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**Functional characterization of a peptide
fragment, Os(3-12), derived from the
carboxy-terminal region of a defensin from
the tick *Ornithodoros savignyi***

By

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**Submitted in partial fulfilment of the degree Magister Scientiae
Biochemistry**

In the Faculty of Natural and Agricultural Sciences

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Pretoria

18 May 2015

SUBMISSION DECLARATION

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SUMMARY

The rapid increase of multi-drug resistant bacteria and associated deaths has stimulated research into the development of novel therapeutic options. Antimicrobial peptides (AMPs) display a high therapeutic potential in solving this problem. Research focuses on new ways to enhance the antibacterial activity of AMPs and this includes the amidation of the C-terminus. Once the structure of an AMP is altered it is necessary to reevaluate the properties of this AMP compared to the unaltered peptide. In this study, a peptide fragment Os(3-12), based on a defensin from the tick *Ornithodoros savignyi*, was amidated at the C-terminus. The effect of C-terminal amidation on the structural, antibacterial, cytotoxic and antioxidant activities of Os(3-12)NH₂ was investigated and compared to Os(3-12) as well as the parent peptide Os. Mode of action related to membrane permeabilization was evaluated. The effect of serum and on the antibacterial activity of Os(3-12)NH₂ was also determined.

Circular dichroism experiments indicated Os(3-12) and Os(3-12)NH₂ to be unstructured in sodium dodecyl sulphate micelles and 50% trifluoroethanol, unlike Os which was predominantly α -helical. Although still less potent than Os, the determined minimum bactericidal concentration (MBC) for each peptide indicated that amidation increases the bactericidal activity of Os(3-12) by 16-fold against *Escherichia coli* and by 8-fold against both *Pseudomonas aeruginosa* and *Bacillus subtilis*. In comparison amidation enhanced the activity of the peptide towards *Staphylococcus aureus* by only 2-fold. The kinetics of bactericidal activity revealed that Os(3-12)NH₂ killed *E. coli* within 10 minutes and *B. subtilis* within 60 minutes. SYTOX green was applied to evaluate the effects of the peptides on the membrane integrity of the bacterial cells. LL-37, a peptide known to disrupt microbial membranes, induced membrane permeabilization of both *E. coli* and *S. aureus* membranes. Both Os and Os(3-12)NH₂ were found to also cause membrane permeabilization of these bacteria, albeit not to the same extent as LL-37, thus suggesting possible internal targets subsequent to membrane permeabilization. In the presence of 30% human serum and a physiological salt mixture comprising of 145 mM NaCl, 2.5 mM CaCl₂ and 1 mM MgCl₂ the bactericidal activity of Os(3-12)NH₂ was lost. The amidated peptide was found to be non-toxic towards human erythrocytes and Caco-2 cells. Os(3-12)NH₂ showed strong antioxidant activity and was found to be 15-fold more active than glutathione (GSH), a known antioxidant.

In conclusion Os(3-12)NH₂ has been identified as a multifunctional AMP that is nontoxic to mammalian cells. However the therapeutic potential of Os(3-12)NH₂ may be restricted to topical applications due to the peptide's inactivity under physiological conditions. Although Os(3-12)NH₂ causes membrane permeabilization, indications are that there are additional intracellular targets that need to be identified.

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ABBREVIATIONS

AA	Amino acid
AAPH	2,2'-azobis (2-aminopropane) dihydrochloride
AMPs	Antimicrobial peptides
AUC	Area under curve
CaCl₂	Calcium chloride
Caco-2	Colorectal adenocarcinoma cells
CD	Circular dichroism
CFU	Colony forming unit
CO₂	Carbon dioxide
CV	Crystal violet
DNA	Deoxyribonucleic acids
Df	Dilution factor
EDTA	Ethylenediaminetetraacetic acid
e-NAP-2	Equine-neutrophil activating peptide-2
FB	Fat body cells
FDA	Food and Drug Administration
G+	Gram-positive bacteria
G-	Gram-negative bacteria
GSH	Glutathione
H	Haemocytes
HD5	Human defensin 5
HD6	Human defensin 6
His	Histidine
HL	Haemolymph
αHNP-1	Alpha human neutrophil peptide-1
Iso-PBS	Isotonic phosphate buffer saline
KCL	Potassium chloride
kDA	Kilodaltons
LB	Luria Bertani
LC	Lethal concentration
L-CPL	Left handed circularly polarized light
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MG	Midgut
MgCl₂	Magnesium chloride
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	Mass spectrometry
MW	Molecular weight
NaCl	Sodium chloride
NaP	Sodium phosphate buffer

NJ	New Jersey
OD₆₀₀	Optical density at 600 nm
OMΔC	AMP from <i>Ornithodoros moubata</i> without the cysteine residues
ORAC	Oxygen radical absorbance capacity
OsDef1	<i>Ornithodoros savignyi</i> defensin isoform one
OsDef2	<i>Ornithodoros savignyi</i> defensin isoform two
Os	Carboxy-terminus of OsDef2 isolated from <i>Ornithodoros savignyi</i>
PAΔC	<i>Pyrrhocoris apterus</i> without the cysteine residues
PAO1	Strain type of <i>Pseudomonas aeruginosa</i>
Phe	Phenylalanine
PMN	Polymorphonuclear
BLAST-PSI	Position Specific Interated Basic Local Alignment Search Tool
PSIPRED	Protein structure prediction
P4	Longcin derivative
P9A	AMP isolated form the silk moth <i>Hyalophora cecropia</i>
P9B	AMP isolated form the silk moth <i>Hyalophora cecropia</i>
R-CPL	Right handed circularly polarized light
RNA	Ribonucleic acids
SCAC	Stomoxys calcitran without the cysteine residues
SDS	Sodium dodecyl sulphate
SG	Salivary gland
TE	Trolox equivalent
TFE	Triflouroethanol
TLR	Toll-like receptors
tPMP-1	Tropomyosin-1
USA	United States of America
UV spectroscopy	Ultraviolet spectroscopy
WHO	World Health Organization
WT	Whole tick

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CHAPTER 1

INTRODUCTION

1.1 Problem statement

Infectious diseases have been regarded as one of the leading causes of death worldwide since the 20th century when chronic degenerative diseases struck developed countries (Barreto *et al.*, 2006). These diseases arise due to host invasion with pathogenic microorganisms such as bacteria (Tenover and Hughes, 1996; Gibbons, 1989), fungi (Groll *et al.*, 1996), protozoans (Stuart *et al.*, 2008; Zuckerman, 1975) or viruses (Clarke *et al.*, 1959; Barry *et al.*, 2015). The search for novel therapeutic compounds against infectious diseases is imperative to find as the rate of resistant microbes drastically accumulates (Walsh, 2004; Projan, 2003; Shlaes, 2003). The ability of pathogenic microorganisms to decrease the effectiveness of antimicrobial agents poses an urgent threat to society as novel infections have emerged and old infections re-surfaced (Cassell and Mekalanos, 2001). According to the World Health Organization (WHO), an estimate of 57 million deaths occur throughout the world each year of which more than 25% are directly caused by infectious diseases (WHO, 2004).

Bacterial pathogens responsible for infectious diseases are a major focus area in medical research as a number of bacterial strains are increasingly evolving into multidrug-resistant forms (Weinstein *et al.*, 2005). This resistance evolution poses an urgent threat to society as treatment options are limited. Therapeutic failure stems from the negligence of antibiotic usage in humans and in agricultural feed (Bantar *et al.*, 2003). The unnecessary and irresponsible exposure of pathogenic microbes to particular drugs has paved the way for microbial adaptation to these antibiotics by genetic mutations. These mutations are the key features of pathogenic resistance and are subsequently passed on to other microbes via horizontal gene transfer adding to multidrug-resistance (Mølbak, 2004). The term ‘superbug’ refers to the continuous accumulation of these genes within microorganisms enhancing their resistance and is associated with increased morbidity and mortality (Davies and Davies, 2010).

Many efforts to combat resistant pathogens has mainly evolved around Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. Gram-positive bacteria pose a public health threat in terms of increased morbidity, mortality, expenditure,

patient management and health implementation control measures. As these bacteria are mainly associated with nosocomial infections, novel therapeutic drugs have been developed and are still being developed to target these pathogens (Woodford and Livermore, 2009). Another urgent threat prevailing is the rapid increase of multidrug-resistant Gram-negative bacilli as the development of novel therapeutic compounds to treat these pathogens has stagnated over the last half century (Xu *et al.*, 2014). Gram-negative bacteria generally associated with disease are *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa* and carbapenem-resistant *Acinetobacter spp* (Rice, 2007). Such infections are mainly found in critically ill patients especially those with ventilator-associated pneumonia. These pathogens develop drug resistance via three basic mechanisms: inactivation of a target drug, alteration of a drug target site or prevention of drug access to target (Waterer and Wunderink, 2001).

As the rate of multidrug-resistance against both Gram-positive and Gram-negative bacteria continues to exceed the development of novel therapeutic compounds, a pre antibiotic era threatens to become a reality (Giske *et al.*, 2008; Davies and Davies, 2010). In order to prevent a non-antibiotic era from prevailing, the development of new antimicrobial agents is urgently required. Antimicrobial peptides (Ou *et al.*) constitute a promising alternative in treating multidrug-resistance. These peptides exhibit potent antimicrobial potential against a wide range of pathogenic microorganisms ranging from bacteria, fungi, viruses and parasites (Hancock and Lehrer, 1998). Unlike conventional antibiotics which are target specific in their mode of action, AMPs employ a non-specific, rapid killing mechanism against pathogens by perturbing their cell membrane reducing the risk of resistance development (Epanand and Vogel, 1999).

1.2 Antimicrobial peptides as novel therapeutic candidates

AMPs are a diverse group of gene-encoded oligopeptides found in all organisms ranging from prokaryotes to eukaryotes. These molecules are evolutionary conserved components of the innate immune system for a number of complex organisms exhibiting a broad spectrum of antimicrobial potential (Giuliani *et al.*, 2007).

The innate immune system is a well-designed system forming the initial line of defence against foreign pathogens. This system comprises of two main types of components: the receptor component and the effector component. The receptors are expressed by a variety of mobilised cell types such as the phagocytic, epithelial, macrophage, neutrophil and mast cells. Some of the

receptors expressed are toll-like receptors (TLRs), C lectin receptors and scavenger receptors. These receptors are programmed to recognise unique microbial components which then activate the effectors and mediators to initiate a rapid response. The effector molecules involve the complement cascade, cytokines, chemokines, superoxides, nitric oxide, prostaglandins, acute phase proteins and AMPs. Effector molecules may be brought to the sites of invasion via mobile cells such as mast cells, phagocytes, natural killer cells, eosinophils and basophils (Oppenheim *et al.*, 2003). The effector molecules may also rapidly neutralize invaders and subsequently attract phagocytes to the sites of infection where such pathogenic microorganisms are then engulfed (Zasloff, 2007).

AMPs are important effectors and regulators of the innate immune system where aside from their antimicrobial potential they are immunomodulatory (Hancock, 2001; Tokumaru *et al.*, 2005). These peptides are involved in the increase of chemokine production upon invasion (Charlet *et al.*, 1996), exerting pro- and anti-apoptotic effects on different immune type cells, recruiting phagocytes (Zanetti, 2004) and promoting adaptive immunity (Banchereau and Steinman, 1998). Certain eukaryotic cells involved in the production of AMPs are the epithelial cells of the gastrointestinal and the genitourinary systems (Niyonsaba *et al.*, 2002), lymphocytes (Oppenheim *et al.*, 2003) and phagocytes (Hancock and Scott, 2000) where AMP expression varies from being constitutive to inducible (Hultmark *et al.*, 1980). An example of AMP constitutive expression is the murine homologue of human beta-defensin 2, named mouse beta-defensin 3, transcribed in low levels within the epithelial cells. Upon infection with *P. aeruginosa* PAO1, mRNA levels of mBD-3 were up-regulated not only in the large airways but in the small bowel and liver (Bals *et al.*, 1999). An example of AMP inducible expression is the AMPs isolated from the silk moth *Hyalophora cecropia*, P9A and P9B. The expression of these AMPs were induced within the haemolymph upon vaccination with *Enterobacter cloacae* (Hultmark *et al.*, 1980).

The ability of AMPs to induce microbial killing resides in their physicochemical characteristics. AMPs are generally small (containing not more than 100 amino acid residues), amphipathic molecules comprising of a number of cationic residues and a substantial proportion of hydrophobic residues (Giuliani *et al.*, 2007). Though these peptides are generally cationic, a small minority are known to be anionic and these are mainly isolated from mammals (Harris *et al.*, 2009). As this study mainly focuses on the cationic AMPs, the importance of structure and content of these peptides will be described in greater detail.

The diversity found amongst the amino acid sequences of AMPs is essential for their broad spectrum of antimicrobial activity. On the basis of their secondary structure, cationic AMPs may be grouped into four major classes: β -sheet containing peptides, α -helical peptides, mixed containing both β -sheet and α -helical structures or extended, as shown in Figure 1.1 (Jenssen, 2009). The α -helical and β -sheet structures are generally the most common. Other key features of AMPs such as size, charge, hydrophobicity, amphipathicity and peptide self-association are also responsible for the antimicrobial potential these peptides exhibit (Nissen and Nes, 1997).

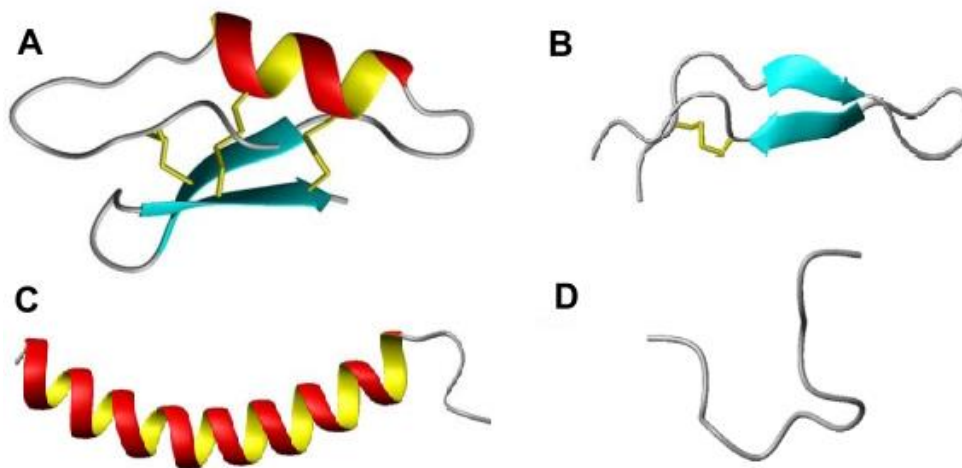


Figure 1.1: AMP class models. Protein models representing the secondary structural differences of the four major classes of AMPs: **A**-represents a mixed structure (Plectasin, a defensin-like molecule), **B**-represents the β -sheet (lactoferricin), **C**-represents an α -helical shape (human cathelicidin LL-37) and **D**-represents the extended shape (lactoferricin) (Jenssen, 2009).

The prime factors responsible for the physical characteristics and antimicrobial potential of AMPs are the amino acid composition and sequence. These two factors govern the overall charge, hydrophobicity and structure of AMPs directly impacting their antimicrobial potential (Hancock *et al.*, 2006).

Charge is an essential factor for AMPs as it governs their attraction towards bacterial membranes. The AMP charge content generally ranges from +2 to +11. The higher the charge, the stronger the electrostatic attraction of the peptides. Too strong of an attraction however poses certain limitations to AMP activity. An overall charge of +7 seems to be the threshold for antimicrobial activity as beyond this, the strong electrostatic forces prevent the formation of AMP secondary structures limiting the peptides from permeabilizing microbial membranes (Tossi *et al.*, 1994). The amino

acids responsible for the cationic nature of these peptides are the charged amino acid side residues such as Arg, Lys and His (Yeaman and Yount, 2003). These amino acids are reported to be necessary for the peptide-membrane interaction and maintain the antimicrobial activity of AMPs irrespective of their size or secondary structures. Peptides comprising of the amino acid Arg exhibit a more stable electrostatic attraction towards negatively charged bacterial membranes than peptides comprising of the other charged amino acid residues. The enhanced antimicrobial potential of peptides comprising of Arg is due to the higher acid dissociation constant of the amino acids guanidinium group (Woods and Ferré, 2005).

The overall hydrophobic content of AMPs determines their partitioning into the lipid bilayers of microbial membranes. The non-polar side chains of amino acids such as: Pro, Trp, and Phe are generally responsible for the hydrophobic nature AMPs exhibit and constitutes 50% of their overall amino acid sequence. This content governs the specificity and antimicrobial efficiency of α -helical peptides. These side chains are the lipophilic anchors required for membrane permeabilization and disruption (Pasupuleti *et al.*, 2012; Yeaman and Yount, 2003). Previous studies have reported peptides comprising of Trp to be more efficient in interacting with bacterial membranes in comparison to other non-polar side chains. The efficient membrane interaction of this amino acid residue is due to its side chain being much more bulkier than the other amino acid residues (Liu *et al.*, 2007). Different bacterial strains however, respond differently to varying levels of AMP hydrophobicity where increasing the hydrophobic content above optimum levels results in a loss of antimicrobial activity as well as peptide specificity. The loss of specificity of AMPs forces the peptides to bind to mammalian cell membranes, increasing their toxicity levels which as a result limits their clinical application (Pasupuleti *et al.*, 2012; Pasupuleti *et al.*, 2008).

To conclude, the number of charged and hydrophobic residues as well as their positions and secondary structural conformations are essential factors governing the antimicrobial mode of action AMPs exhibit (Khandelia *et al.*, 2008).

1.3 Mode of action

The initial target site in the antibacterial mechanism of AMPs is the cell membrane. The amphipathic nature of AMPs denotes their ability to bind and interact with the negatively charged cell membranes of bacterial pathogens via an electrostatic interaction (Hancock and Rozek, 2002).

Bacteria are unicellular organisms that comprise of no nuclear membrane, mitochondria, Golgi bodies or endoplasmic reticulum. Their outer cell wall entails distinct physical differences categorizing bacteria into two basic types: Gram-positive and Gram-negative. The different cell wall constituents in Gram-positive and Gram-negative bacteria are shown in Figure 1.2 below. The Gram-positive cell wall constitutes of a thick peptidoglycan layer surrounding the cytoplasmic barrier, whereas the Gram-negative cell wall constitutes of a thin peptidoglycan layer and an outer membrane (Schaechter *et al.*, 2006).

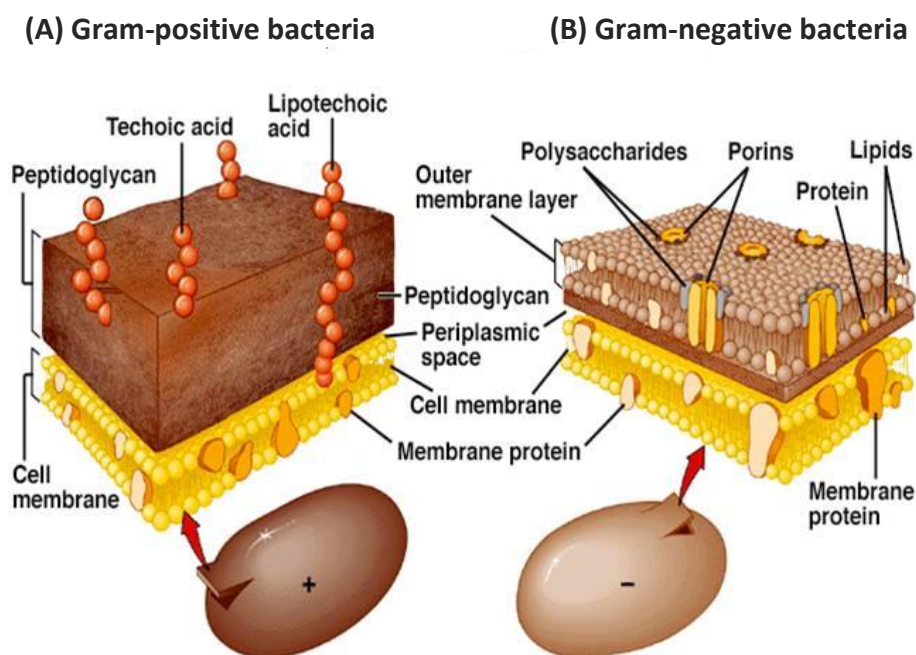


Figure 1.2: Bacteria cell wall differences. A graphical representation of the cell wall differences between (A) Gram-positive and (B) Gram-negative bacteria (Hayat, 2013).

