biological activity. In this study, two AMPs were used: sC18, which is a short C-terminal fragment of the cationic antimicrobial peptide cathelicidin (CAP18) that binds lipopolysaccharide (LPS), and the LL-37 peptide, which also belongs to the group of cathelicidins and shows activity against a wide spectrum of Gram negative and positive bacteria. Conjugation of these peptide-salt conjugates occurred by coupling reactions, and antimicrobial activity was conducted against a wide range of bacteria where the best MIC value was found to be0.2–0.5 μM against all tested strains [167]. Imidazolium cations in their liquid ionic state function as modifying agents, and in conjugation with AMPs, they can confer their charge for effective membrane penetration. These properties of Imidazolium cations would make them ideal candidates in the formulation of hydrophobic AMPs. Future studies should explore using these cations to improve the conductivity of peptides for effective membrane penetration, and encapsulating AMP-Imidazolium conjugates for improved delivery.

The conjugation of various classes of materials to AMPs using different conjugation strategies all have the same goal of providing a plausible vehicle capable of permeating the bacterial cellular membrane, delivering the desired compound or AMP to the intracellular targets, protecting the AMP from the action of degradative enzymes, enhancing biocompatibility and overcoming pharmacological barriers. Conjugations make use of intrinsic chemical routes that require an understanding of both the AMP and conjugate materials involved in terms of whether or not they would synergistically produce the desired biological outcome as well as knowledge of the biological target with its inherent pathogenicity and intracellular biochemical pathways, which can dispel foreign substances, leading to resistance.

As a result of the enhanced biological activity of several conjugates compared to their parent compounds, it is anticipated that their encapsulation into nano systems would provide improved antimicrobial agents that have the ability to permeate the bacterial membrane and interrupt intracellular targets. The formulation of these conjugates in nano systems would also allow for a controlled and sustained release to their specific target sites, reduced toxicity, protection against degradative enzymes, increased bioavailability and high loading capacity, as seen with their parent AMP [168,169].

7. Nano-carriers employed in the delivery of AMPs

Despite their considerable antimicrobial activity, the application of AMPs in clinical settings has been limited by their potential toxicity and vulnerability to chemical degradation by proteases [47]. The encapsulation of AMPs, including their conjugates in suitable nano-carriers, has the potential to target the infection site of bacteria, overcome side effects and protect them from enzymatic degradation [170]. Encapsulating AMPs for effective delivery has been successful in several nanostructures such as liposomes, micelles, nanofibers, metallic nanoparticles (MNPs) and hydrogel nanoparticles [171,172]. Table 4 summarizes the different nano drug delivery systems that have been used thus far for encapsulating AMPs. The main experimental findings, with each of the nano carrier systems reported thus far for AMPs are summarised below.

7.1. Liposomes

Liposomes are spherical vesicles with at least one lipid bilayer and have been used extensively in drug delivery research. They have been found to reduce toxicity, prolong drug half-life, as well as biocompatible and biodegradable properties which makes them very attractive for biomedical investigation [173,174]. They have also been seen to improve the delivery of bioactive molecules by functioning as circulating micro-reservoirs for sustained release [175]. Liposomes can encapsulate both hydrophilic and hydrophobic materials by nature,

Table 4
Nano-carriers used for the delivery of AMPs.

Nano-carrier	AMPs	Spectrum of activity	Main findings & applications	Ref
Liposomes	Nisin	Activity against *S. mutans*	nisin encapsulated in two lipid molar formations: DPPC/DPPG, DPPC/DPPE, DPPC/SPS had greater In vitro activity against the tested strain.	[227]
	Nisin	Activity against L. *lactis*	nisin-loaded liposomes were successful in controlling food spoilage and pathogenic organisms.	[228]
Micelles	KSLW-SSM	Activity against *S. epidermidis*	KSLW loaded in the SSM carrier had no effect on bacterial load in stressed mice.	[187]
Nanofibers	AMP motif (Cys-KR12)	Activity against *P. aeruginosa, S. aureus E. coli* and *S. epidermidis*	Nanofibers containing Cys-KR12 displayed potent antimicrobial activity against the tested strains & activities on keratinocytes, fibroblasts, & monocytes, which play important roles in the wound healing process.	[229]
	Bacteriocin	Activity against L. *sakei, E. faecium*	The activity of plantaricin 423 in nanofibers was more potent than its activity in the cell free supernatant	[230]
Metallic NPs	NK-2 and LLKKK-18 AMPs coupled to a silver Nanoparticle	Activity against *M. smegmatis*	AMP NK-2 displayed improved killing effect with the AgNPs; NP-1 and NP-2 at a 0.5-ppm concentration. Peptide LLKKK-18 exhibited activity with NP-2 at 0.5-ppm against *M. smegmatis*.	[195]
	Cecropin-melittin (CM) conjugated to gold nanoparticles	Activity against *S. aureus; E. coli; K. pneumonia; P. aeruginosa,* multidrug-resistant *E. coli; S. haemolyticus*	CM conjugated to AuNPs were found to be more effective in bacterial membrane penetration than the unbound CM and were not toxic to human cells	[199]
Hydrogels	Novel AMP immobilized on a soft hydrogel network	Activity against *S. aureus* and *S. epidermidis*	Hydrogel with higher AMP concentration led to a greater antimicrobial activity with cidal effects identified for 6, 8, & 10 wt % hydrogels.	[231]
	PEP8R	Activity against *E. coli, P. aeruginosa* and *S. aureus*	The AMP was more active against the Gram-positive bacteria	[232]

making them ideal nano structures for the delivery of amphiphilic compounds [176]. One of the challenges that hinders the activity of the liposomal formulation includes the premature release of the payload before the target site is reached [177,178]. In addition, higher liposomal volumes are necessary when the efficiency of the encapsulation is low for clinical dosage [179]. In separate studies Benech et al. (2002) and Were et al. (2004) studied the activity of nisin encapsulated in liposomes, and found that the encapsulation conferred a higher entrapment efficiency with lower susceptibility to destabilization by nisin. The formulation also had an enhanced efficacy against *L. monocytogenes* compared to the free nisin [180,181]. Using another AMP, Alipour et al. (2008) and Alipour et al. (2009) encapsulated polymyxin B which is a cationic AMP with liposomes targeting resistant Gram-negative bacterial infections. The MICs of the liposomal polymyxin B against Gram-negative strains was significantly lower than the free polymyxin B [182,183]. In the above studies, the activity of the liposomal formulations of polymyxin B was higher that the activity of the un-encapsulated. These four studies only reported antimicrobial activity, encapsulation of AMPs into liposomes and entrapment. No other characterisation data such as release kinetics, essential for formulation optimisation was reported. However, in a more recent study by Ron-Doitch et al. (2016) formulation characterisation was more extensive. They encapsulated the AMP LL-37 in liposomes, and studied its cellular uptake, *in vitro* cytotoxicity and physicochemical properties. The cytotoxicity results showed minimal cytotoxicity in HaCaT cells. From the cellular uptake results (Fig. 4), LL-37 liposomal formulation was taken up more rapidly than the free AMP in all the time periods. The time-dependant uptake of the encapsulated LL-37 was observed as being higher than that of the unbound LL-37 [135]. These studies show the importance of AMP-liposomal formulations in delivering AMPs and the effectiveness of liposomes as delivery vehicles.

7.2. Micelles

Since their discovery in 1984, micelles have gained much attention as nano-based drug delivery systems, especially for poorly water-soluble drugs [184]. Owing to their size, ability to solubilize hydrophobic drugs and achieve target or site-based drug delivery, micelles continue to show great potential as vectors for drug delivery [185]. Micelles possess distinctive structural properties that comprise of two or more hydrophilic and hydrophobic blocks with different solubility ratios in aqueous environment, which makes them effective for drug delivery [186]. Liu et al. (2009) studied cationic AMPs which are capable of self-assembly as potential antimicrobial agents. TEM imaging confirmed the formation of micelles, and the *in vitro* results showed that these micelles had broad spectrum activity with low MIC values [101]. Using another micelle formation strategy, Williams et al. (2012) performed a study on sterically stabilized phospholipid micelles of an antimicrobial wound healing adjunct. The study aimed to examine whether the association of a cationic decapeptide with sterically stabilized nano-micelles (SSMs), would improve stability and *in vivo* antimicrobial effect. *In vitro* assays against *S. epidermidis* reflected reduced activity of the cationic decapeptide in SSM solution, however the *in vivo* studies in animal model

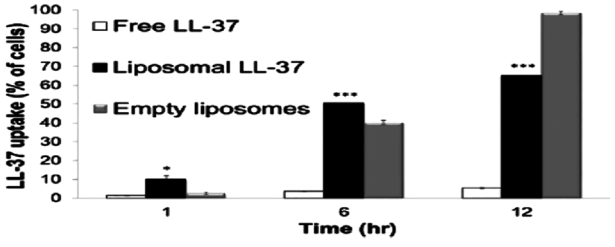

Fig. 4. LL-37 uptake by HaCaT cells (shown as % of cells engulfing treatment) following empty liposomes, free LL-37, or liposomal LL-37. Permission granted [151].

with decapeptide-nano micelles preparations presented no differences in microbial load at post-operative time points. We believe that the loss of activity could be due to electrostatic interactions of the decapeptide with the anionic surface of SSM [187]. The above studies employed micelles to improve activity. To explore another application for micelles Black et al. (2012) reported the self-assembly of peptide amphiphile micelles with the aim of promoting a protective immune response *in vivo*. The formation of the micelles was confirmed by TEM imaging. These peptide amphiphile micelles were found to offer *in vivo* protection from tumours by stimulating T_c cells This study confirmed the use of the peptide amphiphiles in self assembled micelles as a new class of growing nanoparticles that have the ability to induce an immune response [188]. Clearly this study showed that, although these micelles are effective in the entrapment and delivery of AMPs, the choice of the micelle system should be chosen very carefully so that the surface charge of micelles don't interfere with the AMP activity.

7.3. Nanofibers

Nanofibers (fibers with diameters less than 100 nm), which are a product of polymers treated specifically to form filaments, possess great prospective to be used for delivering AMPs and/or AMP-conjugates [189]. These fibers are produced by electro-spinning, which uses electric force to draw charged threads of polymer solution [190], and are developed from both natural and synthetic polymers, such as chitin, chitosan, polyurethane, poly(L-lactic acid) and poly-vinyl alcohol. Nanofibers have large surface to volume ratio and surface-modification possibilities, which make them ideal for AMP-loading and delivery [191]. Heunis et al. (2010) described a novel approach in AMP delivery, where they were incorporated into nanofibers for wound dressings. The AMP plantaricin 423 was encapsulated in nanofibers that were produced by the electro-spinning of polyethylene oxide (PEO). The PEO mobilised AMP showed high activity against *E. faecium* and *L. sakei*. Nanofibers are therefore thought to be the ideal matrix for the immobilization of AMPs, and/or their encapsulation for effective delivery to skin infection [191].

7.4. Metallic nanoparticles

Metallic nanoparticles (MNPs), such as those derived from noble metals, including gold and silver, may serve as potential nano carriers for AMPs and their conjugates. Owing to their large surface area and surface charge, most MNPs easily attach to the surface of bacterial membranes by electrostatic interactions, and thereby interrupt the integrity of the membrane [149,192]. Noble metals are resistant to oxidation and corrosion, which this makes them ideal for nanoparticle formation and reduced toxicity, as most nanoparticles accumulate in the liver, spleen and lymph nodes [193]. Functionalized nanoparticles can be formed by processes such as coupling or adsorption of specific molecules (e.g. AMPs onto MNPs surfaces), with the aim of producing a synergistic approach for the antimicrobial activity of both the NPs and the selected AMP [194]. Silver nanoparticles (AgNPs) have been extensively studied and found to possess potent antimicrobial activities, having been used for decades, especially as antibacterial agent [195]. The mechanism of action has been thought to be directed towards the bacterial cell wall and membrane perturbation, as well as acting on intracellular targets [196]. MNPs have a higher positive zeta potential, promote membrane lysis and penetration when they interact with the negatively charged bacterial membrane [197]. Liu et al. (2013) used a cell penetrating peptide (G3R6YGRKKRRQRRR) which was then used to form silver nanoparticles (AgNPs). The nanoparticles were found to be active against the Gram-positive *B. subtilis* and the Gram-negative *E. coli* [198]. In contrast to the previous studies where the AMPs were used to form the silver nanoparticles, Pal et al. (2016) conducted a study on the activity of an AMP conjugated to a silver nanoparticle against *E. coli,* and the nano-conjugate was reported to enhance

Table 5
List of FDA-Approved Nanomedicines Stratified by Material Category (adapted from D Bobo et al., 2016) [212].

Name	Material description	Nanoparticle advantage	Indication(s)	Year(s) approved
Polymer Nanoparticles – synthetic polymer particles combined with drugs or biologics				
Adagen®/pegademase bovine (Sigma-Tau Pharmaceuticals)	PEGylated adenosine deaminase enzyme	Improve circulation time and decreased immunogenicity	Severe combined immunodeficiency disease (SCID)	1990
Cimzia®/certolizumab pegol (UCB)	PEGylated antibody fragment (Certolizumab)	Improved circulation time and greater stability *in vivo*.	Crohn's disease Rheumatoid arthritis Psoriatic Arthritis Ankylosing Spondylitis	2008 2009 2013 2013
Copaxone®/Glatopa (Teva)	Random copolymer of L-glutamate, L-alanine, L-lysine and L-tyrosine	Large amino-acid based polymer with controlled molecular weight and clearance characteristics	Multiple Sclerosis (MS)	1996
Eligard® (Tolmar)	Leuprolide acetate and polymer (PLGH (poly (DL-Lactide-*co*-glycolide))	Controlled delivery of payload with longer circulation time	Prostate Cancer	2002
Macugen®/Pegaptanib (Bausch & Lomb)	PEGylated anti-VEGF aptamer (vascular endothelial growth factor) aptamer	Improved stability of aptamer as a result of PEGylation	Macular degeneration, neovacular age-related	2004
Mircera®/Methoxy polyethylene glycol-epoetin beta (Hoffman-La Roche)	Chemically synthesized ESA (erythropoiesis-stimulating agent)	Improved stability of aptamer as a result of PEGylation	Anemia associated with chronic kidney disease	2007
Neulasta®/pegfilgrastim (Amgen)	PEGylated GCSF protein	Improved stability of protein through PEGylation	Neutropenia, Chemotherapy induced	2002
Pegasys® (Genentech)	PEGylated IFN alpha-2a protein	Improved stability of protein through PEGylation	Hepatitis B; Hepatitis C	2002
PegIntron® (Merck)	PEGylated IFN alpha-2b protein	Improved stability of protein through PEGylation	Hepatitis C	2001
Renagel®[sevelamer hydrochloride]/ Renagel®[sevelamer carbonate] (Sanofi)	Poly(allylamine hydrochloride)	Increase circulation and therapeutic delivery	Chronic kidney disease	2000
Somavert®/pegvisomant (Pfizer)	PEGylated HGH receptor antagonist	Improved stability of protein through PEGylation	Acromegaly	2003
Oncaspar®/pegaspargase (Enzon Pharmaceuticals)	Polymer-protein conjugate (PEGylated L-asparaginase)	Improved stability of protein through PEGylation	Acute lymphoblastic leukemia	1994
Krystexxa®/pegloticase (Horizon)	Polymer-protein conjugate (PEGylated porcine-like uricase)	Improved stability of protein through PEGylation; introduction of unique mammalian protein	Chronic gout	2010
Plegridy® (Biogen)	Polymer-protein conjugate (PEGylated IFN beta-1a)	Improved stability of protein through PEGylation	Multple Sclerosis	2014
ADYNOVATE (Baxalta)	Polymer-protein conjugate (PEGylated factor VIII)	Improved stability of protein through PEGylation	Hemophilia	2015
Liposome formulations combined with drugs or biologics				
DaunoXome® (Galen)	Liposomal Daunorubicin	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Karposi's Sarcoma	1996
DepoCyt© (Sigma-Tau)	Liposomal Cytarabine	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Lymphomatous meningitis	1996
Marqibo® (Onco TCS)	Liposomal Vincristine	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Acute Lymphoblastic Leukemia	2012
Onivyde® (Merrimack)	Liposomal Irinotecan	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Pancreatic Cancer	2015
AmBisome® (Gilead Sciences)	Liposomal Amphotericin B	Reduced nephrotoxicity	Fungal/protozoal infections	1997
	Liposomal Morphine sulfate	Extended release	Analgesia (post-operative)	2004
Visudyne® (Bausch and Lomb)	Liposomal Verteporfin	Increased delivery to site of diseased vessels; photosensitive release	Macular degeneration, wet age-related; myopia; ocular histoplasmosis	2000
Doxil®/Caelyx™ (Janssen)	Liposomal doxorubicin	Improved delivery to site of disease; decrease in systemic toxicity of free drug.	Karposi's Sarcoma; Ovarian cancer; multiple myeloma	1995 2005 2008
Abelcet® (Sigma-tau)	Liposomal Amphotericin B lipid complex	Reduced toxicity	Fungal infections	1995
Curosurf®/Poractant alpha (Chiesei farmaceutici)	Liposome-proteins SP-B and SP-C	Increased delivery for smaller volume; reduced doxicity	pulmonary surfactant for Respiratory Distress Syndrome	1999
Micellar nanoparticles combined with drugs or biologics				
Estrasorb™ (Novavax)	Micellar Estradiol	Controlled delivery of	Menopausal therapy	2003

(*continued on next page*)

Table 5 (*continued*)

Name	Material description	Nanoparticle advantage	Indication(s)	Year(s) approved
		therapeutic		
Protein nanoparticles combined with drugs or biologics				
Abraxane®/ABI-007 (Celgene)	Albumin-bound paclitaxel nanoparticles	Improved solubility; improved delivery to tumour	Breast cancer NSCLC Pancreatic cancer	2005 2012 2013
Ontak® (Eisai Inc)	Engineered Protein combining IL-2 and diphtheria toxin	Targeted T-cell specificity; lysosomal escape	Cutaneous T-Cell Lymphoma	1999
Nanocrystals				
Emend® (Merck)	Aprepitant	Surface area allows faster absorption and increases bioavailability	Antiemetic	2003
Tricor® (Lupin Atlantis)	Fenofibrate	Increases bioavailability simplifies administration	Hyperlipidemia	2004
Rapamune® (Wyeth Pharmaceuticals)	Sirolimus	Increased bioavalibility	Immunosuppresent	2000
Megace ES® (Par Pharmaceuticals)	Megestrol acetate	Reduced dosing	Anti-anorexic	2001
Avinza® (Pfizer)	Morphine sulfate	Increased drug loading and bioavailability; extended release	Psychostimulant	2002 (2015)
Focalin XR® (Novartis)	Dexamethyl-phenidate HCl	Increased drug loading and bioavailability	Psychostimulant	2005
Ritalin LA® (Novartis)	Metyhlphenidate HCl	Increased drug loading and bioavailability	Psychostimulant	2002
Zanaflex® (Acorda)	Tizanidine HCl	Increased drug loading and bioavailability	Muscle relaxant	2002
Vitoss® (Stryker)	Calcium phosphate	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2003
Ostim® (Heraseus Kulzer)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2004
OsSatura® (IsoTis Orthobiologics)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2003
NanOss® (Rti Surgical)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2005
EquivaBone® (Zimmer Biomet)	Hydroxyapatite	Mimics bone structure	Bone substitute	2009
Invega® Sustenna® (Janssen Pharms)	Paliperidone Palmitate	Allows slow release of injectable low solubility drug	Schizophrenia Schizoaffective Disorder	2009 2014
Ryanodex® (Eagle Pharmaceuticals)	Dantrolene sodium	Faster administration at higher dses	Malignant hypothermia	2014
Inorganic and metallic nanoparticles				
Nanotherm® (MagForce)	Iron oxide	Allows cell uptake and introduces superparamagnetism	Glioblastoma	2010
Feraheme™/ferumoxytol (AMAG pharmaceuticals)	Ferumoxytol SPION with polyglucose sorbitol carboxymethylether	Magnetite suspension allows for prolonged steady release, decreasing number of doses	Deficiency anemiairon deficiency in chronic kidney disease (CKD)	2009
Venofer® (Luitpold Pharmaceuticals)	Iron sucrose	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	2000
Ferrlecit® (Sanofi Avertis)	Sodium ferric gluconate	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1999
INFeD® (Sanofi Avertis)	Iron dextran (low MW)	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1957
DexIron®/Dexferrum® (Sanofi Avertis)	Iron dextran (high MW)	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1957
Feridex®/Endorem® (AMAG pharmaceuticals)	SPION coated with dextran	Superparamagnetic character	Imaging agent	1996 (2008)
GastroMARK™; umirem® (AMAG Pharmaceuticals)	SPION coated with silicone	Superparamagnetic character	Imaging agent	2001 (2009)

biological activity [132]. This study showed another approach of conjugation which utilises the potency of the AgNP together with the AMP and this provides new insights of AgNP-AMP interactions and how this strategy could be used to enhance biological activity. Gold nanoparticles (AuNPs), which are synthesized by the reduction of $HAuCl_4$, have lower toxicity than other nanoparticles due to their noble characteristics [199]. AuNPs-AMP conjugates also possess several advantages, such as protecting the AMP from enzymatic degradation, and do not prevent the AMP from folding into its biologically active conformation [200]. In a study by Casciaro et al. (2016), the AMP Esculentin-1a(1–21)NH_2, which was derived from a frog skin, was coated with gold nanoparticles and evaluated against *P. aeruginosa*. The antibacterial results showed that the AMP coated nanoparticles were more potent than the free peptide [201]. In contrast to the studies above, Rai et al. (2016) used a one-step synthesis approach to conjugate the AMP, cecropin-melittin, to gold nanoparticles. The MIC values were also found to be higher than the free AMP [90]. Pradeepa et al. (2017) conducted a study on the application of Nisin gold nanoparticles as a potent antimicrobial agent against *E. faecalis* and *S. aureus*. The synthesized nanoparticles were found to be non-toxic with less hemolycic activity and lower MIC values that Nisin alone [202].

