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Anti-inflammatory properties of peptides 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 requirements for the degree

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SUMMARY

Lipopolysaccharides (LPS), when released from the outer membrane of Gram-negative bacteria, bind to macrophages, inducing production of pro-inflammatory cytokines and mediator molecules. In addition, associated overproduction of free radicals causes oxidative stress and is a major factor for triggering local inflammation causing tissue and cellular damage. Several cationic antimicrobial peptides (AMPs), besides antimicrobial activity, also possess anti-inflammatory and antioxidant properties. The advantage of this multifunctionality is that while the infection is cleared, inflammation and associated free radical cellular and tissue damage is reduced.

In this study the potential anti-inflammatory and antioxidant properties of synthetic AMPs, Os and Os-C, derived from a defensin (OsDef2) previously identified in the tick, *Ornithodoros savignyi*, were investigated. Os is derived from the carboxy-terminal of OsDef2, whereas Os-C is an analogue of Os from which the Cys residues were omitted. Both Os and Os-C were found to bind LPS *in vitro* by measuring their ability to inhibit the LPS-induced activation of *Limulus* amoebocyte lysate (LAL) enzyme. The binding of free LPS caused the loss of both Os and Os-C bactericidal activity against *Escherichia coli* (*E.coli*) confirming the affinity of both peptides for LPS. Like glutathione (GSH), Os scavenged nitric oxide (NO), which is possibly due to the presence of three Cys residues and the formation of nitrosothiols with NO. Melittin (control) and Os-C, peptides lacking Cys residues, were found to be poor NO scavengers.

RAW 264.7 cells exposed simultaneously to LPS/IFN-γ and Os or Os-C showed that both peptides attenuated the LPS-induced production of NO, as well as TNF-α, in a dosage-dependant manner. In contrast, a 90 minute pre-incubation of RAW 264.7 cells with peptides, followed by LPS/IFN-γ stimulation, showed that both peptides, had a lesser effect on the inhibition of NO production when compared to controls. Although the control peptides, melittin and polymyxin B, had higher NO inhibition activity, both were associated with a loss of cellular viability. The results of the pre-incubation experiments indicated that Os and Os-C, besides binding to LPS, might have specific intracellular sites of action that allow for inhibition of LPS mediated NO formation. In contrast to both melittin and polymyxin B, Os and Os-C were found to be non-toxic towards the RAW 264.7 cells even at peptide concentrations 132 and 57 times higher than the minimum bactericidal concentration (MBC), respectively. Os was found to possess higher cellular antioxidant activity compared to Os-C, which may be attributed to the presence of antioxidant

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amino acids. This study identifies the multifunctional properties of Os and Os-C, however the specific mode of action of each peptide needs to be further elucidated.

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ABBREVIATIONS

AAPH - 2,2'-Azobios(2-amidinopropane) dihydrochloride
ECACC - European Collection of Cell Cultures
CD14 - Cluster of differentiation 14
CD11b/CD18 - Cluster of differentiation 11b molecule/cluster of differentiation 18
CFU - Colony forming unit
DAB - Diaminobutyric acid
DCFH-DA - Dichlorofluorescein diacetate
DMEM - Dulbecco's modified Eagle's medium
DTT - Dithiothreitol
E.coli - *Escherichia coli*
FITC - Fluorescein isothiocyanate
GSH - Glutathione
hBD2 - Human β-defensin 2
HDPs - Host defense peptides
HRP - Horseradish peroxidase
IκB - Inhibitor of nuclear factor NFκB
IKK - IκB kinase
IL-1 - Interleukin-1
IL-6 - Interleukin-6
IFN-γ - Interferon-γ
iNOS - Inducible nitric oxide synthase
IRAK - Interleukin-1 receptor-associated kinase
IRF-3 - Interferon regulatory factor-3
LAL - *Limulus* amoebocyte lysate
LB - Luria-Bertani
LBP - LPS-binding protein
LPS - Lipopolysaccharides
Mal - MyD88 adaptor-like molecule
MAP - Mitogen-activated protein
MBC - Minimum bactericidal concentration
MD-2 - Lymphocyte antigen 96
MSR - Macrophage scavenger receptor

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MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

MyD88 - Myeloid differentiation primary response protein 88

NaP - Sodium phosphate buffer

NED - N-(1-naphthyl)ethylenediamine dihydrochloride

NF-κB - Transcription factor nuclear factor kappa B

NO - Nitric oxide

NOD - Nucleotide-binding oligomerization domain

OsDef1 - *Ornithodoros savignyi* defensin isoform 1

OsDef2 - *Ornithodoros savignyi* defensin isoform 2

PAMPs - Pathogen-associated molecular patterns

PBS - Phosphate buffered saline solution

PKR - RNA-dependent protein kinase

PMN - Polymorphonuclear leukocytes

pNA - p-Nitroaniline

PRR - Pattern recognition receptors

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RP-HPLC - Reverse-phase high-performance liquid chromatography

SA - Sulphanilamide

SEM - Standard error of the mean

SNP - Sodium nitroprusside

TAK1 - Transforming-growth-factor-β-activated kinase 1 kinase activity

TMB - 3,3’5,5’-tetramethylbenzidine

TIR domain - Toll/Interleukin-1 domain

TIRAP - TIR domain-containing adaptor protein

TLRs - Toll-like receptors

TNF-α - Tumour necrosis factor-α

TRAF6 - Tumour necrosis factor receptor associated factor 6

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CHAPTER 1: INTRODUCTION

1.1 Rational of study

Innate immunity is known to be triggered by conserved bacterial components that interact with receptors on the host cells, activating a pro-inflammatory cascade (Bowdish and Hancock, 2005). Sepsis is a life-threatening condition that occurs when oxidative stress and inflammatory responses associated with the pathogenesis of infection are not contained at a local site and spread throughout the body via the circulatory or lymphatic system (Madigan, *et al.*, 2003). Septic shock has a high mortality rate of 40-70% (Lever and Mackenzie, 2007). Despite our ability to treat bacterial infections with antibiotics, it has been found that in some cases antibiotic-induced release of LPS may contribute to sepsis itself (Brandenburg, *et al.*, 2010; Byl, *et al.*, 2006). The advances in improving the survival rate of patients with severe sepsis has been unsatisfactory. According to Bowdish and Hancock (2005) different treatments have had some success in reducing the risk of death due to sepsis, for example, inhibitors of pro-inflammatory cytokine production (Polderman and Girbes, 2004), endotoxin antibodies (Polderman and Girbes, 2004), extracorporeal endotoxin removal (Shoji, 2003) and LPS agonists (Hawkins, *et al.*, 2004). Host defence peptides (HDPs) are a series of peptides that form part of the innate immune system and have numerous roles in innate defences of the host as reported by Steinstraesser, *et al*. (2009). Studies have shown that these peptides, also known as cationic host-defence antimicrobial peptides (AMPs), contain in addition to their antimicrobial activity, the ability to suppress harmful inflammatory/septic responses and are also involved in promoting wound healing (Easton, *et al.*, 2009; Mookherjee and Hancock, 2007).

Synthetic peptides derived from cationic host-defence AMPs exhibiting multiple properties, such as anti-inflammatory and antioxidant activities, are promising leads not only for the development of novel anti-sepsis agents, but hold the potential for a wide range of applications (Li, *et al.*, 2012). Ticks only rely on an innate immune defence for protection against microbes and therefore produce a variety of AMPs, like defensins. Tick defensins could potentially function as templates for the development of multifunctional peptides. Previous research has shown that a synthetic peptide, Os, derived from the carboxy-terminal of a tick defensin (OsDef2) and an analogue in which the three cysteine residues were omitted, Os-C, were bactericidal to both Gram-positive and Gram-negative bacteria, non-toxic to mammalian cells and exhibited antioxidant activity (Prinsloo, *et al.*, 2013).

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Multifunctional peptides hold potential for the treatment of bacterial infections and may prevent inflammation and oxidative damage associated with these conditions (Sawa, *et al.*, 1998). In this study the anti-inflammatory properties of Os and Os-C were investigated using LPS-stimulated RAW 264.7 macrophages as an *in vitro* model of inflammation. In addition the protective effect of these peptides against oxidative damage of RAW 264.7 cells was evaluated.

1.2 Host immunity against invading pathogens: adaptive and innate immune responses

The immune system of multicellular organisms protects them from pathogens through the generation of a range of cells and molecules that are able to recognize and eliminate these foreign invaders. The immune system can be divided into two categories; innate immunity and adaptive immunity (Kindt, *et al*., 2007). The innate immune response which is the first line of defence of a host does not target a particular pathogen and can respond within minutes to hours of infection. The adaptive immune response is directed at specific invaders, and when it is the first exposure, the response may take days (Silverthorn, 2007). Recognition of a pathogen by the innate immune system causes the elevation of an effective adaptive immune response.

Innate immunity consists of physical (skin and mucous membranes), chemical (acidity of the stomach contents and soluble molecules that possess antimicrobial activity) and cellular barriers (cells with sensitive receptors that perceive microbial products and initiate a counter attack). The innate immune response prevents infection in damaged tissue by attracting immune cells and chemical mediators to the site, producing a physical barrier to slow down the spread of infection and establishing tissue repair once the infection is under control. Within the blood and/or tissue fluids there are soluble components, for example small cationic host-defence AMPs that play an important role in this primary defence (Madigan, *et al.*, 2003).

The innate immune response is nonspecific for the recognition of stimulatory molecules, which include bacterial LPS, as well as other molecular patterns associated with pathogens. A limited diversity is observed due to the restricted number of germ line-encoded receptors and lack memory response abilities (Kindt, *et al*., 2007; Hancock and Diamond, 2000). It also consists of a perfect self-versus nonself discrimination capability. The innate immune response consists of several major cell types such as: monocytes, macrophages, neutrophils, natural killer cells and dendritic cells. These cells have various functions in the innate immunity, which includes processes such as phagocytosis, oxidative and non-oxidative attack, secretion of cytokines and interferons, antigen

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presentation, promotion of an inflammatory response and providing co-stimulatory signals (Kindt, *et al*., 2007).