Bacterial membranes are negatively charged due to their surface residues. Gram-positive bacteria obtain their negative charge from the teichoic acid residues residing on the surface of their peptidoglycan layer and Gram-negative bacteria obtain their negative charge from the lipopolysaccharide (LPS) residues presented on their outer membranes. As the outer membrane of Gram-negative bacteria generally functions as a size-selective, sieve-like permeability barrier, the bacterium in addition has other secondary protective measures such as the periplasmic β -lactamase. As a result, Gram-negative bacteria are much more difficult to target and are more resistant to majority of current antibiotics (Hancock, 1997). The plasma membrane for both bacterial cell types is generally surrounded by a thick mucus-like layer of polysaccharides that creates a ‘capsule’.

Surface capsular polysaccharides generally protect pathogenic microorganisms from host immune responses by masking antigen proteins (Merino and Tomás, 2001).

The overall charge of the membrane lipids on cell surfaces is essential in driving AMP selectivity. Eukaryotic membranes are generally zwitterionic comprising of phosphatidylcholine and sphingomyelin in the outer leaflets of their membranes and phosphatidylethanolamine and phosphatidylserine in their inner leaflets of their membranes, stabilized by cholesterol molecules (Pushpanathan *et al.*, 2013). The charge differences between prokaryotic and eukaryotic membranes ensure eukaryotic membranes are not targeted by AMPs (Dathe and Wieprecht, 1999; Matsuzaki, 1999).

Besides peptide charge being essential for the bactericidal mechanism of action of AMPs, other factors such as size, primary sequence, conformation, hydrophobicity, amphipathicity and peptide concentration governs their bactericidal mode of action (Friedrich *et al.*, 2000). Most AMPs generally adopt a dynamic interchange in their structures upon bacterial membrane interaction (Sansom, 1998). Depending on the physicochemical properties of AMPs, the peptides have the ability to induce bacterial killing via membrane interaction in two ways: membrane permeabilization or intracellular translocation inhibition.

1.3.1 Membrane permeabilization

The overall composition and structure of AMPs is essential to induce membrane permeabilization. In order for membrane permeabilization to occur, the peptides undergo the following phases: the attraction phase (the electrostatic attraction of AMPs towards negatively charged bacterial membranes), the attachment phase (the peptides dynamic interchange in structure), the peptide insertion and membrane permeability phase (Brogden, 2005).

Before AMPs attach to the outer bacterial membranes, the peptides first traverse capsular polysaccharides in the attachment phase. The LPS molecules on the outer membranes of Gram-negative bacteria are cross-bridged by divalent cations. These divalent cations stabilize the outer membrane by preventing the LPS molecules from repelling one another (Mayers, 2009). AMPs displace these cations such as Mg^{2+} and Ca^{2+} from their relative binding sites and enter the bacterial cell via a 'self-promoted uptake' mechanism limited to Gram-negative bacteria (Sawyer *et al.*, 1988). Displacing these cations results in pores destabilizing the bacterial cell membrane allowing

the subsequent uptake of the peptides into the cells. These pores lead to more peptides entering the cells and more ions and metabolites leaking out causing membrane depolarisation and the induction of bacteria killing. AMPs therefore promote their own uptake against Gram-negative bacteria instead of diffusing through the outer membrane through water channels as occurs with other antimicrobial agents (Brogden, 2005; Laverty *et al.*, 2011; Mayers, 2009). An effective self-promoted uptake mechanism depends on the characteristic LPS traits of the bacterium (Mayers, 2009). The interaction of AMPs with Gram-positive bacteria is voltage dependent. The electrochemical gradient ($\Delta\psi$) found on the surface of Gram-positive bacteria is positively correlated with peptide binding (Matsuzaki *et al.*, 1997). Previous studies have indicated that this transmembrane potential varies amongst different AMPs where a substantial gradient is required for optimal bactericidal activity (Yeaman *et al.*, 1998; Yeaman and Yount, 2003).

The concentration of AMPs on the bacterial surface is an essential factor to consider in terms of permeabilization. The peptide ratio on the surface of the membrane determines the model employed to induce pore formation in the insertion phase. These models are the aggregate, toroidal-pore, carpet and barrel-stave models as shown in Figure 1.3A-D.

The aggregate model (Figure 1.3A) results in pore formation via the peptides reorienting themselves to form aggregates which span the bacterial membrane. This pore formation results in lipid-peptide micelle complexes which have no exact orientation. The toroidal-pore model (Figure 1.3B) entails the peptides inserting perpendicularly into the bacterial cell membrane due to the electrostatic interactions between the hydrophilic regions of the peptides and the polar head groups of the membrane. The hydrophobic regions of the peptides bend the lipid monolayers forming a water pore aligned by the lipid head groups and the inserted peptides. This toroidal pore model allows more peptides to enter into the cell. In the barrel-stave model (Figure 1.3C), the attached peptides bound to the bacterial membrane start to aggregate. This aggregation leads to the insertion of the peptides perpendicularly into the lipid bilayer resulting in pore formation. Staves are formed in a barrel shaped cluster whereby the hydrophilic regions of the peptides interact with the lumen of the pore and the hydrophobic regions of the peptides interact with the lipid tails. The carpet model (Figure 1.3D) exhibits membrane disruption by orientating the peptides parallel to the membrane, forming extensive layers symbolically representing a carpet. Above a certain threshold concentration, micelles are formed leading to a detergent-like activity and thus pores being formed within the membrane.

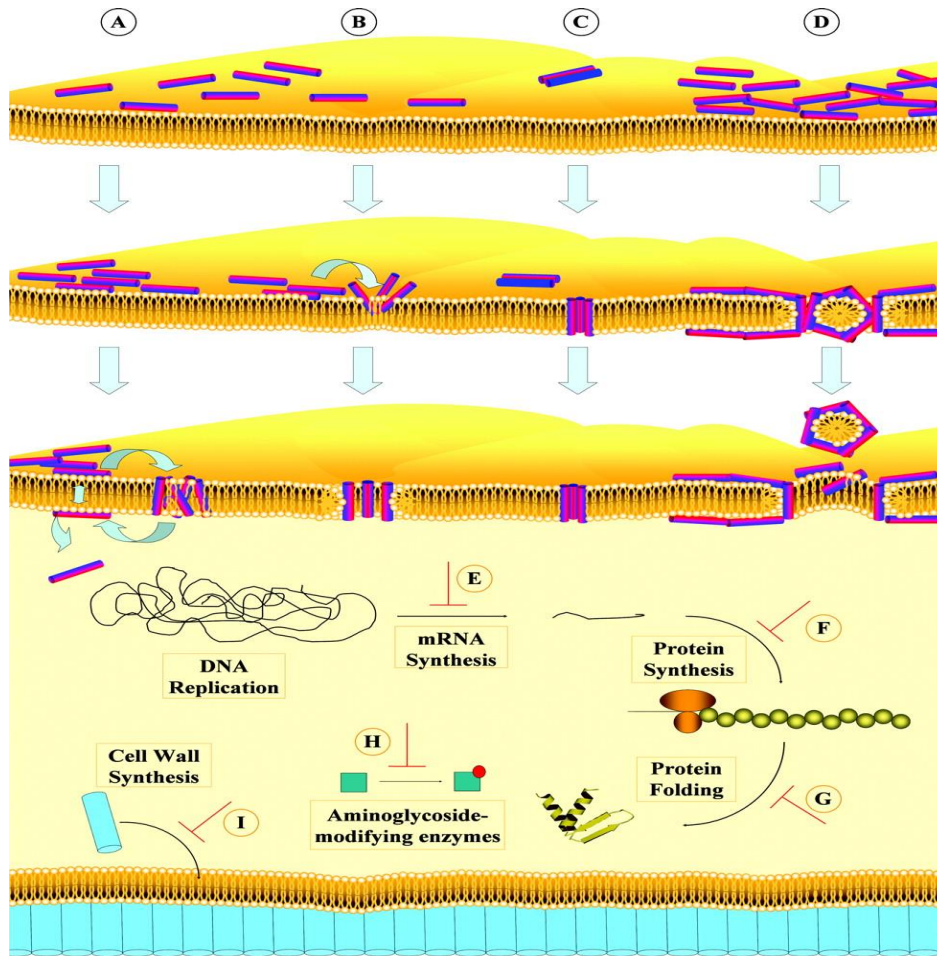


Figure 1.3 : Mode of action of AMPs. A-D: Pore formation (A) The aggregate model, (B) toroidal-pore model, (C) the barrel-stave and (D) the carpet model. E-I: Intracellular targets. Inhibition of (E) mRNA synthesis (F) protein synthesis (G) protein folding (H) enzymes (I) cell wall synthesis (Jenssen *et al.*, 2006).

The general system employed by each model is that at low peptide/lipid concentrations, the peptides bind parallel to the lipid bilayer. As this ratio begins to increase, peptides start to move perpendicularly to the membrane and at high concentrations their perpendicular conformations insert into the bilayer forming pores. As each of the respective models differs significantly in pore formation, their common characteristics are that pore formation disrupts the bacterial membrane integrity resulting in bacterial death (Brogden, 2005; Laverty *et al.*, 2011).

1.3.2 Intracellular translocation inhibition

Membrane permeabilization is just one of the mechanisms AMPs employ to induce cell death. AMPs also have the ability to translocate the cell membrane and inhibit essential life processes of the bacterium. These involve inducing bacterial cell damage by inhibiting either the protein, DNA,

RNA or cell wall synthesis of the bacterium (Figure 1.3E-I). Some peptides may even have multiple intracellular targets inducing a mixed multi-hit mechanism for bacterial cell death. Irrespective of the mechanism employed, the bacterial membrane still remains to be the initial mode of contact for these peptides (Guilhelmelli *et al.*, 2013).

Before intracellular killing can occur, AMPs have to be first taken up by the bacterial cells. As the initial contact is the cell membrane, cellular uptake by the membrane can occur via two mechanisms: direct penetration or endocytosis. AMP killing via endocytosis involves macropinocytosis or receptor-mediated endocytosis (Jones, 2007). Macropinocytosis is a process which occurs as the membrane folds inwards to form vesicles which pinch off with the help of dynamin proteins. The pinched off vesicles have a single membrane and are known as macropinosomes. Receptor-mediated endocytosis involves pits formed on the membrane via proteins such as clathrin and caveolin which eventually bud off in the inner side of the cell forming vesicles (Madani *et al.*, 2011).

Once the peptides have been taken up by the bacterial cells, they may either inhibit bacterial DNA, RNA or protein synthesis resulting in bacterial death. There have been numerous reports on various AMPs binding to intracellular bacterial targets. Indolicidin, isolated from bovine neutrophils, is reported to induce bacterial cell death by binding to DNA with a specific sequence (Hsu *et al.*, 2005), whereas apidaecins, short Pro-Arg rich fragments isolated from insects, inhibit protein synthesis (Castle *et al.*, 1999). tPMP-1 and aHNP-1 derived from the human immune system, inhibit microbial DNA and protein synthesis within a minute once the peptides have entered the bacterial cells (Xiong *et al.*, 1999).

Some AMPs may inhibit microbial protease synthesis. For example equine neutrophil antimicrobial peptide 2 (e-NAP-2), isolated from equine neutrophils, inhibits microbial serine proteases (Couto *et al.*, 1993) and histatin 5, isolated from human saliva, induces periodontal tissue destruction from *Bacterioides gingivales* by inhibiting the bacterial proteases (Nishikata *et al.*, 1991). AMPs with multiple targets such as the seminal plasmin AMP, derived from bull semen, inhibits RNA polymerases and RNA synthesis (Scheit *et al.*, 1979). Other AMPs may activate autolysin proteins within target cells which also lead to pathogen death (Chitnis *et al.*, 1990).

1.4 AMPs in clinical development

Since the early discoveries of the antimicrobial potential of AMPs, this field of research has undergone extensive growth to date and there are approximately a 1000 known AMPs. AMPs have been isolated from vertebrates, invertebrates, plants and microbes (Fjell *et al.*, 2007). Using these templates, *de novo* sequences are now designed from natural sequences or engineered from combinatorial libraries (Rathinakumar *et al.*, 2009).

The success of AMP research is attributed to their structural diversity rendering them excellent candidates in solving the microbial resistance situation. Conventional antibiotics inhibit microbes by employing mechanisms which either inhibit the pathogens protein, cell wall or nucleic acid synthesis or target specific enzymes, proteins or receptors. The nonspecific, rapid membrane permeabilization mechanisms AMPs employ, decreases the chances of resistance development, thus rendering AMPs excellent therapeutic candidates (Aoki and Ueda, 2013).

AMPs have the potential to be developed into a variety of therapeutic compounds. These peptides can either act as single anti-infective agents to treat infections or in combination with other therapeutic compounds to exhibit a synergistic effect. AMPs can also be administered as immunostimulatory agents enhancing the innate immune system of the host or neutralizing endotoxins such as LPS, preventing fatal complications such as septic shock (Wang, 2010).

Although a number of AMPs are under clinical trials, none as yet have been approved by the Food and Drug Administration (FDA) due to a few limitations the peptides hold such as manufacturing costs, peptide instability under physiological conditions and toxicity to host cell components (Gordon *et al.*, 2005). The only accepted AMP thus far is nisin, an AMP derived from bacteria (Pathan *et al.*, 2010). A brief summary of some of the AMPs currently in clinical trial phases, their origins, indication as well as the phase of clinical trial evaluation is presented in Table 1.1.

Table 1.1 AMPs currently in clinical trials (Fox, 2013)

Product	Description	Indication	Phase
Magainin	Skin of the African clawed frog (<i>Xenopus laevis</i>)	Diabetic foot ulcers	3
Omiganan	Derived from indolicidin	Rosacea	2
OP-145	Synthetic 24-mer peptide derived from LL-37	Chronic bacterial middle-ear infection	2
Novexatin	Cyclic cationic peptide	Fungal infections of the toenail	1/2
Lytixar	Synthetic, membrane-degrading peptide	Nasally colonized MRSA	1/2
NVB302	Class B lantibiotic	<i>Clostridium difficile</i>	1
MU1140	Lantibiotic	MRSA and <i>C. difficile</i>	
Arenicin	21 amino acids; rich in Arg and hydrophobic AA	Gram (+) bacteria	Preclinical
Avidocin and purocin	Modified R-type bacteriocins from <i>P. aeruginosa</i>	Narrow spectrum antibiotic for human health and food safety	Preclinical
IMX924	Synthetic 5-amino-acid peptide innate defence regulator	Gram (+) bacteria and Gram (-) bacteria (improves survival and reduces tissue damage)	Preclinical

Abbreviations: MRSA: Methicillin-resistant *Staphylococcus aureus*; Gram (+) bacteria: Gram-positive bacteria; Gram (-): Gram-negative bacteria

The sensitivity of AMPs to changing environments such as pH (Lee *et al.*, 1997), serum, salts (Bals *et al.*, 1998) and proteases (Rozek *et al.*, 2003) arises from their overall structure. Potential toxicity towards host cell components may also be a hindering factor due to the hydrophobic nature of these peptides (Marr *et al.*, 2006). These minor setbacks due to the changing peptide environment can be surpassed via peptide modifications. For therapeutic application, mg amounts per kg body weight per day would be required to treat patients. The ability to produce this yield by chemical synthesis is rather costly (Pasupuleti *et al.*, 2012). Another alternative method to synthesizing AMPs is recombinant expression techniques. Though manufacturing costs would be lowered (Luan *et al.*, 2014), several technical difficulties may follow depending on the microorganism involved in the expression experiment, especially with the production of larger proteins. With *E. coli* models, some factors of concern are inefficient disulphide bond formation, inadequate protein folding (De Marco, 2012) and endotoxin interference (Saida *et al.*, 2006).

In attempt to overcome peptide synthesis costs, the design of shorter peptide fragments from natural AMPs is desirable. The loss of amino acid residues in the process however may have variable impacts on the antimicrobial potential of the peptides. Shorter peptide fragments derived from the

C-terminal regions of natural AMPs have been reported to maintain their antimicrobial activity irrespective of specific sequences or disulphide bond formation (Varkey *et al.*, 2006). P4 is a synthetic 20 amino acid residue peptide fragment corresponding to the main active region of longicin, a defensin peptide obtained from the hard tick *Haemaphysalis longicornis*. Both longicin and P4 exhibited antibacterial, antifungal and antiparasitic activities and remained non-haemolytic extending their therapeutic value (Tsuji *et al.*, 2007). Wang and Zhu (2011) reported that shorter peptide fragments derived from the C-terminal region of the defensin scapularisin-20, isolated from the tick *Ixodes scapularis*, exhibited a wide spectrum of antimicrobial potential against Gram-positive and Gram-negative bacteria. An even shorter amidated peptide fragment spanning the amino acid region 26-39 of scapularisin-20 (14 amino acids, charge +4) exhibited a more potent antimicrobial potential against Gram-positive bacteria (LC = 10-20) than Gram-negative bacteria (LC=15-33). Both peptide fragments were non-haemolytic against mouse erythrocytes (Wang and Zhu, 2011; Zhu *et al.*, 2007). Varkey and colleagues (2006) investigated the antimicrobial activities of synthetic peptides spanning the C-terminus regions of several defensins isolated from the firebug *Pyrrhocoris apterus* (PA Δ C; 17-mer with a net charge of +6), the stable fly, *Stomoxys calcitrans* (SC Δ C; 18-mer with a net charge of +3), and the tick *O. moubata* (OM Δ C; 20-mer with a net charge of +5; isoform C). These synthetic peptides lacked the Cys residues (Δ C) unlike their parent peptides and displayed variable antimicrobial potential. PA Δ C exhibited comparable antimicrobial potential against Gram-positive and Gram-negative bacteria (Lethal concentration (LC) = 5 μ M). OM Δ C was more active against Gram-negative bacteria (LC = 5 μ M) than Gram-positive bacteria (LC = 20 μ M). SC Δ C however was not active against most bacterial species except for *Bacillus subtilis* (LC = 40 μ M) (Varkey *et al.*, 2006).

In order to improve the antimicrobial potential and environmental sensitivities of smaller peptide fragments derived from natural AMPs, the addition of non-natural amino acids (e.g. D-amino acids, N-methyl and α -methyl amino acids) to their sequence or modifications to their terminal ends are generally implemented. These strategies involve natural post-translational modifications such as: cyclization, C-terminal amidation, terminal capping, disulphide bond formation, amino acid substitutions, non-natural amino acid additions or peptide bond modifications by nitrogen alkylation (Wang, 2012; Pasupuleti *et al.*, 2012). A common post-translational modification commonly employed to AMPs synthetically to enhance their antimicrobial activity is C-terminal amidation (Strandberg *et al.*, 2009).

1.5 Terminal capping: Amidation of the C-terminal related to AMPs

In the search for novel therapeutic options, numerous studies have targeted the cationicity of AMPs to improve the antimicrobial activity and reduce the cytotoxicity of these peptides. By increasing the charge of AMPs, the electrostatic attraction of the peptides towards bacterial membranes is enhanced. As the cationicity of AMPs is generally increased by the addition of basic amino acid residues such as Arg, Lys and His, it is also increased by the addition of an amide moiety to the C-terminal end (Dennison *et al.*, 2009). An increase in charge via amidation is obtained as the amide moiety eliminates the negative charge at the C-terminal peptide region.