These strategies of utilising AMPs to reduce silver nitrate to form

AgNPs, or conjugating AMPs to AgNPs or AuNPs, combines the high surface charge of these metallic nanoparticles together with the high positive charge of AMPs, which will form a molecule well capable of bacterial membrane lysis [203].

7.5. Hydrogel nanoparticles

Hydrogels are 3-dimensional, cross-linked networks of water-soluble polymers that can be formulated by various approaches, including microparticles, slabs, coatings, films and more specifically, nanoparticles [204]. Hydrogels are used in a wide array of applications, such as in cellular immobilization, tissue engineering and regenerative medicine [205]. The physiochemical makeup of hydrogels has generated much interest for their use as drug delivery systems. They have a highly porous structural makeup, this feature being important, as it allows drug loading into the matrix of the gel and consequent drug release [204,206]. Hydrogel nanoparticle in drug delivery offer mostly pharmacokinetic benefits, as their formulations allow drugs to be slowly eluted, thus retaining a high concentration of the drug in the nearby tissues over a prolonged period of time [207]. Hydrogels are also thought to possess high biocompatible properties due to their high-water content, and their physiochemical resemblance to the native extracellular matrix [208]. Various materials have been exploited so far for the preparation of hydrogels with AMPs to target different organisms. Rajan et al. (2014) designed a study for the controlled release of the AMP subtilosin from polyethylene glycol-based hydrogels, and showed that it was able to inhibit the growth of *G. vaginalis,* with a reduction of 8 log10 CFU/ml [209]. Håkansson et al. (2014) reported on the formulation of the AMP PLX150 in hydroxypropyl cellulose gel (HPC) to target surgical site infections. The PLX150-HPC combination killed more than 95% of *S. aureus,* and presented a dose-dependent activity with a slow release of the AMP from the HPC hydrogel on the wound site [210]. In a study by Babavalian et al. (2015), the AMP CM11 was incorporated into alginate sulfate hydrogels to target MRSA. The MIC and MBC activity of the CM11 peptide were 2–32 mg/L and 16–64 mg/L respectively, and 50% of the CM11 peptide was released from the hydrogel in the first week [211]. Since hydrogel formulations allow sustained release of drugs, incorporation of AMPs into these systems would offer prolonged AMP release at target sites and retain high AMP concentration in the nearby tissues.

Nanocarriers have shown to increase the biocompatibility of AMPs, shield them from degradative enzymes as well as allowing enhancing their release into specific target sites. These nano drug delivery systems should be exploited further for AMP conjugates, with the aim of improving targeted conjugate delivery into intracellular targets and developing plausible nanomedicines. Table 5 reveals a list of FDA approved nano products derived from various materials [212].

8. Conclusions and future perspectives

The continuous evolution of pathogenic bacteria has led to an ongoing search for novel ways to combat antimicrobial drug resistance. The development of resistance to new antibiotic derivatives, and the inability of antibiotics to reach infection sites at effective concentration, pose a major threat to infection control and prevention. However, new approaches, such as the design and application of AMPs, individually or as conjugates and their delivery in nano-carriers, offer promising alternatives to main-stream antibiotics.

Molecular dynamics of AMPs with model membranes and QSAR approaches to identify or understand descriptors in AMPS for activity is important for the eventual design of novel AMPs including their conjugates and nano delivery systems to achieve optimal efficacy and safety. Molecular dynamic studies have been useful in confirming the successful penetration of AMPs across different types of cell membranes. It has also identified key interactions of AMPs with membrane components for penetration. Molecular dynamic studies have has been further successfully exploited for understanding the stability of AMPs with conjugates as well as their stability and encapsulation into nanocarriers. QSAR approaches on AMPs to date have identified the possible structural or physicochemical descriptors integral in influencing activity against bacteria. However, a considerable gap remains in utilising molecular modelling approaches to study the AMP interaction with model membranes. Structure activity relationship of AMPs with respect to membrane penetration is also lacking. Although limited, the findings so far can guide scientists to: 1) design new AMPs with optimal activity 2) select specific sites for conjugation to various compounds without losing their activity and 3) design and select suitable nanocarriers based on the physicochemical descriptors of the AMPs identified. Whilst understanding penetration of the AMPs through membranes is important, the interaction of AMPs as well as AMP-conjugates with intracellular organelles is also critical because it can elucidate the effectiveness of the AMP and AMP-conjugate strategy in being delivered across the membrane for action on intracellular targets to maximise activity. Since much of the MD and QSAR studies so far have focused on the interaction of AMPs with biological membranes, future studies should therefore explore the mechanism of action of the AMPs and their conjugates with intracellular targets such as the nucleus and mitochondria. Whilst computational modelling of nano carriers with AMPs are beginning to emerge, it needs to be extended further to AMP-conjugates in nanocarriers. Molecular modelling studies should be used to identify formulations that can maximise encapsulation and stability of the nanocarriers and also mechanistically explain their formation and release kinetics. MD simulations should be further explored in order to investigate the binding affinities of AMPs with their conjugates. We believe that these MD mechanistic studies will also elucidate the extent of encapsulation with respect to AMP-conjugates in nano systems to further tell us the best nano carrier which can be used to deliver these conjugates.

In this review the potential of AMP as conjugates with antibiotics, polymers and other classes such as DNA, salts and phenolic based compounds to potentiate antibacterial activity have been successfully demonstrated. Of the 4 groups of AMPs i.e. β-sheet, α-helical, loop and extended peptides the α-helical classes have been the most widely studies structural groups of AMPs used for conjugation to various classes of materials. The widely used AMPs used for conjugation are the indolicidin and they were found to have potency across both gram positive and negative bacteria. It is suggested that other classes of materials such as natural compounds from plant extracts and metal complexes could also be potential conjugate components and should be explored. With the conjugation strategy only preliminary characterisation studies such as structural confirmation, *in vitro* antimicrobial activity, cytoxicity and haemolytic studies, cellular uptake have been reported. Further characterisation studies such as *in vivo* assays and skin lesion studies is required to confirm the efficacy and safety of these conjugates. Further, patient administration of the AMP-conjugates will require its incorporation into a suitable delivery system. Therefore, extensive physicochemical and *in vitro/in vivo* characterisation of drug delivery systems for AMP-conjugates need to be undertaken. Degradation studies of AMP-conjugates as they enter the bacterial membrane should also be conducted, as this will allow for structural manipulation in the design process, and the application of additive factors directed at degradative enzymes. This would greatly enhance the applications of AMPs and broaden their scope in finding therapeutic agents.

Encapsulation of AMPs into five different classes of nano carriers i.e. liposomes, micelles, nanofibers, metallic nanoparticles and hydrogels so far have been successfully achieved with enhanced activity and sustained release. The α-helical group of AMPs only have so far been explored for delivery via nano carriers. However, again these studies are limited in their characterisation which is essential to ensure safety, quality and efficacy for regulatory approval. Future studies should clearly focus on experimental designs to rationally optimise

formulations. Also, in depth characterisation studies to determine the solid phase transformation properties, release kinetics, physicochemical stability, cell uptake mechanisms and *in vivo* efficacy and toxicity testing in animal models should be performed. In addition to encapsulation of the free AMPs into conventional lipid or polymer nanocarriers, the strategy of incorporating the AMP as a structural component of a lipid or polymer which then self assembles to form a nanosystem can also be a novel alternative.

The interest in AMPs as therapeutic agents has gained much interest recently, with studies having shown that more than 60 AMPs have reached the market, with some undergoing clinical trials [213], and many having distinguished themselves to be the new front runners in antimicrobial drug development. The current emerging data on AMP-conjugates and nano-delivery of AMPs further demonstrate their potential to be highly effective and advantageous in treating patients suffering with bacterial infections of both susceptible and resistant nature. Collaborations amongst a highly multidisciplinary team of researchers is therefore highly warranted to realise the future commercialisation of AMP-conjugates and AMP nano delivery systems.

Based on the approaches stated in this review, which utilises various strategies in AMP development, it is envisaged that in the next decade we can expect a rise in AMP based antibiotics that will have the ability to circumvent drug resistance. Owing to the emergence of AMP conjugation to various compounds, we believe that AMP development will go beyond the scope of targeting pathogenic bacteria. As natural AMPs possess immunomodulatory functions, we believe that future studies will focus on molecular mimicry by AMPs for T-cell activation, as well as the nano delivery of AMP conjugates to release them to specific immune cells for enhanced immunity.

Collaborations amongst a highly multidisciplinary team of researchers is therefore highly warranted to realise the future commercialisation of AMP-conjugates and AMP nano delivery systems.

Declaration of interest sections

The author reports that there is no conflict of interest on this paper.

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CHAPTER 3, Experimental Paper

Antimicrobial cell penetrating peptides (aCPPs) with bacterial cell specificity: Pharmacophore modelling and QSAR

3.1. Introduction

This research article focuses on the cell penetrating ability of antimicrobial cell penetrating peptides (aCPPs) based on their therapeutic index (TI). This chapter addresses Aim 1, Objectives 1- 3 and is a first authored experimental paper published in the Journal of Biomolecular Structure and Dynamics. This research article focuses on the cell penetrating ability of antimicrobial cell penetrating peptides (aCPPs) based on their therapeutic index (TI). This chapter further highlights the development of a QSAR model which can estimate the antimicrobial potential (MIC) of a set of aCPPs based on their TI using PHASE 3.0 suite to perform the 3D-QSAR studies. Research outputs from the chapter includes; published in an ISI international journal: Journal of Biomolecular Structure and Dynamics (Impact Factor = 2.15) and the data from this chapter has also been presented in one international conference and one local conference.

- Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation. Mbuso Faya, Rahul S. Kalhapure, Dinesh Dhumal, Nikhil Agrawal, Calvin Omolo, Krishnacharya G. Akamanchi & Thirumala Govender. Journal of Biomolecular Structure and Dynamics.
  International conference: Nano Africa, 23-25 April 2018, Durban South Africa, (senior Author). Poster Presentation (appendix A)
- Antimicrobial cell penetrating peptides with bacterial cell specificity: Pharmacophore modelling, Quantitative Structure Activity Relationship and Molecular Dynamics Simulation. Mbuso Faya, Rahul S. Kalhapure, Dinesh Dhumal, Nikhil Agrawal, Calvin Omolo, Krishnacharya G. Akamanchi & Thirumala Govender. Journal of Biomolecular Structure and Dynamics.
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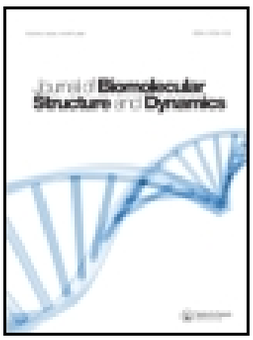

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Antimicrobial cell penetrating peptides with bacterial cell specificity: pharmacophore modelling, quantitative structure activity relationship and molecular dynamics simulation

Mbuso Faya[a], Rahul S. Kalhapure[a]*, Dinesh Dhumal[b], Nikhil Agrawal[a], Calvin Omolo[a], Krishnacharya G. Akamanchi[b] and Thirumala Govender[a]

[a]Department of Pharmaceutical Sciences, University of KwaZulu-Natal, Private Bag, Durban, South Africa; [b]Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai, India

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ABSTRACT

Current research has shown cell-penetrating peptides and antimicrobial peptides (AMPs) as probable vectors for use in drug delivery and as novel antibiotics. It has been reported that the higher the therapeutic index (TI) the higher would be the bacterial cell penetrating ability. To the best of our knowledge, no in-silico study has been performed to determine bacterial cell specificity of the antimicrobial cell penetrating peptides (aCPP's) based on their TI. The aim of this study was to develop a quantitative structure activity relationship (QSAR) model, which can estimate antimicrobial potential and cell-penetrating ability of aCPPs against *S. aureus*, to confirm the relationship between the TI and aCPPs and to identify specific descriptors responsible for aCPPs penetrating ability. Molecular dynamics (MD) simulation was also performed to confirm the membrane insertion of the most active aCPPs obtained from the QSAR study. The most appropriate pharmacophore was identified to predict the aCPP's activity. The statistical results confirmed the validity of the model. The QSAR model was successful in identifying the optimal aCPP with high activity prediction and provided insights into the structural requirements to correlate their TI to cell penetrating ability. MD simulation of the best aCPP with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer confirmed its interaction with the membrane and the C-terminal residues of the aCPP played a key role in membrane penetration. The strategy of combining QSAR and molecular dynamics, allowed for optimal estimation of ligand-target interaction and confirmed the importance of Trp and Lys in interacting with the POPC bilayer.

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3D QSAR; pharmacophore modelling; therapeutic index (TI); molecular dynamics simulation; antimicrobial peptide

Abbreviations: A: hydrogen bond acceptor; aCPPs: antimicrobial cell penetrating peptides; AMPs: antimicrobial peptides; ANN: artificial neural network; AntiBP2: antibacterial peptide prediction; APD2: antimicrobial peptide database; COM: centre of mass; CPPs: cell penetrating peptides; F value: Fisher test; H: hydrophobic group; IC_{50}: half maximal inhibitory concentration; LA: lipid A; LINCS: linear constraint solver; LPS: lipopolysaccharides; Lys: lysine; MD: molecular dynamics; MIC: minimum inhibitory concentration; NPT: isothermal–isobaric ensemble; NVT: canonical ensemble; PLS: partial least squares; PME: particle mesh Ewald; PO_4: phosphate group; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Q^2: cross-validation coefficient; QSAR: quantitative structure activity relationship; R: aromatic ring; R^2: correlation coefficient; RMSE: root-mean squared error; SD: standard deviation; TI: therapeutic index; Tis: therapeutic indices; Trp: tryptophan; vdW: van der Waals

1. Introduction

The resistance to antibiotics by bacteria poses a considerable threat to global health (Davies & Davies, 2010; Sengupta, Chattopadhyay, & Grossart, 2013, highlighting the need to urgently develop novel antibacterial agents. Bacterial resistance has prompted a search of natural inhibitors, leading to the use of antimicrobial peptides (AMPs) (Wang, Zeng, Yang, & Qiao, 2016), which form an integral part of innate immunity (Branco, Viana, Albergaria, & Arneborg, 2015; Bolintineanu, Hazrati, Davis, Lehrer, & Kaznessis, 2010; Wiesner & Vilcinskas, 2010). AMPs are small, cationic and amphiphilic molecules that are found in several classes of both prokaryotes and eukaryotes. They have structures such as linear α-helical peptides, β-sheet globular arrangements, and peptides with uncommon sequences including tryptophan and proline (Carnicelli et al., 2013; Michael Henderson & Lee, 2013; Vale, Aguiar, & Gomes, 2014; Wang et al., 2012). They have been seen to be structurally similar to cationic cell penetrating peptides (CPPs), and while their mechanism of action has not been elucidated, it is thought that they primarily target the bacterial cell membrane (Bhonsle, Venugopal, Huddler, Magill, & Hicks, 2007; Lee, Hall, & Aguilar, 2016; Schmidt & Wong, 2013). Cationic AMPs can bind to

CONTACT Thirumala Govendera govenderth@ukzn.ac.za; Rahul S. Kalhapure kalhapure@ukzn.ac.za; rahul.kalhapure@rediffmail.com
*Present address: School of Pharmacy, University of Texas at El Paso, 500 W University Ave, El Paso, TX 79968, USA.

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lipopolysaccharides (LPS) or lipid A (LA) of Gram-negative bacteria. This action leads to membrane permeation through self-promoted uptake and trans-membrane channel formation via a "barrel-stave" or toroidal pore mechanism, or through membrane destruction via a carpet-like mechanism (Mishra et al., 2013; Wang et al., 2012). Several studies have suggested that the success of AMP activity is mediated by its ability to aggregate on the surface of the membrane of bacteria, or to traverse the bacterial cell membrane and interrupt intracellular targets (Carmona-Ribeiro & de Melo Carrasco, 2014; Da Costa, Cova, Ferreira, & Vitorino, 2015; Guilhelmelli et al., 2013; Lv et al., 2014; Malanovic & Lohner, 2015; Tang, Shi, Zhao, Hao, & Le, 2008). However, membrane penetration/disintegration has been reported to be the primary mechanism of action of these cationic AMPs (Huerta-Cantillo & Navarro-García, 2016; Ong, Wiradharma, & Yang, 2014; Porto, Silva, & Franco, 2012; Tsai et al., 2009).

AMPs function with a great deal of similarity to CPPs and share important features that include short sequence lengths (∼10–40 residues), net positive charge, and an arrangement of amino acids with a substantial content of non-polar residues. All of these are considered to promote aCPP interaction and insertion with the hydrophobic core of the bacterial membrane bilayer. Small sequence modifications of CPPs can alter their biological effect from cell-penetrating to antimicrobial or vice versa, leading to the formation of antimicrobial cell penetrating peptides (aCPP), which are cell penetrating peptides with antimicrobial properties (Bahnsen, Franzyk, Sandberg-Schaal, & Nielsen, 2013). These peptides have a dual effect which offers bacterial membrane penetration together with antimicrobial activity, as seen with the aCPP penetration (Bahnsen, Franzyk, Sayers, Jones, & Nielsen, 2015; Henriques, Melo, & Castanho, 2006; Pushpanathan, Gunasekaran, & Rajendhran, 2016; Splith & Neundorf, 2011). Other examples of aCPPs include Bac7, which binds to bacterial ribosomal proteins and inhibits protein synthesis, and pep-1-K, which has a high membrane perturbing activity (Bobone et al., 2011; Mardirossian et al., 2014). The aCPPs have been widely reported as antibacterial agents, and as part of conjugates, such as drugs and polymers, and are utilised for the sole purpose of enhancing biological activity(Arnusch et al., 2012; Eckhard et al., 2014; Maekawa et al., 2015; Souto et al., 2013). There have also been reports of aCPPs being used as delivery systems to carry cargo across bacterial membranes (Carmona-Ribeiro & de Melo Carrasco, 2014; Eriksen, Skovsen, & Fojan, 2013; Kingsbury, Boehm, Mehta, Grappel, & Gilvarg, 1984). This is important, as it allows for the dual approach of cell penetration and the release of conjugates to their respective intracellular targets, as well as the biological activity of the aCPPs themselves. The aCPPs offer promising prospects to be utilised as alternative agents to known antibiotics. This is due to their ability to permeate the bacterial cell membrane and its cationic charge, which allows them not only to form pores on the bacterial cell membrane but also to traverse this layer to interfere with intracellular targets (Delcour, 2009; Schmidt & Wong, 2013). Another feature that makes them attractive is their ability to carry cargo across the bacterial cell membrane (Aparoy, Reddy, & Reddanna, 2012; Burns, McCleerey, & Thévenin, 2016), this strategy being useful to deliver conjugates, such as drugs and polymers. Continued research in these areas is required to identify the optimal aCPPs with high therapeutic indices (TIs). Tools to facilitate the design of potent and selective aCPPs, either as antibiotic entities themselves, or as components of pharmaceutical materials such as polymers, or as ligands for drug delivery carriers are essential to optimise their applications.

Quantitative structure–activity relationships (QSAR) is a useful tool in the rational design of potent and selective aCPPs (Mollica et al., 2018). Frequently used predictive tools, such as AntiBP2 and APD2 databases, are based on sequence analysis and physicochemical features, whereas other predictive models outline more structural-descriptor insight required for the designing of novel AMPs by outlining specific descriptors such as polarity of amino acids, free energy, hydration, and isoelectric point which are all properties responsible for biological activity (Vora et al., 2018; Wang et al., 2012). One such model based on inductive descriptors was developed by Cherkasov (2005), where the prediction was based on artificial neural network (ANN) for a series of newly synthesized polypeptides (Cherkasov, 2005; Torrent, Andreu, Nogués, & Boix, 2011). Taboureau et al. (2006) used GRID to generate 3D descriptors and built a high-performance QSAR model for novispirin AMPs (Taboureau et al., 2006). Fjell et al. (2009) also carried out an ANN virtual screening using physicochemical descriptors to screen for potential AMPs (Fjell et al., 2009). QSAR is an important tool, as it allows for the accurate design and structural elucidation of the descriptors responsible for peptide activity (Porto et al., 2012). This computational tool uses specific physicochemical descriptors that are directly responsible for the mechanism of action of aCPPs (Torrent et al., 2011; Vishnepolsky & Pirtskhalava, 2014). This quantitative method of predicting activity is used to design or modify aCPPs to elucidate their antibacterial activity.