Soluble and/or membrane-bound mediators containing pattern recognition receptors (PRRs) recognize bacterial motifs called pathogen-associated molecular patterns (PAMPs), which are structural components of bacteria, such as LPS, lipoteichoic acid, peptidoglycan and bacterial flagellin (Akira and Takeda, 2004; Thomma, *et al*., 2011). The main steps used by the innate immune system to attack infection are the following: PAMPs are recognized by the complement system mediators causing the activation of the complement cascade leading to lysis of the pathogen, opsonisation and promotion of phagocytosis. Recognition triggers inflammation and the secretion of immuno-regulatory molecules that result in immune clearance (Kindt, *et al*., 2007).

Immature macrophages and dendritic cells have receptors that detect microbial products, of which Toll-like receptors (TLRs), are important due to the fact that these receptors allow the detection of a broad spectrum of pathogens. Activated macrophages will cause phagocytosis and produce toxic chemicals that facilitate the destruction of the phagocytosed microbes, as well as secretion of cytokines to induce inflammatory responses (Kindt, *et al*., 2007). Dendritic cells internalize the antigen at the site of infection and present the antigen to T cells in the lymphoid tissue. This will direct the adaptive immune response towards a particular pathogen. The dendritic cells also secrete cytokines that are accountable for the initiation of inflammatory responses (Cohen, 2002; Kindt, *et al*., 2007). The innate immune responses are activated through signal transduction pathways. The microbial products bind to PRRs causing signals to be transmitted through the interactions of specific intramolecular molecules. This will allow the defence mechanisms to clear the invading pathogen as a consequence of these signals (Kindt, *et al*., 2007).

1.3 Inflammatory response as a result of infection

Inflammation is a non-specific reaction that occurs as a result of exposure to a harmful, opportunistic pathogen or toxin or due to tissue injury that induces a cascade of events known as an inflammatory response (Kindt, *et al*., 2007). This response is an ever-present characteristic of the host defence against invading microorganisms and combats the early stages of infection to initialize tissue repair. Distinctive, localized, inflammatory responses will result in redness, swelling, pain and heat at the site of infection (Madigan, *et al*., 2003). Inflammation is an important response of the infected organism that is associated in wound healing and is involved in

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the clearance of infection (Vetriselvan, *et al.*, 2013). Cytokines and mediator molecules, such as chemokines, bradykinin, thrombin, plasmin and anaphylatoxins, are just some of the molecules that are released by the innate and adaptive system that may trigger or develop specific aspects of the inflammatory response (Kindt, *et al*., 2007).

Inflammation can be classified as acute and chronic. Acute inflammation occurs as long as the injurious stimuli is present and ceases once the stimulus has been removed with the processes mentioned above. Chronic inflammation is a pathological condition that occurs as a result of simultaneous and continued activation of inflammation and tissue damage that is characterised by the infiltration of mononuclear immune cells such as macrophages, monocytes and leukocytes. In an attempt to reverse tissue damage the body respond with angiogenesis and fibrosis (Vetriselvan, *et al.*, 2013). The inflammatory response that was initially started to fight infection eventually leads to organ damage and finally septic shock if left untreated (Qureshi and Rajah, 2008).

1.3.1 The role of LPS in inflammation in macrophages

The bacterial cell membrane of Gram-negative bacteria contains outer and cytoplasmic membranes that are separated by a thin cell wall composed of peptidoglycans (**Figure 1.1**) (Cohen, 2002; Madigan, *et al*., 2003). The outer membrane is anchored to the cytoplasmic membrane by lipoproteins present in the periplasm. The outer membrane of Gram-negative bacteria is a lipid bilayer that consists of an inner leaflet containing phospholipids and an outer leaflet containing LPS. In addition to its endotoxic and immunogenic properties, LPS also prevents the entry of harmful substances into the bacterial cell. Entry of nutrients, such as glucose, into the bacteria occurs through proteins called porins that form transmembrane diffusion channels (Ding, *et al.*, 2003; Li, *et al.*, 2004).

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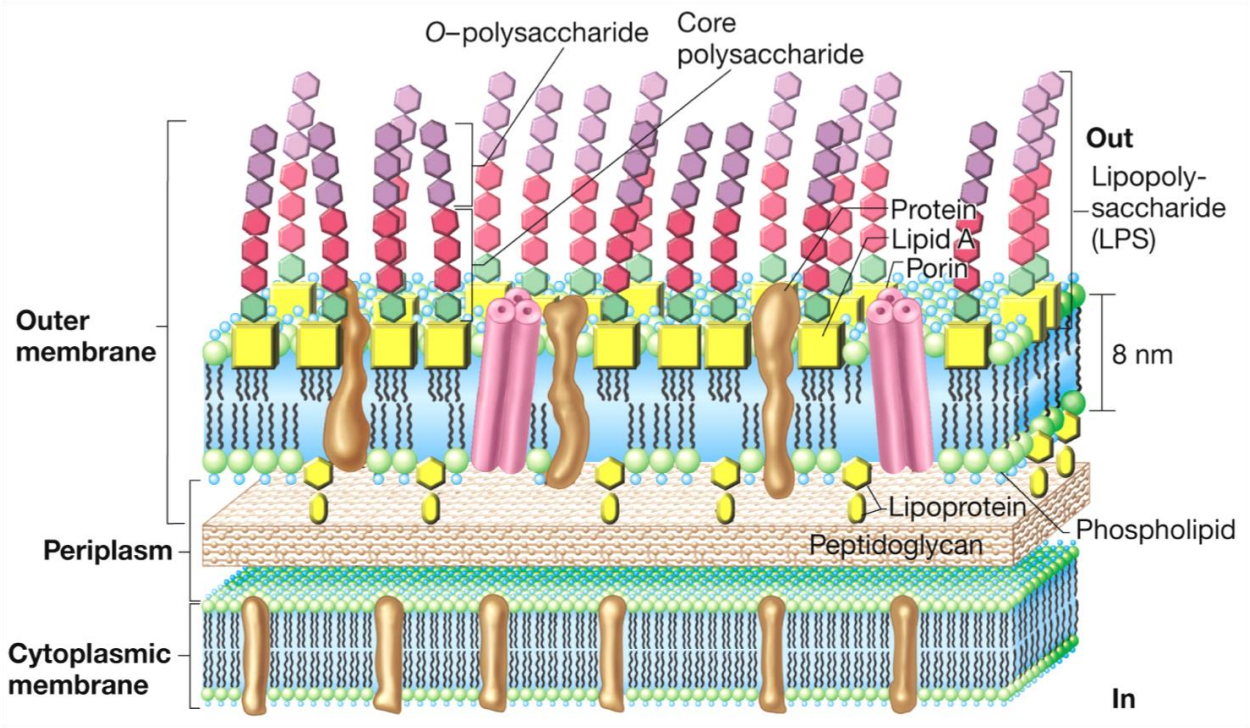

Figure 1.1: Schematic representation of a Gram-negative cell envelope. The arrangement of lipopolysaccharide, lipid A, phosholipid, porins and lipoprotein in the outer membrane of Gram-negative bacteria. (Madigan, *et al*., 2003).

LPS is a very potent soluble molecule of bacteria that induces cytokine production leading to inflammatory responses (Dinarello, 1997). LPS present in the outer membrane is made up of two components, the lipid A and polysaccharide component. The latter consists of two portions, the core polysaccharide and the O-specific polysaccharide. The O-specific polysaccharide (or antigen), a chain of repeating oligosaccharides of three to eight units, is strain specific and determines the serological identity of different bacterial strains. The core polysaccharide portion consists of 2-keto-3-deoxyoctulosonic acid (KDO), heptose, glucose, galactose and N-acetyl-D-glucosamine. The lipid A portion of LPS consists of D-glucosamine and a variable number of fatty acid chains. The phosphorylated β1,6 linked D-glucosamine of lipid A is attached to the polysaccharide portion through KDO (**Figure 1.2**) (Ding, *et al.*, 2008; Hitchcock, *et al.* 1986; Madigan, *et al*., 2003; Netea, *et al.*, 2002).

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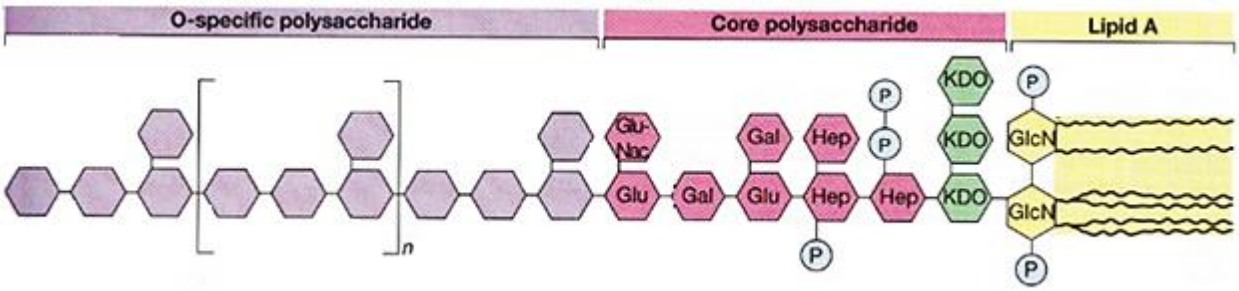

Figure 1.2: The structure of a lipopolysaccharide of Gram-negative bacteria. The major components of LPS consist of the following sequence, O-specific polysaccharides-core polysaccharides-lipid A). Ketodeoxy-octonate (KDO); Heptose (Hep); D-glucose (Glu); D-galactose (Gal); heptose (Hep); N-acetyl-D-glucosamine (GlcNac); D-glucosamine (GlcN); phosphate (P) (Madigan, *et al*., 2003).