Amidation of the C-terminus under natural circumstances involves the oxidative decarboxylation of an additional C-terminal Gly residue in a two-step enzymatic process. Amidation increases the peptides stability by protecting the peptide from the cleavage by carboxypeptidases. The added amide moiety also provides an extra hydrogen bond for α -helical formation (Andreu and Rivas, 1998).

C-terminal amidation has been proven to enhance the antimicrobial activity of natural and synthetic AMPs (Dennison *et al.*, 2009). Synthetic C-terminal amidation can be found amongst a number of AMPs such as the cecropins, dermaceptins, clavenin, melittin, PGLa and PR-39. (Andreu and Rivas, 1998). The impact of this amidation effect on the peptides antimicrobial and cytotoxic potentials vary due to their structural diversity as a result of their amino acid compositions and lengths (Dennison *et al.*, 2009). In regards to this, C-terminal amidation of the dermaseptin S3, a family of AMPs isolated from the skin of tree frogs belonging to the *Phyllomedusa* genus, showed an enhanced bactericidal potential against a wide range of pathogenic bacteria with a reduced haemolytic potential. C-terminal amidation of eumenitin, an AMP derived from the venom of the solitary eumenine wasp *Eumenes rubronotatus* (Konno *et al.*, 2006) however, reduced the haemolytic potential of the peptide as well as the bactericidal potential (Kim *et al.*, 2011).

1.6 Defensins

One of the major classes of AMPs is the defensins, found throughout the phylogenetic tree. Defensins were first identified in human neutrophils. These AMPs are small cationic molecules and are regarded as one of the most abundant groups of AMPs found amongst mammals, insects and plants (Jenssen *et al.*, 2006). Defensins comprise of 18-45 amino acid residues and form part of a cysteine rich cationic family without glycosyl or acyl side chain modifications (Chrudimska *et al.*,

2010; Ganz *et al.*, 1985). Defensins are produced as pre-propeptides of approximately 8 kDa and comprise of a number of isoforms. These AMPs are generally cleaved through a conserved C-terminal cleavage motif known as the furin cleavage site (RVRR), resulting in two 4 kDa mature peptide fragments (Kopáček *et al.*, 2010). These functionally matured peptides usually contain 6 Cys residues that form intra-molecular disulphide bridges. The disulphide bonding patterns found amongst defensin peptides render them more stable under physiological serum conditions (Andes *et al.*, 2009). The secondary and tertiary structures of defensins show similarities to that of membrane modulators (Chrudimska *et al.*, 2010). Defensins also aid in wound healing by stimulating host immune processes responsible for tissue organization (Ulm *et al.*, 2012). Some defensins are constitutively expressed (human neutrophil defensins, α -defensins) within the immune system whereas others are expressed in response to inflammatory stimuli or microbial invasion (α -defensins HD5 and HD6) (Lachmann and Oldstone, 2006).

Vertebrate defensins are divided into the following subfamilies depending on the alignment of their disulphide bridges as shown in Figure 1.4: α -defensins, β -defensins and θ -defensins as well as insect defensins (Mehra *et al.*, 2012). The structures of the α - and β -defensins differ in the positions of their Cys residues as well as their disulphide bonds. Both α - and β -defensins have a triple stranded sheet with a distinct defensin fold. The α -defensins comprise of 29-35 amino acid residues and are generally shorter than the β -defensins which comprise of 36-42 amino acid residues. α -Defensins exist as prepropeptides found in mature polymorphonuclear (PMN) cells and Paneth cells whereas β -defensins are constitutively expressed in the epithelium where expression may be increased in response to infection or inflammation. The disulphide bonding patterns of α -defensins is generally a 1-6, 2-4, 3-5 linkage, whereas β -defensins generally form a 1-5, 2-4, 3-6 disulphide bond linkage (Schutte *et al.*, 2002; Schneider *et al.*, 2005). θ -Defensins are cyclic peptides formed by the splicing and cyclization of two 9 amino acid segments of α -defensins and were first identified in Rhesus macaque monkey leukocytes. Invertebrate defensins comprise of the α -helix being disulphide bonded to a β -sheet with a Cys linkage (1-4, 2-5, 3-6) differing from vertebrate defensins (Ganz, 2003). Insect defensins are generally transcribed and released into the fat body or haemolymph in response to septic injury or constitutively expressed in the haemolymph and released after immune threats (Hoffmann and Hetru, 1992).

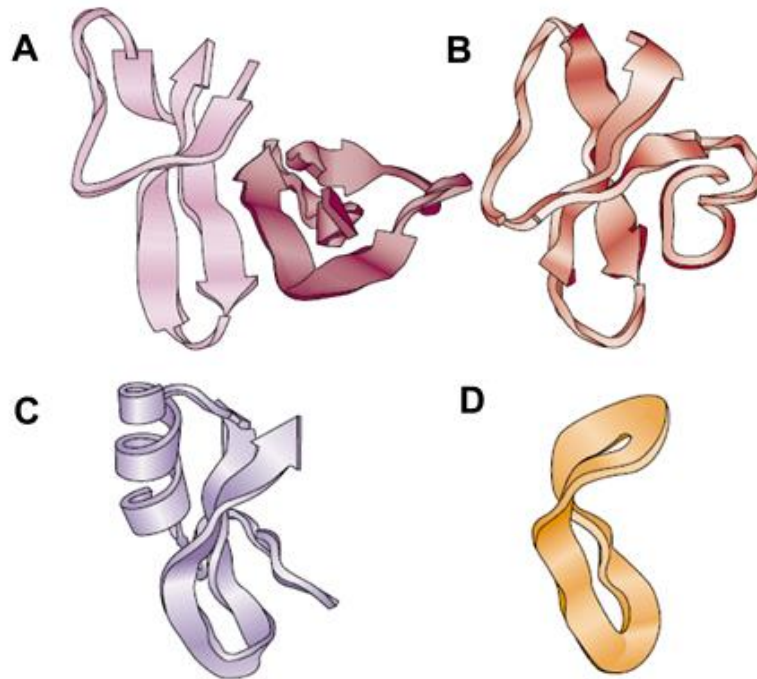


Figure 1.4: Overall secondary conformation of the defensin subfamilies: (A) α -defensins (B) β -defensins (C) insect defensins and (D) θ -defensins (Ganz, 2003).

Defensins are active against bacteria, fungi and viruses (Gao *et al.*, 2009), under optimal conditions such as low ionic strength and low concentrations of divalent cations, plasma proteins and other interfering substances (Kagan *et al.*, 1990). The functional region of defensins responsible for their antimicrobial potential resides in the C-terminal β -sheet domain known as the Y-core motif. This region varies in sequence, length and defensin function (Wang and Zhu, 2011). Antimicrobial action is induced by targeting the membranes of pathogens (Kagan *et al.*, 1990). Human defensin HNP-1, derived from human neutrophils, induces bactericidal activity against *E. coli* by targeting both the outer and inner membranes (Lehrer *et al.*, 1989).

As invertebrates rely solely on their innate immune system for immune protection, AMPs play a major role in the immunity of these organisms. Almost all AMPs are produced by invertebrate fat body cells and haemocytes, and are later released into the haemolymph in response to septic injury (Rodriguez de la Vega *et al.*, 2004). AMPs are also found in the midgut and epithelial tissues as effector molecules providing protection against external microbial invasion. Invertebrate defensins have been isolated from insects, scorpions, mussels, horseshoe crabs and ticks. To date, over 150 defensins from insects are known (Coutinho *et al.*, 2008).

1.7 Tick defensins

Ticks are obligate hematophagous arthropods forming part of the invertebrate group, arachnid (Hajdušek *et al.*, 2013). Ticks are categorized into three major families: the *Ixodidae* (hard ticks), the *Argasidae* (soft ticks) and the *Nuttalliellidae* (confined to southern Africa), all of which are versatile arthropod disease vectors, able to transmit pathogens ranging from bacteria, viruses, protozoa, fungi and nematodes to their hosts. The feeding on blood is necessary for the development and reproduction of ticks. Tick immunity is well designed with effective cellular and humoral responses to protect them against pathogens. A major feature of their innate immune system is the synthesis and release of AMPs that play an essential role in the innate defence system (Taylor, 2006). Defensins are the main family of AMPs found within ticks.

The first record of a tick defensin was derived from the haemolymph of *Ornithodoros moubata* in 2001. This defensin was identified from the cloning and sequencing of two isoforms found in the haemolymph (Nakajima *et al.*, 2001). To date, a number of defensins have been isolated from several tick species exhibiting a spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeast, protozoa and fungi. A few defensin peptides isolated from ticks exhibiting antibacterial and antifungal activity are listed in Table 1.2 and Table 1.3.

Tick defensins are constitutively expressed in a variety of tissues and have been isolated from the haemolymph, midgut, salivary glands and fat body cells of both hard and soft ticks (Kopáček *et al.*, 2010). Nakajima *et al.* (2002) have concluded the predominant location of tick defensin like genes to be the midgut. The various defensins isolated from ticks indicates their role in host immunity.

Despite the fact that ticks are able to harbour and transmit diseases, they are efficient in protecting themselves against pathogenic microbes with the aid of AMPs (Chrudimska *et al.*, 2010). Ticks therefore are a natural, valuable source for obtaining these therapeutic peptides (Lu *et al.*, 2010)

Table 1.2: Defensins isolated from ticks (Adapted from: (Cheng, 2010))

Tick	AMP	Source	Antimicrobial activity			Reference
			+G	-G	Fungi	
<i>Ornithodoros moubata</i>	Defensin A	MG	Y	N	N	(Nakajima <i>et al.</i> , 2001, Nakajima <i>et al.</i> , 2002)
	Defensin B	MG	Y	N	N	(Nakajima <i>et al.</i> , 2001, Nakajima <i>et al.</i> , 2002)
	Defensin C	MG	Y	N	N	(Nakajima <i>et al.</i> , 2001, Nakajima <i>et al.</i> , 2002)
	Defensin D	FB	Y	N	N	(Nakajima <i>et al.</i> , 2001, Nakajima <i>et al.</i> , 2002)
	Rabbit α -HS	MG	Y	N	N	(Nakajima <i>et al.</i> , 2003)
<i>Dermacentor variabilis</i>	Varisin	HL	Y	Y	N	(Sonenshine <i>et al.</i> , 2002)
	Defensin-1	MG, FB	Y	N	N	(Ceraul <i>et al.</i> , 2007)
	Defensin-2	MG,FB	Y	N	N	(Ceraul <i>et al.</i> , 2007)
<i>Haemaphysalis longicornis</i>	Gut-defensin	MG	Y	Y	N	(Zhou <i>et al.</i> , 2007)
	Hisal-defensin	SG	Y	Y	N	(Zhou <i>et al.</i> , 2007)
	Longicin	MG	Y	Y	Y	(Tsuji <i>et al.</i> , 2007)
	Longicornsin	SG	Y	Y	Y	(Lu <i>et al.</i> , 2010)
<i>Ixodes sinensis</i>	Ixosin	SG	Y	Y	Y	(Yu <i>et al.</i> , 2006)
	Ixosin	SG	Y	Y	Y	(Liu <i>et al.</i> , 2008)

Note; Y: Yes, N: No, G+: Gram-positive bacteria, G-: Gram-negative bacteria, FB: Fat body, H: Haemocytes, HL: Haemolymph, MG: Midgut, SG: Salivary gland.

1.8 Background of this study

This study mainly focuses on the soft tick, *Ornithodoros savignyi*, commonly known as the sand tampan, endemic to the arid and semi-arid regions of Africa (Mans and Neitz, 2004). As this livestock parasite is known to be a rich source in bioactive molecules, it was used as a model for tick investigations of which several anti-haemostatic components have been described (Maritz-Olivier *et al.*, 2007).

From the haemolymph of immune challenged *O. savignyi*, a Gram-positive AMP was purified (Olivier, 2002). With the use of Edman sequencing, the N terminal sequence of this peptide was determined. This partial sequence was then used to design primers for the cDNA cloning of a midgut defensin in *O. savignyi* of which two full length defensin isoforms, *O. savignyi* defensin isoform one (OsDef1) and two (OsDef2) were identified (Table 1.4) (M. Botha, Honours project). Both defensin isoforms were 42 amino acid residues long with the mature peptide represented by 37 of these residues. Both these defensin isoforms were active against Gram-positive bacteria (Prinsloo *et al.*, 2013). OsDef2 (+6) was chosen as a template for the design of shorter peptide fragments as it was more active than OsDef1 (+5). Based on these findings, Os was derived from the last 22 amino acid residues of the C-terminus of OsDef2.

Table 1.3: Structural characteristics of study peptides

Peptide	Sequence	Charge	MW ^a	No. of AA ^b
OsDef1	GYGCPFNQYQCHSHCSGIRGYRGGYCKGAFKQTCKCY	+5	4172.7	37
OsDef2	GYGCPFNQYQCHSHCKGIRGYKGGYCKGAFKQTCKCY	+6	4185.8	37
Os	KGIRGYKGGYCKGAFKQTCKCY	+6	2459.9	22

^aMW: Theoretical molecular weight of peptides obtained from: Innovagen [<http://www.innovagen.com>]

^bAA: amino acids

The difference in amino acid sequence between Os Def1 and OsDef2 are highlighted in red, charged amino acid sequences are highlighted in blue, Cys residues are highlighted in green

The antibacterial, antioxidant and cytotoxic activities of Os was evaluated. The 22 amino acid length sequence was predominantly α -helical in structure and was active against Gram-positive and Gram-negative bacteria. Os was non-lytic against human erythrocytes as well as the SC-1 and Caco-2 cell lines (Prinsloo *et al.*, 2013). When investigating the bactericidal potential of Os under physiological sodium chloride (NaCl) conditions, Os remained active against *E. coli*, however lost activity against *S. aureus*. In the presence of 30% human serum, Os remained active against *S. aureus* however displayed a 32-fold reduction in bactericidal activity against *E. coli* (Prinsloo, 2013). The peptide fragment was found to have antioxidant potential greater than glutathione (GSH), a known antioxidant (Prinsloo *et al.*, 2013).

As shorter peptide fragments are desirable templates for therapeutic drug design in order to reduce AMP manufacturing costs, Os was further investigated structurally to determine the peptide's active regions responsible for bactericidal activity. A series of synthetic overlapping 10-mer peptides plus the N and C terminal fragments based on the structure of Os were then investigated for bactericidal activity against *B. subtilis* and *E. coli*. Two peptides, Os(3-12) and Os(11-22) were found to be active with MBCs in the range of 30-60 µg/ml (Table 1.5).

Table 1.4: Properties of the synthetic overlapping peptide fragments based on the structure of Os (Odendaal, 2013)

Peptide	Sequence	No of AA ^a	Net charge	Turbidity assay (MBC µg/ml) ^b	
				<i>B. subtilis</i>	<i>E. coli</i>
Os	KGIRGYKGGYCKGAFKQTCKCY	22	+6	3.75	1.9
Os(1-10)	KGIRGYKGGY	10	+3	>120	>120
Os(3-12)	IRGYKGGYCK	10	+3	30	30
Os(5-14)	GYKGGYCKGA	10	+2	>120	>120
Os(7-16)	KGGYCKGAFK	10	+3	>120	>120
Os(9-18)	GYCKGAFKQT	10	+2	>120	>120
Os(11-20)	CKGAFKQTCK	10	+3	>120	>120
Os(13-22)	GAFKQTCKCY	10	+2	>120	>120
Os(11-22)	CKGAFKQTCKCY	10	+3	30	60

^a AA: Amino acids

^b MBC: Minimum bactericidal concentration

1.9 Aims of this study

The shorter peptide fragments, Os(3-12) and Os(11-22) were not as potent as the parent peptide Os. The purpose of this study was to investigate whether amidation of the C-terminus of Os(3-12) would increase the peptide's antibacterial potency. The antibacterial, antioxidant and cytotoxic activities of Os(3-12)NH₂ were determined and compared with the amidated peptide as well as Os.

More specifically the objectives of this study were to:

1. Elucidate the secondary structure of Os(3-12)NH₂
2. Determine the MBC of Os(3-12)NH₂ against Gram-positive and Gram-negative bacteria
3. Determine the killing kinetics of this peptide in both Gram-positive and Gram-negative bacteria
4. Investigate whether membrane permeabilization by Os(3-12)NH₂ and Os in both Gram-positive and Gram-negative bacteria is a possible mode of action
5. Evaluate the bactericidal activity of Os(3-12)NH₂ under serum and salt conditions
6. Determine the cytotoxic potential of Os(3-12)NH₂ in human erythrocytes and Caco-2 cells

7. Establish whether Os(3-12)NH₂ possess antioxidant activity

1.10 Outputs

OMAR ISMAIL N, SERUM J, BESTER MJ, NEITZ AWH & GASPAR ARM (2014). Functional characterization of an amidated synthetic peptide, Os(3-12), derived from the tick *Ornithodoros savignyi* Isoform 2 (OsDef2). 25th South African Biochemistry and Molecular Biology (SASBMB) congress, Goudini Spa. Western Cape. 6-9 July 2014 (P-173. p. 152).

CHAPTER 2

MATERIALS AND METHODS

2.1 Peptides and their preparation

Os(3-12)NH₂ was purchased from LifeTein (NJ, USA). Os(3-12) and Os were purchased from GenScript (New Jersey, USA). The purity and molecular mass for each peptide was determined by their respective suppliers using reverse-phase HPLC and mass spectrometry. Control peptides such as melittin, LL-37 and glutathione (GSH) were purchased from Sigma Aldrich (RSA). The source and the role of each control peptide used in this study is presented in Table 2.1.

Table 2.1: Control peptides used in this study

Peptide name	Source	Role in study	References
Melittin	European honey bee, <i>Apis mellifera</i>	Secondary structure determination, cytotoxicity and membrane permeabilization assays	(Vogel and Jähnig, 1986)
LL-37	Cathelicidin AMP from human neutrophils	Membrane permeabilization assay	(Oren and Shai, 1997)
GSH	Natural antioxidant in the body	Antioxidant assay	(Sen, 1999)

UV absorbance was used to determine the concentrations of peptides; Os(3-12)NH₂, Os(3-12), Os and melittin. UV absorbance is a technique used to determine protein concentration based on the extinction coefficients of the Tyr and Trp residues within the peptide sequence. The lyophilized peptides were dissolved in sterile distilled water by gentle inversion. A 20 times dilution was prepared to determine the absorbance value using a UV visible recording spectrophotometer (Shimadzu UV-160A, 1 cm quartz cuvette, Kyoto, Japan). The protein concentration (mg/ml) was calculated using the following Beer-Lambert law based equation:

$$c = \frac{Abs \times Df \times MW}{(nTyr)(\epsilon_{Tyr}) + (nTrp)(\epsilon_{Trp})}$$

where c is the peptide concentration in mg/ml, Abs is the absorbance read at 280 nm, Df is the dilution factor, MW is the molecular weight of the peptide, n is the number of Tyr and Trp residues and ϵ is the extinction coefficient values of Tyr and Trp which are 1200 and 5560 AU/mmol/ml, respectively.

This method could not be applied to LL-37 (human cathelicidin) and GSH since these peptides do not contain Tyr and Trp. The peptide concentration for GSH was determined gravimetrically using the molecular weight of the peptide. The peptide concentration of LL-37 was determined using the Bradford assay.

The Bradford assay is a method involving the binding of the dye Coomassie Brilliant Blue G-250 to proteins. Coomassie Brilliant Blue G-250 has the ability to exist in three forms: the cationic (red), neutral (Greenfield) or anionic (blue) form. The dye is initially found in the cationic form allowing the binding to basic amino acids in proteins, mainly Arg, converting the dye to the anionic form. The anionic form is then detected at 595 nm. By creating a protein standard of known concentrations, the unknown concentration of interest can be determined from the equation generated. The Bradford assay was performed according to the Quick Start™ Bradford Protein Assay kit (Bio-Rad, RSA). A standard curve was created using the general standards supplied by the kit. The protein concentration of LL-37 was then calculated using the equation obtained from the graph: $y = 0.0005x + 0.318$.