Moreover, pharmacophore models can be generated based either on the ligand or the target, which identifies the groups in the former that are responsible for the potency or the binding targets with respect to the target site (Xie, Qiu, & Xie, 2014). QSAR models developed for antimicrobial peptides correlate their structural features with antimicrobial activity (Toropova, Veselinović, Veselinović, Stojanović, & Toropov, 2015). It has been reported that the higher the TI, the higher the bacterial cell penetrating ability (Aoki & Ueda, 2013; Matsuzaki, 2009; Tripathi, Kathuria, Kumar, Mitra, & Ghosh, 2015). To the best of our knowledge, till date no QSAR models have been used to predict the bacterial cell specificity of a CPP based on its TI. The use of QSAR as a chemo-informatic tool to predict the TI of potential aCPPs by analysing available experimental data will therefore hasten the design and synthesis of novel aCPPs specific for bacterial cell, leading to development of efficient antibacterials. The TI compares the amount of a therapeutic agent to the amount that causes toxicity (Muller & Milton, 2012; Tamargo, Le Heuzey, & Mabo, 2015), with a high TI being preferable for a drug to

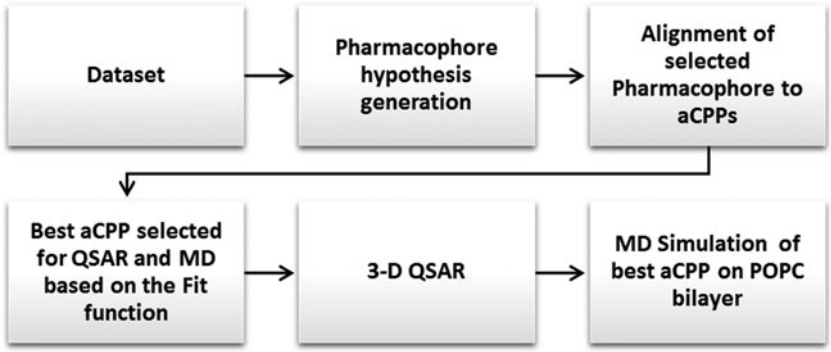

Figure 1. Workflow for identification of best aCPP for membrane penetration.

have a favourable safety profile. It is important in pharmacotherapy as an essential tool for therapeutic drug monitoring to ensure a greater therapeutic benefit without resulting in undesired toxicity (Tamargo, Le Heuzey, & Mabo, 2015). This study aimed to develop a QSAR model to validate the cell penetrating ability of aCPPs based on their TI. The TI was calculated by correlating the minimum inhibitory concentration (MIC) of the aCPPs to their effect on eukaryotic cells, with an indicative TI being calculated by relating the cell viability EC_{50} value to the MIC value. Pharmacophore hypothesis generation and 3D-QSAR were used to understand the cell penetrating ability of the aCPPs utilised based on their TIs. Finally, the validated pharmacophore model was used to identify the best aCPP and from the dataset obtained. The obtained hits were further examined based on the fit function, and the best fit was further tested using molecular dynamics to study its membrane penetrating ability. Molecular dynamic (MD) simulations are widely applied to understand the atomic-level information peptides structures (Agrawal & Skelton, 2016, 2018) and aCPP's interactions with the membranes (Arasteh & Bagheri, 2017; Mizuguchi & Matubayasi, 2018; Velasco-Bolom, Corzo, & Garduño-Juárez, 2017). Thus, an integrated approach comprising of pharmacophore modelling and MD simulation were employed to identify the best aCPPs with optimal membrane penetrating ability across a POPC bilayer membrane. POPC which is a phospholipid that is ubiquitous in cell membranes, contains a phosphatidylcholine (PC) component which provides a structural framework and functions as a permeability barrier (Koymans et al., 2015). POPC has also been found in numerous lipid mixtures used to mimic bacterial cell membranes (Raymonda, Almeida, & Pokorny, 2017). Therefore, in the context of molecular dynamics, this phospholipid serves as a good template for simulations studies to predict ligand–membrane interactions.

2. Materials and methods

2.1. Dataset

The data set was obtained from Park et al. (2009) and Bahnsen et al. (2013), with the TI being calculated and the synthesized peptides showcasing the potent MIC values against *S. aureus* (Bahnsen et al., 2013; Park et al., 2009). The obtained data was randomly divided into 21 training set compounds, with seven being reserved for a test set. The biological activities ($-\log IC_{50}$) of both datasets were similar, suggesting that the dataset was reasonable. The peptides' 3D structures which served as ligands were generated in Maestro 9.8 molecular modelling package from Schrodinger. Figure 1 describes the computational workflow conducted in this study.

2.2. PHASE methodology

PHASE 3.0 was used for pharmacophore-based alignment and utilized for the QSAR model development (Dixon et al., 2006). Default pharmacophoric features used to develop the pharmacophore model included a hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic (H), negative (N), positive (P) and aromatic ring (R). Five steps were used in the process of developing a pharmacophore model, which include ligand preparation, creating pharmacophore sites from a set of features, discovering common pharmacophore, scoring the hypotheses, and building of the QSAR model. The maximum and minimum number of sites was set to five to discover a common pharmacophore. The size of the box of the pharmacophore was set to 2 Å, with the top-ranking hypotheses selected for 3D QSAR analysis, for which grid spacing was 1 Å and the maximum partial least squares (PLS) was set to 3.

2.3. Pharmacophore hypothesis generation

PHASE is an important tool in the identification of 3D structural arrangements of the ligand functional groups, which are common and responsible for inducing biological activity (Kaur, Sharma, & Kumar, 2012). For site generation, the default pharmacophoric features were utilised. The variant AAHRR, for which all the compounds were matched, was searched to generate the best common pharmacophore hypothesis (AAHRR.114). The hypothesis AAHRR.114 was selected as most appropriate as it has the highest survival score (3.984) for common pharmacophore hypothesis, which gives the best alignment of the active ligands. This alignment also gives the fitness to all the inhibitors, while the best aligned ligand gives the maximum fitness. The evaluation of the newly formed common pharmacophore was achieved by comparing the experimental and the calculated activities for the training set molecules. Common

pharmacophore of significant statistical values was selected for molecular alignments.

2.4. Pharmacophore model validation

The aim of the pharmacophore generation was to develop a QSAR model that was statistically significant both internally and externally (Kaur et al., 2012), where the evaluation and predictability of the model being achieved by external validation. A scatter plot of experimental versus predicted activity for the training set showed a substantial linear correlation and a slight difference between the experimental and predicted activity. External validation was used to determine the efficacy of the model. The dataset obtained was separated into training and test set where validation of the model (AAHRR) for the test set was judged by the cross-validation coefficient (Q^2). R^2 was determined for the training set which depicted relevance of the model. The *F* value (which assesses the statistical significance) and Pearson-*R* (which measures the strength of the linear relationship between two variables) indicated greater confidence of the model, where a higher *F* value implies a more significant correlation and a Pearson-*R* value closer to 1 indicates a strong positive linear correlation. Standard deviation (SD) and Root-mean squared error (RMSE) were calculated which reflected good stability of the model. Model validation is an important step during pharmacophore design, as it determines the success, accuracy and reliability of the developed model (Meraj et al., 2013).

2.5. Molecular dynamic simulations

2.5.1. Peptide structure prediction

The 3D structure of "KLWKLWKKWLK" aCPP was predicted using PEP-FOLD server, which uses a de-novo approach for predicting peptide structure from amino acid sequences (Figure 2(B)). PEP-FOLD server uses a greedy algorithm driven by a coarse-grained force field for predicting the 3D model of a peptide (Shen & Maupetit, 2012).

2.5.2. Molecular dynamics simulations

POPC bilayer was constructed using the CHARMM-GUI membrane builder and contains a total of 128 lipid molecules (Wu et al., 2014) (Figure 2(C)). The peptide was placed more than 5 Å away from any lipid molecules of the upper leaflet of the bilayer. The Charmm36 force field was used for peptide and POPC bilayer system, which was solvated using TIP3P water model (Huang & MacKerell, 2013). The system contains a total of 7572 water molecules, and 5 Cl^- ions were added to neutralize the system. The system was energy minimized using the steepest descent algorithm, and 500 ps simulated annealing under the isobaric–isothermic (NPT) conditions was performed to equilibrate the water molecules around the lipid head group atoms (Bixon & Limn, 1966). The system was further equilibrated for 100 ps with canonical (NVT) ensemble, followed by 1000 ps with NPT ensemble and a 50 ns production run was performed in the NPT ensemble. The hydrogen bond lengths of peptide and lipid molecules were constrained using the LINCS algorithm (Hess & Fraaije, 1997). Particle mesh Ewald (PME) method was used for calculation of long-range electrostatic interactions (Darden et al., 2007). The van der Waals (vdW) and electrostatic interactions were calculated using a cut-off of 1.2 nm. The Parrinello–Rahman method was used for pressure coupling and the Nose–Hoover thermostat was used for temperature coupling (Braga & Travis, 2014; Parrinello & Rahman, 1995). The simulation was performed at a pressure of 1 bar and a temperature of 323 K using the GROMACS package (Hess & Fraaije, 1997).

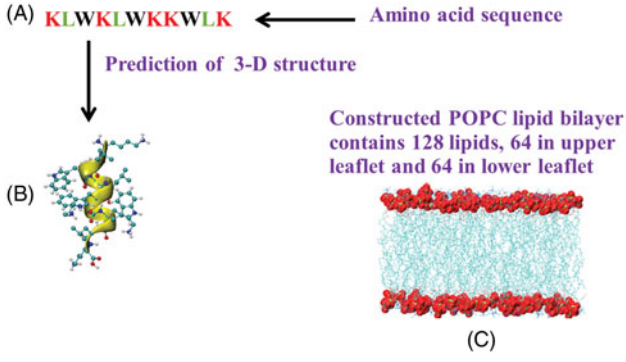

Figure 2. (**A**) Representation of the single amino acid code of the aCPP peptide. (**B**) Representation of the predicted 3-D structure of aCPP and (**C**) shows the constructed POPC lipid bilayer, P atoms have been shown in VdW sphere.

3. Results

Upon completion of the pharmacophore identification process, 65 variant hypotheses were generated. In this study, 28 aCPPs were used to predict activity using PHASE and fitness score determined (Table 1), with molecules 9, 10, 19, 20, 23 and 28 not being picked up by the system. The most appropriate pharmacophore model (AAHRR.114) to predict aCPP activity had a five-point hypothesis that consisted of two hydrogen bond acceptor (A), one hydrophobic group (H) and two aromatic ring features (R), as shown in Figure 3. Pharmacophore hypothesis scoring values are shown in Table 2. Compound 25 had the best alignment on the pharmacophore AAHRR as shown in Figure 4, and the distance between the sites in the pharmacophore is shown in Figure 5. Alignment of both active and inactive molecules to the hypothesis AAHRR.114 is shown in Figures 6 and 7, respectively. A depiction of the cubes produced for the highest active molecule (compound 25) in the present 3D-QSAR is shown in Figure 8(A–E), where the blue cubes indicate favourable effect on activity and red cubes indicate unfavourable effect. For the 3D-QSAR model generation, the PHASE descriptors were considered as independent variables and the activity values as dependent variables in deriving the 3D-QSAR models by the PLS regression method. The 3D-QSAR was evaluated by the Fisher test (*F*), correlation coefficient (R^2) and Pearson-*R*. Table 2 outlines the summary of the 3D-QSAR results. The statistical results of the model exhibited an R^2 value of .9016, RMSE = 0.5911, Q^2 = 0.5311, SD = 0.2072, variance ratio (*F*) = 36 and Pearson-*R* = .847. The validation of the above model was achieved by predicting

Table 1. aCPPs for training and test set (Bahnsen et al., 2013; Park et al., 2009)

Entry	Peptide	Set	Therapetic index (TI)	Biological activity		PHASE predicted activity (−log IC_{50})	Fitness score
				MIC (μM) against *S. aureus*	(−log IC_{50})		
1	Penetratin	Training set	4.4†	64	1.806	1.44	0.32
2	penArg	Training set	2.3†	16	1.204	1.30	0.31
3	pen13	Training set	>3.3†	256	2.408	2.07	0.22
4	Pen13Arg	Training set	1.1†	32	1.505	1.86	0.23
5	Penshuf	Training set	0.3†	64	1.806	1.89	0.68
6	PenshufLeu	Training set	0.6†	16	1.204	1.37	0.76
7	PenshufLysLeu	Training set	1.0†	32	1.505	1.48	0.76
8	PenshufArgLeu	Training set	0.3†	16	1.204	1.33	0.76
9	WR8	Training set	10.1†	44	ND	ND	ND
10	Tat13	Training set	<3.3†	>256	ND	ND	ND
11	K8W3	Training set	200*	4	0.602	0.59	2.51
12	KL7W3	Training set	200*	4	0.602	0.53	2.97
13	K6L2W3	Training set	400*	2	0.301	0.51	2.98
14	K3L5W3	Training set	0.8*	4	0.602	0.54	2.99
15	K2L6W3	Training set	0.05*	32	1.505	0.58	2.97
16	R8W3	Training set	100*	8	0.903	0.81	2.46
17	R6L2W3	Training set	100*	4	0.602	0.66	2.50
18	O6L2W3	Training set	200*	4	0.602	0.56	0.72
19	O6X2W3	Training set	100*	4	ND	ND	ND
20	R6L2W3-D	Training set	200*	2	ND	ND	ND
21	Indolicidin	Training set	12.5*	4	0.602	0.53	0.35
22	PenLys	Test set	>3.9†	256	2.408	1.45	0.32
23	PenLeu	Test set	>15.6†	64	ND	ND	ND
24	pen13Lys	Training set	>3.9†	256	2.408	2.02	0.23
25	**K5L3W3**	**Test set**	**50***	**2**	**0.602**	**0.52**	**3**
26	K4L4W3	Test set	3.1*	2	0.602	0.53	2.99
27	R7LW3	Test set	200*	4	0.301	0.70	2.48
28	K6L2W3-D	Test set	100*	4	ND	ND	ND

ND, not determined.
†Values taken as such from the literature.
*Values calculated by dividing %cell viability by MIC against *S. aureus*.
[a]Represents peptide sequences: I, Isoleucine; R, Arginine; K, Lysine; M, Methionine; L, Leucine; Q, Glutamine; W, Tryptophan; F, Phenylalanine; O, Pyrrolysine; N, Asparagine; P, Proline; X, Any amino acid.

Figure 3. Pharmacophore hypothesis (AAHRR). Purple sphere—A, green sphere—H and brown ring—R.

Table 2. Summary of 3D-QSAR results

PLS statistical parameters	Results	PLS statistical parameters	Results
Number of molecule in training set	18	R^2	.9016
Number of molecule in test set	6	Q^2	0.5311
Number of PLS factors	3	Standard deviation (SD)	0.2072
Root-mean squared error (RMSE)	0.5911	Variance ratio (*F*)	36
		Pearson-*R*	.847

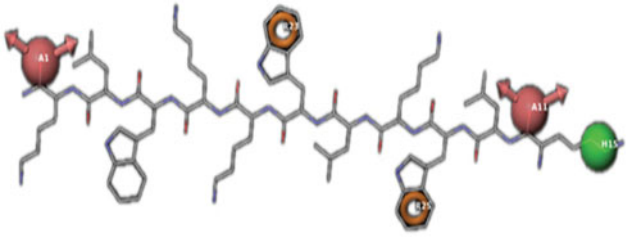

Figure 4. The best common pharmacophore hypotheses for compound 25.

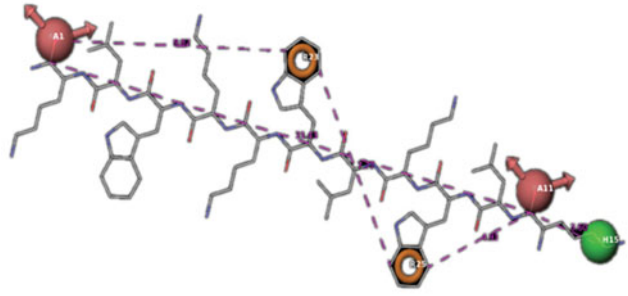

Figure 5. The pharmacophore hypothesis showing distance between the pharmacophoric sites of compound 25.

the biological activities of the training set molecules, as indicated in Table 1. To further confirm the 3-D QSAR results, compound 25 which was considered as the best aCPP based on the fit function was further analysed for its membrane penetrating ability by MD simulation. Figure 9 shows the representative images from the simulation at different time points. The time evolution of the distance of each residue of the aCPP from the POPC bilayer showed that the peptide formed strong interactions with the POPC bilayer at two different time points, one at approximately 28 ns and remained bound until 50 ns (Figure 10). The next time period we observed the peptide to be bound at approximately 109 ns and remained bound until 200 ns. During this time, we observed Lys-1 inserted into the PO4 groups of the membrane (Figure 11). To further observe the closest residues during the binding, average distances for each residues for

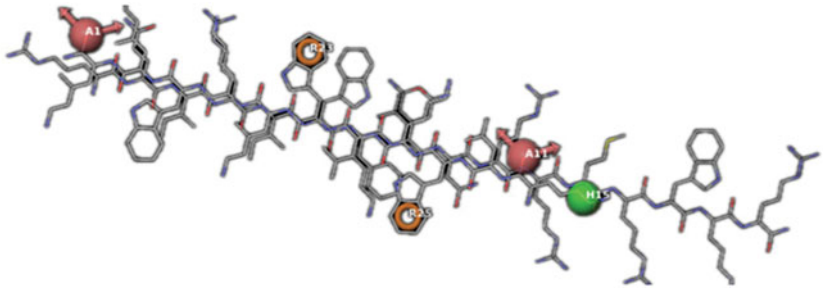

Figure 6. Alignments of active molecules.

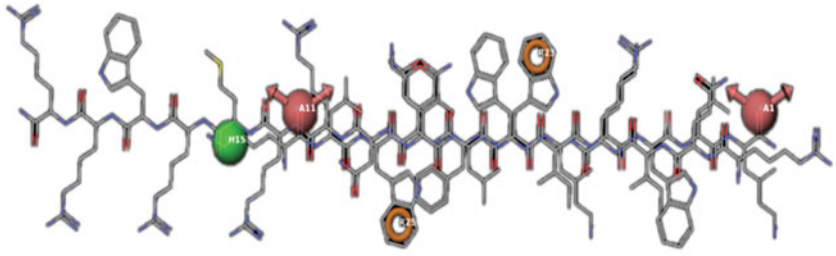

Figure 7. Alignments of inactive molecules.

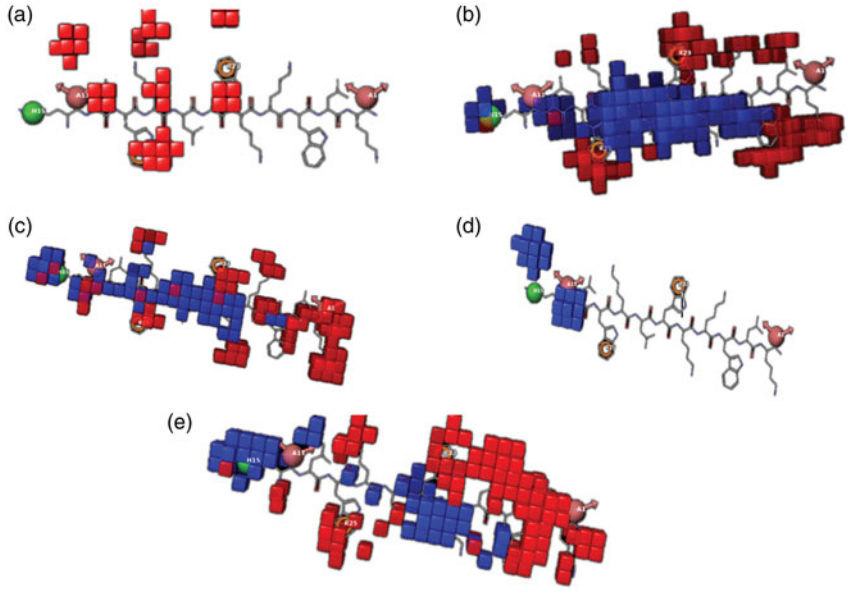

Figure 8. The 3D QSAR model based on compound 25 illustrating (**A**) hydrogen bond donor groups, (**B**) hydrophobic groups, (**C**) Electron withdrawing groups, (**D**) other effects and (**E**) combined effects.

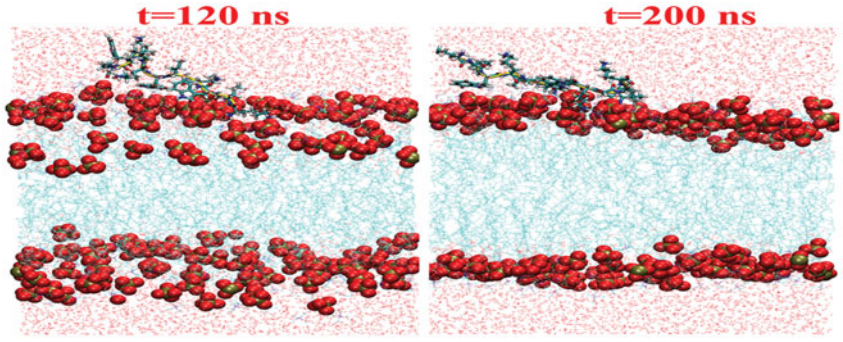

Figure 9. Two representative images of aCPP-POPC lipid bilayer interaction showing interaction, one at 120 ns and at 200 ns. PO_4 atoms of bilayer have been shown in VDW representation and aCPP peptide has been shown in cartoon representation.