LPS is released from bacteria by numerous ways, for instance when bacteria undergo cell division, cell death or when bacterial infections are treated with antibiotics (Hancock and Scott, 2000; Rosenfeld, *et al.*, 2006). Released LPS binds to LPS-binding protein (LBP), forming a LPS-LBP complex (**Figure 1.3**). The complex then interact with a cluster of differentiation 14 (CD14) molecule that is the primary receptor of LPS (Lin, *et al.*, 2008). CD14 can be membrane bound through glycosylphosphatidylinositol linked molecules present on the cell surfaces of macrophages, but can also be soluble and be present in the tissue fluid (Cohen, 2002). Toll-like receptor signalling is initiated by the binding of the LPS-LBP-CD14 complex to the extracellular portion of the transmembrane protein, Toll-like receptor 4 (TLR4). This occurs together with an extracellular protein, lymphocyte antigen 96 (MD-2), which is responsible for the correct positioning of TLR4 on the cell surface (Cohen, 2002).

On the cytoplasmic side, the TLR contains a protein domain called the Toll/IL-1 (TIR) domain. The signal-induced assembly involves the binding of TIR to interleukin-1 receptor-associated kinase (IRAK), which is facilitated by myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP), also known as MyD88 adaptor-like molecule (Mal). This process is inhibited by Toll-interacting protein (Tollip) that prevents signalling (Cohen, 2002; Akira and Takeda, 2004).

IRAK consist of IRAK1 and IRAK4. During the signalling cascade IRAK4 phosphorylates its partner, IRAK1, providing a docking site for the tumour-necrosis-factor-receptor-associated factor 6 (TRAF6). The resulting IRAK1:TRAF6 complex dissociates from IRAK4 and binds to another IRAK1 protein kinase to allow the activation of the transforming-growth-factor-β-activated kinase 1 (TAK1) kinase activity (Cohen, 2002; Akira and Takeda, 2004; Kindt, *et al*., 2007). TAK1 is

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essential for the phosphorylation-mediated activation of the mitogen-activated protein kinase (MAPK) pathway and transcription factor nuclear factor kappa B (NF-κB) pathway.

In the NF-κB pathway, IκB kinase (IKK) is phosphorylated by TAK1, which then phosphorylates inhibitor of nuclear factor NF-κB (IκB) of the IκB:NF-κB complex. This causes the transcription factor, NF-κB, to be released which migrates to the nucleus. In the nucleus it initiates the transcription of genes encoding cytokines, adhesion molecules and other effectors of the innate immune response (Kindt, *et al*., 2007).

Also depicted by **Figure 1.3** are other cell surface molecules which also have the capacity to bind LPS, such as macrophage scavenger receptors (MSRs), CD11b/CD18 (cluster of differentiation 11b molecule/ cluster of differentiation 18,) and ion channels. The MyD88-independent pathway allows TIRAP to signal through a RNA-dependent protein kinase (PKR) and interferon regulatory factor-3 (IRF-3). Nucleotide-binding oligomerization domain (NOD) proteins, NOD1 and NOD2, are present in the cytosol of the cell and recognize LPS, allowing another means for the macrophages to respond to the bacterial infection (Cohen, 2002).

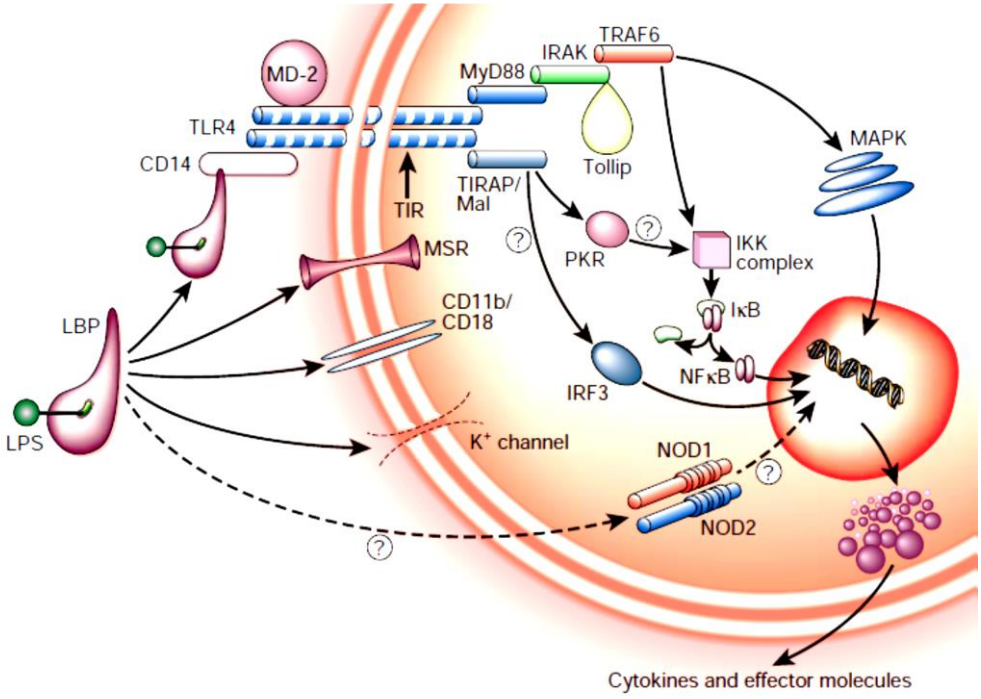

Figure 1.3: Cell-surface recognition mechanisms and intracellular signalling of LPS. Primary detection mechanism by macrophages is via the LBP-LPS complex and TLR4-MD-2 signalling complex. Other surface molecules that detect LPS are MSRs, CD11b/CD18 and ion channels. Intracellular signalling is dependent on the intracellular TLR domain (TIR), IRAK, MyD88 and TIRP that result in the production of pro-inflammatory cytokines and mediator molecules. Additionally, LPS binds to receptor proteins, NOD proteins, present in the cytosol (Cohen, 2002).

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Activated macrophages have an increased level of phagocytic activity, destroy ingested microorganisms more readily in phagosomes and secrete mediators for inflammation. Classic pro-inflammatory cytokines and mediator molecules released by macrophages are tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and NO. Overstimulation of macrophages by LPS leads to unregulated expression and secretion of pro-inflammatory cytokines and mediator molecules that plays a critical role in the pathogenesis of septic shock (Cohen, 2002; Rosenfeld, *et al.*, 2006).

1.3.2 Effects of macrophage released pro-inflammatory cytokines and effector molecules

As a result of macrophage activation, these cells release pro-inflammatory cytokines (IL-1, IL-6, IL-12, IL-15, IL-18 and TNF- α), lipid mediators (platelet activating factor, prostaglandins and tissue factor) and free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is important to note that upon activation of macrophages, the large range of products released have multiple targets and as a result are involved in several parallel mechanisms. For example, TNF-α and IL-1, are prototypic cytokines that are secreted shortly after LPS exposure and in turn activate an entire secondary system which results in various responses (Cohen, 2002; Kindt, *et al*., 2007).

To be more specific, TNF-α and IL-1, act together to induce coagulation and increase vascular permeability, assist in the synthesis of acute-phase proteins, promote fever by affecting the thermoregulatory center in the hypothalamus, as well as facilitating the activation of T- and B-cells. They likewise induce the increased expression of adhesion molecules, as well as, allowing the migration of neutrophils, monocytes and leukocytes into the tissue at the site of infection (Kindt, *et al*., 2007). The function of the initial defence of a host is to eliminate invading organisms and thus acute inflammation is a minor consequence for effective defence. It is in the case of systemic inflammation that large amounts of TNF-α and IL-1 are released into the bloodstream and mediate many of the immunopathological features of LPS-induced shock (Cohen, 2002; Dinarello, 1997; Ilieva, *et al.*, 2004; Madigan, *et al*. ,2003). IL-18, a regulatory cytokine, induces the production of interferon-gamma (IFN-γ) by T cells and NK cells with sequentially upregulates surface expression of TLR-4, MD-2 and MyD88 in macrophages (Bosisio, *et al.*, 2002).

NO is an effector molecule that is important for the regulation of inflammation and is known to mediate vascular permeability, vasodilation and is involved in tissue damage at sites of infection

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(Guzik, *et al.*, 2003). Inducible nitric oxide synthase (iNOS) within macrophages oxidizes L-arginine to yield L-citrulline and NO (Dinarello, 1997; Guzik, *et al.*, 2003) (**Figure 1.4**).

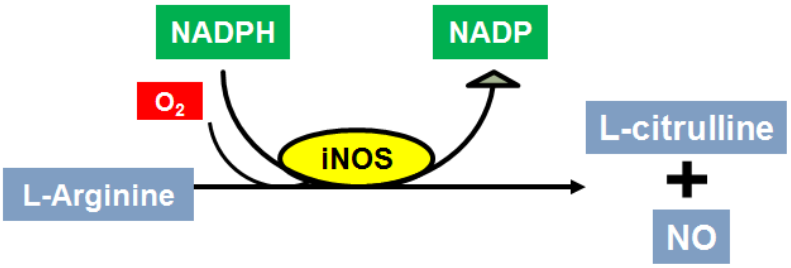

Figure 1.4: Production of NO from L-Arginine.

The enhanced expression of iNOS, in addition to LPS stimulation, is also attributed to the local availability of inflammatory cytokines like TNF-α, IL-1 and IFN-γ (Guzik, *et al.*, 2003; Heba, *et al.*, 2001). IFN-γ have been shown to stabilize iNOS mRNA by suppressing iNOS mRNA degeneration. In RAW 264.7 macrophage cells, a study indicated the synergism between LPS and IFN-γ is more successful in supplying a more favourable and sustainable source of NO due to the enhancement of iNOS transcription (Evans, 1996; Vodovotz, *et al.*, 1993; Xie and Nathan, 1994).

The production of NO by these activated macrophages serves to protect the host against microorganisms but when overproduced it can suppress the macrophages and contribute towards the inflammatory destruction of infected tissue (Vodovotz, *et al.*, 1994). In the case of systemic inflammation that results in septic shock, NO has been shown to cause the decrease in arterial pressure, as well as, systemic vascular resistance (Dinarello, 1997). To confirm this, Mashimo and Goyal (1999) utilized iNOS deficient mice to study the effects of the enzyme, iNOS. The results obtained indicated that the knockout mice were more susceptible to inflammatory damage and tumours, but more resistant to septic shock.