All peptides were diluted to 1.2 mg/ml, filter sterilized using 0.45 µM Acrodisc syringe filters (Separations, RSA), aliquoted (20 µl/vial) and stored in the freezer at -20 °C.

2.2 PSIPRED: Secondary structure prediction of peptide

The PSIPRED program is a highly accurate secondary structure prediction server which predicts the secondary structure of proteins based on their primary sequence. This prediction server holds a Q3 score of 76.5%., the highest score obtained for accuracy on any server published to date (McGuffin *et al.*, 2000). PSIPRED involves a two feed-forward neural network to perform analysis on outputs obtained from PSI-BLAST (Position Specific Integrated BLAST) (Altschul *et al.*, 1997). This secondary structure program was used to predict the secondary structure of Os(3-12)NH₂, Os(3-12) and melittin (Jones, 1999).

2.3 Secondary structure analysis using circular dichroism

Circular dichroism (CD) is a spectroscopic technique aiding in the rapid determination of the secondary structures and folding properties of proteins. CD is also used to study various protein interactions (Greenfield, 2006).

The absorption of circularly polarized light occurs in molecules with more than one chiral chromophore. Chiral chromophores are mirror image isomers which differ in their interactions with polarized light and each other. A primary use of CD is aiding in the determination of the secondary structures of the chiral chromophores. Since the amino acids which make up protein molecules are chiral, CD is generally used to determine protein secondary structure (Greenfield, 2006).

CD is defined as the difference in absorption in right handed circularly polarized light (R-CPL) from left handed circularly polarized light (L-CPL) of the chiral chromophores. These absorption measurements are carried out on the visible and ultra-violet regions of the electromagnetic spectrum where electronic transitions can be measured. The CD signal can either be positive or negative depending on whether the L-CPL is greater or lesser than the R-CPL (Fasman, 1996). Each secondary structure of a protein molecule has a specific CD signature which can be easily identified. The various secondary structures of protein molecules are the α -helix, β -sheet and randomly coiled conformations. The specific characteristic profile and magnitude on a CD spectrum of these secondary structures can be seen in Figure 2.1. The α -helical structure generally exhibits a positive mean residue ellipticity value at 190 nm and a negative ellipticity value at 209 and 222 nm. The β -sheet structure generally exhibits a positive ellipticity at 195-200 nm and a negative ellipticity at 210-220 nm. A randomly coiled structure generally exhibits a negative ellipticity at 200 nm. As the secondary structures of protein molecules are environment sensitive, CD is used to observe these structural differences under different environmental conditions such as temperature, pH, ionic strength, in the presence of solutes and small molecules (Corrêa and Ramos, 2009).

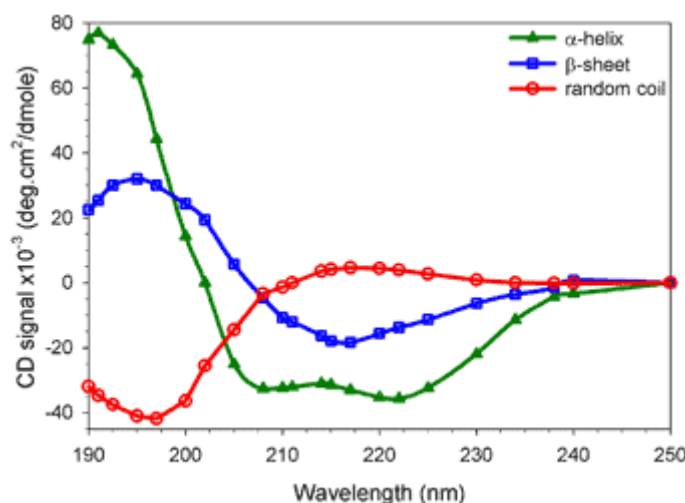


Figure 2.1: Characteristic far-UV CD spectra for peptide secondary structures. The overall CD spectra for the secondary structures: α -helix (Greenfield), β -sheet (blue) and randomly coiled (red) showing their characteristic ellipticity peaks (Khan, 2015).

In this study, CD was used to determine the secondary structures of Os(3-12)NH₂, Os(3-12) and melittin (positive control) in order to evaluate the effect of amidation on the secondary structure of Os(3-12)NH₂ compared to Os(3-12) and Os. Far-UV CD spectroscopy was used to evaluate the effect of two different solutions, sodium dodecyl sulphate (SDS) and trifluoroethanol (TFE) on the peptide's secondary structure in a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan), over the 180-250 nm range with a path length of 0.2 cm. The scans were carried out at 20 °C with a scan speed of 200 nm/min, a data pitch of 0.1 nm and a bandwidth of 0.5 nm. Peptides were prepared (total volume 200 µl) in water, 25 mM SDS and 50% TFE to obtain a final peptide concentration of 50 µM. All data acquired for the CD spectra above 800 volts was omitted due to unreliability. The results obtained were the averages of 10 scans corrected for solvent effects. The signals were converted to mean residue ellipticity, $[\theta]$, using the following equation:

$$[\theta] = \frac{100 \times \theta}{c n l}$$

where c is the peptide concentration in millimolar, θ is the measured ellipticity in millidegree, n is the number of residues and l is the path length (cm).

2.4 Bacterial strains and their preparation

Escherichia coli (ATCC 700928), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (13933), and *Staphylococcus aureus* (U3300) were used for the antibacterial assays. Bacterial strains were streaked on to Luria Bertani (LB) agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.2% (w/v) bacteriological agar, pH 7.5) and grown overnight at 37 °C. A subculture was prepared from the overnight bacterial culture and used for the antibacterial assays.

2.4.1 Bacterial overnight culture

Several colonies of each streaked strain were picked and inoculated in 25 ml LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) at 37 °C for a period of 14-16 hours.

2.4.2 Bacterial subculture

Each bacterial overnight culture was subcultured and grown until each strain had reached their relevant exponential phases. The OD₆₀₀ values for each strain at their respective exponential phases were previously determined in our laboratory using the bacterial standard curve assay (Prinsloo, 2013). The initial concentration (cells/ml) for each bacterial strain was then theoretically calculated by dividing the OD₆₀₀ value obtained for each bacterium by the respective gradient derived from this assay.

The bacterial subculture was prepared by diluting each bacterial overnight culture a 100 times (100 µl: 9900 µl) in LB broth for 2-3 hours at 37 °C in a shaking incubator until the respective OD₆₀₀ values (*E. coli* ~0.6; *S. aureus* and *P. aeruginosa* ~0.5; *B. subtilis* ~0.4) were reached. As each bacterial strain was now in their mid-exponential phase, the bacteria were washed three times which entailed centrifuging the bacterial subculture at 14500 x g for 2 minutes for *E. coli*, *P. aeruginosa* and *S. aureus*; 5 minutes for *B. subtilis*, and re-suspending the pellets in 10 mM sodium phosphate (NaP) buffer (0.002 M H₂NaO₄P.H₂O, 0.008 M HNa₂O₄P.2H₂O, pH 7.4) for Gram-negative bacteria and 1% LB in NaP buffer for Gram-positive bacteria. The bacteria were diluted to 1 X 10⁶ cells/ml using the initial number of cells obtained from the OD₆₀₀ value. The dilution factor was calculated using the following equation:

$$Df = \frac{OD_{600}}{m \text{ for bacterial strain}} \times \text{final concentration needed (CFU/ml)}$$

where Df is the dilution factor, OD₆₀₀ is the optical density for the respective bacteria grown for 2-3 hours, m is the gradient obtained for the respective bacteria ($m_{E. coli} = 2 \times 10^{-9}$, $m_{P. aeruginosa} = 3.8 \times 10^{-9}$, $m_{S. aureus} = 3.67 \times 10^{-9}$, $m_{B. subtilis} = 3 \times 10^{-9}$), OD₆₀₀/m = initial cells/ml concentration), and the final required concentration of the bacterial concentration (generally 1x10⁶ CFU/ml).

2.5 Antibacterial activity

The bactericidal potential of Os(3-12)NH₂ was evaluated using the colony forming unit (CFU) assay. This assay was used to determine the minimum bactericidal concentration (MBC) of the peptide against the selective bacterial strains in order to determine its potency (Hiemstra and Zaat, 2013). The MBC for Os(3-12)NH₂ against the four selected bacterial strains was then compared to the parent peptide Os and the unamidated peptide Os(3-12).

The CFU assay was performed according to the slightly modified method of Tsuji *et al.* (2007) and Varky *et al.* (2006). The MBC for Os(3-12)NH₂ was determined by incubating the peptide, concentration range of 0.06-120 µg/ml (10 µl) with each bacterial subculture diluted to 1 x 10⁶ cells/ml (90 µl) in polypropylene tubes for 2 hours at 37 °C in a shaking incubator. The incubated samples were subsequently diluted 500 times in NaP buffer for Gram-negative bacteria and 1% LB in NaP buffer for Gram-positive bacteria, plated out onto LB agar plates and incubated overnight for 16-18 hours at 37 °C. A growth control was calculated to contain 180 CFU and the percent killing was calculated in terms of the respective growth control. The MBC was defined as the lowest concentration at which 99% bacterial killing was observed.

2.6 Kinetics of bacterial killing

The killing kinetics provides information on the time that a compound takes usually at its MBC to kill all bacteria (Boswell *et al.*, 1997).

The bacterial subcultures (*E. coli* and *B. subtilis*), diluted to 1 x 10⁶ CFU/ml as done previously for the CFU assay, were incubated at the respective MBC for Os(3-12)NH₂ in NaP buffer for *E. coli* and NaP buffer supplemented with 1% LB for *B. subtilis*. Aliquots (10 µl) of this mixture were removed at fixed time intervals (0, 5, 10, 30, 60, and 120 minutes) and diluted 500 times in NaP buffer for *E. coli* cells and LB for *B. subtilis* cells. These diluted samples were then plated out onto LB agar plates and incubated at 37 °C for a period of 16-18 hours. The number of colonies that formed after the overnight incubation were known as colony forming units (CFU) and were counted and expressed as log CFU/ml vs. time (in minutes) using the following formula:

$$\log CFU/ml = \frac{\text{No. of colonies counted}}{0.1 \text{ ml}} \times Df's$$

In the above formula, the number of colonies were divided by 0.1 ml as 100 µl were plated for each bacterial strain. Df's represents the dilution factors used in the assay i.e. 500 x and $\frac{10}{9}$ x dilutions.

2.7 Membrane permeabilization

SYTOX green is a cationic cyanine dye with a molecular weight of approximately 900 Da, generally used in bacterial assays to determine bacterial cell viability. SYTOX green is non-permeable to intact cells however, once cell membranes have been compromised, this nucleic acid stain has the ability to enter the cells and bind to their DNA exhibiting a >500-fold enhanced

fluorescence upon excitation with an argon ion laser at 488 nm or any other 450-490 nm source (Rathinakumar *et al.*, 2009, Roth *et al.*, 1997). Therefore, the non-permeable nature of SYTOX green is an excellent indicator to elucidate the mode of action of AMPs as most often the main targets for inducing bacterial death is membrane permeabilization.

The SYTOX green uptake assay was therefore used to elucidate the mode of action of Os and Os(3-12)NH₂ by measuring end-point fluorescence of the DNA-binding dye SYTOX Green (Life Technologies, Carlsbad, CA, USA). Experimental conditions such as bacterial number, SYTOX green concentration and preincubation times were optimized. The known membrane permeabilizing AMPs; LL-37 and melittin were used as positive controls (Turner *et al.*, 1998; Tosteson and Tosteson, 1981).

The SYTOX green assay was performed according to the modified method of Bourbon *et al.* (2008). The bacterial subcultures: *S. aureus* and *E. coli* were grown until their respective exponential phases were reached. The cells were thereafter centrifuged (1600g x for 10 minutes), washed and resuspended in NaP buffer. Washed cells were diluted to a cell density of 1 x 10⁶ cells/ml. Diluted cells (80 µl) were incubated in a shaking incubator with 10 µl of (v/v) 1 µM SYTOX green (Initial SYTOX green solution: 5 mM in DMSO, 10 µM SYTOX green dye stock solution prepared: 2 µl SYTOX green in 998 µl dH₂O, stock solution used) in a 600 µl Eppendorf tube for 15 minutes to allow for equilibration. Melittin, LL-37, Os and Os (3-12)NH₂ were subsequently added at a concentration range of 0.06-120 µg/ml (10 µl per concentration) to the bacteria-SYTOX green solution and further incubated for 30 minutes. This solution was then transferred to a 96 well Costar black plate (Nunc, Thermo Fisher Scientific, NYC) where end point fluorescence was measured using the fluorescent plate reader, the SpectraMax® Paradigm®, multi-mode detection platform (Separations, RSA). The SpectraMax® Paradigm® was programmed as follows: excitation (504 nm) and emission (544 nm) wavelengths; 96 well costar black/clear bottom plate; off stop and go at 140 ms; 1 mm away from plate with a medium shake for 5 seconds before reading. All raw data were exported to excel and data was expressed as relative fluorescence units (RFUs).

2.8 Effects of serum and salt on the MBC of Os(3-12)NH₂

The suppression of the bactericidal activity of AMPs under serum and salt conditions has been regarded as a major hindering factor in their clinical development as novel therapeutic compounds

(Maisetta *et al.*, 2008). Novel AMPs functional under physiological serum and salt conditions are excellent therapeutic candidates for systemic use. The effect of human serum and salt on the bactericidal activity of Os(3-12)NH₂ was therefore determined.

The exponentially growing bacteria subcultures were washed thrice and resuspended in NaP buffer for *E. coli* and NaP buffer supplemented with 1% LB for *S. aureus*. The bacteria were further diluted to obtain a cell density of 1.125×10^6 CFU/ml. This diluted bacterial subculture (80 μ l) was then incubated with aliquots (10 μ l) of Os(3-12)NH₂ over a final concentration range of 0.06-120 μ g/ml containing 10 μ l (i) 30% human serum (pooled lyophilized human serum obtained from Sigma Aldrich (RSA)); (ii) a physiological salt mixture [final concentration 145 mM NaCl, 2.5 mM CaCl₂ and 1 mM MgCl₂ (Klabunde, 2011)] at 37 °C in a shaking incubator. Incubated samples were subsequently diluted 500 times in NaP buffer for *E. coli* and LB for *S. aureus*. Of the 500 times dilution, 0.1 ml was plated onto LB agar plates and incubated overnight at 37 °C for 16-18 hours. The new MBC for Os(3-12)NH₂ under physiological conditions was then determined with respect to the bacterial growth control as described previously for the CFU assay.

2.9 The lytic effect of Os(3-12)NH₂ on human erythrocytes

The erythrocyte is a differentiated cell type that lacks nuclei and organelles. Erythrocytes have typical eukaryotic bilayer membranes. Damage to the cell membrane causes increased leakage of the cellular content specifically of haemoglobin that absorbs strongly at 500-600 nm.

The haemolytic activity of Os(3-12)NH₂ and melittin (positive control) against human erythrocytes was performed using the haemolysis assay according to the modified method of Stark *et al.* (2002). Blood was collected from the veins of healthy, consenting donors using a sterile needle connected to a 5 ml EDTA vacuum extraction blood tube (Ethical clearance obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria). Erythrocytes were collected by centrifugation at 2750 x g for 2 minutes. The subsequent plasma and buffy coat were removed. The erythrocytes were then washed with an equal volume of isotonic phosphate buffered saline solution (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH₂PO₄.2H₂O, 8.1 mM Na₂HPO₄, pH 7.4; (Iso-PBS)) and 90 μ l of this volume of erythrocytes were thereafter incubated with 10 μ l of the peptides Os(3-12)NH₂ and melittin (final concentration range 0.25-100 μ M) for 30 minutes at 37 °C. The samples were then centrifuged at 2750 x g. The supernatants (75 μ l) were collected, placed into the wells of a 96 well micro-titre plate and the absorbance measured using a BioTek plate reader (Vermont, USA) at

570 nm. Cells incubated with 2% SDS and Iso-PBS buffer represented the positive control 100% and negative control 0% haemolysis respectively. The results were expressed as percentage haemolysis relative to the SDS control using the following formula:

$$\% \text{ Haemolysis} = \frac{[Abs(\text{peptide}) - Abs(\text{negative control})]}{[Abs(\text{positive control}) - Abs(\text{negative control})]} \times 100$$

where Abs = absorbance at 570 nm.

2.10 Cytotoxicity of Os(3-12)NH₂ against Caco-2 cells

Cell lines are typical eukaryotic cells that undergo metabolism and cell division *in vitro*. Cytotoxic assays aid in assessing the safety of compounds by determining their ability to damage cells leading to cell death (Vaucher *et al.*, 2010). Cell death is associated with loss of cellular content and/or detachment of cells. The crystal violet assay is a quantitative analysis of the relative density of cells adhering to a micro titre plate indirectly indicating cell toxicity due to reduced CV staining of nuclear cell DNA (Cerovsky *et al.*, 2008). The CV dye is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) which is a cationic dye that binds DNA and negatively charged proteins (Gillies *et al.*, 1986). This fixed dye, upon solubilization, correlates with cell number, where a reduction in staining indicates cytotoxicity (Thomas *et al.*, 2004; Rothman, 1986; Castro-Garza *et al.*, 2007).

The cytotoxic effect of Os(3-12)NH₂ was evaluated against Caco-2 cells. Caco-2 (adult, *Homo sapiens*, epithelial, colorectal adenocarcinoma, doubling time 62 hours) cells (6×10^4) were plated in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum before being incubated with Os(3-12)NH₂ and melittin at a concentration range of (1.9-120) µg/ml for 48 hours at 37 °C. In order to fix the cells, 10 µl of 20% paraformaldehyde was added to each well for 30 minutes at 37 °C, at a final concentration of 2%. The fixative and medium was then removed and the cells were blotted dry. The cells were then stained with 0.1% CV (200 µl) solution made in formic acid [(0.1% (v/w) CV in 200 mM formic acid, pH 3] for 30 minutes at room temperature. After 30 minutes, the plate was washed with distilled water to remove excess dye and blotted dry. Bound dye was then extracted from the cells via solubilization with acetic acid (50 µl of 10% solution in distilled water). The absorbance of the resulting solution was measured at 630 nm and cell number expressed as a percent relative to the control where no peptide was added to cells.

2.11 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay determines the antioxidant activity of biological fluids, cells and synthetic compounds. This assay measures the loss of fluorescein fluorescence over time due to peroxy-radical formation caused by the degradation of 2,2'-azobis (2-aminopropane) dihydrochloride (AAPH). A water soluble vitamin E analogue namely Trolox is used as a positive control that inhibits fluorescein decay in a dose-dependent manner.

Peroxy radicals (ROO-) are formed from the breakdown of AAPH at 37 °C. These peroxy radicals oxidize fluorescein (3', 6'-dihydroxy-3H-spiro[2-Benzofuran-1,9'-xanthen]-3-one) to a non-fluorescent product. Antioxidant substances have the ability to suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. This fluorescence signal is measured over a time period by excitation at 485 nm and emission at 538 nm. The concentration of antioxidant in the test sample is directly proportional to the fluorescence intensity observed throughout the course of the assay and is assessed by comparing the net area under the curve (AUC) to that of the known antioxidant, Trolox (Figure 2.2). The antioxidant potential of Os(3-12)NH₂ and the tripeptide, reduced glutathione (GSH) was investigated

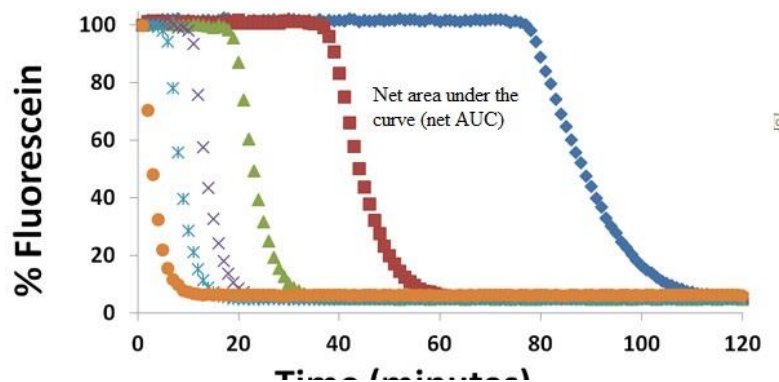


Figure 2.2: A typical graph obtained from the ORAC experiment. Percent fluorescence is measured over time for each compound tested. The longer the graph takes to slope the higher the antioxidant potential, e.g. the blue line. The area under the curve is then compared to that of trolox, the known vitamin E analogue. Adapted from Brescia (2012).