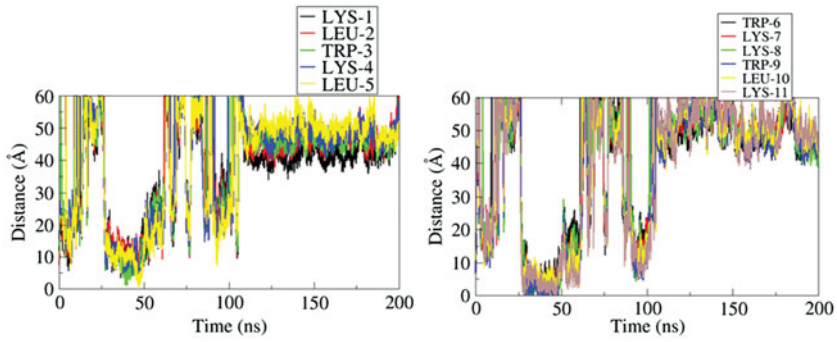

Figure 10. Time evolution of centre of mass (COM) distance between each residue of peptide with the phosphate (PO_4) group of upper leaflet.

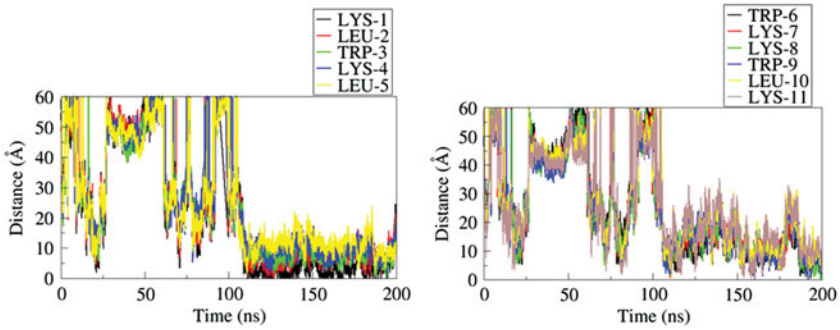

Figure 11. Time evolution of centre of mass (COM) distance between each residue of peptide with the phosphate (PO_4) group of lower leaflet.

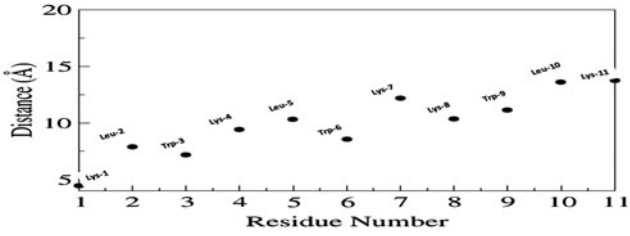

Figure 12. Average distance for the last 20 ns for each residue.

the last 20 ns (180–200 ns) were calculated (Figure 12). As in our simulation we have used periodic boundary conditions (PBC), we observed the peptide to interact with both the lower and upper leaflet of the membrane. Therefore, we have calculated the COM between both leaflets and peptide separately. The last 20 ns (180–200 ns) average distances of each residues (Figure 12) reveal that the Lys-1, Leu-2, Trp-3 and Trp-6 were the closest to the membrane during the binding. The MD simulations therefore confirmed the interaction of compound 25 with the POPC bilayer.

4. Discussion

In this study, pharmacophore and QSAR model development was performed by PHASE. Several hypotheses were generated, with the top-ranking ones being subjected to 3D-QSAR analysis, where the grid spacing was set to 1 Å and the maximum PLS factors set to 3. Partial least squares (PLS) is a statistical method which is used to find the relationship between two matrices and/or finds a linear regression model between variables. The first hypothesis, AAHRR.114, was found to be the best, being characterized by a high survival score (3.984) and an R^2 of .9016. AAHRR 114 featured two hydrogen bond acceptor (A), one hydrophobic group (H) and two aromatic ring features (R). A R^2 value greater than .5 and close to 1 confirmed the models predictive ability for the compounds(Frimayanti, Yam, Lee, & Othman, 2011). Q^2 measures the robustness and predictive power of the QSAR model and must be $\geq$0.5 but lower that the R^2 value (Veerasamy et al., 2011). Compound 25 was the best and comprised of the amino acids, lysine (K-5), leucine (L-3) and tryptophan (W-3), with a positive net charge of +5 and a total hydrophobic ratio of 57%. There are several reports on peptides which are rich in the amino acids lysine, leucine and tryptophan being specific for bacterial membrane penetration that support our findings (Jin et al., 2016; Kim et al., 2013; Nguyen et al., 2010; Su, Doherty, Waring, Ruchala, & Hong, 2009). The positive charge allows the aCPP to interact with the negatively charged bacterial cell membrane whereas the hydrophobicity allows the aCPP to penetrate deeper into the hydrophobic core of the bacterial cell membrane causing membrane lysis and pore formation (Chen et al., 2007). Figure 8(A–E) represents features responsible for activity (blue cubes) and those which attenuate activity (red cubes). Features responsible for activity are specifically represented in Figure 8(B and C) whereas Figure 8(A and D) show features that attenuate activity. Substitutions at the domains represented by the red cubes with amino acids which will increase the aCPP's cationicity will confer favourable activity (Faraz, Verma, & Akhtar, 2016; Mehta, Khokra, Arora, & Kaushik, 2012).

The MD simulation which ran for 200 ns showed spontaneous insertion of the aqueous phase aCPP into the upper and lower leaflet region of the lipid bilayer. The last 20 ns of

the simulation revealed that the Lys-1, Leu-2, Trp-3 and Trp-6 were the closest to the membrane during the binding, revealing the importance of these amino acids in membrane penetration based on their net charge. The importance of charged residues in membrane penetration is well known (Futaki, 2005; Herce & Garcia, 2008; Nakase, Takeuchi, Tanaka, & Futaki, 2008; Persson, Esbjo, Gokso, Lincoln, & Norde, 2004). It could be significant since we observed in our study that the C terminal region (residue 6–11) has more positive charge compared with the N terminal region (residue 1–5), which assisted the C terminal region to insert into the bilayer. Charged Lys side chains have been seen to possess high p*K*a values and this allows them to form strong electrostatic interactions with membranes, which leads to membrane penetration (Li, Vorobyov, & Allen, 2013). Trp is particularly prevalent among naturally occurring antimicrobial peptides and can strongly interact with hydrophobic membrane components, thus leading to increased antimicrobial activity (Bi, Wang, Dong, Zhu, & Shang, 2014; Li et al., 2013). The MD studies confirmed the importance of Trp and Lys residues in interacting with the POPC lipid bilayer, which allows the peptide to penetrate the model membrane.

5. Conclusion

This study presented the ligand-based pharmacophore and 3-D QSAR model which gave important structural-binding features of aCPPs acting as *S. aureus* antagonists based on their TI. Pharmacophore modelling compares activities with the 3-D arrangement of various physicochemical features. The hypothesis AAHRR.114 was found to be the most appropriate pharmacophore model to determine the best compound with potent activity. AAHRR.114 contains two hydrogen bond acceptors, one hydrogen bond donor, two hydrophobic regions, and one aromatic ring features. The AAHRR.114 model was able to predict the activity of the aCPPs, and the validation results provide additional confidence. The best aCPP was found to be compound 25, with a fitness score of 3 and the PHASE predicted activity of 0.52 being better that the experimental activity (0.602). The proposed 3D-QSAR model AAHRR.114 was useful in estimating the antimicrobial potential and cell penetrating ability of aCPPs, confirm the relationship between the TI and aCPPs, where aCPPs with a higher TI showed good activity and PHASE was also able to predict the possible descriptors responsible for activity. This QSAR approach in analysing aCPP cell penetration, by observation of its TI, can be used for future studies to explore specific descriptors responsible for biological activity that also accounts for cell penetration. Membrane penetration study using MD simulation also revealed the aCPP-POPC bilayer interaction, resulting in the aCPP insertion across the bilayer. The combination of these two computational studies will also lead to the rational design of optimal and novel aCPPs for therapeutic activity and for peptide-conjugate delivery.

Disclosure statement

No potential conflict of interest was reported by the author.

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CHAPTER 4, Experimental Paper

Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

4.1 Introduction

This chapter addresses Aim 2, Objectives 1 – 6 and is a first authored experimental article that has been completed and communicated to the journal of submitted to European Journal Pharmaceutics and Biopharmaceutics (impact factor 4.491), an ISI international journal (manuscript ID: EJPB_2018_1382). This research article focuses on the encapsulation of novel AMPs with vancomycin (VCM) and oleic acid (OA) in a liposomal system to target MRSA infections, and characterization and evaluation of bare novel AMPs activity in comparison to the encapsulated materials to enhance antimicrobial activity. Data from this chapter has also been presented in one conference:

- Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against methicillin-resistant *Staphylococcus aureus* (MRSA). Mbuso Faya[1], Calvin A. Omolo [1], Fernando Albericio[1], Beatriz G. de la torres[1], Heba A. Hazzah[3], Ruma Maji[1] , Pavan Walwaker[1], Chunderika Mocktar[1], Bongani Nkambule[2], Thirumala Govender[*,1] University of KwaZulu-Natal Nanotechnology Platform Workshop, 22nd November 2017. Oral Presentation

Supramolecular Lipidation of Novel Antimicrobial Peptides Enhances Antimicrobial Activity Against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Mbuso Faya*[,1], Heba A. Hazzah[3,4], Calvin A. Omolo[1], Nikhil Agrawal[1], Ruma Maji[1], Pavan Walwaker[1], Chunderika Mocktar[1], Bongani Nkambule[2], Fernando Albericio[3], Beatriz G. de la Torre[4], Thirumala Govender[*,1]

[1]Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa

[2]School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa.

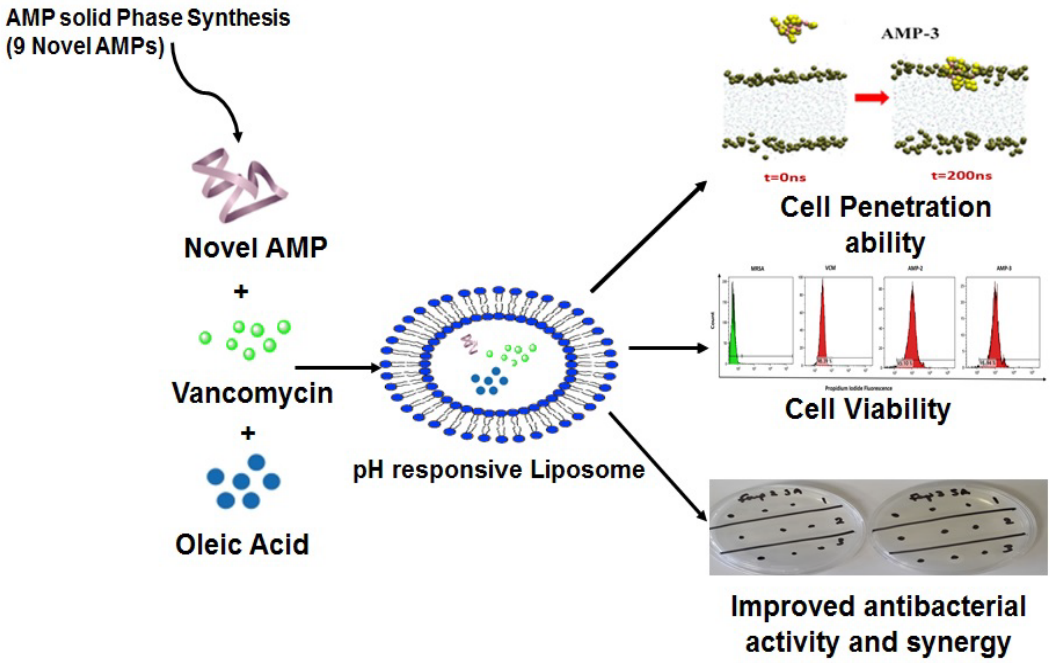
[3]Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa.

[4] KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal Durban 4041, South Africa.

*Corresponding author.

Email address: mbuso.faya@gmail.com/govenderth@ukzn.ac.za

Graphical Abstract



Abstract

Antimicrobial peptides (AMPs) have the ability to penetrate and transport cargo across bacterial cell membranes. These AMPs have been labelled as exceptional candidates to function in drug delivery, however their antimicrobial activity can be attenuated due to innate peptide resistance mechanisms found in bacterial cells. The aim of this study was to investigate the effectiveness of supramolecular lipidation of novel AMPs for enhanced MRSA activity. The supramolecular lipidation strategy was carried out through the formulation of liposomes by thin layer film hydration methodology, containing phosphatidylcholine, cholesterol, oleic acid (OA), novel AMP as well as vancomycin (VCM). Characterization of the AMPs and liposomes involved high performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) for peptide purity and mass determination. Dynamic light scattering (DLS) was used to determine size, polydispersity and zeta potential. TEM (surface morphology), broth dilution, flow cytometry (antibacterial activity), MTT assay, hemolysis and intracellular antibacterial studies were also determined. The size, PDI and zeta potential of the drug loaded AMP_2-Lipo-1 was 102.6±1.81, 0.157±0.01 and -9.81±1.69 respectively, while AMP_3-Lipo-2 drug loaded formulation corresponded to 146.4±1.90, 0.412±0.05 and -4.27±1.25, respectively at pH 7.4. However, in acidic pH for both formulations, we observed an increase in size, PDI and a switch to positive zeta potential, which indicated the pH responsiveness of our liposomal systems. *In vitro* antibacterial activity against *S. aureus* and MRSA revealed that liposomes had enhanced activity at pH 6 compared to pH 7.4. *In vitro* cytotoxicity results showed percentage viability ranging from 80-85% for all cell lines employed and both liposomes showed haemolysis of less than 1% at, which indicated its non-toxicity to red blood cells (RBCs). The therapeutic benefit conferred by AMP_2-Lipo-1 and AMP_3-Lipo-2 was evaluated on MRSA infected HEK-293 cells. From this intracellular study it was observed that the liposomal formulations had good activity without affecting the cell line The AMP_2-Lipo-1 and AMP_3-Lipo-2 liposomal formulation were seen to possess better activity than their parent AMPs. This showed that the supramolecular assembly can potentially be used to enhance activity and penetration of AMPs thereby improving the treatment of bacterial infections.

Keywords: AMPs, supramolecular lipidation, membrane insertion, MRSA, liposomes, pH response

1. Introduction

The emergence of drug resistance over the years has prompted a need for novel compounds to combat infectious disease[1]. Treatment options targeting MRSA infections has proven to be challenging due to limited viable therapeutic routes that bypass resistance [2]. Resistance to antibiotics is generally linked to the failure of antibiotic treatment, where formerly curable infections are now part of the leading cause of high morbidity rates and escalation of healthcare costs (WHO, 2014)[4][5]. Over the years there has been a rise in deaths caused by MRSA infections and this is prevalent in both community and in nosocomial settings[6][7]. This occurrence has led to the over utilisation of VCM as it has been deemed to be the last remaining resort to treat MRSA [8]. The prevalent use of VCM has resulted in the emergence of Vancomycin resistant *Staphylococcus aureus* [9][10]. Even with the great prospects antibiotics have offered towards therapy due to bacterial infections, several limitations have been observed with the current dosage forms. These include insufficient concentration at target sites, poor antibiotic penetration, side effects and poor compliance[11]. These limitations have led to resistance of antibiotics by microorganisms causing infections on a global scale[12]. The application of fatty acids such as oleic acid (OA) in targeting pathogenic microorganisms have also been to be effective[13]. However *in vitro* studies have shown that the activity of OA is augmented when incorporated into liposomes compared to free OA[14].

AMPs have been seen to possess antimicrobial properties and could possibly mitigate the limitations of conventional antibiotics[15][16][17][18]. The warranted attraction towards AMPs is due to their capability to alter the conformational integrity and penetrability of bacterial cell membranes by triggering membrane disruption, fusion, or translocation[19]. These AMPs tend to be amphipathic with a high positive zeta potential due to the distribution of charged amino acids in their chain[20]. They also readily accumulate at the lipid membrane interface, which can lead to membrane partitioning or cellular internalization[21][22]. They also have been reported to confer little haemolysis making them ideal candidates in drug delivery as well as novel antibiotics[23][24][25]. Another factor that that adds to the disease burden is the proliferation of intracellular infections[26]. This is caused by poor delivery of antibiotics into host cells, resulting in inadequate bacterial clearance which has the potential to cause bacteria to develop resistance mechanisms[27]. A study conducted by Schlusselhuber et al., 2013 reported on an α-helix AMP (eCATH1) and its ability to confer bactericidal effects against *Rhodococcus equi* in macrophages[28]. Similarly, Brinch et al. (2010) reported on the effectiveness of the AMP, NZ2114 effectiveness against intracellular *S. aureus* in human and mouse monocytes[29]. All these facts highlight the need for the identification of novel and new

AMPs to widen the pool of available antimicrobial agents that are effective against sensitive and resistant MRSA.

The delivery of AMPs or antibiotics into specific target sites within the body is one of the main challenges for successful therapeutics[30]. Nano drug delivery systems can enhance delivery of AMPs by protecting then against degradative enzymes and conferring high solubility and carrier capacity as well as efficient delivery at target site[31][32]. Specifically pH responsive systems that can target infection sites at acidic conditions offer great exploits in controlling the spread of infection[33]. At acidic pH, the nano system swells and eventually bursts to release the inner contents at the infection site[34]. A widely used nano system for AMPs involves the application of liposomes which are sphere-shaped vesicles which possess at least one lipid bilayer[35]. Due to their biocompatibility and biodegradability, they have been broadly used in drug delivery investigations and found to reduce toxicity and prolong drug half-life[36][37][38]. Currently, liposomes have been used to encapsulate AMPs and/or antibiotics for their delivery at desired target sites[30][39]. In both cases this results in higher antimicrobial activity and sustained release with offers better therapeutic potential compared to non-encapsulated AMPs and antibiotics[40][41]. Co-delivery of AMPs with other agents such as antibiotic drugs and non-drug antimicrobial agents may potentiate antibacterial activity by multiple mechanisms of action targeting the bacteria and thereby reducing the ability of bacteria to develop resistance[42]. Indeed, co-delivery of antibiotics such as Vancomycin with agents such as linolenic acid have improved activity against bacteria such as MRSA and *S. aureus*[13]. Thus far, there is no report on co-delivery of AMPs with antibiotics drugs and other non-drug antimicrobial agents in a single delivery system to target MRSA.

One of the current strategies used to target resistant microorganisms is supramolecular lipidation which involves the non-covalent assembly of different moieties into a system [43]. This technique has been widely used in drug delivery to improve biological activity and to increase affinity for lipid membranes[44]. The application of supramolecular lipidation would offer great prospects in lowering therapeutic doses without affecting the integrity of the designed AMPs and also enhances the activity of AMPs[45]. In the context of this research, we propose a supramolecular lipidation assembly, employing three different agents encapsulated in a liposome without any covalent bonding to target MRSA infections. To our knowledge, o such approach has been described in literature for a lipid-based AMP delivery system.

In this study we propose the encapsulation of novel AMPs with VCM and oleic acid in a liposomal system to target MRSA infections. Therefore, the aim of this study was to design and synthesize novel AMPs and employ them to decorate vancomycin and oleic acid containing liposomes to achieve pH responsiveness. In this current research we have designed nine Novel AMPs and two of them (EKKRLLKWWR and KWWKLLRKKR) were selected based on their antimicrobial activity to predict their bacterial membrane penetrating ability through molecular dynamics and flow cytometry. These AMPs were further encapsulated in a liposomal formulation containing OA and vancomycin at the core to form a pH responsive drug delivery system for infection targeting and treatment of intracellular infections, leading to enhanced antimicrobial activity. We envisage that at basic pH, the AMPs will form ion pairing with the negatively charged OA incorporated in the liposomes while at acidic pH the OA gets protonated and the ion pair is broken leading to pH responsiveness. To the best of our knowledge, this is the first report of such a technique to improve AMPs antimicrobial activity. This assembly is expected to enhance activity of the AMPs and offer synergistic properties with the encapsulated moieties. *In vitro* and *in silico* findings from the synthesis of the AMPs, liposomal decoration and antimicrobial activity are herein reported in this paper.

2. Experimental section

2.1 *Materials and Methods*

Rink amide paramethylbenzhydrylamine (MBHA) resin and 9 fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Iris Biotech, Germany. Other reagents used for peptide synthesis included trifluoroacetic acid (TFA; Sigma), piperidine (Merck), DIC (purchased from GL Biochem), oxyma (Luxemburg BioTechnologies) and dimethylformamide (DMF peptide synthesis grade; Sigma). All other reagents were of analytical grade. Mueller Hinton Agar (MHA) (Biolab, South Africa), Nutrient Broth (Biolab, South Africa), Nutrient Agar (Biolab, South Africa) and Mueller Hinton Broth 2 (MHB) (Sigma-Aldrich, USA) were used in the antibacterial testing studies and tested against *Staphylococcus aureus* (ATCC 25922) and MRSA (ATCC BAA-1683^{TM}). Cholesterol, oleic acid, phosphatidylcholine (Sigma-Aldrich, USA) as well as VCM (Sinobright Import and Export Co., Ltd. China) were used in the formulation of liposomes. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Merck Chemicals (Germany). Propidium iodide dye cell viability kit was purchased from Thermofisher (USA).