It has been found that macrophage activity is also regulated through the generation of toxic mediators such as ROS and RNS (Syahida, *et al.*, 2006). ROS released as a result of a respiratory burst in leukocytes is involved in the killing of pathogens and is a normal by-product of metabolism. According to Guzik (2003), intracellular ROS may also act as secondary messengers in inflammatory signal transduction due to the ability of ROS to stimulate transcription factors which lead to an increase in the expression of cytokines and chemokines involved in the inflammatory response. It is important to note that inflammatory NO reacts with superoxide

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radicals (O_2^-) forming peroxynitrite ($ONOO^-$) that mediates the toxic effect of NO (Hsu and Wen, 2002). Overproduction of free radicals promotes oxidative stress and is thus a major factor for triggering local inflammation and tissue damage by oxidation of cell membrane components, DNA and proteins.

1.4 Role of cationic host defence AMPs during infection and inflammation

HDPs, also known as cationic host-defence AMPs, are associated with innate immunity and as mentioned have been shown to play a major role in the innate immune response of the host due to their ability to modulate the innate immune responses against pathogens (Finlay and Hancock, 2004; Steinstraesser, *et al*., 2009). During infection AMPs will act locally at the site of infection causing disruption of the pathogen cell membrane, generation of intracellular effects which inhibit DNA, RNA and protein synthesis and the activation of antimicrobial enzymes that cause lysis of the organism (Kindt, *et al*., 2007).

AMPs are present in all species of life: bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals (Hancock and Diamond, 2000) as depicted in **Figure 1.5**. Within a single organism a variety of different classes of peptides with variations in each class can be present. Reasons for this diversity might be that some peptides work in synergy with each other against invading microorganisms. Differences might also exist due to the production of these peptides by different cell types (Hancock and Diamond, 2000).

Two categories of AMPs have been identified, cationic and anionic peptides, of which the former is the largest category (Lai *et al*., 2004). Cationic AMPs are small (below 25-30 kDa), amphipathic peptides and are diverse in structure, as well as sequence (Bulet, *et al.*, 2004). These peptides have been found to have an extensive range of functions that provide protection to the host against a variety of pathogens. This is due to their antimicrobial activity and are able to limit sepsis by reducing the endotoxin-induced inflammatory responses (Easton, *et al.*, 2009; Mookherjee and Hancock, 2007). AMPs also have been shown to have cytotoxic activity against a variety of cancers (Mader and Hoskin, 2006).

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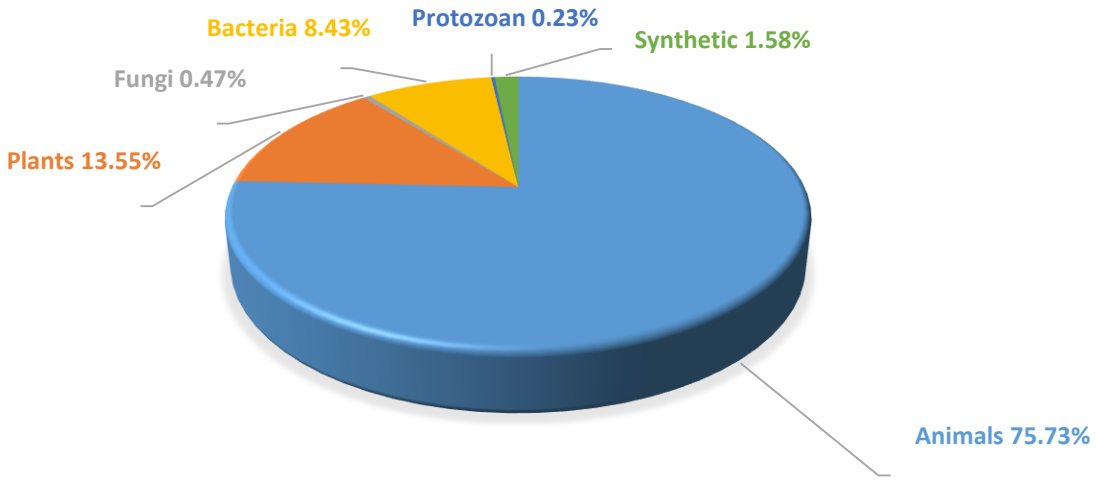

Figure 1.5: Sources of host-defence AMPs. Data obtained from The Antimicrobial Peptide Database (available at: http://aps.unmc.edu/AP/facts.php).

One of the proposed roles of cationic AMPs in acute inflammation (**Figure 1.6**) is the regulation of direct killing of a pathogen through lysis of bacterial cells, neutralization of LPS and regulation of macrophage responses to the LPS stimuli (**Figure 1.6a**). AMPs also stimulate mast cell degranulation which results in the release of histamine that causes increased permeability of blood vessels around the infected tissue. In addition, AMPs also increase chemotaxis of neutrophils and polymorphonuclear leukocytes (PMN), and may aid in antigen presentation towards T-helper cells as well as facilitate leukocyte recruitment to the site of infection. Cationic peptides also promote the adhesion of PMN to phagocytes and non-opsonic phagocytosis (**Figure 1.6b**). AMPs also inhibit fibrinolysis together with plasminogen activator inhibitor-1 to reduce the spreading of bacteria. Fibroblast chemotaxis and growth is promoted resulting in wound healing. AMPs also take part in the inhibition of proteases that leads to the inhibition of tissue injury and are involved in apoptosis of pathogen infected host cells (**Figure 1.6c**).

When acute inflammatory responses are unable to clear the bacterial infection, chronic inflammation and the adaptive immune responses are initiated (Hancock and Diamond, 2000). The proposed roles of cationic AMPs in chronic inflammation (**Figure 1.6**) include acting as chemotaxins for monocytes and recruiting T-cells, enhancing chemokine production and the proliferative response of T-helper cells. In addition AMPs also suppress macrophage signaling towards LPS, thereby reducing cytokine production and switching on specific macrophage genes. AMPs also participate in the stimulation of apoptosis of macrophages and activate lymphocytes for the potential elimination of infected cells (**Figure 1.6d**) (Ganz and Lehrer, 1998; Hancock and Diamond, 2000).

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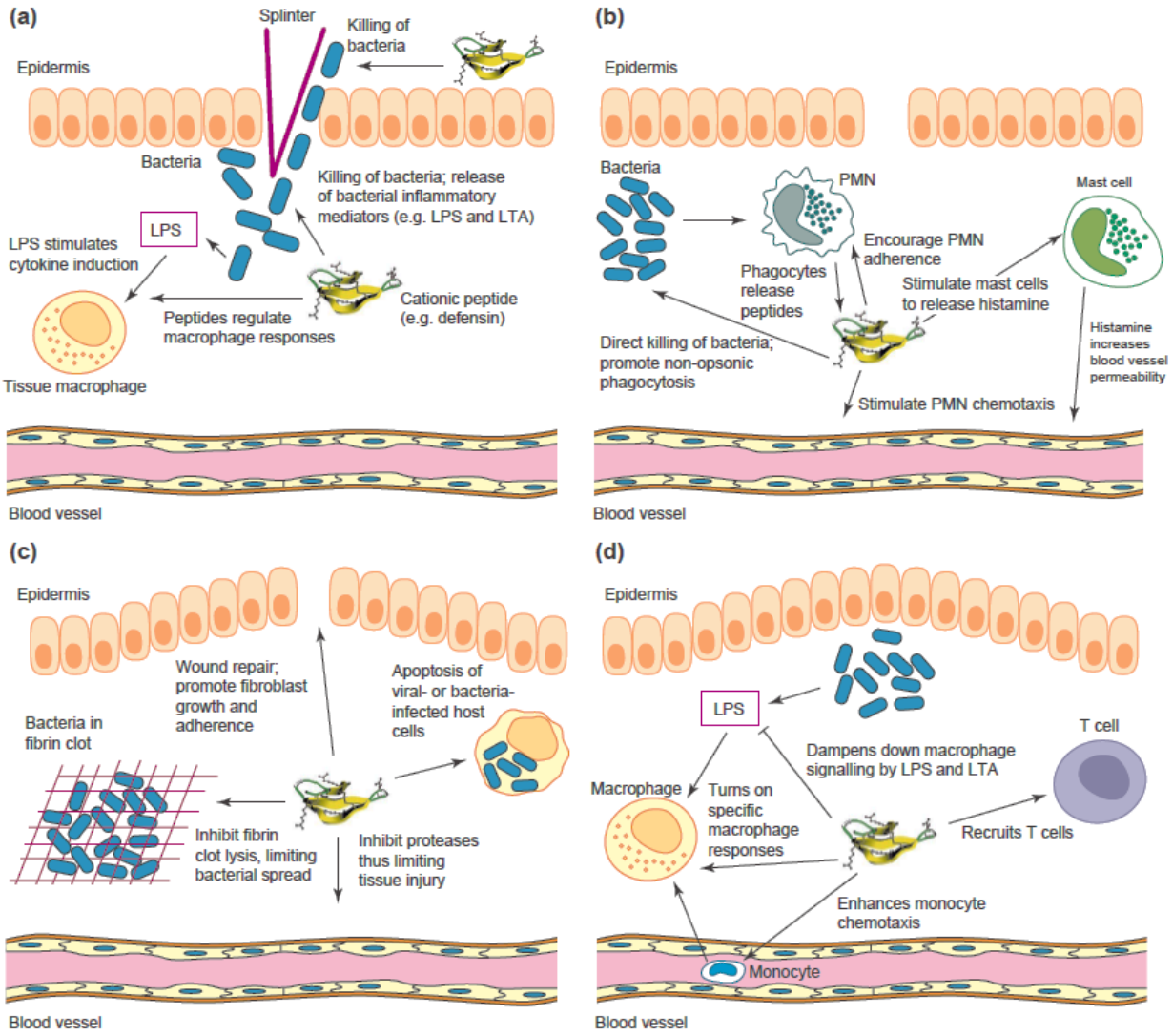

Figure 1.6: The proposed role of AMPs in infection and wound healing (a-d) as well as associated acute (a-c) and chronic (d) inflammation (Hancock and Diamond, 2000)**.**

1.5 Cationic host defence peptides as potential therapeutic drugs

AMPs are found in epithelial and mucosal cells, and in organs like the gut, lungs, kidneys and skin (Cohen, 2002). As described in section 1.4 (**Figure 1.6**), wounds are sites of increased risk for infection as well as associated acute and chronic inflammation. The misuse of antibiotics has led to the development of antimicrobial resistance that is problematic when trying to manage infections and this has motivated the search for new anti-infective agents (Gordon, *et al.*, 2005; Jerala and Porro, 2004). Cationic peptides were initially identified as antimicrobial agents that display antibacterial, antiviral, anti-parasitic and antifungal activities. These peptides have also been identified to have additional types of activities, such as anti-cancerous, anti-inflammatory and antioxidant activities. Listed in **Table 1.1** are the activities of several of these peptides found

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in vertebrates, invertebrates and pathogens (Gordon, *et al.*, 2005; Hancock, 2001; Marr, *et al.*, 2006; Yi, *et al.*, 2014).