This assay method was based on the method of Ou *et al.* (2002) . A serial dilution of Trolox (vitamin E analogue) in distilled water: 0-800 μ M was used as the standard, with distilled water

used as the blank. Two serial dilutions of the peptides were prepared, Os(3-12) ranging from 0.125-5 μ M and GSH ranging from 5-20 μ M; fluorescein was added (final concentration 0.139 nM) followed by AAPH (final concentration of 0.11 μ M). Fluorescence was measured every 5 minutes for 4 hours on a FLUOstar OPTIMA plate reader (BMG Lab Technologies, Offenburg, Germany). A wavelength of 485 nm was used as the excitation wavelength and emission was measured at 520 nm. All raw data was exported to an Excel spreadsheet for calculations. The AUC for each sample was calculated as ($\text{netAUC} = \text{AUC}_{\text{antioxidant}} - \text{AUC}_{\text{blank}}$), and the final results were expressed as μ M TE (Trolox Equivalent).

2.12 Data and statistical analysis

All experimental measurements were performed in triplicate and the data is representative of three independent experiments. Data were analyzed using a T-test and One-way ANOVA with Dunetts post-test using GraphPad Prism (GraphPad software, v6.04, La Jolla California, USA. [URL:http://www.graphpad.com/faq/viewfaq.cfm?faq=1362](http://www.graphpad.com/faq/viewfaq.cfm?faq=1362)) to obtain statistical significance when necessary.

CHAPTER 3

RESULTS

3.1 Physicochemical properties of peptides

The physicochemical properties of the peptides used in the study are presented in Table 3.1. These include the parent peptide Os (the active region of OsDef2), Os(3-12) and the amidated peptide Os(3-12)NH₂. Control peptides of the study were melittin and LL-37. The parent peptide Os comprises of 22 amino acid residues while the controls melittin and LL-37 comprise of 26 and 37 amino acids, respectively. Os(3-12) and Os(3-12)NH₂ are only 10 amino acid residues long.

Table 3.1: Physicochemical parameters of peptides

Peptide	Sequence	Charge ^a	MW ^a	pI ^a	%[H] ^b
Os	KGIRGYKGGYCKGAFKQTCKCY	+6	2459.95	9.93	41
Os(3-12)	IRGYKGGYCK	+3	1144.36	9.95	40
Os(3-12)NH ₂	IRGYKGGYCK-NH ₂	+4	1143.38	10.34	40
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	+5	2846.46	12.43	46
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	+6	4493.33	11.10	35

^a Data obtained from Innovagen [<http://www.innovagen.com>]

^b Hydrophobic amino acid percent in peptide sequence obtained from LifeTein [<http://www.lifetein.com/peptide-analysis-tool.html>]

The overall physicochemical properties of AMPs depends on their amino acid compositions. Due to the amidated C-terminus of Os(3-12), the overall charge of the peptide, isoelectric point (pI) as well as molecular weight increased slightly in comparison to the unamidated Os(3-12). As Os(3-12) comprises of 1 Arg residue and 2 Lys residues, Os(3-12)NH₂ comprises of the same with an increase in charge due to C-terminal amidation eliminating the negative charge at the C-terminus region. Os still holds the highest charge due to the 6 charged amino acid residues it contains (5 Lys residues and 1 Arg residue). The net charge of Os compared to melittin and LL-37 showed to be of a similar magnitude where Os and LL-37 (5 Arg and 6 Lys, eliminating 5 negatively charged residues) exhibited overall charges of +6 and melittin (3 Lys and 2 Arg residues) of +5.

Both Os(3-12) and Os(3-12)NH₂ comprised of the same hydrophobic content as no extra hydrophobic amino acids were added to the peptide sequence, similar to Os. In a comparative analysis of the study peptides, melittin is regarded as the most hydrophobic peptide exhibiting a hydrophobic content of 46%.

3.2 PSIPRED: Secondary structure prediction of peptide

The secondary structure of Os(3-12)NH₂ was predicted using the PSIPRED program (Figure 3.1). In order to determine the impact of amidation on secondary structure, this program was used to make predictions on the secondary structures of Os(3-12) and Os(3-12)NH₂. Melittin is well known to adopt an α -helical structure when placed in a membrane mimicking environment, therefore it was used as a positive control in both the secondary structure prediction and determination investigations (Vogel and Jähnig, 1986).

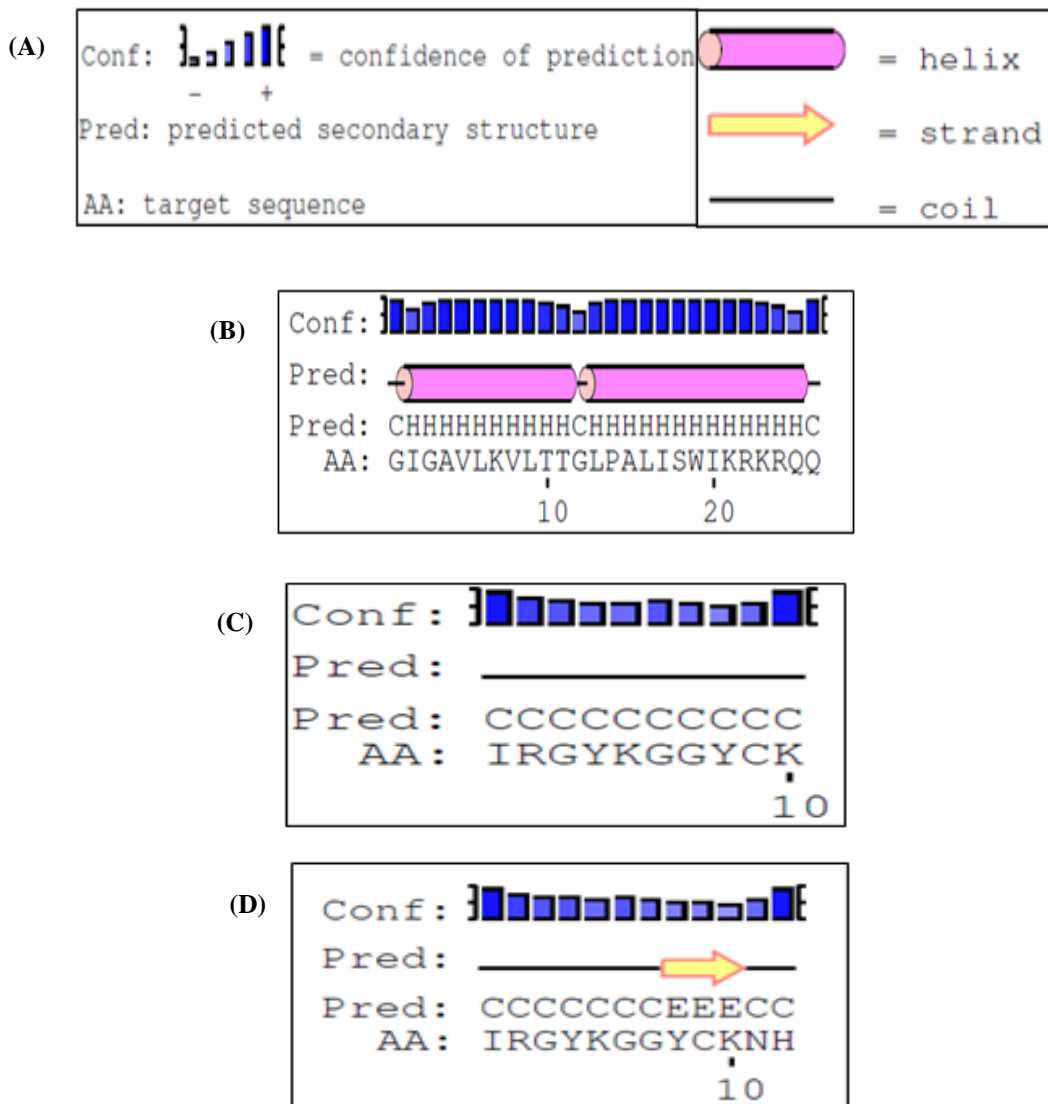


Figure 3.1: Secondary structure predictions of respective peptides using the PSIPRED program. (A) PSIPRED legend (B) Melittin (C) Os(3-12) and (D) Os(3-12)NH₂

PSIPRED confirmed the secondary structure of melittin to be α -helical. Os(3-12) was predicted to be coiled and Os(3-12)NH₂ was predicted to be coiled with a slight change in conformation towards the C-terminus, where it was shown to exhibit a strand conformation.

3.3 Secondary structure analysis using circular dichroism

CD is a widely used technique to determine the effects of the environment on the structural conformation and stability of peptides. Most AMPs are generally unstructured in solution but tend to fold into their respective secondary structures when placed in a membrane mimicking environment (Tossi *et al.*, 2000). CD is commonly employed to detect secondary structural differences of AMPs in SDS micelles and TFE. SDS is a detergent which mimics an anionic membrane environment allowing AMPs to adopt their secondary structural conformations (Tulumello and Deber; 2009, Radziszewsky *et al.*, 2007). TFE is an organic, water-miscible liquid widely used as a structure-inducing co-solvent allowing the formation of stable secondary structures in aqueous solutions (Kwon *et al.*, 1998).

The secondary structures of Os(3-12) and Os(3-12)NH₂ were investigated using CD. Melittin was once again used as a positive control as it is known to be α -helical when placed in a membrane mimicking environment (Vogel and Jähnig, 1986). The CD spectra for the synthetic peptides; Os(3-12), Os(3-12)NH₂ and melittin in water, 25 mM SDS and 50% TFE solutions were determined (Figure 3.2).

The CD spectrum for melittin (Figure 3.2A), obtained in water, exhibited its lowest ellipticity values at 190 nm and 200 nm and thereafter maintained a negative ellipticity for the remainder of the spectral range, clearly indicating melittin to be randomly coiled in water. The CD spectrum obtained for Os(3-12) and Os(3-12)NH₂ in water (Figure 3.2A) were similar to one another and appeared the peptides appeared to be unstructured for the entire spectral range. In the 50% TFE and 25 mM SDS solutions, the CD spectrum for melittin exhibited two minimum values at 208 nm and 222 nm, characteristic for an α -helical conformation (Figure 3.2B and C). The CD spectrum for Os(3-12) and Os(3-12)NH₂ dissolved in 50% TFE and 25mM SDS remained unstructured despite being placed in a membrane mimicking environment (Figure 3.2B and C). There also appeared to be no difference in the secondary structures of Os(3-12)NH₂ compared to the unamidated peptide fragment Os(3-12).

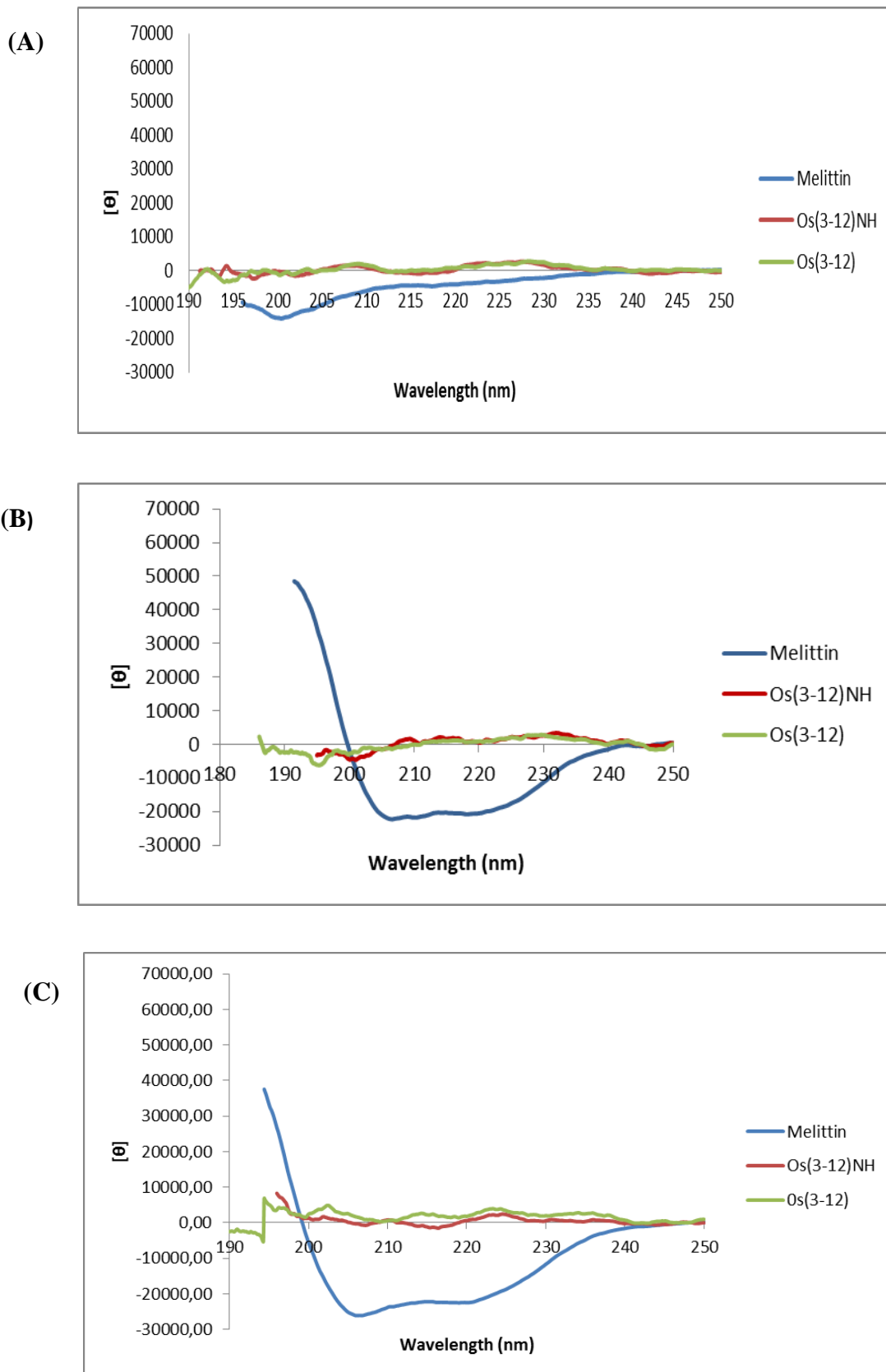


Figure 3.2: CD spectra of Os(3-12) and Os(3-12)NH₂. The CD spectra for 50 μ M Os(3-12) (Greenfield), 50 μ M Os(3-12)NH₂ (red) and 50 μ M melittin (blue) was observed in: (A) water, (B) 50% TFE and (C) 25 mM SDS.

3.4 Antibacterial activity

The MBC for Os(3-12)NH₂ was determined using the CFU method in order to evaluate the bactericidal potential of the amidated peptide (Table 3.2). The MBC values obtained for Os(3-12)NH₂ ranged from 3.75-30 µg/ml (3.28-26.24 µM) against the tested Gram-positive and Gram-negative bacteria.

Table 3.2: Minimum bactericidal concentration (MBC) values of the respective peptides in NaP buffer

Bacterial strain	MBC (µg/ml)		
	Os ^a	Os(3-12) ^a	Os(3-12)NH ₂
<u>Gram- positive bacteria</u>			
<i>B. subtilis</i>	1.88 (0.77 µM)	60 (52 µM)	7.50 (6.56 µM)
<i>S. aureus</i>	15 (6.10 µM)	60 (52 µM)	30 (26.24 µM)
<u>Gram- negative bacteria</u>			
<i>E. coli</i>	1.88 (0.77 µM)	60 (52 µM)	3.75 (3.28 µM)
<i>P. aeruginosa</i>	0.94 (0.38 µM)	60 (52 µM)	7.50 (6.56 µM)

The MBC values obtained for Os(3-12)NH₂ are the results of three independent experiments

^aThe MBC values for Os (Prinsloo *et al.*, 2013) and Os(3-12) (Odendaal, 2013) were included in the table for comparison purposes.

The amidated peptide was more active against Gram-negative bacteria than Gram-positive bacteria where the most potent bactericidal activity was seen to be against *E. coli* with an MBC value of 3.75 µg/ml (3.28 µM). The peptide was the least active against *S. aureus* (30 µg/ml; 26.2 µM). The overall MBC values for Os(3-12)NH₂ showed to be more active than the unamidated peptide fragment Os(3-12) with MBC values 16-fold more active against *E. coli*, 8-fold more active against *B. subtilis* and *P. aeruginosa*, and 2-fold more active against *S. aureus*.

The amidated peptide was however slightly still less active than the parent peptide Os whose MBC values ranged from 0.94-15 µg/ml (0.38-6.10 µM) compared to Os(3-12)NH₂.

3.5 Kinetics of bacterial killing

The killing kinetics for Os(3-12)NH₂ was evaluated against *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) in order to determine the killing efficiency of the peptide at the respective MBC

values (Figure 3.3). For *E. coli*, the peptide at 3.75 µg/ml (3.28 µM) killed all cells within 10 minutes. For *B. subtilis*, the peptide at 7.5 µg/ml (6.56 µM), killed all cells within 60 minutes. As a result, the peptide was much more efficient in killing *E. coli* than *B. subtilis*. The time required to eliminate all the bacteria was however not as rapid as the parent, Os, which killed both *E. coli* and *B. subtilis* cells within 5 minutes (Prinsloo *et al.*, 2013).