2.2 *AMP Design, Synthesis and purification*

2.2.1 Peptide design

The AMPs were designed using a data filtering technology (DFT)[46] (**Figure 1**). A set of antimicrobial peptides having gram positive activity were selected and mutants were generated (APD online tool). A total of 86 AMPs made up our database. From our database, we limited peptide length to 10 amino acid residues and a charge of ≥ +4. Frequency of amino acids, structure type and hydrophobicity were considered and a limit of hydrophobic percentage was set to range between 40% to 60%. Another important filter, Motif was considered which refers to a cluster of amino acid residues that occur frequently in natural AMPs. From this filtration technology, it was observed that the frequency of LL, KK and WW occurred frequently in the selected AMPs. The novel peptides were checked on APD on-line tool for novelty as well as charge, hydrophobicity. The best designed peptides were also checked for cell penetrating ability (*CPPpred on-line tool*) as well as haemolytic activity (*HemoPI on-line tool*) [47].

2.2.2 *Synthesis and purification of peptides*

The designed peptides were synthesised by solid phase peptide synthesis (SPPS) on Rink amide MBHA resin. All syntheses were carried out under microwave (MW) conditions using a CEM Liberty Blue system by standard Fmoc/*t*-Bu methodology by means of DIC/Oxyma Pure™ as coupling reagents. Peptides were prepared in a 0.1 mmol scale and a 5-fold excess of reagents were used. A solution of 20% piperidine in DMF was used for the Fmoc removal in each step. To obtain fluorescent peptides to one half of the peptidyl resin 5(6)-carboxyfluorescein was coupled using 10 equiv and equimolar amount of DIC/Oxyma Pure™. All peptides (Fluorescent and not) were cleaved and final deprotected by treatment with TFA-TIS-H_2O (95:2.5:2.5) for 1 h at room temperature. Thereafter precipitation was carried out by addition of chilled diethyl ether, taken up in water or 10% acetic acid. Crude peptides were purified by semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC). Purity was confirmed by analytical high-performance liquid chromatography (HPLC) and characterization by liquid chromatography mass spectrometry (LCMS).

2.3. Antibacterial activity

2.3.1 Determination of Minimal inhibitory concentration (MIC)

Briefly, the bacterial cultures were grown in Mueller–Hinton Broth, with (MHB) appropriate dilutions made to achieve 5×10^5 colony forming units per mL (CFU/mL) of bacteria. Serial

dilutions of the plain AMPs, AMP_2-Lipo-1, AMP_3-Lipo-2 and bare VCM were carried out in MHB broth and incubated with bacterial cultures containing 5×10^5 colony forming units per mL (CFU/mL) for 18 h in a shaking incubator at (1000 rpm) 37° C. 10 µL of the serial dilutions were spotted on Mueller–Hinton Agar (MHA) plates and incubated for a further 18 h. The minimum concentration at which no visible bacterial growth was observed and was considered to the MIC and cell viability was evaluated via flow cytometry.

2.3.2 Intracellular studies on MRSA infected HEK-293 cell line

Briefly, HEK 293 cells were seeded in 96-well plates at a density of 5×10^4 cells/ml per well for 24 h in DMEM medium containing 10% DMEM without any antibiotics. Overnight culture of MRSA was washed thrice with centrifugation at 3000 rpm at 4 ºC, resuspended and diluted with DMEM medium at a cell density of 10^{12} cells/ml. HEK-293 cells were infected with MRSA at a multiplicity of infection (MOI) of 100. After 2 h of incubation, the infected cells were washed 7 times with DMEM medium containing 10% FBS to remove extracellular bacteria. The last medium for washing was plated on MHA plate to make sure all extracellular cells had been removed. In each well, VCM loaded AMP_2-Lipo-1(7.81 µg/mL) and AMP_3-Lipo-2 (3.9 µg/mL) and bare VCM (3.9 µg/mL) were added at concentrations of 5 times the MIC and untreated cells were incubated for 22 h. Thereafter, the cells were lysed triton X for 10 min in distilled water. The suspensions were serially diluted and spread on MHA agar plates to determine the number of viable MRSA by colony counting, where the colony forming units (CFU) were calculated by the following equation;

$$\mathrm{CFU/mL} = \frac{\text{number of colonies x dilution factor}}{\text{volume of the culture plate}}$$

2.4 Cell penetration studies

2.4.1 Molecular dynamics simulation

The 3D-structure of AMP-2 and AMP-3 were predicted using the PEP-FOLD server[48]. The server uses a de-novo method for predicting peptide structures from amino acid sequences and a greedy algorithm driven by a coarse-grained force field for predicting the 3D model of a peptide[49][50]. Charmm-GUI martini maker was used to convert the all-atom peptides to coarse-grained model[51]. POPC bilayer was constructed using the CHARMM-GUI Martini maker bilayer builder option and constructed bilayer contains a total of 128 lipids, 64 lipids in each leaflet. The bilayer was equilibrated for 100 ns before using for the peptide-membrane simulations. The peptides were placed more than 25 Å from the top leaflet of the POPC bilayer.

The AMP-2 system contained the AMP-2, 3001 water molecules, POPC bilayer, and 4 Cl^- ions were added to neutralize the system. The AMP-3 system contained AMP-3, 27773 water molecules, POPC bilayer, and 6 CL^- ions were added to neutralize the system. The two systems were energy minimized using the steepest descent method[52] and further equilibrated for 500 ps. All simulations were performed at 310.15 K temperature and 1 atm pressure. The velocity rescale method[53] was used for temperature and the Parrinello-rahman[54] method was used for the pressure coupling. 1.0 ps time was used for temperature coupling and 12.0 ps was used for the pressure coupling and semi-isotropic pressure coupling type was employed. For VdW interactions. cut-off scheme was employed and Potential-shift-Verlet was used for vdw-modifier and 1.1 nm cut-off was used for rdw. For coulomb type, reaction-field scheme was used, and 1.1 nm cut-off used for r coulomb. The Leap-frog algorithm was used for the integration of Newton's equation of motion with an integration time of 20 fs. Martini version 2.2[55] was used for amino acids and Martini version 2.0[56] was used for lipids, water, and Ions. GROMACS simulation package[57] was used for simulations and each simulation was performed for 200 ns resulted in a total of 400 ns.

2.4.2 Cell membrane penetration with Flow cytometry

The membrane penetration of the two AMPs (AMP-2 and AMP-3) was evaluated on MRSA membranes using flow cytometry. Briefly, MRSA cells were first harvested at log phase, and then washed twice with PBS. The washed cells (5×10^5 CFU/mL were mixed with the labelled and non-labelled AMPs in PBS, and the non-labelled AMPs were incubated with PI solution (5 µL) for 30 min at RT. 50 µL of each sample mixture was transferred into sample vials with each tube containing 350 µL of the sheath fluid and vortexed for 1 min. The cell-penetrating efficiency of both AMPs was also investigated by FACScan analysis, via the influx of carboxyfluorescein-labeled AMPs into bacterial cells. The PI fluorescence was excited by a 488-nm laser and collected through a 617 nm bandpass filter (red wavelength). The untreated MRSA cells were considered to be the negative control. The BD FACSCANTO II (Becton Dickinson, CA, USA) equipment was used for flow cytometry. Instrumentation settings included sheath fluid flow rate of 16 mL/min, a sample flow rate of 0.1 mL/min. Data with fixed cells were collected using a flow cytometer software (BD FACSDIVA V8.0.1 software [USA]). The voltage settings used for fluorescence-activated cell sorting (FACS) analysis were: 731 (forward scatter [FSC]), 538 (side scatter [SSC]) and 444 for PI. The bacteria were initially gated using forward scatter, and cells of the appropriate size were then gated and at

least 10,000 cells collected for each sample in triplicate. To avoid any background signals from particles smaller than the bacteria, the detection threshold was set at 1,000 in SSC analyses.

2.5 *In vitro* Cytotoxicity

Adenocarcinomic alveolar basal epithelial cells (A 549), HEK-293 and HeLa cell lines were employed for cytotoxicity studies. The cell lines were grown exponentially at 37°C in a humidified atmosphere of 5% CO_2. The AMPs were dissolved in milli-Q water, and dilutions of concentrations of 20, 40, 60, 80 and 100 µg/mL were prepared. The three cell lines were seeded equivalently (2.5×10^3) into 96-well plates and incubated for 24 h. The wells were replenished with fresh culture medium (100 µL per well) together with the appropriate concentration of the test solutions to obtain the final concentrations. The positive control comprised of wells that contained cells with culture medium only, while the negative control contained wells with culture medium only excluding cells. After 48h incubation, the culture medium and test materials were removed and replaced with 100 µL of fresh culture medium and 100 µL of MTT solution (5 mg/mL in PBS) in each well. After 4 h of incubation, the media and MTT solution was removed, and solubilization of MTT formazan was achieved by adding 100 µL of dimethyl sulfoxide. The optical density of each well was measured on a microplate spectrophotometer (spectrostar nano, Germany) at an absorbance wavelength of 540 nm. All the experiments were performed with six replicates. The percentage cell viability was calculated as follows.

$$\% \text{ Cell viability} = \left(\frac{\text{A549 nm treated cells}}{\text{A549 nm untreated cells}}\right) X\ 100\%$$

2.6 *In vitro* Haemolysis

A previously described method was used for the determination of percentage haemolysis[58]. Briefly, freshly collected sheep blood was washed three times with autoclaved phosphate buffer saline (PBS, pH 7.4) by centrifugation at 2800 rpm for 5 min. Plain AMPs (AMP-2 and AMP-3) and liposomes (AMP_2-Lipo-1 and AMP_3-Lipo-2) were diluted with the PBS to a concentration ranging from 0.025 to 0.2 mg/ml for each sample. The RBC suspension (0.2 ml) was added to 1.8 ml of each sample and left for incubation at 37 ◦C for 30 min. Thereafter the samples were then centrifuged at 3000 rpm for 10 min. Spectrophotometric readings of the supernatant of each sample at different concentrations were taken for the determination of hemoglobin release. To obtain 0% and 100% haemolysis, 0.2 ml of RBC suspension was added

to 1.8 ml PBS and distilled water, respectively. The degree of haemolysis was calculated by the following equation:

$$\%\ \text{Haemolysis} = \left(\frac{\text{ABS-ABS}_0}{\text{ABS100-ABS}_0}\right) X\ 100\%$$

where ABS_{100} and ABS_0 are the absorbances of the solution at 100% and 0% haemolysis, respectively.

2.7 Preparation and characterization of liposomes

2.7.1 Preparation of liposomes

Liposomes were prepared by a thin layer film hydration method[59]. Briefly, liposomes were prepared using phosphatidylcholine (PC), cholesterol, oleic acid (OA), AMP and a fixed amount of drug, where blank formulation was without the presence of the drug. The lipid mixture was dissolved in 5 mL of chloroform and the solvent evaporated using a rotary evaporator to obtain the thin film and further dried overnight in a desiccator. The dried lipid film was rehydrated with 5 mL of water in the presence of VCM and the resulting lipid suspension was vortexed for 1 min and followed by probe sonication for 7 mins at 30% amplitude to produce the desired liposomes (AMP_2-Lipo-1 and AMP_3-Lipo-2).

2.8 Characterisation of the decorated liposomes

2.8.1 Size, Polydispersity Index (PI), Zeta Potential (ZP) and morphology

The size, PDI, and ZP of the liposomes were analyzed by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK), with all measurements being performed in triplicate. The morphology was examined using high resolution transmission electron microscopy on a Jeol, JEM-1010 (Japan) transmission electron microscopy (HRTEM). The liposomes were diluted appropriately then mounted onto the surface of a copper grid, and the excess sample blotted off using a filter paper, then dried at ambient temperature. The images were captured at an accelerating voltage of 200 kV.

2.8.2 Entrapment efficiency (% EE)

Ultrafiltration method was used for the determination of the entrapment efficiency. 2 mL of the liposomal formulations were placed in Amicon® Ultra-4 centrifugal filter tubes (Millipore Corp., USA) of 10 kDa pore size and centrifuged at 3000 rpm at 25 °C for 30 min. The amount of free VCM in the filtrate was analysed by HPLC (Shimadzu, Japan) analysis, with a UV

detection wavelength of 230 nm. The mobile phase consisted of a mixture of 6.8mg KH_2PO4 dissolved in 1L H_2O and Methanol (79:21 v/v), pumped through a Nucleosil 100-5 C18 column (150 mm X 4.6 mm internal diameter) at a flow rate of 1 mL/min, with an injection volume of 100 µL. The linear regression coefficient (R^2) of 0.9997, was used to obtain the unknown concentrations values. The % EE was calculated using the following equation:

$$\%EE = \left(\frac{\text{Weight of VCM in nanoparticles}}{\text{Weight of VCM added}}\right) X\ 100\%$$

3. Results and Discussion

3.1 Design, synthesis and characterisation of AMPs

AMP screening and design:

AMPs were designed using the module CellPPD in the antimicrobial database (APD)[60] (**Figure 1**). The design strategy allowed for the placement of LL, KK, WW flanked by charged amino acids for the first 3 sequence, followed by placement of LL, KK, WW motifs interchangeably flanked by polar amino acids for the next three sequences and lastly flanking the motifs interchangeably by hydrophobic amino acids to total a sequence chain of 10 amino acid residues. This *in silico* method was developed to accelerate the prediction and design of optimal cell penetrating peptides (CPPs). CellPPD which is a support vector machine (SMV)-based model, allows for the generation of all possible single mutant analogues and predicted whether they were cell penetrating or not. Furthermore, all the important physico-chemical properties like net charge, hydrophobicity, structure type, molecular weight were estimated. The net charge of all AMPs ranged between + 4 to + 7 which is an important factor for interacting with the negatively charged bacterial membrane. The hydrophobic percentage of the AMPs ranged between 40-60%. This is an important determinant of their distribution between the lipid bilayer of the bacterial membrane and the aqueous core. The AMPs also showed α-helix structural conformation, with the exception of AMP-4 and AMP-5 which had a non-helixbeta and non-alphabeta conformation, respectively. Cell penetrating ability and haemolytic activity probability scores range between 0 and 1, where closer to 1 meant a strong probability to penetrate and haemolytic whereas 0 being very unlikely to be cell penetrating and non-haemolytic (**Table 1**).

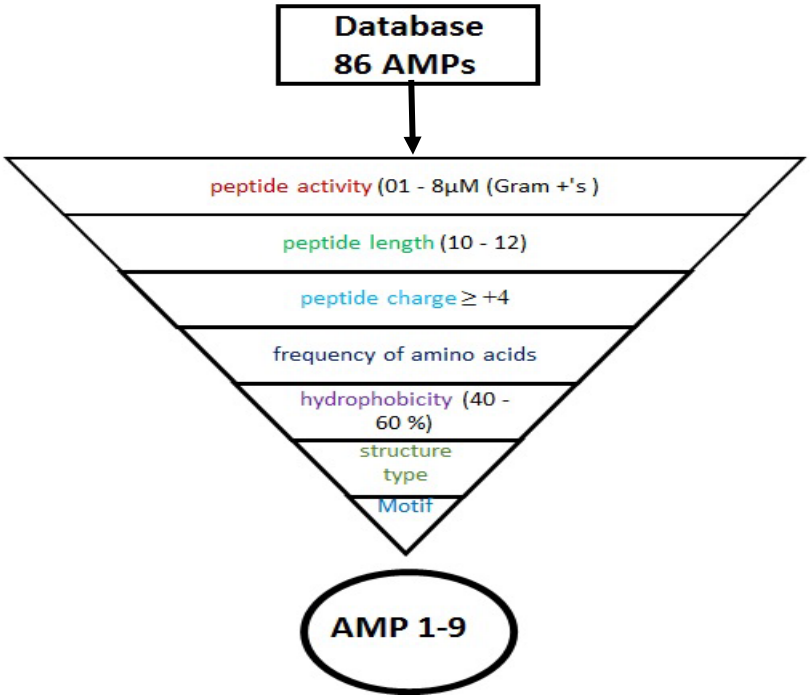


Figure 1: Database filtering technology with peptide filters[46]

AMP synthesis:

Peptides and fluorescein labelled peptides were synthesized by standard SPPS methodology. All of them were purified to homogeneity by semi-prep HPLC to purities higher than 85% and their identity was confirmed by mass spectrometry (appendix B and C). Sequences and theoretical properties are shown in **Table 1**.

Table 1: Novel Antimicrobial peptides

Name	Peptide Sequence	Net Charge	Hydroph-obicity	Structure	Mol weight (calc)	Cell Penetrating ability (*CPPpred*)	Haemolytic activity (*HemoPI*)
AMP-1	H-AALRKKDWWK-NH_2	+4	40%	helix	1300.5	0.908	0.50
AMP-2	H-EKKRLLKWWR-NH_2	+5	40%	helix	1441.8	0.878	0.50
AMP-3	H-KWWKLLRKKR- NH_2	+7	40%	helix	1440.8	0.877	0.56
AMP-4	H-QLLWKKRWWR- NH_2	+5	50%	nonhelixbeta	1498.8	0.868	0.48
AMP-5	H-KKKSLLRWWW- NH_2	+5	50%	nonalphabeta	1429.7	0.867	0.49
AMP-6	H-KWWRLLHKKQ- NH_2	+5	40%	helix	1421.7	0.823	0.48
AMP-7	H-KLLRKKFWWG- NH_2	+5	50%	helix	1360.7	0.809	0.53
AMP-8	H-RKKALLRWWV- NH_2	+5	60%	helix	1355.8	0.781	0.49
AMP-9	H-IWWFLLRKKR- NH_2	+5	60%	helix	1445.9	0.771	0.49

3.2 Antimicrobial activity Results

3.2.1 MIC determination

The bare AMPs had better activity towards Gram positive bacteria (*S. aureus* and MRSA) compared to Gram negative bacteria (*E.coli*). From this panel of AMPs, it was observed that AMP-2 and AMP-3 had better MICs compared to the rest of the AMPs. AMP-2 had an MIC of 125 µg/mL and AMP-3 MIC was 62.5 µg/mL against MRSA. In the case of *S. aureus,* AMP-2 MIC was 125 µg/mL where AMP-3 MIC was 31.25 µg/mL (**Table 2**). Based on these results, AMP-2 and AMP-3 were chosen to evaluate their antimicrobial potential in a liposomal nano system against MRSA. The first AMP-based liposomal formulation (AMP_2-Lipo-1) MIC values were 1.95 µg/mL and 3.9 µg/mL at pH 6.0 and at pH 7.4, respectively. AMP_3-Lipo-2 had an MIC of 0.48 and 3,9 µg/mL at pH 6.0 and at pH 7.4, respectively **(Table 4).** The MIC value for VCM was 15.65 µg/mL against MRSA at both pH. It is envisaged that the augmented activity of this supramolecular liposomal assembly over VCM could be due to the combination of the cationic AMPs offering a membrane permeation ability with the OA and VCM released at the target site in a sustained manner. The AMPs assist the VCM to kill bacteria by selectively forming pores on the bacterial membrane through disruptive “lytic” or pore-forming “ionophoric” mechanisms[61]. This action of AMPs allow the VCM to effectively bind to the C-terminal D-Ala-D-Ala of the pentapeptide of lipid II and the nascent peptidoglycan, inhibiting both transpeptidation and transglycosylation during the peptidoglycan synthesis[62]. Oleic acid (OA) also plays a major role in this liposomal assembly as it has been widely used in different formulations due to its non-toxicity, bio-compatibility, bio-degradability, permeation enhancement and displays antibacterial activity[63]. The fusion of the liposomes with the bacterial membranes cause the entrapped OA to be released into the bacterial membranes or the intracellular environment, resulting in higher local antimicrobial concentration and more efficient bactericidal activity while allowing VCM to disrupt the bacterial cell wall biosynthesis. Liposomal size plays an important role in their physicochemical properties and biological functions as a drug delivery vehicle[64]. Smaller liposomes (diameter, < 50 nm) are unstable and likely to agglutinate with others due to their high surface tension[65]. In contrast, larger liposomes (diameter, >200 nm) are usually stable but may have difficulty in cell penetration[66]. Liposomes with moderate size range will have relatively prolonged stability, preserve the capability to fuse with bacterial or cell membranes,

and possess good penetration ability[67][68]. These findings highlight the applicability of the supramolecular liposomal assembly to enhance the activity of AMPs against MRSA infections.

Antimicrobial studies of controls were also conducted to isolate and establish the antibacterial efficacy our two liposomes (**Table 4**). The bare AMP-2 (125 µg/ml) had better activity than AMP_2-lipo-1, encapsulating the AMP-2 only (AMP_2-Lipo-1+AMP-2). The liposomal formulation which encapsulated all three agents, (AMP_2-Lipo-1+OA+VCM) showed enhanced activity (3.9 µg/ml) compared to the bare AMP-2. In addition, AMP_2-Lipo-1 +VCM (3.9 µg/ml) had better activity than AMP_2-lipo-1+AMP and the AMP_2-Lipo-1+OA showed no activity.

A similar trend was observed with AMP-3 (62.5 µg/ml) which had better activity than AMP_3-Lipo-2 which encapsulated the AMP-3 only. However, the AMP_3-Lipo-2 + OA+VCM (3.9 µg/ml) showed enhanced activity than the bare AMP-3. The liposomal formulation containing the AMP-3 + VCM (7.8 µg/ml) had better activity than the formulation containing only the AMP-3. The AMP_3-Lipo-2 +OA+VCM (3.9 µg/ml) also showed good activity compared to the AMP_3-Lipo-2 +AMP-3 and the formulation containing the OA only, showed no activity. In both liposomes which encapsulated all three agents, (AMP, OA and VCM), the activity was better compared to the bare AMPs which validates the supramolecular approach in enhancing the activity of AMPs. Furthermore, pH responsiveness was evaluated for the final formulations (**Table 4)**. It was observed that both liposomes which contained a final mixture of AMP+OA+VCM, had had better activity at pH 6.0 compared to pH 7.4.