Table 1.1: Summary of the activities of some cationic peptides obtained from various sources (Hancock, 2001; Yi, *et al.*, 2014).

Name	Source	Peptide activity
Vertebrates		
Indolicidin	Bovine	Anti-bacterial (Gram (+) and Gram (-)); Antifungal; Antiviral; Anti-cancer; Anti-parasitic
HNP1	Human	Anti-bacterial (Gram (+)); Chemotactic
Magainins	Frog	Anti-bacterial (Gram (+) and Gram (-)); Antifungal; Wound healing
Protegrin	Pig	Anti-bacterial (Gram (+) and Gram (-)); Antifungal
PR39	Pig	Wound healing
Invertebrates		
CEMA	Synthetic	Anti-bacterial (Gram (+) and Gram (-)); Antifungal; Anti-cancer; Anti-endotoxic
Coprisin	Beetle	Anti-bacterial (Gram (+) and Gram (-)); Antifungal
Melittin	Honey bee	Anti-bacterial (Gram (+) and Gram (-)); Antifungal; Antiviral; Anti-cancer; Anti-parasitic
Polyphemusin	Horseshoe crab	Anti-bacterial (Gram (+) and Gram (-)); Antifungal; Antiviral; Anti-cancer; Anti-endotoxic
Royalisin	Honey bee	Anti-bacterial (Gram (+))
Pathogens		
Gramicidin S	Bacteria	Anti-bacterial (Gram (+) and Gram (-)); Antifungal
Polymyxin B	Bacteria	Anti-bacterial (Gram (-)); Anti-endotoxic

The advantages of multifunctional activity are that the wound becomes free of infection and inflammation, as well as associated formation of free radicals is reduced. Furthermore, due to the

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emergence of resistance of drugs against a broad spectrum of bacterial species, there is an increase in the need to find new strategies and types of molecules different to the conventional antibiotics to combat potentially harmful infections.

There are several advantages in using AMPs instead of antibiotics in treatment of bacterial infections, namely: (i) bacteria develop less resistance towards AMPs than towards antibiotics; (ii) AMPs are active against a larger spectrum of bacteria, fungi, viruses and eukaryotic parasites; (iii) AMPs have shown activity in very low concentrations; and (iv) AMPs can be chemically modified in many ways (Lazarev and Govorun, 2010). Unfortunately, limitations exist in using AMPs as therapeutic agents. Some bacteria have shown to develop mechanisms to evade direct killing by AMPs. Bacteria have the potential to reduce the net ionic charge of their cell envelope causing a decrease in affinity towards AMPs. AMPs are vulnerable towards proteolytic degradation and are targeted by several microbial proteases. A number of AMPs have been shown to be cytotoxic at high concentrations and high costs are involved in the production of new synthetic AMPs that are less cytotoxic. Under physiological conditions many AMPs lose their antimicrobial activity, but retain their immunomodulatory properties to provide protection against invading pathogens (Steinstraesser, *et al.*, 2009).

Cationic peptides provide templates for design of novel drugs that can be used for defence/treatment in other organisms. Cathelicidins are AMPs that are present in neutrophils and have been found in a number of species to contain LPS-binding and neutralization abilities (Bowdish and Hancock, 2005). Initial studies showed LPS binding to CD14 and LBP is inhibited by LL-37, a human cathelicidin, blocking LPS-induced pro-inflammatory cytokine production in macrophages (Nagaoka, *et al.*, 2001; Scott, *et al.*, 2000). *In vivo* studies showed that LL-37 decreased the production of TNF-α induced by LPS and also prevented lethality in animal models with sepsis (Scott, *et al.*, 2002). For the treatment of venous leg ulcers, phase I and II clinical studies on LL-37, showed that the drug improved the wound healing rate and no tolerability concerns were observed. Currently the drug is undergoing further studies to determine its anti-infective and anti-endotoxic properties. (Pergamum announces final data from Phase I/II study; http://www.pergamum. com/blog/pergamum-announces-final-data-phase-iii-study-ll-37-patients-chronic-leg-ulcers/, accessed 10 August 2014).

A novel synthetic cationic peptide made by Action Pharma A/S, called AP-214, derived from the HDP α-melanocyte-stimulating hormone and fused to hexalysine at its C-terminal has completed

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phase II clinical trials (Yeung, *et al.*, 2011). The objective of the study was to determine whether the candidate drug could prevent organ failure after surgery. Currently the results of the study are not released. (Clinical trial information: NCT00903604, http://clinicaltrials.gov/ct2/show/record/NCT00903604, accessed 10 August 2014).

Omiganan or MX-226 is a synthetic peptide derived from indolicidin (Yeung, *et al.*, 2011), developed by Migenix, has entered phase III clinical trials and has demonstrated to have topical antiseptic properties as well as the ability to prevent catheter infections (NCT00027248 and NCT00231153). Another phase III clinical trial demonstrated that this peptide suppressed inflammation caused by rosacea, indicating anti-inflammatory properties (NCT00608959).

The bactericidal/permeability increasing protein (BPI) recombinant protein fragment, $rBPI_{21}$, is being developed by Xoma Ltd. Initial *in vitro* studies showed a reduction in LPS-induced E-selectin production as well as TNF-α production in mononuclear cells. There was also a decrease in the production and release of NO and pro-inflammatory cytokines from monocytes/macrophages (Hancock, 2000; Bowdish and Hancock, 2005). The *in vivo* studies showed that after $rBPI_{21}$ administration, there was an increase in the survival rate in both the rat and mouse models. In phase I and II clinical trials, paediatric patients suffering from meningococcal sepsis, had a better clinical outcome, with a mortality rate of 3.5% compared to untreated patient deaths of 22% (Hancock, 2000; Bowdish and Hancock, 2005). Phase III trials showed increased survival and reduction in mortality indicators. The drug candidate also showed remarkable anti-endotoxic activity.

Examples of cationic peptides involved in pharmaceutical development are shown in **Table 1.2**.

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Table 1.2: Sources, activity, application and clinical results of tested AMPs (Fox, 2013; Harrison, 2014; Afacan, *et al.*, 2012).

Peptide	Source of characteristics	Treatment	Clinical phase
AP214	Derived from α-MSH*	Sepsis-induced kidney failure	IIb
CUBICIN	Daptomycin, lipopeptide	Injection	III
Lytixar	Synthetic, membrane-degrading peptide	i) Nasal MRSA infection ii) Topical	I and II IIa
MU 1140	Lantibiotic	Gram-positive bacteria (MRSA) **	Pre-clinical
Novexatin	Cyclic, cationic peptide	Fungal toenail infections	I and II
OP-145	Derived from LL-37	Middle-ear infection	I and II
Pexiganan	Frog skin, derived from magainin-2	Topical antibiotic	III
Talactoferrin	Derived from lactoferrin	i) Non-small lung cancer ii) Diabetic ulcers and renal cancer iii) Sepsis	I, II and III

*α-melanocyte-stimulating hormone
**Methicillin-resistant *Staphylococcus aureus*

In contrast to conventional antibiotics that kill bacteria through lysis resulting in the release of immune-stimulatory bacterial components, AMPs from various sources have shown to have endotoxic binding activity and/or reduce LPS-induced pro-inflammatory cytokine and NO release (Bowdish and Hancock, 2005; Periti and Mazzei, 1999). From this it is clear that cationic peptides have therapeutic potential. Application can be broadened if factors, such as safety, stability and modes of delivery of the parent (lead) compound and synthetic derivatives are explored in different tissue (Hancock, 2000).

Unfortunately, to date none of the identified peptides from various sources and synthetic peptides subjected to clinical trials has made it past the later stages (Gordon, *et al.*, 2005; Harrison, *et al.*, 2014). Many AMPs under controlled experimental conditions have confirmed reproducible *in vitro* efficacy, but in clinical trials do not translate as successful therapeutic agents when compared to conventional antibiotics. Regrettably, these AMPs are tested for their activity against microbial cultures and enter pre-clinical and clinical trials without being fully optimized (Fox, 2013; Gordon,

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et al., 2005). Various naturally occurring AMPs have been studied for their antimicrobial and therapeutic potential and an important class of these is the defensins.

1.6 Defensins

Defensins are a major group of cationic AMPs that are present in all species of life including vertebrates, invertebrates and plants (Hancock and Diamond, 2000). These open-cyclic cysteine-rich peptides have been structurally divided into subgroups, i) mammalian defensins consist of β-sheet structures, ii) horseshoe crab, scorpion, spider and plant defensins possess β-hairpin fold structures and iii) invertebrate defensins display cysteine-stabilized αβ motifs (Baumann, *et al.*, 2010; Bulet, *et al.*, 2004).