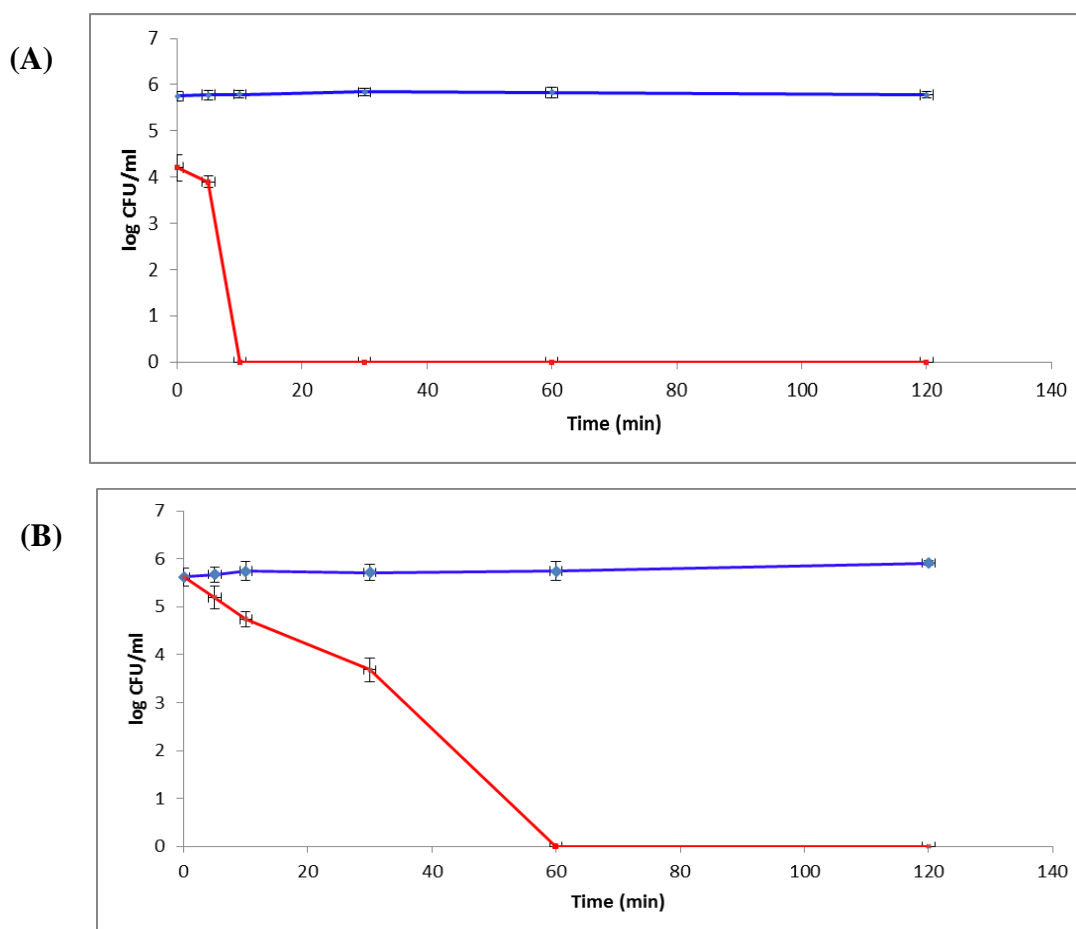


Figure 3.3: The killing kinetics of Os(3-12)NH₂ against the respective bacteria. The killing kinetics was determined at the MBC of each peptide for the respective bacteria using the colony forming unit (CFU) assay to determine the decrease in log CFU/ml with time. Blue line represents the untreated bacterial cells, red line represents Os(3-12)NH₂. Os(3-12)NH₂ was incubated with **(A)** *E. coli* at the MBC of 3.75 µg/ml (3.28 µM); **(B)** *B. subtilis* at the MBC of 7.5 µg/ml (6.56 µM), over a time interval range. Error bars represent the standard error of mean of two independent experiments.

3.6 Membrane permeabilization

As AMPs induce bacterial killing either via membrane disruption or intracellular translocation, inhibiting intracellular life targets of the bacterium, a first step to gain insight into the killing mechanisms of AMPs is to investigate their ability to permeabilize the cell wall of bacteria. The

mode of action for Os and Os(3-12)NH₂ was investigated using a non-membrane permeabilizing dye; SYTOX green. Melittin and LL-37, two known membrane lytic peptides were used as positive controls in this study. For these experiments, the concentration range 0.06-120 µg/ml for all four peptides were investigated against *S. aureus* and *E. coli*.

In *S. aureus*, the control LL-37 exhibited an increase in fluorescence across the concentration range 0.06-3.75 µg/ml, melittin from 0.06-1.88 µg/ml, Os from 0.06-3.75 µg/ml and Os(3-12)NH₂ from 0.06-60 µg/ml. Maximal fluorescence for Os(3-12)NH₂ and Os was observed to be close to the MBC values of the peptides (Figure 3.4). For all the peptides tested, fluorescence decreased at higher concentrations. The control peptides LL-37 ($p < 0.0001$) and melittin ($p < 0.01$) exhibited significant membrane permeabilization in comparison to the untreated cells. Os(3-12)NH₂ ($p < 0.01$) at the concentration range 0.47-120 µg/ml and Os ($p < 0.05$) at the concentration range of 0.47-60 µg/ml also exhibited significant membrane permeabilization in comparison to the untreated cells. These results indicated that Os and Os(3-12)NH₂ disrupt the membrane integrity of *S. aureus* cells albeit not the extent of LL-37.

In *E. coli*, all peptides induced SYTOX green uptake (Figure 3.5). Both control peptides LL-37 and melittin exhibited significant membrane permeabilization ($p < 0.0001$) compared to the untreated cells. Os(3-12)NH₂ ($p < 0.01$) and Os ($p < 0.001$) exhibited significant membrane permeabilization, but to a lesser extent than LL-37. A decrease in fluorescence was observed for all peptides at higher concentrations, and for Os and Os(3-12)NH₂ the decrease occurred at concentrations greater than their MBCs (Table 3.2).

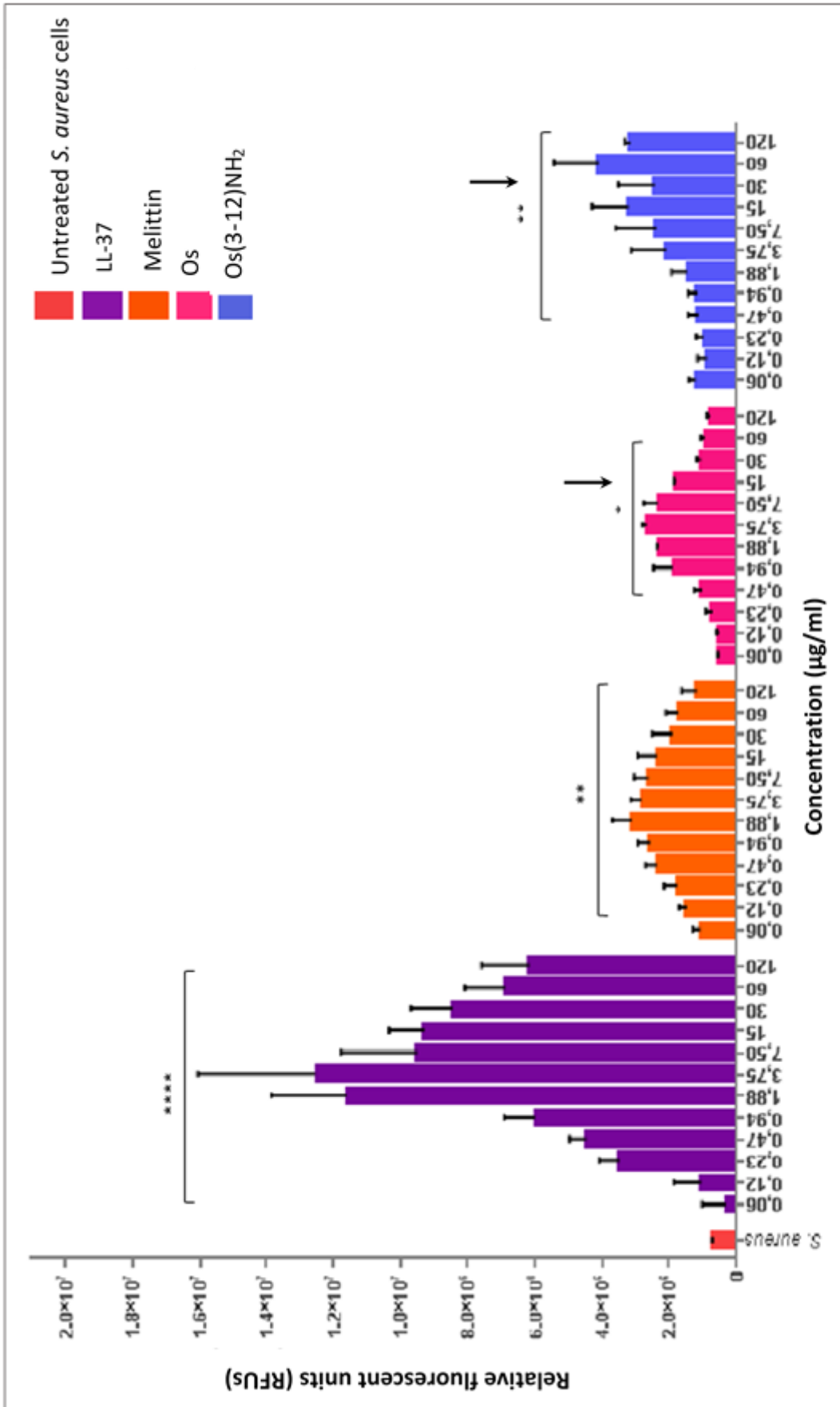


Figure 3.4: SYTOX green uptake detection for *S. aureus* membrane permeabilization. The graph represents untreated *S. aureus* cells (red), the two control peptides LL-37 (purple) and melittin (orange), and the study peptides Os (pink) and Os(3-12)NH₂ (blue). *S. aureus* cells were equilibrated with the SYTOX green dye for 15 minutes. A concentration range of 0.06-120 µg/ml for each AMP was incubated with the bacterial cells for 30 minutes where end point fluorescent readings were subsequently recorded in response to bacterial membrane permeabilization. Relative fluorescent units (RFUs) expressed represent SEM where n=3. Arrows represent the MBC for Os (15 µg/ml) and Os(3-12)NH₂ (30 µg/ml). One way ANOVA was applied to detect the statistical difference of the RFUs obtained for each peptide concentration relative to the untreated (*S. aureus*) cells where *p<0.05, **p<0.01 and ****p<0.0001.

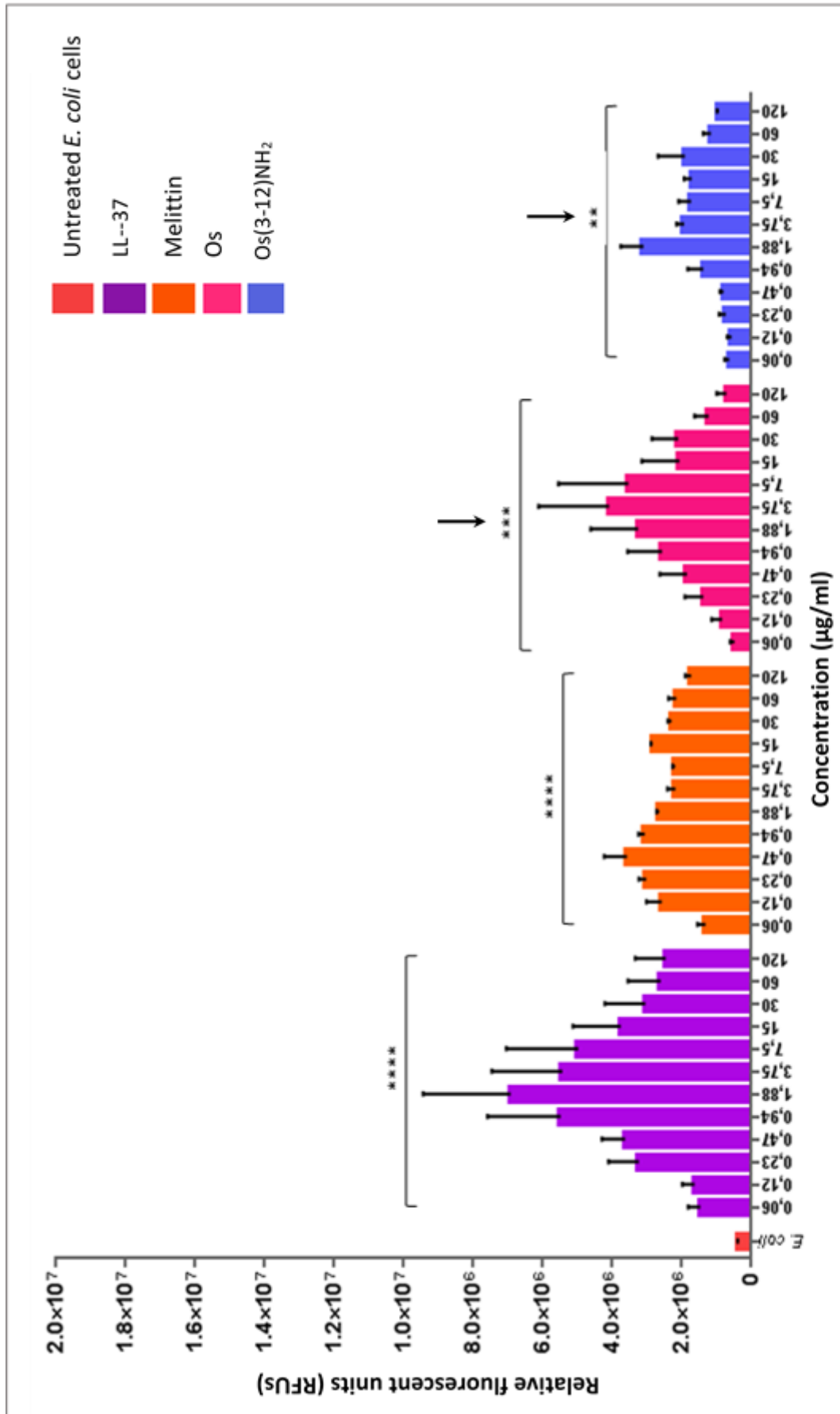


Figure 3.5: SYTOX green uptake detection for *E. coli* membrane permeabilization: The graph represents untreated *E. coli* cells (red), the two control peptides: LL-37 (purple) and melittin (orange), and the study peptides Os (pink) and Os(3-12)NH₂ (blue). *E. coli* cells were equilibrated with the SYTOX green dye for 15 minutes. A concentration range of 0.06-120 µg/ml for each AMP was incubated with the bacterial cells for 30 minutes where end point fluorescent readings were subsequently recorded in response to bacterial membrane permeabilization. Relative fluorescent units (RFUs) expressed represent SEM where n=3. Arrows represent the MBC for Os (1.88 µg/ml) and Os(3-12)NH₂ (3.75 µg/ml). One way ANOVA was applied to detect the statistical difference of the RFUs obtained for each peptide concentration relative to the untreated (*E. coli*) cells where **p<0.01, ***p<0.001 and ****p<0.0001.

3.7 Effects of serum and salt on the MBC of Os(3-12)NH₂

Though AMPs have the potential to serve as antimicrobial agents, the majority of these peptides lose activity under physiological conditions thereby hampering their application for systemic usage (Huang *et al.*, 2011). For this reason the bactericidal activity of Os(3-12)NH₂ was investigated in the presence of 30% human serum as well as a salt mixture comprising of the following salts at physiological concentrations; 145 mM NaCl, 2.5 mM CaCl₂ and 1 mM MgCl₂ (Klabunde, 2011) using the CFU method (Table 3.4 and Table 3.5).

In the presence of 30% human serum (Table 3.3), unlike the parent peptide Os, the amidated peptide had lost all bactericidal activity against both *E. coli* and *S. aureus*. The bactericidal potential of the amidated peptide fragment was also evaluated against *B. subtilis*. Due to the bactericidal properties of human serum, the growth of *B. subtilis* cells was completely inhibited in the presence of serum alone since the untreated *B. subtilis* cells were unable to grow and was thus omitted from this study.

Table 3.3: Bactericidal potency evaluation of Os(3-12)NH₂ in the presence of serum

Bacteria	Os ^a		Os(3-12)NH ₂ ^b	
	MBC in NaP	MBC in 30% serum	MBC in NaP	MBC in 30% serum
<i>E. coli</i>	1.88 (0.77 μM)	60 (24.40 μM)	3.75 (3.28 μM)	>120 (105 μM)
<i>S. aureus</i>	3.75 (1.52 μM)	3.75 (1.52 μM)	7.50 (6.56 μM)	>120 (105 μM)

^a MBC values for Os obtained from: (Prinsloo, 2013)

^b Values represent the MBC of Os(3-12)NH₂ and are the results of three independent experiments

To investigate whether the salts present in serum are responsible for the loss of activity in the presence of serum, the bactericidal activity was evaluated in the presence of a salt mixture. The presence of salt also negatively impacted the bactericidal activity of Os(3-12)NH₂ against *E. coli* and *S. aureus* (Table 3.4).

Table 3.4: Bactericidal potency evaluation of Os(3-12)NH₂ in the presence of salt

Bacteria	MBC in NaP ^a	MBC in salt mixture ^b
<i>E. coli</i>	3.75 (3.28 μM)	>120 (105 μM)
<i>S. aureus</i>	7.5 (6.56 μM)	>120 (105 μM)

^aValues represent the MBC of Os(3-12)NH₂ and are the results of three independent experiments
^bSalt mixture comprised of 145 mM NaCl, 2.5 mM CaCl₂ and 1 mM MgCl₂

3.8 Haemolytic activity

For therapeutic development, it is essential that peptides are not toxic to mammalian cells. The cytotoxicity of Os(3-12)NH₂ was firstly evaluated for their ability to cause lysis to human erythrocytes. The haemolytic ability was evaluated over a concentration range of the peptide (0.25-100 μM) melittin (Figure 3.6), a known haemolytic peptide (Zhu *et al.*, 2007). Os(3-12)NH₂ was non-haemolytic over the concentration range tested (0.25-100 μM). Melittin on the other hand exhibited a dosage effect in response to haemolysis, resulting in complete lysis of human erythrocytes at concentrations greater than 25 μM.

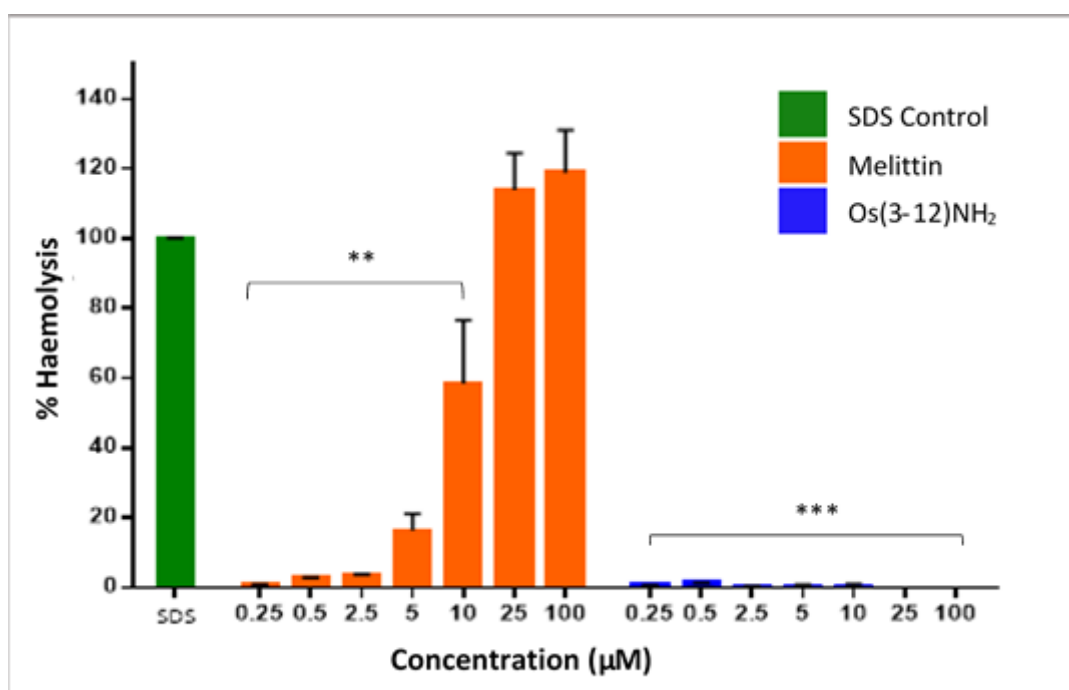


Figure 3.6: Haemolytic evaluation of Os(3-12)NH₂ against human erythrocytes. The haemolytic activity of Os(3-12)NH₂ (blue) and the positive control melittin (orange) against human erythrocytes. The %Haemolysis obtained for both peptide fragments was calculated relative to SDS (Greenfield), the 100% haemolysis. Error bars here represent the standard error of mean where n=3 and haemolysis significance was evaluated in relation to SDS (2 tailed t-test, p<0.05).

3.9 Cytotoxicity of Os(3-12)NH₂ against Caco-2 cells

The cytotoxic potential of Os(3-12)NH₂ was further evaluated in Caco-2 epithelial cells using the CV assay. The toxic effects of Os(3-12)NH₂ was again compared to melittin (positive control), the known lytic AMP. Melittin and Os(3-12)NH₂ were evaluated over a concentration range of 1.9-120 µg/ml (Figure 3.7). Os(3-12)NH₂ maintained a 100% cell number over the entire concentration range investigated. Melittin however exhibited a dosage effect in response to cell viability with an approximate 35% reduction in cell number at 30 and 120 µg/ml (**p<0.01).