Table 2: Novel Antimicrobial peptide MICs

Peptide Name	Peptide Sequence	MRSA (mg/ml)	*S. aureus* (mg/ml)	*E. coli* (mg/ml)
AMP-1	AALRKKDWWK	N/A	N/A	N/A
AMP-2	EKKRLLKWWR	125	125	500
AMP-3	KWWKLLRKKR	62.5	31.25	250
AMP-4	QLLWKKRWWR	N/A	N/A	N/A
AMP-5	KKKSLLRWWW	N/A	N/A	N/A
AMP-6	KWWRLLHKKQ	500	500	500
AMP-7	KLLRKKFWWG	N/A	250	125
AMP-8	RKKALLRWWV	250	N/A	500
AMP-9	IWWFLLRKKR	500	N/A	500

Table 3: MICs of controls and liposomes

Peptide Name	MRSA (μg/ml) – pH 7.4
VCM	15.625
OA	1250
Bare AMP-2	125
Bare AMP-3	62.5
AMP_2-Lipo-1 + AMP-2	N/A
AMP_2-Lipo-1 + OA	N/A
AMP_2-Lipo-1 + VCM	3.9
AMP_2-Lipo-1 + OA+VCM	3.9
AMP_3-Lipo-2 + AMP-3	N/A
AMP_3-Lipo-2 + OA	N/A
AMP_3-Lipo-2 +VCM	7.8
AMP_3-Lipo-2 + OA+ VCM	3.9

Table 4: pH responsiveness

Peptide Name	MRSA (μg/ml) – pH 6.0	MRSA (μg/ml) – pH 7.4
AMP_2-Lipo-1	1.95	3.9
AMP_3-Lipo-2	0.48	3.9

3.2.2 Synergism studies

To further evaluate the effectiveness of this supramolecular assembly to enhance antimicrobial activity of novel AMPs, the fractional inhibitory concentration (FIC) was determined (**Table 5)**. The FIC is defined by the estimation of interaction between two or more drugs which are intended to be used in combination towards a specific target. The equations used to calculate the ΣFIC is shown below:

$$\Sigma\text{FIC} = \frac{\text{MIC of liposome}}{\text{MIC of VCM}}$$

Table 5: FIC of liposomas at 2 pH's

	pH 6.0	**pH 7.4**
$FIC_{AMP2\text{-}lipo\text{-}1}$	0.12	0.25
$FIC_{AMP3\text{-}lipo\text{-}2}$	0.03	0.25

From the described parameters (**Table 6)**, we have observed a synergistic effect for AMP_2-Lipo-1 (0.12 at pH 6.0 and 0.25 at pH 7.4) as well as for AMP_3-Lipo-2 (0.03 at pH 6.0 and 0.25 at pH 7.4), where the FIC index for both formulations were found to be ≤ 0.5 at both pH's.

Table 6: FIC index

Index	Results
≤0.5	Synergistic
>0.5-1	Additive
>1 but <2	Indifference
≥2	Antagonistic

3.2.3 Intracellular activity

The therapeutic benefit conferred by AMP_2-Lipo-1 and AMP_3-Lipo-2 was evaluated by determining their efficacies against intracellular MRSA[69][70][71]. A cell culture assay was established whereby HEK-293 cells were infected with MRSA for 2 h. After washing the extracellular bacteria, bare VCM or AMP_2-Lipo-1 and AMP_3-Lipo-2 nanoparticles were added to the cells. The ability of the nanoparticles to kill intracellular MRSA was determined by colony counting. No decrease in the CFU counts of MRSA cells was observed with untreated HEK-293 cells as well as with the application of bare VCM. AMP_2-Lipo-1 and AMP_3-Lipo-2 had lower CFU counts compared to VCM. AMP_3-Lipo-2 which initially had better MICs showed a significant decrease in CFU counts at 5 times the MIC (**Figure 2**). Even though MRSA is thought be an extracellular pathogen, it can invade a variety of cell types where it escapes from the endosomes/phagosomes and proliferates within the cytoplasm. Due to the acidic nature of the phagosomes, it is thought that the liposomes broke down to release the entrapped AMP and VCM, which led to the observed decrease in CFU counts. Intracellular MRSA infections may contribute to persistence and relapses of infection after treatment, due to the poor penetration of antibiotics in the intracellular compartments[72]. Using the human embryonic kidney HEK-293 cells we demonstrated that the liposomal assembly can be delivered within the intracellular compartment more effectively than VCM and would therefore be more effective in killing intracellular MRSA.

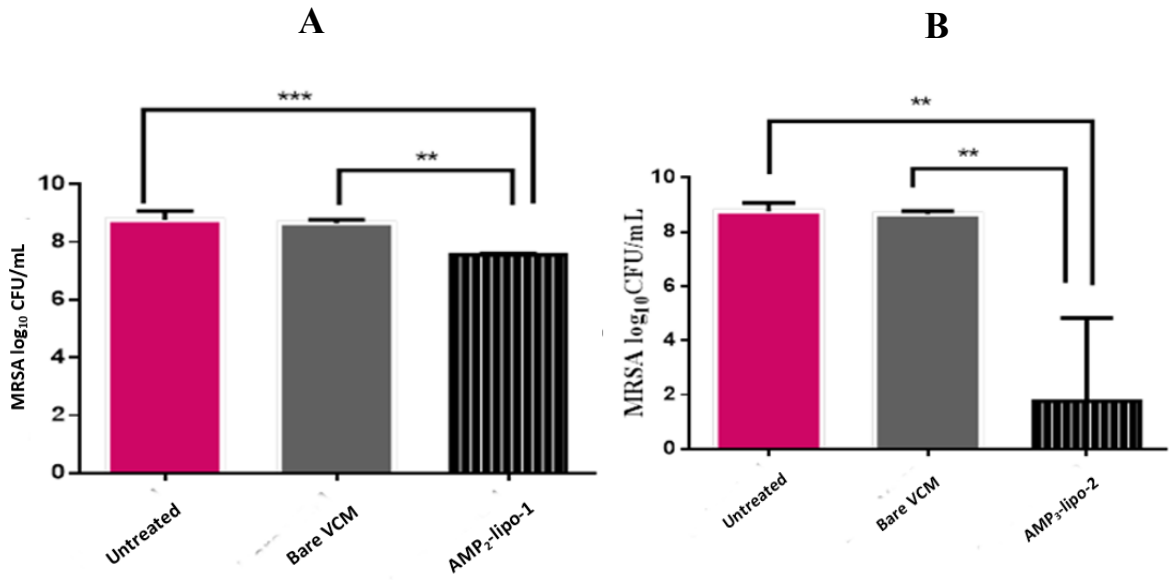


Figure 2: Intracellular activity on MRSA infected HEK 293 cells. A (AMP_2-Lipo-1) and B (AMP_3-Lipo-2)

3.3 Cell penetration of the AMPs

3.3.1 Molecular dynamics (insertion of the AMPs into a model membrane)

To evaluate the process of cell membrane penetration of the AMPs, MD simulations were performed on POPC bilayer membrane[73][74]. Visual inspection of trajectories of the two AMPs revealed AMP-2 and AMP-3 inserted in to the POPC bilayer (**Figure 3**). Z-axis centre of mass distance (COM) between peptides/each residues and PO4 beads of interacting leaflet was calculated using the in-house tcl script. Average distance for each AMP for last 50 ns simulations were calculated, where it was observed that both AMPs were close to the PO4 beads. To further identify which specific residues penetrated the POPC bilayer, we calculated the average distance for the last 50 ns for each of the AMP residue. For AMP-2, residue Lys3 (1.43 ± 1.07) was found to be the closest to the membrane followed by Trp9, Arg4, Lys7, Lys2. For AMP-3, residue Arg7 (1.37 ± 1.05) was closest to membrane after insertion followed by Lys1, Lys8 and Lys9 **(Table 7**). Several studies have reported on peptides rich in lysine and arginine residues being specific for bacterial membrane association due to their net positive charge, which further supported our findings[75][76]. However, from the MD simulation studies, we can hypothesize that electrostatic interactions that occurs during peptide rearrangement had a role to play in membrane affinity.

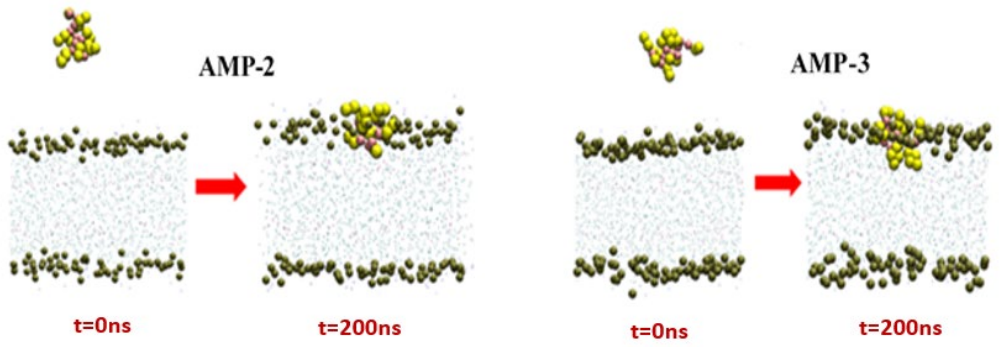


Figure 3: Shows the AMP-2 and AMP-3 simulation systems at t=0 and t=200ns

Table 7: Average distances of residues from PO4 atoms for the last 50 ns

Residue name and residue id (AMP-2)	Average distance last 50ns (Å) (AMP-2)	Residue name and residue id (AMP-3)	Average distance last 50ns (Å) (AMP-3)
GLU1	4.72 ± 1.74	**LYS1**	1.46 ± 1.11
LYS2	2.80 ± 1.80	**TRP2**	5.41 ± 1.73
LYS3	1.43 ± 1.07	**TRP3**	3.53 ± 1.79
ARG4	2.13 ± 1.34	**LYS4**	2.68 ± 1.46
LEU5	5.88 ± 1.76	**LEU5**	2.43 ± 1.47
LEU6	4.99 ± 1.99	**LEU6**	4.56 ± 1.69
LYS7	2.72 ± 1.50	**ARG7**	1.37 ± 1.05
TRP8	3.30 ± 1.98	**LYS8**	1.55 ± 1.13
TRP9	2.11 ± 1.65	**LYS9**	2.37 ± 2.05
ARG10	4.02 ± 2.19	**ARG10**	5.16 ± 2.38

3.3.2 Flow cytometry bacterial cell viability

MRSA bacterial cells were incubated with bare VCM and labelled AMPs for 24 hours. The PI and 6 carboxyfluorescein fluorescent dye were used to determine cell penetration as well as dead cells in the population. The data was captured and analyzed using Kaluza-1.5.20 (Beckman Coulter USA) flow cytometer software. The PI dye is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between the base pairs and its detection indicates either cell death or lack of membrane integrity. In this study, VCM was used as a control to indicate cellular uptake or cell death. The VCM acts by compromising the integrity of the cell wall, which enhances the PI permeability and uptake. Treatment of MRSA cells with the bare VCM and labelled AMPs (AMP-2 and AMP-3), a shift in PI fluorescence was observed (**Figure 4**). From these results it can be deduced that the AMPs had better bacterial killing and cell penetrating ability in the defined

cell population compared to VCM and therefore could be used in liposomal formulations to enhance the activity of VCM.

Furthermore, MRSA was incubated with different concentrations of AMP-2 which had initially showed lower antibacterial activity compared to AMP-3. **Figure 5** shows that increased AMP-2 concentrations resulted in a corresponding increase in membrane disrupted cells, where it was observed that at 62.5 µg/mL or higher, > 90% of MRSA cells were PI+. These results suggest that the AMPs showcase a concentration dependant cell permeation which is an important factor for their utilisation in combinatorial studies.

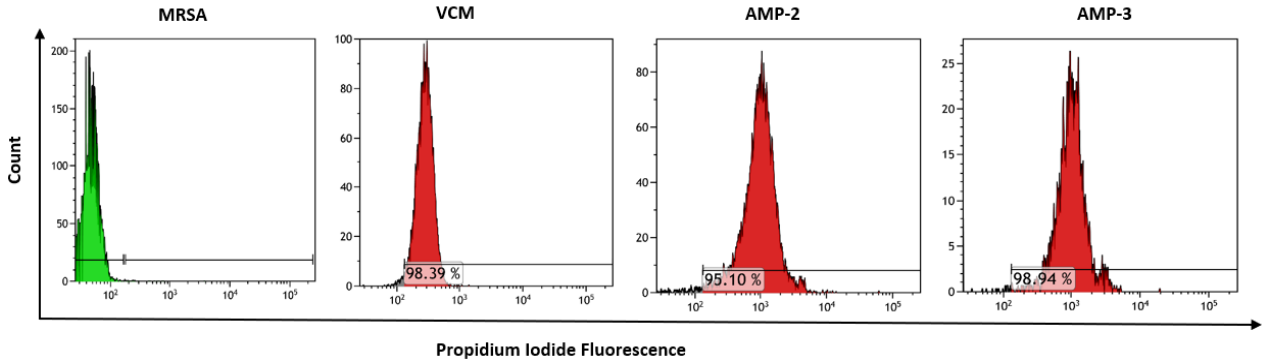


Figure 4: Cell counts vs Propidium uptake. Green represents untreated MRSA (live cells); red represents percentage of uptake in the population after incubation with VCM, AMP-2 and AMP-3.

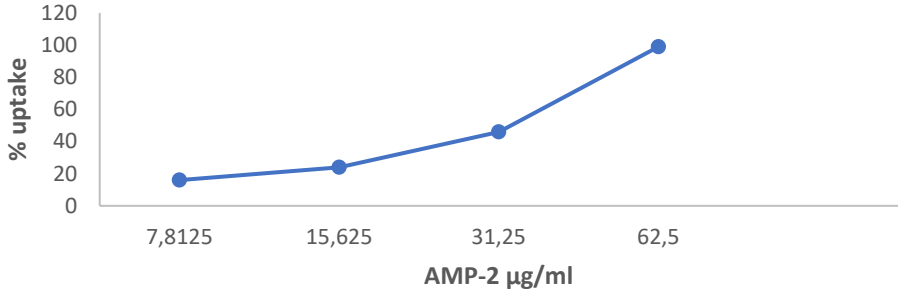


Figure 5: Percentage uptake at different peptide concentration

3.4 *In vitro* Cytotoxicity

The biosafety analysis of AMP-2 and AMP-3 was evaluated on a well-known *in vitro* cytotoxicity assay which assessed cell viability after their exposure to the test antimicrobial peptides[77]. Quantification of viable cells was carried out by an MTT (tetrazolium) cytotoxicity assay by exposing the AMPs to the mammalian cells, where viable cells reduce the tetrazolium dye into an insoluble crystalline formazan. The amount of formazan crystals

formed in the cells is typically comparative to the number of viable cells present. The results obtained showed percentage viability ranging from 80-85% for all cell lines employed in this study (**Figure 6**). This percentage viability displayed by AMP-2 and AMP-3 was above the minimum requirements for biocompatibility and toxicity regulatory requirements for synthesized biomaterial[78][79][80]. These findings suggest that these peptides are safe for biomedical applications and to be utilised in conjunction with other materials.

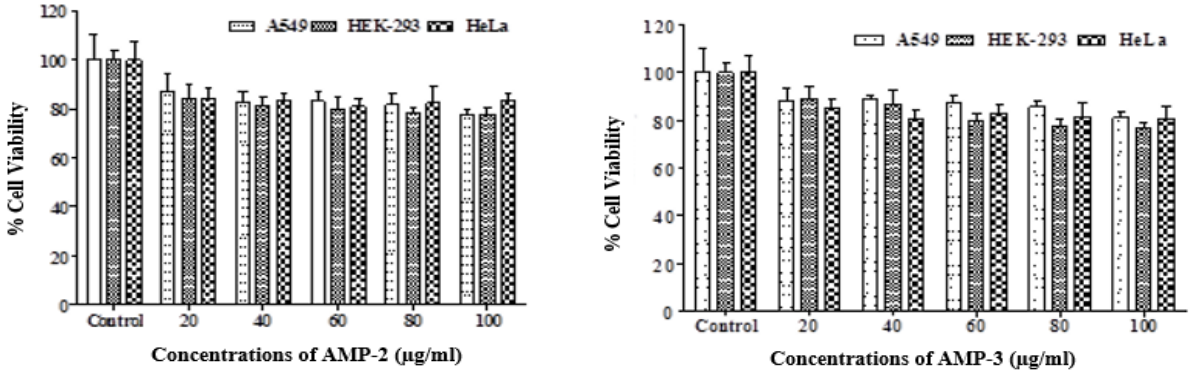


Figure 6: Cytotoxicity evaluation of AMP-2 and AMP-3 at different concentrations A549, HEK293 and HeLa cells

3.5 Haemolysis results

Both naked AMPs and liposomes (AMP_2-Lipo-1 and AMP_3-Lipo-2) reflected haemolysis of less than 1% at a concentration of 0.2 mg/ml, which indicates their non-toxicity to red blood cells (RBCs). Peptides are known to lyse RBCs and therefore their design has to take into account their chain length, type of amino acids present as well as their cationicity. Controls were dH_2O and PBS pH 7.4 which were also maintained at 0.2 mg/ml concentration. Visually it was observed that dH_2O lysed the RBCs since water is hypotonic to RBCs whereas the PBS had similar haemolysis to that of our two formulations of less than 1% (**Figure 7**).

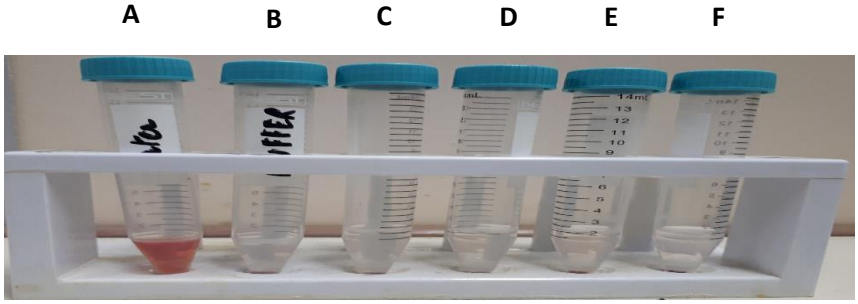


Figure 7. Determination of haemolysis. **A** Haemolysed RBC after placing in hypotonic solution, **B** non-haemolysed RBC after placing it in isotonic buffer solution, **C, D, E, F** no signs of haemolysis after treating the RBC with AMP2, AMP3, AMP2-Lipo-1 and AMP3-Lipo-2.

Preparation and characterisation of the peptide decorated liposomes

Characterization was carried out in terms of size, polydispersity index (PDI) and Zeta potential (ZP), (**Table 8**). The size, PDI and ZP at pH 7.4 of the drug loaded liposomes (**AMP_2-Lipo-1 and AMP_3-Lipo-2**) were 102.6±1.81, PDI of 0.157±0.01 and -9.81±1.69 and 146.4±1.90, PDI of 0.412±0.05 and -4.27±1.25 for **AMP_2-Lipo-1 and AMP_3-Lipo-2** respectively. However, when the liposomes were placed in pH 6.0 it was observed that both liposomal formulations had an increase in size and decrease in negative charge. **AMP_2-Lipo-1** had a size of 387.4±51.11and PDI of 0.81±0.03 and a zeta potential of -2.19±0.57 whereas **AMP_3-Lipo-2** had a size of 229.4±13.8 and PDI of 0.74±0.01, with a zeta potential of 0.14±0.31. measurements were also made at pH 4.5 where **AMP_2-Lipo-1** had a size of 192.4±6.9 and PDI of 0.49±5.24, with a zeta potential of 1.50±0.31. **AMP_3-Lipo-2** reflected similar changes at pH 4.5 where the size was 218.6±6.18 and PDI of 0.63±0.02, with a zeta potential of 1.80±2.21. The observed differences (particle swelling and charge switch) at the three different pH is indicative of pH responsiveness of our formulation and therefore could function considerable well in drug delivery. This liposomal structure offers unique physicochemical properties for carrying and delivering VCM. At physiological pH, this nano formulation will have a slower drug release as the ion pair between OA and AMP still intact. The morphological analysis of the liposomes was conducted using HRTEM (**Figure 8**), where the formed nanoparticles observed to be spherical structures with similar size determined using DLS technique.