In humans, vertebrate defensins contribute to the innate defence against bacteria, viruses and fungi (Kindt, *et al.*, 2007) and enhance host defences by interacting with different cells such as neutrophils, monocytes, macrophages and dendritic cells (Bowdish and Hancock, 2005). Defensins are cationic peptides with a molecular mass of 4-5 kDa (Bals, 2000; Wong, *et al.*, 2007). The defensins are rich in arginine residues, and due to the excess of basic amino acids over acidic amino acids, have net charges of +2 to +7 (Hancock and Diamond, 2000; Kindt, *et al.*, 2007). The two main subfamilies of defensins are α-defensin and β-defensin (Ganz and Lehrer, 1998) that are encoded by single genes on chromosome 8p21-23 (Linzmeier, *et al.*, 1999; Bals, 2000) and a third group, θ-defensins, identified in non-human primates (Tang, *et al.*, 1999). Defensins are synthesized as precursor molecules that consist of a signal sequence followed by a propeptide region. The propeptide region undergoes proteolytic processing to release the mature, biologically active peptide (Hancock and Diamond, 2000). The α- and β-subfamilies display a conserved three disulphide-linked cysteine motif (Wong, *et al.*, 2007). The cysteine residues in α-defensins are linked at Cys1-Cys6, Cys2-Cys4 and Cys3-Cys5 and in β-defensins at Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6. In both groups the triple-stranded anti-parallel β-sheets and tertiary structure is stabilized by disulfide bonds (Ganz, 2003; Ganz and Lehrer, 1998).

Endotoxin-induced inflammatory responses can cause the up-regulation of these AMPs as a response to pro-inflammatory cytokines and mediators, like TNF-α, IL-1 and IL-6 (Hancock and Diamond, 2000). For example, it has been found that the gene expression of human β-defensin 2 (hBD2) is induced by LPS through an epithelial-cell-expressed CD14-mediated signal transduction pathway (Hancock and Diamond, 2000). The signal sequence flanking the hBD2

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propeptide region revealed NFκB-binding sites and has a variety of functions in relation to the immunity of the host (Zasloff, 2002; Finlay and Hancock, 2004). The functions include antimicrobial activity, anti-endotoxin activity, selective modulation of pro-inflammatory responses, promoting wound healing and angiogenesis. In addition, AMPs induce gene expression, enhance protein secretion and influence initiation of the adaptive immunity (Zanetti, 2004; Wong, *et al.*, 2007). Serum defensins can also be used as biomarkers for cancer due to their significantly elevated levels in cancer patients (Arimura, *et al.*, 2004).

Over 1 million species of insects have been identified and are found in all terrestrial environments. This large species number is a good indication of their ability to combat pathogens and to survive different environmental conditions (Andrès, 2012; Bulet, *et al.*, 1999). Several roles of these AMPs have been demonstrated to provide defence against bacteria, fungi, viruses and protozoa (Bulet, *et al.*, 1999). In addition to the killing of invading pathogens, the Toll-like receptor-mediated HDP production, lectin-mediated complement activation, reactive oxygen producing systems and phagocytic systems are also present (Iwanaga, *et al.*, 2005). According to the Antimicrobial Peptide Database the most abundant group of peptides that have been isolated is from insects (**Figure 1.5**). Due to the high structural diversity of these peptides it is easy to realize the massive potential of developing these peptides as anti-infectious therapeutic drugs.

In insects the two major families of AMPs are cecropins and defensins of which defensins are the most widespread AMP characterised (Čeřovský and Bèm, 2014; Hoffmann, 1995). These open-cyclic peptides have a molecular mass of 4-6 kDa. The defensins found in insects have 6 cysteine residues and have a common conserved motif of three disulfide bonds (Bulet, *et al.*, 2004; Čeřovský and Bèm, 2014). The cysteine pairings that occur within these defensins are between Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 (Bulet, *et al.*, 2004), as depicted in **Figure 1.7**.

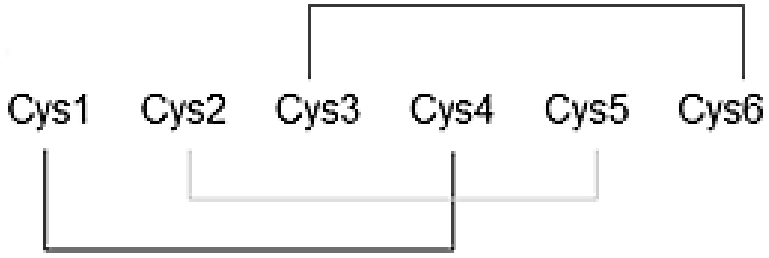

Figure 1.7: Cysteine pairing found within insect defensins. Only cysteine residues are shown for comparative purposes. Cysteine pairing of insect defensin containing 6 cysteine residues.

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Insect defensins consist of three distinct domains: a flexible amino-terminal loop, central amphipathic α-helix and a carboxy-terminal anti-parallel β-sheet (Hoffmann, 1995). The tertiary structure of a defensin (**Figure 1.8**) is obtained as a result of the α-helix forming a disulfide bond between itself and the β-sheet and the amino-terminal forming two disulfide bonds between itself and the β-sheet (Hoffmann, 1995).

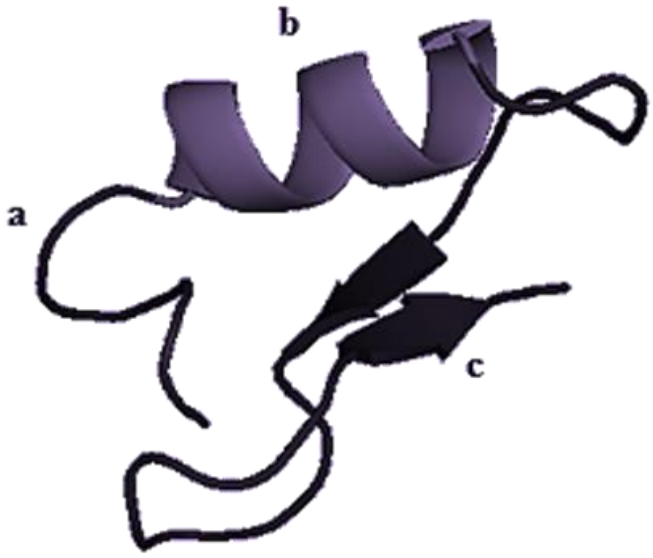

Figure 1.8: Tertiary structure domains of invertebrate defensins. Image was obtained using PyMOL modelling of the amino acid sequence of insect defensin A attained from Phyre[2] (Protein Homology/analogy Recognition Engine V 2.0). Indicated in the figure are the three distinct domains; a) amino-terminal loop, b) α-helix and c) β-sheets.

Defensins have also been identified from the arachnid class of invertebrates such as scorpions, spiders and ticks. The sequences of these defensins have been shown to be 65% similar to other defensins derived from other invertebrates and have the same cysteine pairings (**Figure 1.7**) that occur within insect defensins (Bulet, *et al.*, 2004). The first scorpion defensin that was isolated from *Leiurus quinquestriatus*, a North African scorpion, showed to have bactericidal activity toward Gram-positive bacteria but presented no activity toward Gram-negative bacteria. Lycocitins are AMPs isolated from the venom glands of the spider *Lycosa singoriensis* and have been shown to possess antimicrobial activity towards Gram-positive as well as Gram-negative bacteria and fungi (Budnik, *et al.*, 2004; Kozlov, *et al.*, 2006). Numerous defensins have been isolated from scorpions and spiders and in **Table 1.2** some examples of these defensins and their specificity against various microorganisms are listed (Harrison, *et al.*, 2014). Although the antimicrobial activity of these defensins have been elucidated, very little is known regarding other activities such as anti-inflammatory and antioxidant activity as described for the peptides **in Table 1.3.**

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Table 1.3: Defensins derived from various scorpions and spiders

Species	Name	Antimicrobial activity	Reference
P. imperator[1]	Scorpine	Gram (+) and Gram (-) bacteria; Parasites	Conde, *et al.*, 2000
O. carinatus[2]	Opiscorpine-1	Yeasts; Gram (-) Bacteria	Zhu and Tytgat, 2004
	Opiscorpine-2	Yeasts; Gram (+) and Gram (-) Bacteria	Zhu and Tytgat, 2004
H. laoticus[3]	Heteroscorpine	Gram (+) and Gram (-) Bacteria	Uawonggul, *et al.*, 2007
H. gertschi[4]	HgeScplp1	Gram (+) Bacteria	Diego-Garcìa, *et al.*, 2007
	HgeScplp2	Yeasts; Gram (+) and Gram (-) Bacteria	Schwartz, *et al.*, 2007
T. discrepans[5]	Bactridin-1	Gram (+) and Gram (-) Bacteria	Diaz, *et al.*, 2009
A.gomesiana[6]	Gomesin	Yeast; Gram (+) and Gram (-) Bacteria; Parasites; Fungi	Silva, *et al.*, 2000
	Acanthoscurrin	Gram (-) Bacteria; Fungi	Lorenzini, *et al.*, 2003
O. kitabensis[7]	Oxyopinin 1	Gram (+) and Gram (-) Bacteria; Mammalian cells	Corzo, *et al.*, 2002
C. salei[8]	Cupiennin 1	Gram (+) and Gram (-) Bacteria	Kuhn-Nentwig, *et al.*, 2002

[1]*Pandinus imperator;* [2]*Opistophthalmus carinatus;* [3]*Heterometrus laoticus;* [4]*Hadrurus gertschi;* [5]*Tityus discrepans;* [6] *Acanthoscurria gomesiana;* [7]*Oxyopes kitabensis;* [8]*Cupiennius salei.*

Ticks are small, haematophagous (bloodsucking) arachnids that are external parasites that feed on different hosts like mammals, reptiles and birds (Cabezas-Cruz and Valdès, 2014; Nakajima, *et al.*, 2002). They are vectors of severe diseases in mammals that are caused by viruses and bacteria and are capable of transferring blood-borne diseases without being affected themselves (Chrudimská, *et al.*, 2011; Sonenshine and Hynes, 2008). This is an indication that their immune system provides them with effective mechanisms against potentially harmful microorganisms. In ticks, defensins are mainly expressed in the midgut after blood feeding or pathogen invasion (Chrudimská, *et al.*, 2011; Nakajima, *et al.*, 2002). Defensins play a major role in the innate immunity of ticks (Chrudimská, *et al.*, 2010) and examples are shown in **Table 1.4.**