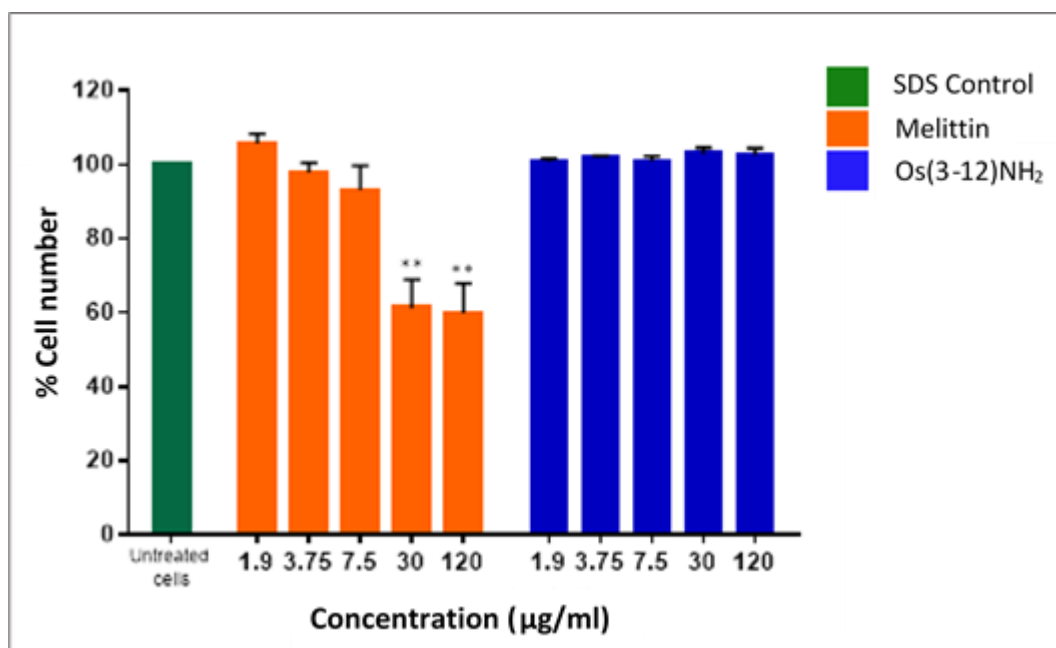


Figure 3.7: The cytotoxic activity of Os(3-12)NH₂ against Caco-2 cells. Cytotoxic effects of Os(3-12)NH₂ (blue), and positive control; melittin (orange) against untreated Caco-2 cells (Greenfield). Error bars represent the standard error of mean where n=3. Statistical analysis using a 2-tailed t-test at melittin concentrations 30 and 120 µg/ml showed the data to be statistically significant, **p< 0.01 to the untreated Caco-2 cells.

3.10 Antioxidant potential

The antioxidant potential for Os(3-12)NH₂ was evaluated using the ORAC assay to determine if Os(3-12)NH₂ was multifunctional just as the parent peptide Os. GSH, a well-known antioxidant, was used as a positive control (Kerksick and Willoughby, 2005). A dose response relationship for GSH and Os(3-12)NH₂ is presented in Figure 3.8.

Both peptides exhibited positive trends showing the antioxidant capacity to increase with an increase in peptide concentration. Os(3-12)NH₂ and GSH. A linear regression analysis shared that

Os(3-12)NH₂ exhibited a steeper gradient (27.839) compared to GSH (1.8329), indicating a 15-fold greater antioxidant potential for Os(3-12)NH₂. Prinsloo *et al.* (2013) determined the molar ratio for the parent peptide Os to be 21.63 μMTE/μM. From this data, a molar ratio of 30.31 μMTE/μM was determined for Os(3-12)NH₂ indicating that the antioxidant activity for Os(3-12)NH₂ is 1.4-fold greater than that of Os.

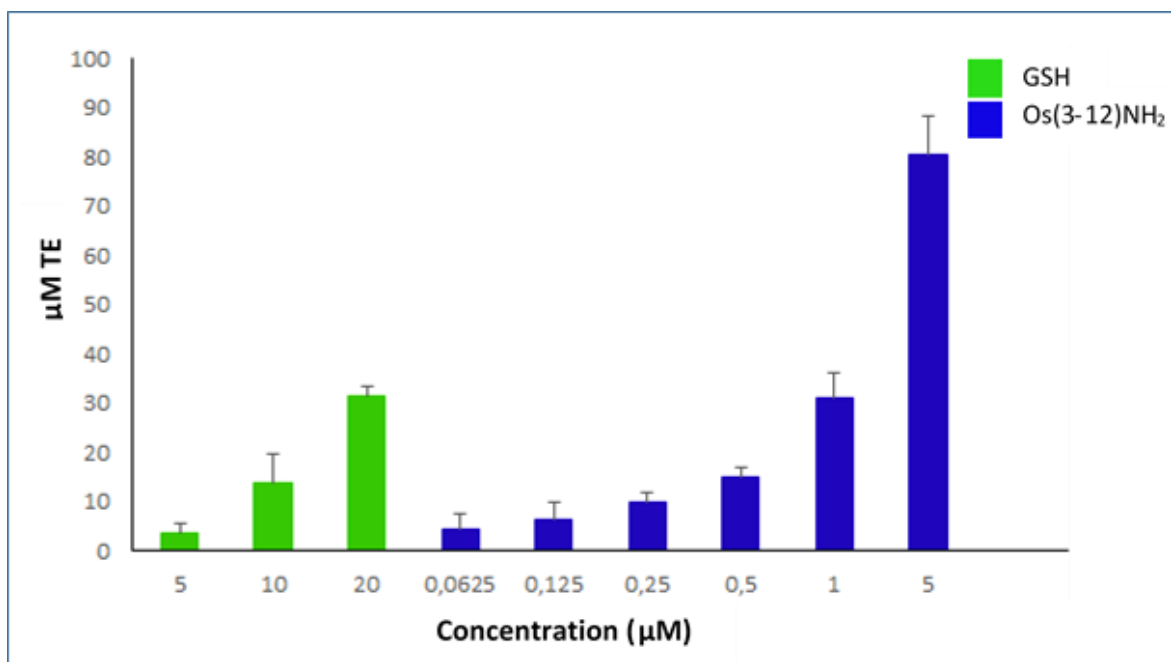


Figure 3.8: Antioxidant activity of Os(3-12)NH₂ and GSH. ORAC for GSH (Greenfield) and Os(3-12)NH₂ (blue) expressed as μM TE. Error bars represent the SEM for three independent experiments each done in triplicates. Equation obtained from a linear regression analysis for Os(3-12)NH₂ was $y = 27.839x + 2.4727$ ($R^2=0.9928$) and GSH was $y = 1.8329x + 5.201$ ($R^2=0.9979$).

CHAPTER 4

DISCUSSION

In an effort to combat the rapid emergence of multidrug-resistant bacteria from prevailing throughout the globe, AMPs prove to be promising leads in the search for innovative antimicrobial agents. The amphipathic nature of these peptides allows them to exhibit a broad spectrum of antimicrobial potential, with a rapid, non-specific mechanism of action resulting in a low rate of resistance development (Huang *et al.*, 2011). Despite the therapeutic value of AMPs, a few setbacks to their clinical development are their manufacturing costs, inactivity under physiological conditions and toxicity towards host cell components (Craik *et al.*, 2013).

In order to reduce synthesis cost, smaller peptide fragments corresponding to the main active regions of natural AMPs are designed (Saido-Sakanaka *et al.*, 2004). The antimicrobial potential of these smaller peptide fragments varies depending on the region of the parent peptides they are derived from. Peptide fragments derived from the C-terminal of natural AMPs are generally more active than their parent compounds (Varkey *et al.*, 2006), whereas truncated peptide fragments display comparable or decreased antimicrobial potential in comparison to their parent compounds (Mor and Nicolas, 1994; Sitaram *et al.*, 1993; Sitaram and Nagaraj, 1990; Perez-Paya *et al.*, 1995). As a result, numerous peptide modifications are applied in an attempt to improve the antimicrobial potencies of truncated peptides. The addition of an amide moiety at the C-terminal region is generally employed to enhance their antimicrobial activity. This modification however has various impacts on the lytic potential of AMPs towards human cells (Dennison *et al.*, 2009; Strandberg *et al.*, 2009).

From the tick *O. savignyi* defensin, the main active regions of the C-terminally derived peptide Os, were identified as Os(3-12) and Os(11-22) comprising of 10 and 12 amino acid residues, respectively. Both these regions were less active than Os when evaluated against Gram-positive and Gram-negative bacteria (Odendaal, 2013). In order to improve the potency of the Os(3-12) region, the peptide fragment was amidated at the C-terminal. Theoretically, it can be predicted that Os(3-12)NH₂ would be more potent than Os(3-12) due to the increase in charge from +3 to +4, as the hydrophobic content of the peptides remained the same. An increase in charge would enhance the electrostatic attraction of the peptide fragment towards bacterial membranes. As these were just

mere theoretical predictions, Os(3-12)NH₂ was evaluated to determine the impact of C-terminal amidation on the structural, antibacterial, cytotoxic and antioxidant functionalities of the peptide fragment.

The secondary structure of AMPs is an essential component to be evaluated in relation to peptide modifications as it governs their antimicrobial activity (Suarez *et al.*, 2005). The secondary structure prediction server PSIPRED confirmed melittin to be α -helical (Figure 3.1B) and predicted Os(3-12)NH₂ and Os to be coiled, with a slight change in conformation at the C-terminal region (Figure 3.1C and D). As these predictions required experimental validation, CD spectroscopy was performed. The CD analysis (Figure 3.2) confirmed the control peptide melittin to be randomly coiled in water and α -helical when placed in both SDS and TFE. The amidated and unamidated peptide fragments were revealed to be irregularly structured when placed both in water, SDS and TFE, with no difference in structure compared to one another. Unlike the parent peptide Os which was randomly coiled in water and predominantly α -helical when placed in a membrane mimicking environment (Prinsloo *et al.*, 2013), the failure of both Os(3-12) and Os(3-12)NH₂ to adopt the α -helical conformation may be attributed to their decreased length.

α -Helices are right handed coils of amino acid residues, where each coil is kept intact due to hydrogen bonds between the backbone amino (N=H) and carbonyl (C=O) groups. The hydrogen and oxygen atoms are attracted to one another due to their partial charges and form exactly every 4 amino acid residues. Every turn in the helix consists of 3.6 residues with the amino acid R-groups pointing outwards (Berg *et al.*, 2002). According to Manning *et al.* (1988), the minimum length requirement of a peptide to produce an α -helix like CD spectrum is 2 to 3 turns comprising of 7-11 amino acid residues. The amino acid content which make up the helix are equally important. The loss of secondary structure for Os(3-12)NH₂ and Os(3-12) is however generally characteristic of small AMPs. AMPs containing 15 amino acid residues or less have been previously demonstrated to adopt poor or irregular structures when placed in membrane mimicking environments (Ladokhin *et al.*, 1999). The inability of the Os(3-12)NH₂ to form a secondary structure when placed in a membrane mimicking environment indicates either the amidated peptide cannot interact strongly with a lipid environment or the peptide has the ability to interact with the lipid membranes without the need for an α -helical conformation (Zelezetsky and Tossi, 2006). Oren *et al.* (2002) have reported unstructured peptides to maintain a useful microbicidal potential with improved peptide

specificity against host cells. These findings imply that even though Os(3-12)NH₂ may not be as potent as Os against bacterial cells, the peptide however would remain nonlytic against host cells.

Due to the lack of the α -helical structure in Os(3-12)NH₂, the bactericidal activity of the peptide was investigated against Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and compared to both Os(3-12) and Os, respectively (Table 3.2). The amidated peptide was more potent than Os(3-12) against the respective bacterial strains evaluated. As the overall hydrophobic contents and secondary structures of both peptide fragments remained the same, the only difference was their net charge (Os(3-12); +3 and Os(3-12)NH₂; +4). The enhanced bactericidal potency of Os(3-12)NH₂ can therefore be attributed to the impact of amidation increasing the overall charge on the peptide by eliminating the negative charge at the C-terminal end (Dennison *et al.*, 2009). Os(3-12)NH₂ was however still not as potent as Os. This decreased potential can be attributed to the loss in secondary structure of the peptide fragment as well as the lower charge as the cationicity of Os is +6 (Deslouches *et al.*, 2005). Nevertheless, Os(3-12)NH₂ is a shorter peptide fragment reducing peptide synthesis costs. The amidated peptide can therefore be further modified to enhance its antimicrobial activity. Shorter peptide derivatives of bovine and murine lactoferricins, comprising of 5-11 amino acid residues, were required to comprise of a minimum of 3 Arg residues and 3 Trp residues in addition to C-terminal amidation for enhanced antimicrobial potential (Strøm *et al.*, 2002). These amino acid residues should be investigated further for their effect on the bactericidal potential of Os(3-12)NH₂.

The amidated peptide can be regarded as a valuable therapeutic source due to its activity against *S. aureus*, *E. coli* and *P. aeruginosa*. These pathogenic bacteria form part of the on-going resistance development cycle. Methicillin-resistant *S. aureus* has moved from being a hospital acquired infection to a community acquired infection with enhanced virulence and transmission characteristics (Davies, 1996; Rice, 2007). *E. coli* has limited therapeutic treatment options to urinary tract infections within communities as it continues to rapidly spread (Pallett and Hand, 2010). A simple burn wound infection caused by *P. aeruginosa* has now evolved into a major nosocomial threat. The persistency of *P. aeruginosa* in cystic fibrosis patients and its ability to escape host immunity is of great concern (Davies, 1996). With no new developments against pathogenic Gram-negative bacteria, their rapid increase in resistance has become a growing problem (Giske *et al.*, 2008). As the amidated peptide was on overall more potent against Gram-negative bacteria than Gram-positive bacteria, the peptide holds potential for further development,

as common anti-Gram-negative bacterial drugs such as sulphonamides, β -lactams and amino glycosides are no longer effective (Davies, 1996).

The low rate of resistance development amongst AMPs can be attributed to their non-specific, rapid killing strategies (Andreu and Rivas, 1998). In order to obtain an indication of just how rapid the AMP killing mechanism is, the killing kinetics for Os(3-12)NH₂ at its MBC for *E. coli* and *B. subtilis* was evaluated. The amidated peptide killed *E. coli* within 10 minutes, whereas *B. subtilis* required 60 minutes (Figure 3.3). Norcia *et al.* (1999) reported conventional antibiotics such as fluoroquinolones, at 8 times their MIC to effectively kill *E. coli* cells after 3 hours and β -lactams (penicillin G and ceftiofur) within 6 hours, where the regrowth of *E. coli* cells were observed after 12 hours. Thus, the killing kinetics observed for Os(3-12)NH₂ at the MBC of the peptide against both *E. coli* and *B. subtilis* was much more rapid and effective than conventional antibiotics.

In a comparison to Os, the parent peptide was found to kill both bacterial cells, *E. coli* and *B. subtilis* at the same rate (5 minutes) (Prinsloo *et al.*, 2013), quicker than the amidated peptide. The killing efficiency of AMPs is governed by a number of factors such as charge, hydrophobicity, amphipathicity and structure (Tossi *et al.*, 2000). As shorter peptide fragments differ in each of these factors, they generally exhibit longer killing times (Zelezetsky and Tossi, 2006). A major factor responsible for the longer killing times observed with the amidated peptide fragment can therefore be attributed to the peptide's loss of α -helical propensity. This loss of secondary structure implies the peptide's interaction with the bacterial lipids is not as strong as that of Os. The importance of structure however in the bactericidal mechanism of action of AMPs against Gram-positive and Gram-negative bacteria differ (Zelezetsky and Tossi, 2006). An increased charge and α -helical content has been positively correlated to enhanced bactericidal activity against Gram-positive bacteria (Giangaspero *et al.*, 2001). As Os(3-12)NH₂ and Os differ in charge and structure, the longer killing time observed for Os(3-12)NH₂ against *B. subtilis* (60 minutes) suggests the peptide eliminates the bacteria by a different mechanism of action. As structural requirements are less stringent in the bactericidal mechanism of action against Gram-negative bacteria (Giangaspero *et al.*, 2001), the 5 minute delay in the killing time of Os(3-12)NH₂ compared to Os against *E. coli* is characteristic of shorter peptide fragments.

The different killing rates observed for Os(3-12)NH₂ against Gram-positive and Gram-negative bacteria and in comparison to Os led to the investigation of the ability of the peptide to cause

membrane permeabilization (Hancock and Rozek, 2002). Membrane permeabilization was investigated with the aid of the SYTOX green dye. SYTOX green is a cationic, cyanine, nucleic acid stain dye which increases in fluorescence when bound to nucleic acids. The dye does not have the ability to enter viable cells with intact membranes, unless the membranes are disrupted (Makovitzki *et al.*, 2006). Fluorescent cells are therefore proportional to the fraction of permeabilized cells within the bacterial population (Hocquellet *et al.*, 2012).

In the membrane permeabilization detection assay, two positive control peptides were required to validate the study findings. These peptides, the human cathelicidin LL-37 and melittin, were chosen based on their permeabilizing capabilities. LL-37, derived from the C-terminal of human cathelicidin CAP18 is a 37 amino acid peptide fragment with a net charge of +6. The active C-terminus is cleaved by serine proteases. The peptide fragment obtained its name from the two leucyl residues at the N-terminus (Sørensen *et al.*, 2001). LL-37 is unstructured in pure water and α -helical in a membrane mimicking environment (Rosenfeld and Shai, 2006). LL-37 is known to be active against a broad spectrum of Gram-positive and Gram-negative bacteria where the AMP induces membrane permeabilization either via the carpet or toroidal pore mechanism (Lee *et al.*, 2011). Melittin is a toxic component derived from the venom of the European honey bee *Apis mellifera*. This 26 amino acid peptide fragment is a cationic (+5), haemolytic peptide where the residues (1-20) are predominantly hydrophobic and (21-26) are predominantly hydrophilic (Dempsey, 1990), resulting in melittin to be highly hydrophobic. This peptide fragment is randomly coiled in water and α -helical when placed in a membrane mimicking environment encouraging tetramer formation at high concentrations (Dempsey, 1990). Melittin is a known membrane permeabilizing agent, inserting into the lipid bilayer forming pores via the toroidal pore mechanism (Allende *et al.*, 2005; Vogel and Jähnig, 1986).

The ability of Os and Os(3-12)NH₂ to induce membrane permeabilization was investigated against *S. aureus* and *E. coli*. In *S. aureus*, both Os(3-12)NH₂ ($p < 0.01$) and Os ($p < 0.05$) exhibited significant membrane permeabilization in comparison to the untreated cells (Figure 3.4). The same was observed for *E. coli* where both Os(3-12)NH₂ ($p < 0.01$) and Os ($p < 0.001$) exhibited significant membrane permeabilization (Figure 3.5). A trend in fluorescence was observed for all peptides evaluated against both bacterial cells where an increase in fluorescence was observed with increased peptide concentrations upto a peak followed by a decline as the peptide concentrations increase. The peak concentration at which maximal fluorescence was observed for both Os and

Os(3-12)NH₂ was found to be close to the MBC values for each peptide. As the MBC value of the study peptides was taken to be the lowest concentration at which 99% killing was observed, the increase in fluorescence at lower concentrations up until this point is expected. The decreased fluorescence observed at the higher concentrations of the peptides emphasizes one of the limitations to the usage of the SYTOX green dye. As permeabilized cells may comprise of intact or damaged DNA, the inability of the dye to bind to damaged DNA confuses a permeabilized cell for an intact cell resulting in a low fluorescence emission (Lebaron *et al.*, 1998). Hence the decrease in fluorescence observed at the higher peptide concentrations corresponds to bacterial killing with damaged or peptide bound DNA. To further explain this theory, increased concentrations of an antimicrobial agent are generally associated with faster killing rates (Vinks *et al.*, 2013). Membrane permeabilization at concentrations greater than the MBC values of the peptides occurs much rapidly than at lower peptide concentrations. Therefore, the decreased fluorescence observed for the peptides can be either due to DNA damage by the rapid permeabilization of the bacterium or peptide binding to DNA preventing SYTOX green binding.