Table 8: DLS determination of size, PDI, Zeta potential

	pH	**Size nm**	**PDI**	**Zeta Potential mV**	**% E.E**
AMP2-Lipo-1 (blank)	**7.4**	112.7±2.50	0.174±0.00	-10.7±2.75	
	6.0	150.8±2.17	0.52±0.06	0.88±0.23	
	4.5	173.8±4.8	0.51±0.03	2.38±0.38	
AMP2-Lipo-1 (drug loaded)	**7.4**	137.6±0.75	0.157±0.01	-9.81±1.69	64.24%
	6.0	387.4±51.11	0.81±0.03	-2.19±0.57	
	4.5	192.4±6.9	0.49±5.24	1.50±0.31	
AMP3-Lipo-2 (blank)	**7.4**	137.6±0.75	0.34±0.01	-6.14±1.42	
	6.0	177.1±3.03	0.45±0.03	2.06±0.54	
	4.5	170.3±5.75	0.496.02	2.82±0.46	
AMP3-Lipo-2 (drug loaded)	**7.4**	146. 4±1.90	0.412±0.05	-4.27±1.25	26.57%
	6.0	229.4±13.8	0.74±0.01	0.14±0.31	
	4.5	218.6±6.18	0.63±0.02	1.80±2.21	

A B

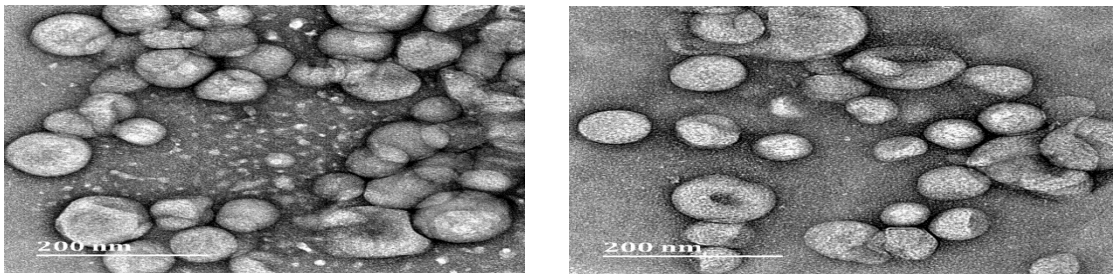


Figure 8: HRTEM of A (AMP_2-Lipo-1) and B (AMP_3-Lipo-2) **– 200nm**

4 Conclusion

We successfully synthesized a panel of nine AMPs from which two were selected based on their antimicrobial activity. We further tested the efficacy of supramolecular lipidation of the two selected novel AMPs for pH responsiveness and enhanced antimicrobial activity against MRSA. AMP_2-Lipo-1 and AMP_3-Lipo-2 formulations were tested for intracellular activity of MRSA infected HEK cell line. The two AMPs were found to be permeable against POPC model membrane and using flow cytometry, it was observed that they possessed good cell penetration against MRSA. This is indicative of the potential of these compounds to carry cargo for intracellular delivery as well as to be used in a nanoparticle assembly to function as probes for sustained membrane penetration while allowing the drug to be released to its target site. However, the MIC values of the prepared liposomes which contained the novel AMPs, OA and VCM showed enhanced activity compared to the bare AMPs. This indicated the advantage of the nano system in improving antimicrobial activity of the AMPs. The intracellular activity of the prepared liposomes also indicated their ability to traverse normal cells and target intracellular MRSA. The cyto-compatibility and haemo-compatibility of AMP_2-Lipo-1 and AMP_3-Lipo-2 formulations projects their safety for IV administration and toward normal cells.

5 Acknowledgements

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6 Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER 5
CONCLUSION

5.1 General Conclusions

Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever increasing range of infections caused by bacteria. AMR is an increasingly serious threat to global public health that requires action across all government sectors and society. Without effective antibiotics, the success of major surgery and cancer chemotherapy would be compromised. The cost of health care for patients with resistant infections is higher than care for patients with non-resistant infections due to longer duration of illness, additional tests and use of more expensive drugs. The exploration and application of AMPs and their encapsulation in nano delivery systems has been seen to be one of the significant areas to improve drug therapy. Therefore, the broad aim of this study was to design, synthesize and evaluate novel AMPs for their bacterial membrane penetration and activity followed by their co-encapsulation with VCM and OA in a liposomal system to enhance their antimicrobial activity.

The specific research aims of this study were therefore to firstly develop a QSAR model which can simultaneously estimate antimicrobial potential and bacterial cell penetrating ability of antimicrobial cell penetrating peptide (aCPPs) against *S. aureus*. Secondly, to design and synthesize novel AMPs and employ them to decorate vancomycin and oleic acid containing liposomes to achieve pH responsiveness for enhanced antimicrobial activity

The main conclusions generated from the research data are summarised below:
Aim 1:

- A total 28 aCPPs were divided into training and test set compounds and PHASE 3.0 suite was used to build a pharmacophore. It was found that the most appropriate pharmacophore model (AAHRR.114) to predict aCPP activity had a five-point hypothesis that consisted of two hydrogen bond acceptor (A), one hydrophobic group (H) and two aromatic ring features (R). AAHRR.114 was selected for QSAR model development and the 3D-QSAR was evaluated by Cross-validation coefficient (Q2), Fisher test (F), Correlation coefficient (R2) and Pearson-R, Standard deviation (SD) and Root-mean squared error (RMSE). Data analysis confirmed the validity of the model. The results from the model

showed that the higher the therapeutic index (TI), the higher the PHASE predicted activity which is consistent with our hypothesis. The optimal aCPP was compound 25 which had the amino acids lysine (K-3), leucine (L), and tryptophan (W-3), which are known to be specific for bacterial membrane penetration. The QSAR model developed was successful in identifying the optimal aCPP with high activity prediction and provided significant insights into the structural requirements to correlate their TI to their cell penetrating ability. The molecular dynamics simulation also confirmed the penetration of the best aCPP obtained from the QSAR study on the POPC model membrane.

- The findings are important for future research in peptide design and will allow researchers to focus on optimizing the TI of aCPPs for bacterial cell specificity. It will also facilitate the synthesis of novel aCPPs for the design and development of aCPP based drug delivery systems. A good TI also implies that the hemolytic index is low. A low hemolytic index suggests that the designed aCPP can be applied in clinical trials.

Aim 2:

- A prediction tool (CellPPD) from the antimicrobial peptide database was used to design the desired AMPs. The designed peptides were synthesised by solid phase peptide synthesis (SPPS) on Rink amide MBHA resin and successfully confirmed by HPLC and LCMS.
- Molecular dynamics simulation was also used to confirm the AMPs ability to cross bacterial membranes. To evaluate the process of cell membrane penetration of the AMPs, MD simulations were performed on POPC bilayer membrane using the GROMACS package. Visual inspection of trajectories of the two AMPs revealed AMP-2 and AMP-3 inserted in to the POPC bilayer, where it was observed that both AMP-2 (1.41 ± 0.97) and AMP-3 (1.42 ± 0.98) were close to the PO_4 beads.
- Flow cytometry was used to determine the ability of the AMPs to penetrate MRSA bacterial cells. The PI fluorescent dye was used to determine cell penetration as well as dead cells in the population. From these results it was deduced that the AMPs had better bacterial killing and cell penetrating ability in the defined cell population compared to VCM and therefore could be used in liposomal formulations to enhance antimicrobial activity.

- Cytotoxicity studies performed using an MTT assay on mammalian cell lines HELA, A549 and HEK-293 revealed that the AMPs were biosafe with percentage viability ranging from 80-85% for all cell lines employed.
- *In vitro* haemolysis using sheep blood revealed that the AMPs had close to zero haemolytic index, revealing their biosafety profile.
- Liposomes encapsulating the AMPs, VCM and OA were prepared by a thin layer film hydration method and further characterized by size, polydispersity index (PDI) and Zeta potential (ZP).
- The size, PDI and ZP at pH 7.4 of the drug loaded liposomes of AMP-2 and AMP-3 (**AMP_2-Lipo-1 and AMP_3-Lipo-2**) were 102.6±1.81 nm, PDI of 0.157±0.01 and -9.81±1.69 mV and 146.4±1.90 nm, PDI of 0.412±0.05 and -4.27±1.25 mV for **AMP_2-Lipo-1 and AMP_3-Lipo-2**, respectively. However, when the liposomes were placed in pH 6.0 it was observed that both the liposomes formulation has an increase in size and decrease in negative charge. **AMP_2-Lipo-1** had a size of 387.4±51.11 nm and PDI of 0.81±0.03 and a zeta potential of -2.19±0.57 mV whereas **AMP_3-Lipo-2** had a size of 229.4±13.8 nm and PDI of 0.74±0.01, with a zeta potential of 0.14±0.31 mV. Measurements were also made at pH 4.5 where **AMP_2-Lipo-1** had a size of 192.4±6.9 nm and PDI of 0.49±5.24, with a zeta potential of 1.50±0.31 mV. **AMP_3-Lipo-2** reflected similar changes at pH 4.5 where the size was 218.6±6.18 nm and PDI of 0.63±0.02, with a zeta potential of 1.80±2.21 mV.
- The observed differences (particle swelling and charge switch) at three different pH is indicative of pH responsiveness of our formulation and therefore could function considerable well in drug delivery.
- The *in vitro* antibacterial studies carried out on bare AMPs showed that they had better affinity towards Gram positive bacteria (MRSA and *S aureus*) compared to Gram negative bacteria (*E.coli*). Furthermore, the two liposomal formulations had better activity at acidic pH compared to VCM, which indicated that they can be used to target bacterial infection sites.
- The therapeutic benefit conferred by AMP_2-Lipo-1 and AMP_3-Lipo-2 was evaluated *in vitro* by determining their efficacies against intracellular MRSA. A cell culture assay was developed in which HEK-293 cells were first infected with MRSA
- AMP_2-Lipo-1 and AMP_3-Lipo-2 had lower CFU counts compared to VCM. AMP_3-Lipo-2 (which initially had better MICs), showed a significant decrease in CFU counts at 5 times the MIC.

The findings of this study therefore confirmed the biosafety profile of the novel AMPs. Furthermore, this study confirmed the ability of the liposomal supramolecular assembly comprising of AMPs, OA and VCM in enhancing antibacterial activity in both extracellular and intracellular settings.

5.2 Significance of the findings in the study

Newly designed AMPs and AMP decorated pH responsive OA and VCM loaded liposomes were synthesized and formulated respectively to challenge antimicrobial drug resistance and limitations of current dosage forms. The significance of the findings in this study include the following:

New pharmaceutical products: Novel AMPs were designed and synthesized and thereafter non-covalently co-encapsulated with VCM and OA in a liposome. These new materials are new pharmaceutical products to combat drug resistance and serve as new drug entities for effective therapeutic outputs.

Improved patient therapy and disease treatment: The supramolecular assembly was capable of enhancing antibacterial activity, therefore it can improve patient therapy and disease treatment caused by drug resistant bacterial infections by augmenting the antimicrobial activity of the encapsulated agents for effective targeting and allowing for minimal doses to be used and improving patience compliance.

Creation of new knowledge to the scientific community:

The following new knowledge was generated in this study:

- Identification of mechanisms, structural features and descriptors required to design optimal novel aCPP materials for lead optimization in drug-candidate discovery.
- Synthetic methods for development of novel AMPs and their evaluation *in vitro* and *in silico* for bacterial membrane penetration can add to the conception of new knowledge.
- Molecular modelling approaches for optimal binding and penetration of the aCPPs and AMPs to the bacterial membrane.
- Co-delivery techniques and identification of methods for the understanding of the synergistic mechanisms of these compounds with drug and non-drug agents in a

liposomal delivery system to target diseases associated with bacterial infections will also contribute to new scientific knowledge.

- The non-covalent interaction between the novel AMPs, VCM and OA in a liposomal system was successfully identified. Their effect of pH responsiveness, the antimicrobial activity, cell penetration using flow cytometry and MD simulations was also identified.
- Antimicrobial activity through the determination of MIC, flow cytometry, haemolysis and intracellular activity successfully showed good antimicrobial activity and high biosafety profiles

Stimulation of new research:

AMPs possess great potential to change antimicrobial therapy and their non-covalent co-delivery with other antibacterial agents will allow them to effectively treat diseases associated with bacterial infections. The proposed research holds great prospects in combating drug resistance for the following reasons:

- The findings are important for future research in peptide design and will allow researchers to focus on optimizing the TI of aCPPs for bacterial cell specificity. It will also facilitate the synthesis of novel aCPPs for the design and development of aCPP based drug delivery systems.
- The proposed supramolecular liposomal assembly can further stimulate the research area of drug, non-drug and peptide encapsulation for enhanced membrane penetration and activity. Furthermore, this research area can be evaluated for enhanced drug delivery and peptide intracellular targeting for combatting drug resistance.

5.3 Recommendations for future studies

Although the designed AMPs and AMP decorated pH responsive OA and VCM loaded liposomes show great prospects in bacterial cell membrane penetration and demonstrated the enhanced ability to combat drug resistance, additional studies are necessary to further explore and improve their potential as alternatives to current dosage forms. The following studies are proposed:

- In the case of the novel AMPs, based on their ability to traverse and carry cargo across cell membranes, specific organelle targeting protocols could be performed to test their efficacy in halting specific biochemical pathways or cellular functions.
- AMPs are known to possess biomimetic ability, and this can allow for further research in this area for apoptotic pathway activation.
- AMPs are also known as host defence proteins, and this can allow for further studies to synthesize biomimetic AMPs to stimulate the multiple immune responses.
- The tagging of AMPs with antibodies for effective neutralization of bacteria and viruses could be performed to target specific sites within the disease-causing agents.
- The developed supramolecular assembly could be utilized with different antibiotics to target both Gram positive and negative bacteria.
- Co-delivery of AMPs with surface enhanced nano systems could be explored effective membrane permeation.
- Encapsulation of drug conjugated AMPs in different nano systems could be performed to offer a bi-directional approach in membrane penetration and intracellular organelle targeting.
- *In vivo* studies could be conducted on both novel AMPs as well as the liposomal assembly to further elucidate their biocompatibility prior to clinical trials.

5.4 Conclusion

The findings from this study demonstrate the capabilities of the novel antimicrobial peptides in bacterial membrane penetration as well as utilization of nano-based drug delivery systems to treat both extracellular and intracellular infections. This current research has made significant strides to circumvent limitations of current dosage forms and further directed way towards a new class of compounds that should be explored in antibiotic therapy. Nanotechnology has played a fundamental role in improving antibiotic therapy by allowing the nano encapsulation of different antimicrobial moieties in a nano-based drug delivery system, as depicted in this study. This has led to a synergistic approach to target infection sites and improved drug delivery. Going forward, this approach will play a pivotal role in the treatment of diseases associated with bacterial infections, thereby reducing the burden of disease globally.

Appendix A

ANTIMICROBIAL CELL PENETRATING PEPTIDES (ACPPS) WITH BACTERIAL CELL SPECIFICITY: PHARMACOPHORE MODELLING, QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP AND MOLECULAR DYNAMIC SIMULATION

Mbuso Faya[1], Rahul S. Kalhapure[1*], Dinesh Dhumal[2], Nikhil Agrawal[1], Calvin Omolo[1], Krishnacharya G. Akamanchi[2], and Thirumala Govender[1*],

[1]Discipline of Pharmaceutical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, South Africa

[2]Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, MAtunga (E), Mumbai, India 400019.

INTRODUCTION AND AIMS

- Current research has shown cell-penetrating peptides (CPPs) and antimicrobial peptides (AMPs) as probable vectors for use in drug delivery and as novel antibiotics. It has been reported that the higher the therapeutic index (TI) the higher is the bacterial cell penetrating ability.
- The aim of this study was to develop a quantitative structure activity relationship (QSAR) model, which can estimate antimicrobial potential and cell-penetrating ability of aCPPs against *S. aureus*, to confirm the relationship between the TI and aCPPs and to identify specific descriptors responsible for aCPPs penetrating ability. Molecular dynamics (MD) simulation was also performed to confirm the membrane insertion of the most active aCPPs obtained from the QSAR study.

METHODS

PHASE alignment of aCPP to Pharmacophore (AAHRR 114)

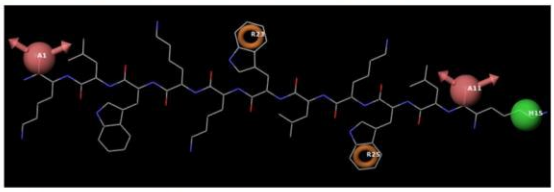

Fig. 1. Schematic representation of aCPP-pharmacophore alignment

Dataset

- The data set was obtained from Park *et al.* (2009) and Bahnsen *et al.* (2013).
- The obtained data was randomly divided into 21 training set compounds, with seven being reserved for a test set.
- The peptides' 3-D structures which served as ligands were generated in Maestro 9.8 molecular modelling package from Schrodinger.

PHASE Methodology

- PHASE 3.0 was used for pharmacophore-based alignment and utilized for the QSAR model development.
- Default pharmacophoric features used to develop the pharmacophore model included a hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic (H), negative (N), positive (P) and aromatic ring (R).
- The variant AAHRR, for which all the compounds were matched, was searched to generate the best common pharmacophore hypothesis (AAHRR.114).
- The hypothesis AAHRR.114 was selected as most appropriate as it has the highest survival score (3.984) for common pharmacophore hypothesis, which gives the best alignment of the active ligands.

Molecular Dynamic Simulations

- 3D-structure of "KLWKLWKKWLK" aCPP was predicted using PEP-FOLD server
- POPC bilayer was constructed using the CHARMM-GUI membrane builder and contains a total of 128 lipid molecules
- The peptide was placed more than 5 Å away from any lipid molecules of the upper leaflet of the bilayer and the simulation ran for 200ns

RESULTS AND DISCUSSION

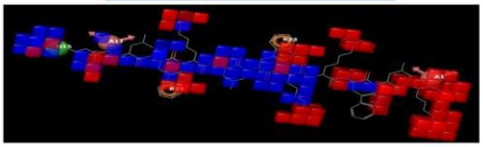

Fig. 2. Features responsible for activity (blue cubes) and those attenuate activity (red cubes).

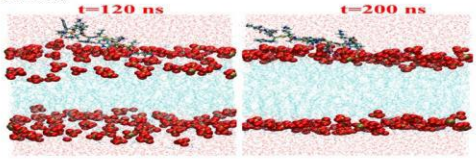

Fig. 3. aCPP-POPC lipid bilayer showing sustained interaction from 120ns-200ns. PO4 atoms of bilayer have been shown in VDW representation and aCPP peptide has been shown in cartoon representation

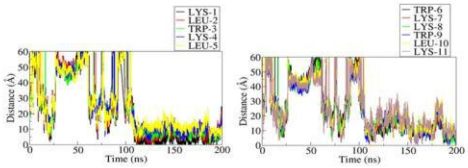

Fig. 4. Time evolution of centre of mass (COM) distance between each residue of peptide with the Phosphate (PO4) group of lower leaflet.

Table 2. Summary of 3D-QSAR results

PLS statistical parameters	Results	PLS statistical parameters	Results
Number of molecule in training set	18	R^2	0.9016
Number of molecule in test set	6	Q^2	0.5311
Number of PLS factors	3	Standard deviation (SD)	0.2072
Root-mean squared error (RMSE)	0.5911	Variance ratio (F)	36
		Pearson-R	0.847

Discussion

- The best compound comprised of the amino acids, lysine (K-5), leucine (L-3) and tryptophan (W-3), with a positive net charge of +5 and a total hydrophobic ratio of 57%.
- The MD simulation showed spontaneous insertion of the aqueous phase aCPP into the lower leaflet region of the lipid bilayer and the C-terminal region (residue 6-11) of the aCPP formed strong interactions compared to the N-terminal (residue 1-5)
- We further observed the positively charged Lys-11, Lys-8, and the non-polar aromatic Trp-9 were closest to the membrane during the binding, revealing the importance of these amino acids in membrane penetration based on their net charge.

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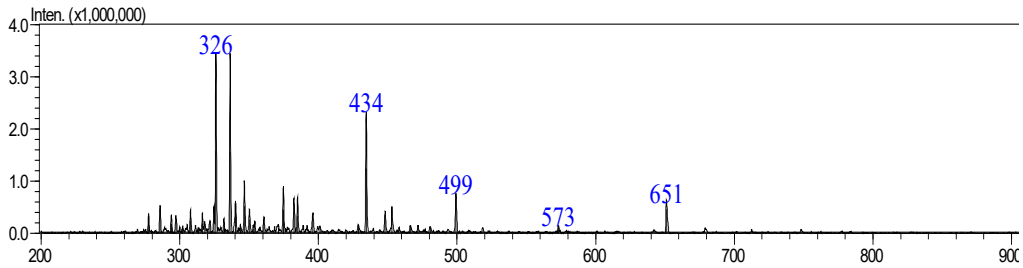
CONCLUSION

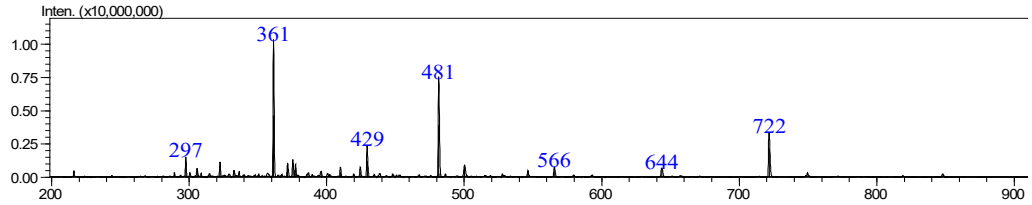
The proposed 3D-QSAR model was useful in estimating the antimicrobial potential and cell penetrating ability of aCPPs and confirmed the relationship between the TI and aCPPs. MD simulation also revealed the aCPP-POPC bilayer interaction, resulting in the aCPP insertion across the POPC bilayer. The combination of these two computational studies will also lead to the rational design of optimal and novel aCPPs for therapeutic activity and for peptide-conjugate delivery.