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Table 1.4: Defensins derived from various tick species

Tick species	Common name	Peptide	Antimicrobial activity	Reference
Ornithodoros moubata	Eyeless tampan	Defensin A	Gram (+) Bacteria	Nakajima *et al*, 2001
		Defensin B	Gram (+) Bacteria	
		Defensin C	Gram (+) Bacteria	
		Defensin D	Gram (+) Bacteria	
Amblyomma hebraeum	Bont tick	ADP-1	Gram (+) and Gram (-) Bacteria	Lai *et al.*, 2004
		ADP-2	Gram (+) and Gram (-) Bacteria	
Boophilus microplus	Cattle tick	Rhimp	Gram (+) Bacteria	Fogaça *et al.*, 2004
Dermacentor variabilis	American dog tick	Varisin	Gram (+) and Gram (-) Bacteria	Sonenshine *et al.*, 2002
Haemaphysalis longicornis	Bush tick	Gut-defensin	Gram (+) and Gram (-) Bacteria	Zhou *et al.*, 2007
		Longicin	Gram (+) and Gram (-) Bacteria; Fungi; Parasites	Tsuji *et al.*, 2007
		Longicornsin	Gram (+) and Gram (-) Bacteria; Fungi	Lu *et al.*, 2010
Ixodes ricinus	Castor bean tick	Ir-Def1	Gram (+) Bacteria	Chrudimská, *et al*., 2011
		Ir-Def2	Gram (+) Bacteria	

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1.7 Background to this study

The AMPs used in this study were derived from a defensin previously identified in the soft tick, *O. savignyi*. This tick is a livestock parasite endemic to arid and semi-arid regions of Africa. Certain fractions from the hemolymph of these ticks, which had been immune challenged, displayed antibacterial activity against Gram-positive bacteria (Olivier, 2002). An amino-terminal sequence obtained from these fractions indicated high homology to scorpion defensins. The molecular characterization of two midgut defensin isoforms (**Table 1.4**) was achieved by designing primers based on this information from the original defensin. OsDef1 and OsDef2 contain 42 amino acid residues, with the mature peptide represented by 37 of these residues. Both isoforms exhibited the cysteine residues characteristic to defensins. The amino acid sequences for the mature forms of OsDef1 and OsDef2 differ only at positions 16 and 22, as indicated in green in **Table 1.5** (Olivier, 2002). The native mature peptide, OsDef1, has a net charge of +5 at a pH of 7 and OsDef2 under the same pH conditions has a net charge of +6.

Table 1.5: Properties of OsDef1, OsDef2, Os and Os-c

Peptide	Sequence	No of amino acids	Charge	MW*
OsDef1	GYGCPFNQYQCHSHCSGIRGYRGGYCKGAFKQTCKCY	37	+5	4172.7
OsDef2	GYGCPFNQYQCHSHCKGIRGYKGGYCKGAFKQTCKCY	37	+6	4185.8
Os	KGIRGYKGGYCKGAFKQTCKCY	22	+6	2459.9
Os-C	KGIRGYKGGY_KGAFKQT_ K _Y	19	+6	2150.5

*Theoretical molecular weight. The differences in amino acid sequence between OsDef1 and OsDef2 are highlighted in green. Cysteine residues are highlighted in red and the positively charged residues are underlined. Red underscore represent omitted cysteine residues.

Synthetic OsDef1 and OsDef2 were found to be active against Gram-positive bacteria (Prinsloo, *et al.*, 2013). From its tertiary structure it can be seen that OsDef2 has a defined α-helix and a β-sheet domain (**Figure 1.9**). The tertiary structure of OsDef2 also consists of an amino-terminal loop, amphipathic α-helix and a carboxy-terminal anti-parallel β-sheet similar to insect defensins.

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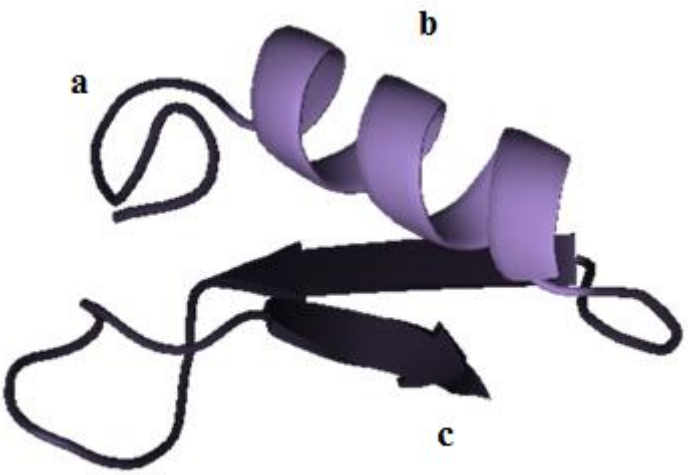

Figure 1.9: Tertiary structure of OsDef2. Image was obtained using PyMOL modelling of the amino acid sequence of OsDef2 attained from Phyre[2] (Protein Homology/analogy Recognition Engine V 2.0). From this tertiary structure it can be seen that OsDef2 has a defined α-helix and a β-sheet domain. The tertiary structure of OsDef2 is, therefore, typical of that of insect defensins. Indicated in the figure are the three distinct domains; a) amino-terminal loop, b) α-helix and c) β-sheets.

OsDef2 was used as a template for the design of shorter peptides, Os and Os-C that have stronger antibacterial activity (Prinsloo, *et al.*, 2013). Their sequences are depicted in **Table 1.5**. Os and Os-C were shown to be active against Gram-positive bacteria and Gram-negative bacteria (MBC values ranging from 0.38 – 6.10 µM). Os showed higher antibacterial activity than the analogue, Os-C, as well as faster killing kinetics against Gram-positive (*E. coli*) and Gram-negative bacteria (*Bacillus subtilis*). Contrary to Os-C, Os retained most of its bactericidal activity in physiological conditions. Both peptides were found to be non-toxic to mammalian cells and no erythrocyte haemolytic activity was observed. Scanning electron microscopy showed that in contrast to melittin, an antimicrobial peptide from bee venom, which caused blebbing of bacterial surfaces, cells exposed to either peptide appeared flattened and empty. Circular dichroism data indicated that in a membrane-mimicking environment, the cysteine-containing peptide has a higher α-helical content and Os-C higher β-sheet content. Moreover, the peptides displayed potent antioxidant activity (Prinsloo, *et al.*, 2013).

1.8 Aims of this study

The aim of this study was to determine the potential anti-inflammatory properties of synthetic peptides, Os and Os-C. In this study the ability of Os and Os-C to bind LPS, scavenge NO and to

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inhibit the production of NO and TNF-α by RAW 264.7 macrophages was evaluated. The effect of both peptides on the viability of RAW 264.7 macrophages was also determined. In addition both peptides were investigated for their protective effects against oxidative damage to RAW 264.7 macrophages.

More specifically the aims were to determine whether:

1. Both Os and Os-C bind to LPS.
2. LPS influences the bactericidal activity of Os and Os-C against *E.coli.*
3. Os and Os-C are able to scavenge NO.
4. Os and Os-C inhibit the production of NO by RAW 264.7 cells.
5. Both peptides affect the viability of RAW 264.7 cells.
6. Os and Os-C inhibit the production of TNF-α by RAW 264.7 cells.
7. Os and Os-C provide protection against free radicals in unstimulated RAW 264.7 cells.

1.9 Outputs

Part of this work described in this dissertation was presented as a poster at a conference:

MALAN, M., SEREM, J.C., BESTER, M.J., NEITZ, A.W.H. & GASPAR A.R.M. (2014). Anti-inflammatory properties of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. South African Biochemistry and Molecular Biology (SASBMB) biennial congress, Goudini Spa, Western Cape, 6-9 July 2014 (P-163, p. 147).

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CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All reagents used were of analytical grade and for all experiments double distilled deionised water (dddH_2O; Millipore system Q, Millipore, USA) was used. Concentrations reported are those used in the final reaction volumes.

E. coli (ATCC 700928) used for the bacterial assay was grown overnight aerobically in Luria-Bertani (LB) broth (1% NaCl, 1% Tryptone, 0.5% yeast, pH 7.4) at 37°C.

The RAW 264.7 macrophage cell line used in this study was semi-adherent from the species *Mus musculus* (mouse) that was isolated from the Abelson murine leukemia virus-induced tumour. The cells were obtained from the European Collection of Cell Cultures (ECACC) supplied by Sigma-Aldrich (RSA). RAW 264.7 cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 1% antibiotics (streptomycin/ penicillin/fungizone) (sDMEM) at 37°C in an atmosphere of 5 % CO_2.

2.2 Culturing of RAW 264.7 macrophages

RAW 264.7 macrophages were incubated at 37ºC in an atmosphere of 5% CO_2 in sDMEM. When the cells were 70% confluent, the medium was removed and scraped loose. These cells were collected using sDMEM then transferred into a 15 ml tube and centrifuged at 850 g for 2 minutes. The supernatant was discarded and the pellet re-suspended in 5ml sDMEM. Low passages are required for successful NO production. The RAW 264.7 cells were used between passages 10-20. The cells were seeded at a concentration of $1x10^6$ cells/ml in sterile 96 well cell culture plates. Wells containing only sDMEM were included as blanks.