The peptides LL-37, melittin and Os exhibit DNA binding capabilities (Prinsloo, 2013; Québatte *et al.*, 2013; Sandgren *et al.*, 2004). Further investigations of the DNA binding capabilities of Os(3-12)NH₂ would clarify the mechanism of these peptides as complete membrane permeabilization may not be necessary for the lethal process. This was observed for the Bac8c peptide (an 8 amino acid AMP derived from Bac2A, a variant of the naturally occurring bovine peptide), where bacterial death occurred at the time of membrane depolarization and not membrane permeabilization. Membrane depolarization is a process by which a reduction in the negatively charged membrane potential of bacteria is observed due to an influx of positive cations. Relating this definition to AMPs, membrane depolarization is known to occur as AMPs bind to bacterial membranes and displace the divalent cations resulting in peptide insertion. Peptide insertion into the bacterial membrane leads to the formation of minor disruptions on the surface membrane and the inhibition of intracellular processes resulting in death before membrane permeabilization has even occurred (Spindler *et al.*, 2011).

The fluorescence intensity of Os was greater in *E. coli* than *S. aureus*, whereas the fluorescence intensity of Os(3-12)NH₂ was greater in *S. aureus* than *E. coli*. The different fluorescence intensities observed for each peptide against both bacteria supports the notion of AMPs employing different killing strategies against Gram-positive and Gram-negative bacteria. AMP insertion into

Gram-negative bacteria basically involves the attraction of the peptides to the LPS molecules on the outer membrane via the self-promoted uptake mechanism (Huang *et al.*, 2011). AMPs interact with Gram-positive bacterial membranes in a voltage-dependant manner (Kordel *et al.*, 1988), where the requirements however for a transmembrane potential differ amongst different AMPs (Friedrich *et al.*, 2000). In addition to the electrostatic attraction of AMPs towards the teichoic acid residues on the surface of the membrane, H-bond formation and cationic electrostatic interactions are highly important due to the large peptidoglycan layer of Gram-positive bacteria. As a result the peptide requirements to permeabilize Gram-positive and Gram-negative bacteria as well as their mechanism of pore formation differs.

An important aspect in the clinical development of AMPs is their activity in a physiological setting. The *in vivo* stability of peptides in blood is well modelled by their *in vitro* stability in serum and plasma (Powell *et al.*, 1993). The amidated peptide lost its activity against *E. coli* and *S. aureus* in the presence of 30% human serum (Table 3.3). The inactivity of AMPs in the presence of serum is a major setback these peptides face in clinical development, thus restricting their use to topical applications (Hancock and Sahl, 2006). The ability to overcome this limitation is to determine the factors responsible for masking the bactericidal potential of the peptides (Huang *et al.*, 2011). As serum is a complex mixture of proteins, enzymes and salts, a number of factors can be responsible for the antimicrobial inhibition of AMPs.

Serum comprises of a number of transport proteins, none as unique as albumin. Albumin is a vital serum transport protein with a high binding affinity under physiological conditions exerting covalent and reversible binding to a range of substances from neutral lipophilic and acidic drug like ligands to positively charged peptides (Kragh-Hansen, 1990). Svenson *et al.* (2007) have studied the interactions of small AMPs with human serum albumin and have confirmed a single binding site on the transport protein with a dissociation constant of 4.3-22.2 μM to be theoretically responsible for 95% of AMP-albumin binding. The authors also confirmed albumin to not have any antimicrobial potential. The strong binding affinity of albumin towards AMPs reduces the peptide concentration available to eliminate bacteria (Svenson *et al.*, 2007). The AMP binding site on albumin was found to be mainly binding site II due to the peptides hydrophobic moieties (Sivertsen *et al.*, 2014). Serum proteases such as trypsin and chymotrypsin may also inactivate the bactericidal potential of AMPs. Although C-terminal amidation should increase the stability of the peptide by offering protection to the C-terminal end, protection by C-terminal amidation alone is negligible as

serum endopeptidases initiate peptide cleavage in the middle of the fragment (Nguyen *et al.*, 2010). Since trypsin cleaves C-terminally at Arg and Lys residues (Brown and Wold, 1973) and chymotrypsin cleaves C-terminally at Phe, Tyr and Trp residues (Hedstrom *et al.*, 1992), these enzymes have the potential of degrading Os(3-12)NH₂ due to the Arg, Lys and Tyr residues found within the sequence of the peptide.

Salt is a major component of serum responsible for the inhibition of the bactericidal activity of AMPs (Guani-Guerra *et al.*, 2010). The importance of salt resistance is illustrated in cystic fibrosis patients and immunocompromised patients where functional AMPs are necessary to combat *S. aureus* and *P. aeruginosa* from residing within the host (Friedrich *et al.*, 1999). Os(3-12)NH₂, however lost its bactericidal activity in the salt mixture comprising of 145 mM NaCl, 2.5 mM CaCl₂ and 1 mM MgCl₂ against *E. coli* and *S. aureus* (Table 3.4). The loss of AMP activity in the presence of salt is a typical characteristic of the antimicrobial potential of AMPs being salt sensitive. The effects of salts on the lipid bilayers reveal cations to influence the electrostatic potential of the lipid bilayer by decreasing the bilayer diffusion capability and fluidity (Böckmann *et al.*, 2003). Cations prefer binding to the lipid head group regions, close to the ester oxygens. This binding complexes the lipid molecules, increasing lipid ordering and decreasing the average lipid surface area. Monovalent cations bind to these regions much faster and deeper than divalent cations (Böckmann and Grubmüller, 2004; Böckmann *et al.*, 2003). AMPs associate with the lipid head groups in a similar manner. The cationic peptides bind to the lipid bilayers via hydrogen bonds, irrespective of their degree of insertion into the bilayer. These hydrogen bonding sites are generally found at the acceptor sites of the phosphate moieties on the lipids and at the acceptor oxygens of the glycerol ester groups. Na⁺ ions affect the hydrogen bonding between peptides and lipids by binding to these hydrogen bonding sites on the lipids (Kandasamy and Larson, 2006). As a result, the cations compete for binding to the same favourable lipid head group sites as AMPs (Lee *et al.*, 1997), interfering with the lipid-AMP interaction (Huang *et al.*, 2011).

The hydrophobic nature of AMPs governs their ability to bind to the zwitterionic host cell membranes. In comparison to melittin, the control peptide of the lytic evaluation studies, Os(3-12)NH₂ was non-toxic against the human erythrocytes (Figure 3.6) and Caco-2 cells (Figure 3.7). The amidated peptide remained nonlytic just as Os (Prinsloo *et al.*, 2013). The lytic potential of AMPs are generally associated with a hydrophobic interaction towards phosphatidylcholine residues on mammalian cells (Matsuzaki *et al.*, 1995). Increasing the hydrophobic content of AMPs

therefore enhances their hydrophobic interaction towards mammalian membranes (Tachi *et al.*, 2002). The main hydrophobic amino acid residue significantly involved in the lytic potential of melittin is Trp, at position 19. The lytic potential of Trp has been reported to be due to the amino acid's ability to target cholesterol within biological membranes via the indole moiety, a similar mechanism utilized by polyene antibiotics and cholesterol. Cholesterol is an essential factor in maintaining the barrier integrity of the epithelial monolayer of Caco-2 cells (Lambert *et al.*, 2005).

Adding to the therapeutic value of Os(3-12)NH₂, the antioxidant potential of the amidated peptide was investigated as the parent peptide Os manifested antioxidant activity (Prinsloo *et al.*, 2013). The amidated peptide displayed antioxidant activity 15-fold greater than that of GSH (Figure 3.8). Compared to the molar ratio for Os of 21.63 $\mu\text{M TE}/\mu\text{M}$, the molar ratio for Os(3-12)NH₂ was calculated to be 30.31 $\mu\text{M TE}/\mu\text{M}$, 1.4-fold greater than that of Os. An evaluation of the antioxidant potential of free amino acids revealed Cys, Met, Trp, Tyr, Phe, His, and Pro to contribute to the antioxidant potential of food derived peptides as well as synthetic natural peptides (Pownall *et al.*, 2010; Erdmann *et al.*, 2006; Hernández-Ledesma *et al.*, 2005). The radical scavenging activities of Tyr, Trp and Cys include proton and hydrogen atom transfer mechanisms by their respective phenolic, indolic and sulfhydryl hydrogens. The electron dense side chains of His, Trp and Met also contribute to radical scavenging (Udenigwe and Aluko, 2011). The antioxidant potential of Os(3-12)NH₂ can be attributed to the 2 Tyr residues and 1 Cys residue the peptide comprises of. Os however comprises of 3 Cys and 3 Tyr residues.

Due to the antioxidant potential of Os(3-12)NH₂, the peptide fragment has the ability to prevent free radicals from forming within the body. Oxidative stress is a major challenge the human body faces daily where reactive oxygen species damage biomolecules from DNA, lipids and proteins (Block *et al.*, 2002). In the long term, the excessive free radical production has the ability to impair metabolic processes leading to cell death (Jiao and Wang, 2000). Oxidative stress is thus one of the main contributors to the ageing process, cancer, cardiovascular diseases as well as Alzheimer's disease (Hybertson *et al.*, 2011). The antioxidant potential of Os(3-12)NH₂ and Os supports the role of AMPs in maintaining redox homeostasis in the midgut of the tick *O. savignyi* (Hajdušek *et al.*, 2013).

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

AMPs possess the potential to combat multidrug-resistant bacteria. The amphipathic nature of these peptides leads to a rapid, nonspecific, killing mechanism active against a broad spectrum of pathogens. A few major setbacks to the clinical development of AMPs however are their expensive synthesis costs, bactericidal inactivity under physiological conditions and toxicity towards host cell components (Huang *et al.*, 2011). As a result, shorter peptide fragments are desirable although may lack in bactericidal potency in comparison to their parent molecules (Perez-Paya *et al.*, 1995; Saido-Sakanaka *et al.*, 2004; Sitaram and Nagaraj, 1990; Sitaram *et al.*, 1993). This study focused on enhancing the bactericidal potential of the synthetic peptide, Os(3-12), derived from the parent peptide Os and investigated the impact of this modification on the amidated peptide's structure, bactericidal, cytotoxic and antioxidant activities.

In the structural analysis, CD data showed both the amidated and unamidated peptides to remain unstructured when placed in a membrane mimicking environment, unlike Os which was predominantly α -helical (Prinsloo *et al.*, 2013). The failure to form a secondary structure in both Os(3-12)NH₂ and Os(3-12) was attributed to the decreased peptide length.

In terms of bactericidal activity, an enhanced activity of Os(3-12) was achieved due to C-terminal amidation. Although still less potent than Os, the determined MBC for each peptide indicated that amidation increased the bactericidal activity of Os(3-12) by 16-fold against *E. coli* and by 8-fold against both *P. aeruginosa* and *B. subtilis*. In comparison amidation enhanced the activity of the peptide towards *S. aureus* by only 2-fold. As both peptide fragments were found to be unstructured with no difference in comparison to one another, the enhanced bactericidal potential of Os(3-12)NH₂ can be attributed to the increase in peptide charge due to amidation. The decreased production costs of Os(3-12)NH₂ further motivates future peptide modifications of the peptide in addition to C-terminal amidation to further enhance the peptide's potency. Strom *et al.* (2002) demonstrated the antimicrobial activity of shorter peptide fragments to improve due to the amino acid substitutions of Arg and Trp in addition to C-terminal amidation. The substitution of these residues to the sequence of Os(3-12)NH₂ should be considered for future studies to enhance the antimicrobial activity of the peptide.

Os(3-12)NH₂ was observed to be more efficient in killing Gram-negative bacteria (10 minutes) than Gram-positive bacteria (60 minutes). As the amidated peptide took much longer to kill Gram-positive bacteria than Gram-negative bacteria, it was concluded that the peptide employs a different killing mechanism against the different types of bacterial cells. In comparison to the parent peptide Os, Os killed both *E. coli* and *B. subtilis* within 5 minutes (Prinsloo *et al.*, 2013). Therefore the longer killing times observed for the amidated peptide implies the peptide's interaction with the bacterial membrane lipids are not as strong as the interaction with Os due to the lack of charge and structure. In order to improve the peptide-membrane lipid interaction of Os(3-12)NH₂, some insight into the bactericidal mechanism of action for both Os(3-12)NH₂ and Os was required.

The SYTOX green uptake assay was employed to investigate both study peptides ability to induce bacterial membrane permeabilization. Both Os(3-12)NH₂ and Os exhibited membrane permeabilization against *S. aureus* and *E. coli*, but to a lesser extent than the control peptide LL-37. The fluorescence intensity differences against Gram-positive and Gram-negative bacteria implies different killing strategies against these bacteria. The fluorescent trend observed by both peptides against *E. coli* and *S. aureus* implies the peptides may exhibit a dual mechanism of action at higher peptide concentrations. This dual mechanism may entail DNA binding capabilities in addition to membrane permeabilization. For this reason, it is essential to determine the DNA binding capability of Os(3-12)NH₂.

As mentioned previously, membrane permeabilization does not necessarily induce bacterial death. Previous reports on the mechanism of action of AMPs have observed membrane depolarization to correlate with bacterial death and not membrane permeabilization. In order to obtain the lethal sequence of events for both Os(3-12)NH₂ and Os, a time dependent study of membrane permeabilization is necessary to perform in order to correlate bacterial membrane permeabilization with the killing kinetics. The information of this sequence of events is essentially important in the investigation against Gram-positive bacteria as the AMP killing event is dependent on the bacterial transmembrane potential.

As AMPs permeabilize *E. coli* membranes via the self-promoted uptake mechanism, the LPS binding capabilities of the peptides should be investigated. A strong AMP-LPS binding leads to an inefficient translocation of the AMPs into the bacterial cells resulting in a loss of bactericidal

activity. In contrast a weaker binding of AMPs towards LPS leads to an efficient translocation of the peptides into the bacterial cells and consequently an increase in bactericidal activity (Chai *et al.*, 2014). This binding affinity may account for the differences in the killing kinetics between Os(3-12)NH₂ and Os, therefore should be further investigated.

The membrane layers between Gram-negative and Gram-positive bacteria differ vastly. Unlike Gram-positive bacteria with just the cytoplasmic membrane, Gram-negative bacteria comprise of an inner and outer membrane. The SYTOX green uptake mechanism against Gram-negative bacteria confirms inner membrane permeabilization of the bacteria as the dye was able to bind to the bacterial cells DNA and fluoresce. In order to understand the peptides mechanism against Gram-negative bacteria, the outer membrane should be investigated as well. The ability of the Os(3-12)NH₂ and Os to permeabilize the outer membrane as well as the rate of permeabilization should be investigated to comprehend whether the peptides binds to DNA or the DNA is damaged due to cell death. The outer membrane permeabilization can be investigated by measuring the activity of the periplasmic β -lactamase with its respective substrate CENTA, a synthesized chromogenic cephalosporin, with a highly reactive β -lactam ring (Bebrone *et al.*, 2001; Marcellini *et al.*, 2009).

AMPs binding to bacterial membranes generally induce changes to the bacterial surfaces. These changes correspond to the pore formation mechanisms the peptides employ and is necessary to determine. Changes in the bacterial morphologies can be evaluated with transmission electron microscopy (TEM) (Makovitzki *et al.*, 2006; Marcellini *et al.*, 2009). Likewise this technique can be used to confirm that these peptides are nonlytic against mammalian cells.

Although C-terminal amidation may improve the bactericidal activity of Os(3-12)NH₂, this modification has a negligible effect in terms of the peptides serum stability (Nguyen *et al.*, 2010). The bactericidal potential of Os(3-12)NH₂ was lost in the presence of 30% human serum and the salt mixture comprising of NaCl, MgCl₂ and CaCl₂. The instability of AMPs under physiological *in vitro* conditions is one of the limiting factors to their clinical development. This factor restricts their application to topical usage. The elucidation of the peptides bactericidal mechanism of action would further aid in the understanding of the peptides serum and salt sensitivities. As serum comprises of a number of salts, proteins, enzymes and other components, it is necessary to identify the specific component responsible for the loss of the bactericidal activity of Os(3-12)NH₂ in order to obtain a more valuable future peptide modification.

Albumin, one of the main biological transporters of serum is an essential factor in the inhibition of the bactericidal activity of AMPs. The transporter protein reduces the concentration of free AMPs able to react with bacterial membranes due to peptide-albumin binding. Investigating how AMPs interact with albumin is necessary for the clinical development of these peptides (Sivertsen *et al.*, 2014). Another factor of human serum responsible for the serum sensitivity of Os(3-12)NH₂ is the peptide's protease susceptibility. L-isomers of amino acid residues found in the peptide's sequence are easy targets for endopeptidases. By replacing these L-isomers with D-isomers, stability is offered to the peptide fragment as the irregular nature of these amino acid residues are much more difficult to hydrolyze (Yin *et al.*, 2013). These unnatural amino acid residues also enhance the bactericidal potential of AMPs (Strömstedt *et al.*, 2009), although may not be very cost effective. Peptide cyclization is therefore another optional modification which also improves the serum stabilities of AMPs and their antimicrobial potential (Nguyen *et al.*, 2010). Cyclization is a good approach to improve the pharmacodynamics of Os(3-12)NH₂. As shorter AMPs are more susceptible to serum and salt inactivation, the capping of the terminal end regions of the peptide fragment with the non-natural bulky amino acid β -naphthylalanine could also be considered for the future improvement of the serum and salt resistance of Os(3-12)NH₂ (Chu *et al.*, 2013).

The nonlytic potential of Os(3-12)NH₂ against human erythrocytes and Caco-2 cells motivates further research to be done in reducing the serum and salt sensitivities of this peptide. As the haemolytic investigation was conducted in the iso-PBS buffer, the presence of Na⁺ ions in this buffer is of concern as the amidated peptide is salt sensitive. As the Na⁺ ionic concentration in the iso-PBS buffer (155 mM), the Na⁺ ionic concentration within the NaP buffer used in the bactericidal investigations (18 mM) and the cell culture media serum and salt composition for the Caco-2 cells may affect the activity of the amidated peptide, a dosage effect of serum and salt on the peptides functionality is imperative to investigate for the future. The peptide's nonlytic potential however can be confirmed due to the peptide's loss of secondary structure and the absence of Trp residues.

The antioxidant activity of Os(3-12)NH₂ was calculated to be 15-fold greater than GSH and 1.4-fold greater than Os. The antioxidant potential of Os(3-12)NH₂ therefore increases the peptide's therapeutic value, rendering Os(3-12)NH₂ multifunctional (Huang *et al.*, 2011; Mangoni *et al.*,

2008). The antioxidant potential of AMPs is a novel finding and is worthy of further investigation especially related to amino acid composition and sequence and measured antioxidant activity.

As the current therapeutic status of the peptide is restricted to topical use due to the inactivity under physiological conditions, the peptide can be used in creams, gels and mouth washes. Some clinical applications would be preventing catheter related infections (Melo *et al.*, 2006), topical creams to prevent acne, oral candidiasis (thrush) as well as implant surface infections (Kazemzadeh-Narbat *et al.*, 2010). Due to the peptide's antioxidant potential, the peptide can be an ingredient of anti-ageing creams and sun screen lotions. In addition this AMP can be used to prevent age-related eye diseases such as the formation of cataracts (Telang, 2013).

In conclusion, the small size of Os(3-12)NH₂, the peptide's potent bactericidal activity against Gram-positive and Gram-negative bacteria, nonlytic potential *in vitro* against human erythrocytes and Caco-2 cells and potent antioxidant potential increase the therapeutic value of this peptide. Further insight into the peptide's bactericidal mechanism of action, antioxidant functionality and physiological sensitivity is essential for future basic and applied research.

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