Appendix B: LCMS Charts

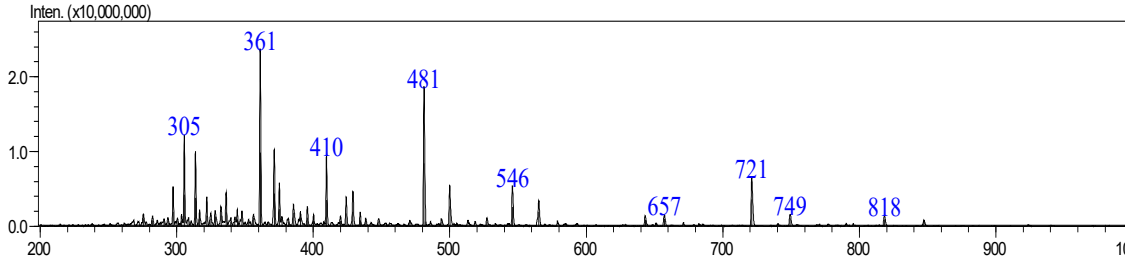
FAMP1: AALRKKDWWK, 1301



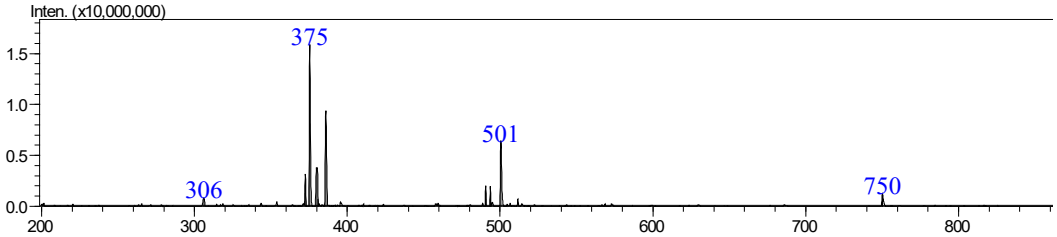
FAMP2: EKKRLLKWWR, 1441.8



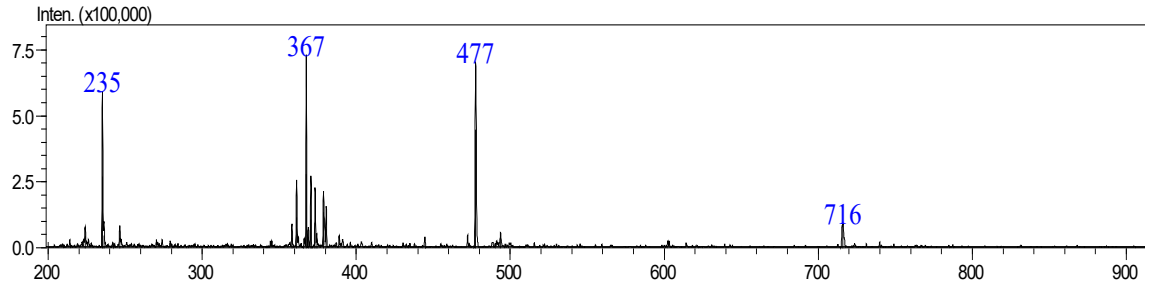
FAMP3: KWWKLLRKKR , 1440.8



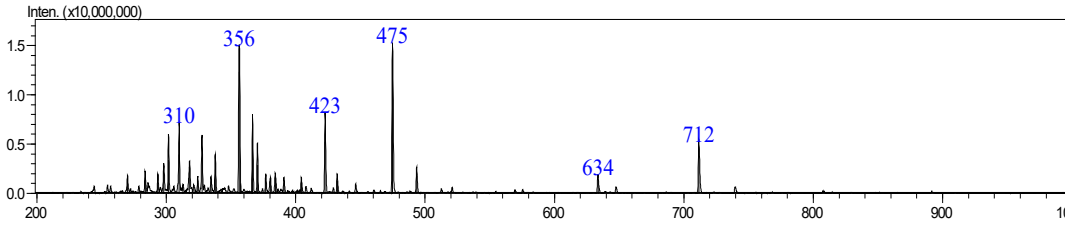
Famp4: QLLWKKRWWR, 1499.9



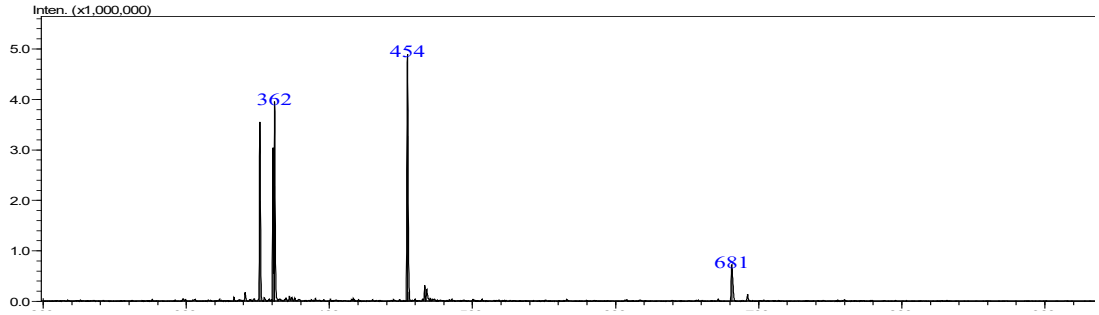
Famp5: KKKSLLRWWW, 1430.9



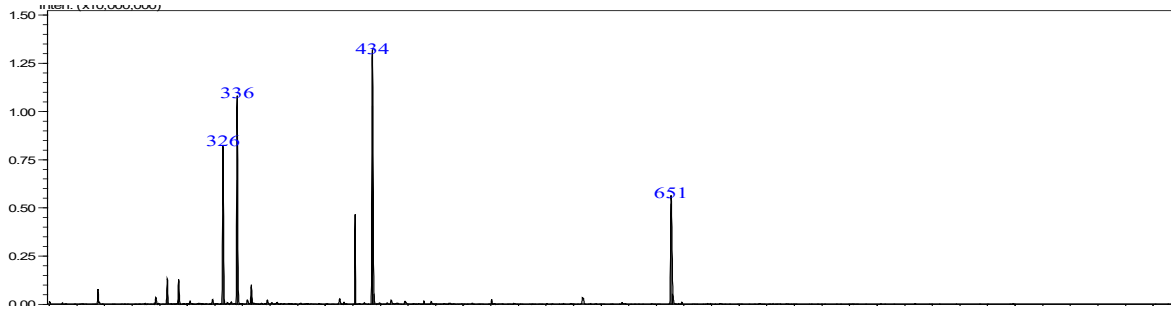
FAMP6: KWWRLLHKKQ, 1422.9



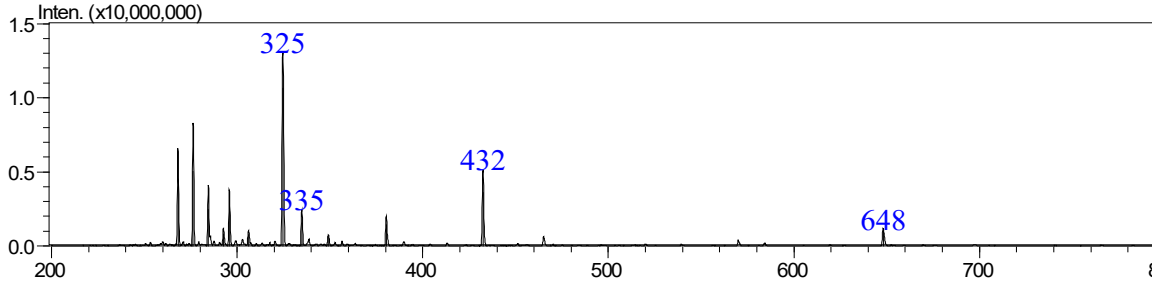
FAMP7: KLLRKKFWWG, 1361.85



FAMP8: KKKAARRWWA, 1300

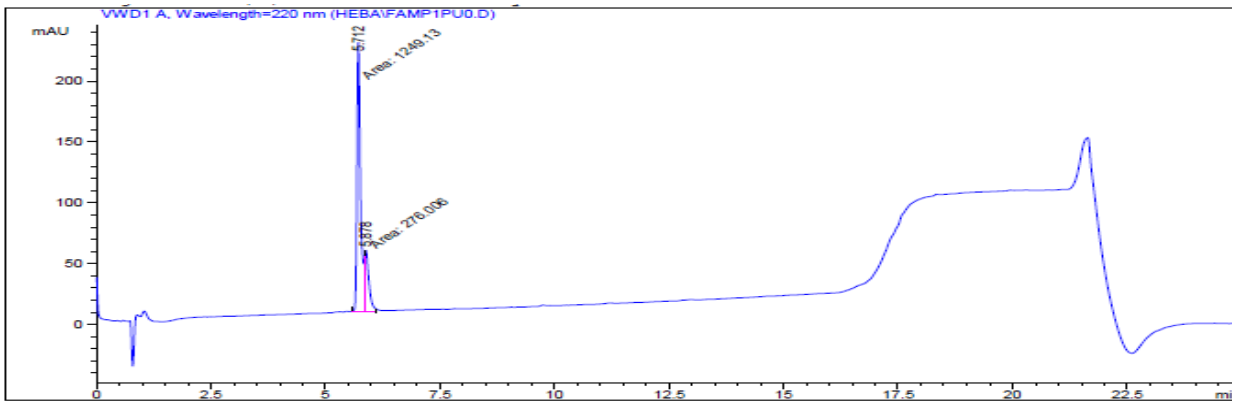


FAMP9:KKKKLLLLRR, 1294.9

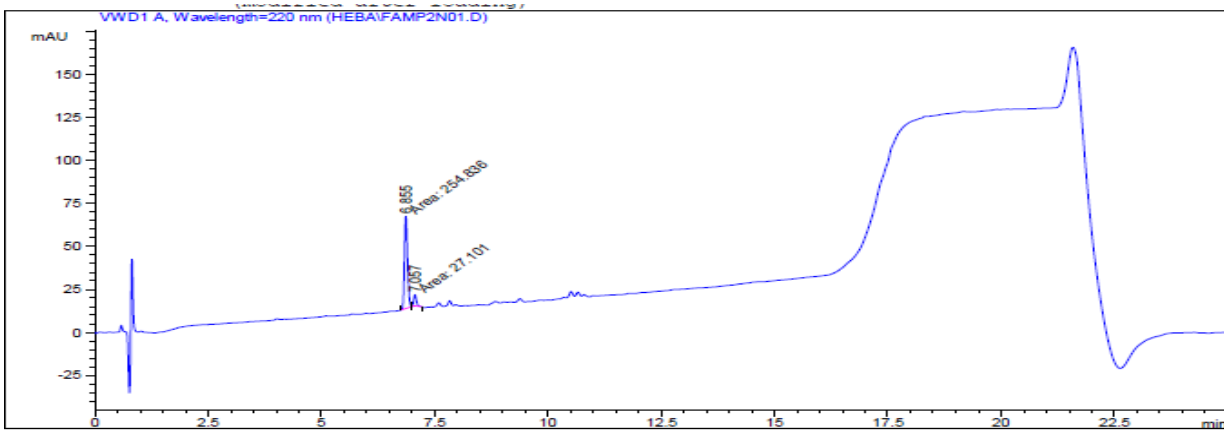


Appendix C: HPLC Charts

AMP 1



AMP 2



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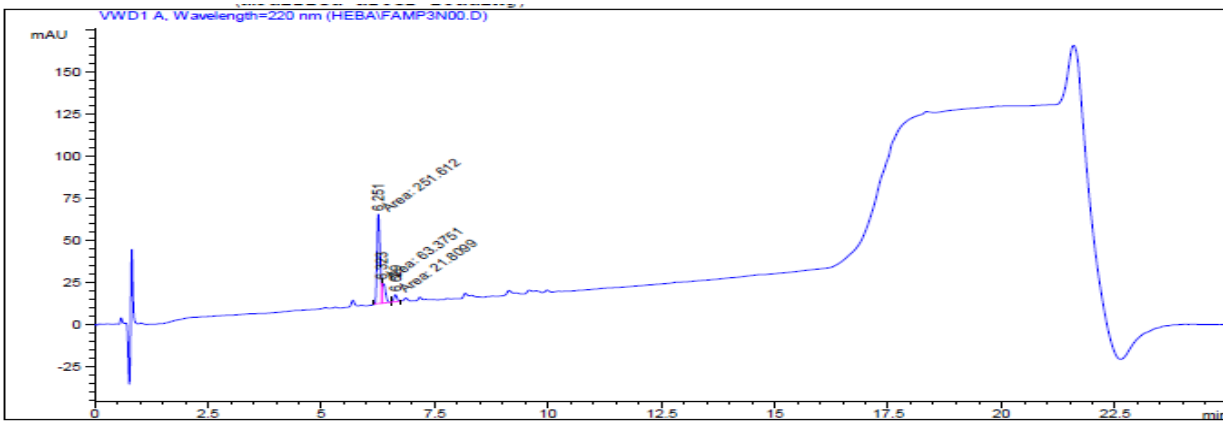

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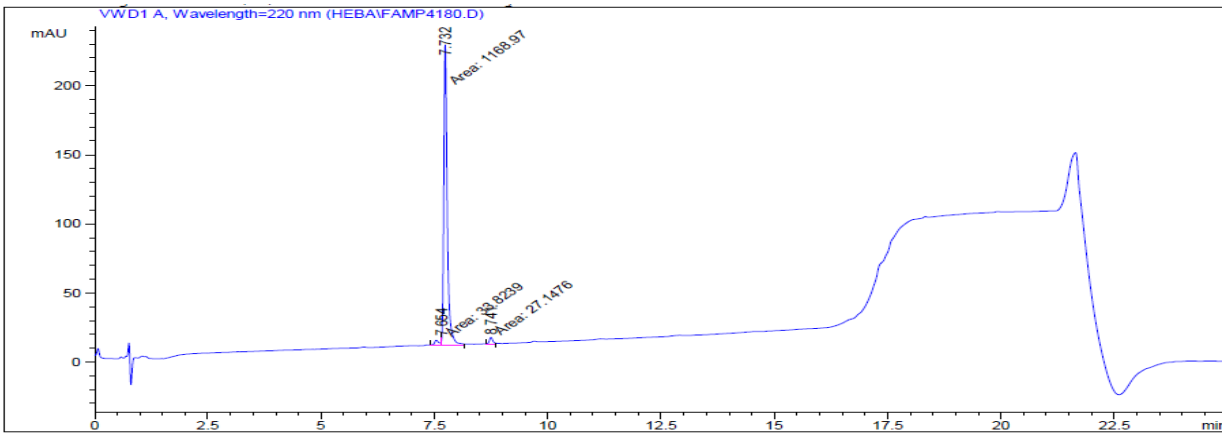

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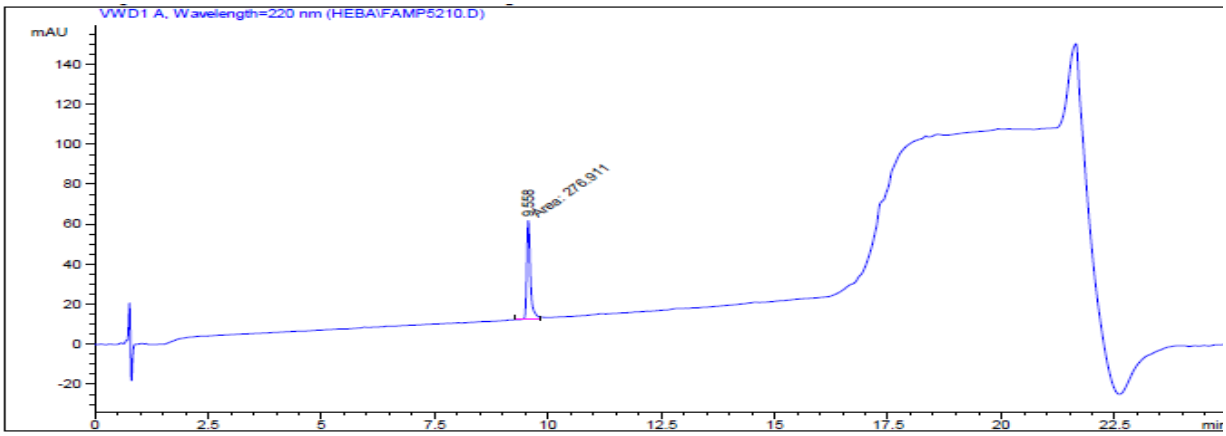

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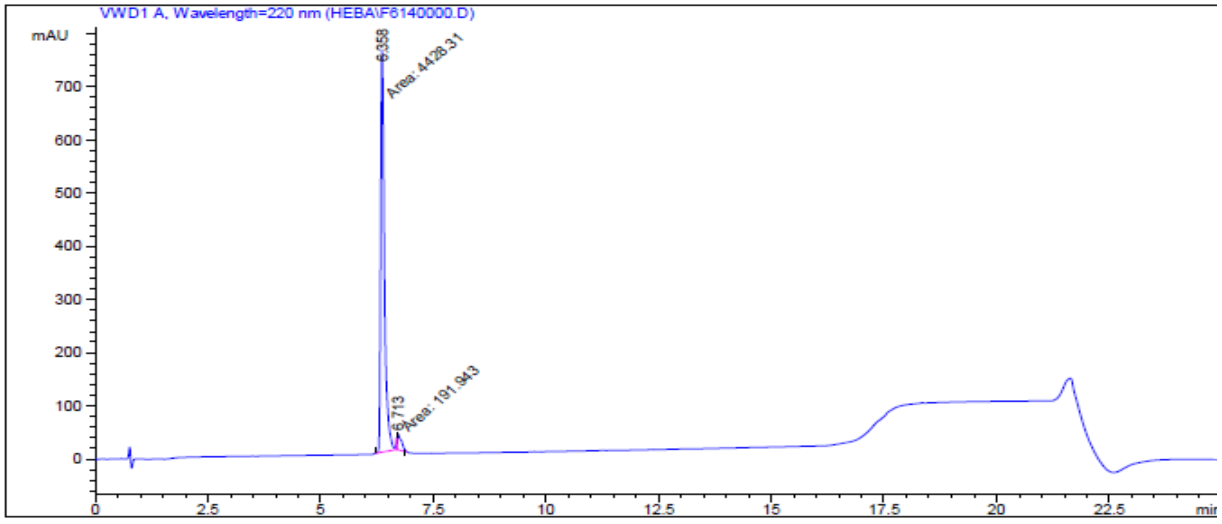

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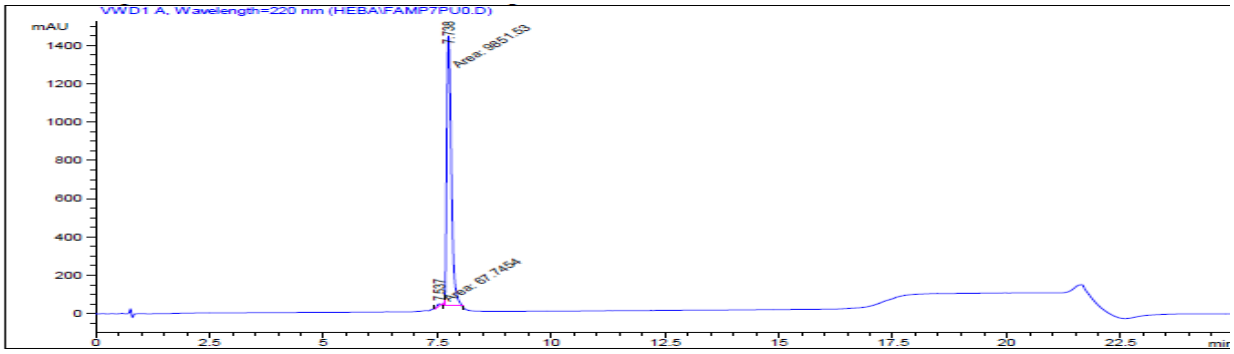

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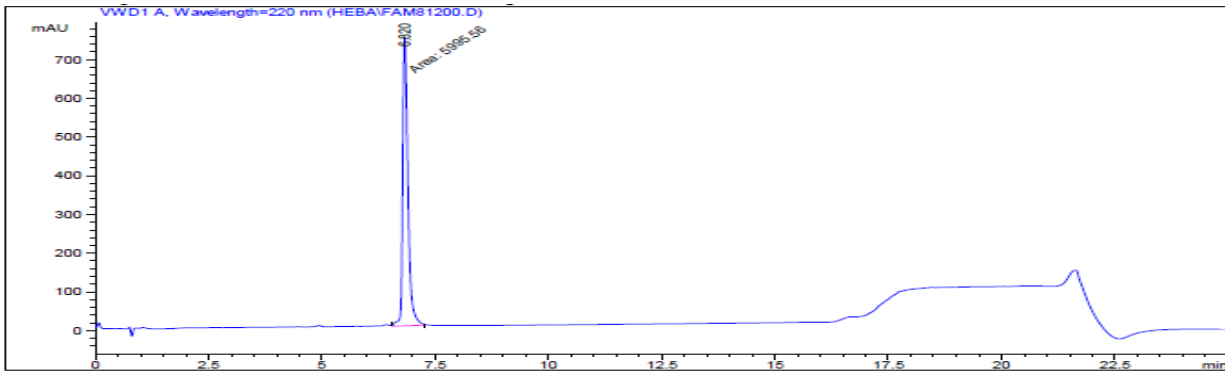

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