2.3 Synthetic peptides and peptide preparation

The properties of synthetic peptides used in this study are given in **Table 2.1**. Os and Os-C were synthesised using FlexPeptide^{TM} technology by GenScript (New Jersey, USA). The purity and molecular mass of these peptides were determined by reverse-phase high-performance liquid

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chromatography (RP-HPLC) and mass spectrometry by the supplier. The reducing agent, dithiothreitol (DTT, 10 nmol), was added to Os prior to lyophilization. Melittin and polymyxin B were purchased from Sigma Aldrich (RSA). The lyophilized peptides may contain between 10% and 70% bound salts and water by weight thus it was essential to determine the concentrations of the peptides. The concentrations of the peptides were determined using the molar extinction coefficient of the chromophoric residues: Tyr (1200 AU/mmole/ml) and Trp (5560 AU/mmole/ml), and the Beer-Lambert Law equation:

$$c = \frac{A \times df \times Mr}{n(\text{Ɛ}\, Tyr) + n(\text{Ɛ}\, Trp)}$$

where c is the peptide concentration (mg/ml); *A* is the absorbance of the peptide measured at 280 nm; df the dilution factor; *Mr* the relative molar mass of the peptide (g/moles) and Ɛ the extinction coefficient (AU/mmole/ml).

Stock solutions of the peptides were prepared in water at a concentration of 1 mM, filter sterilized (0.45 µm membrane) and stored in 20 µl aliquots at -20 °C.

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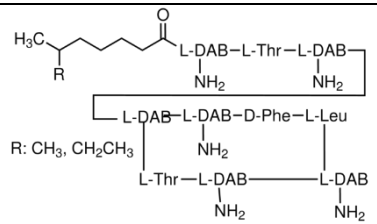
Table 2.1: Properties of the peptides used and their sources

Peptide	Sequence	Nr of amino acids	Net charge	Molecular weight[c]	Source	Use
Investigated peptides						
Os	KGIRGYKGGYCKGAFKQTCKCY	22	+6	2459.9	Carboxy-terminal of OsDef2	Investigation of anti-inflammatory properties
[a]Os-C	KGIRGYKGGY_ KGAFKQT_ K_Y	19	+6	2150.5	Carboxy-terminal of OsDef2 (Cys omitted)	
Control peptides						
[b]Polymyxin B		8	+5	1385.6	*Bacillus polymyxa* strains	Positive control for: • LPS binding • Inhibition of NO and TNF-α production • Cytotoxicity`
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	+5	2846.46	Honey bee venom, *Apis mellifera*	
Glutathione	ECG	3	-1	307.3	-	Positive control for: • NO scavenging • Antioxidant activity

[a] The underscores indicate the omitted cysteine residues

[b] DAB = diaminobutyric acid

[c] Molecular weight (Da) obtained from suppliers by MS analysis

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2.4 LAL chromogenic assay

To determine the ability of Os and Os-C to bind LPS, a kinetic chromogenic *limulus* amoebocyte lysate (LAL) kit (Lin, *et al.*, 2008) obtained from Pierce Biotechnology (Rockford, USA) was used. The addition of LPS activates the proteolytic activity of Factor C. The activated protease catalyses the cleavage of the chromogenic substrate, Ac-Ile-Ala-Arg-*p*-nitroaniline, releasing p-nitroalinine (pNA) resulting in yellow colour formation (**Figure 2.1**).

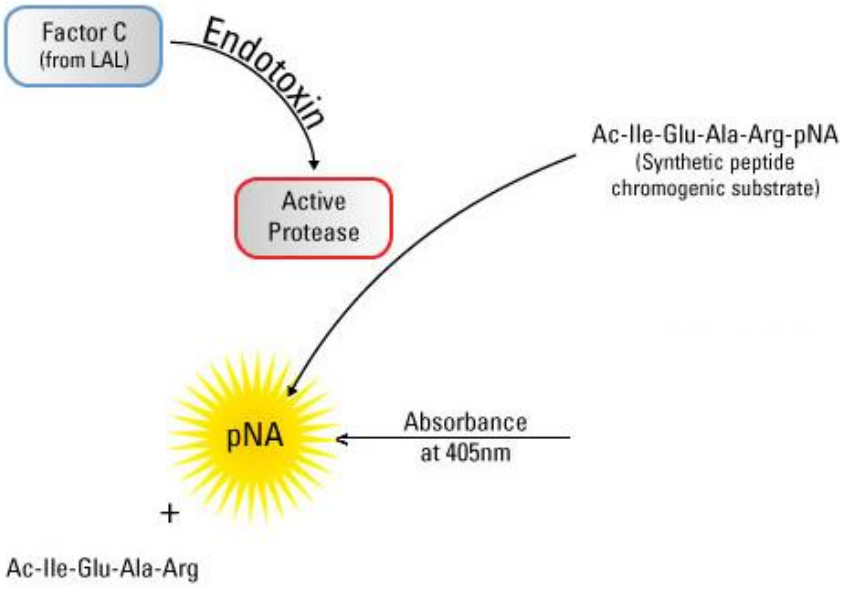

Figure 2.1: Principle of chromogenic LAL assay.

Endotoxin neutralization experiments were carried out following the protocols recommended by the manufacturer. Stock solutions of the peptides were prepared in the pyrogenic water provided with the kit. Various concentrations of peptides (0.098 - 25 µM) were incubated with a constant concentration of LPS (100 ng/ml; 0.01 µM; *E. coli* O111:B4) at 37°C for 5 minutes in wells of an pyrogenic sterile 96-well microtitre plate. A total of 25 µl of this mixture was added to 10 µl of the LAL reagent, and the mixture was incubated for a further 5 minutes at 37 °C. This was followed by the addition of 20 µl of the LAL chromogenic substrate solution which was incubated for an additional 10 minutes to allow yellow colour development. The reaction was terminated by the addition of 25 % acetic acid and the absorbance was measured at 405 nm. The reduction of absorbance as a function of peptide concentration is indirectly proportional to the inhibition of LAL activation by the peptide (Srivastava, *et al.*, 2012). Melittin and polymyxin B were used as positive controls. Three independent experiments were performed and each experiment included

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triplicates for each compound at each concentration. The inhibition of LAL activation was calculated as follows:

$$\%\ \text{Inhibition of LAL activation} = 100 - \left(\frac{\text{Mean absorbance of Sample}}{\text{Mean absorbance of Untreated}} \times 100\right)$$

2.5 Antibacterial assay

The colony forming unit (CFU) method as described by Prinsloo, *et al.* (2013) was used to investigate if in the presence of LPS might reduce the bactericidal activity of Os and Os-C against *E.coli*. LPS has been shown to inhibit or influence the bactericidal activity of antimicrobial peptides (Lin, *et al.*, 2008). It also serves as an indication of whether the LPS might interfere with the action of the peptide *in vivo*, as well as serving as an indirect method of confirming LPS-binding to the peptide. In this experiment the MBCs of Os (1.88 µg/ml; 0.76 µM) and Os-C (3.75 µg/ml; 1.74 µM) were used as determined by Prinsloo, *et al.* (2013). The MBC is the lowest concentration of Os and Os-C that kills 98% of all the bacteria in the culture. The peptide concentrations were kept constant throughout the experiment, and increasing concentrations of solubilised LPS (1.56 – 50 µM), were added. The peptides were pre-incubated with LPS for 2 hours before addition of *E.coli.*

E. coli from stock solutions were streaked on LB agar plates and grown for 14 to 16 hours at 37 °C. Colonies were picked and dispersed in 25 ml LB broth and grown overnight at 37°C shaking at 150 rpm. The overnight culture was then sub-cultured by diluting the bacteria 100 times in LB broth and allowed to proliferate at 37°C until the mid-logarithmic growth phase was reached (OD_{600} = 0.6). The optical density (OD) was measured using an UV-visible recording spectrophotometer (Shimadsu, Japan) and 1cm cuvettes. The bacteria (1 ml) were spun down (14000 *g* for 90 seconds), the supernatant discarded and the pelleted cells washed twice with 0.5 ml sodium phosphate (NaP) buffer (0.1 M $Na_2HPO_4.2H_2O$ and 1 M NaH_2PO_4, made up to 10 mM, pH 7.4). The washed *E.coli* pellet was resuspended in 1 ml NaP buffer. Subsequent bacterial dilution was made from the resulting cell suspension to give approximately a 1.125 x 10^6 CFU/ml. This was determined from the OD_{600} against CFU/ml relationship for *E.coli*, 2 x 10^{-9}, by Prinsloo, *et al.* (2013).

LPS (10 µl) was serially diluted with water spanning a final concentration range of 0.156 - 10 µM and incubated with peptide (10 µl) at their respective MBCs for 2 h at 37°C with shaking at 150

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rpm. A volume of 80 µl of washed bacteria was incubated to pre-incubated samples and further incubated for 2 hours at 37°C with shaking at 150 rpm. Thereafter, the incubated samples were diluted 500 times in NaP buffer and 100 µl of these dilutions was plated on LB agar plates and incubated for 14-16 hours at 37 ^{0}C. Sterile controls containing 100 µl water, NaP buffer and LPS, respectively, was used. In addition a peptide control containing 80 µl NaP buffer, 10 µl water and 10 µl peptide and a growth control containing 80 µl bacteria and 20 µl water were included. After incubation, the samples were diluted 500 times in NaP buffer.

The surviving colonies were counted to determine the number of CFUs for each LPS exposed plate and compared to the growth control and plates that were not exposed to LPS. The bacterial viability was compared to the killing effect of the peptides that were not exposed to LPS. Three independent experiments were performed and each experiment included triplicates for each compound at each concentration. Bacterial viability was calculated as follows:

$$\%\ \text{Bacterial viability} = \left(\frac{\text{Mean CFUs of LPS exposed}}{\text{Mean CFUs of Growth Control}} \times 100 \right)$$

2.6. Determination of NO scavenging activity

To determine the ability of Os and Os-C to scavenge NO generated from sodium nitroprusside (SNP) was determined with a quantitative chemical assay using the Griess reagent (1:1mixture (v/v) of 1% sulphanilamide (SA) and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED) in 2.5% phosphoric acid) with minor modification (Hernández-Ledesma, *et al.*, 2009). Due to the short half-life of NO, it is readily converted to nitrite and nitrate, therefore this assay indicates the indirect measurement of NO present. Nitrite (NO_2^-) reacts with SA, a diazotizing agent, to produce diazonium salt (DS) in the presence of an acidic medium. The coupling reagent, NED, then forms a stable, dark pink azo compound (AC) (**Figure 2.2**) (Giustarini, *et.al*, 2008).

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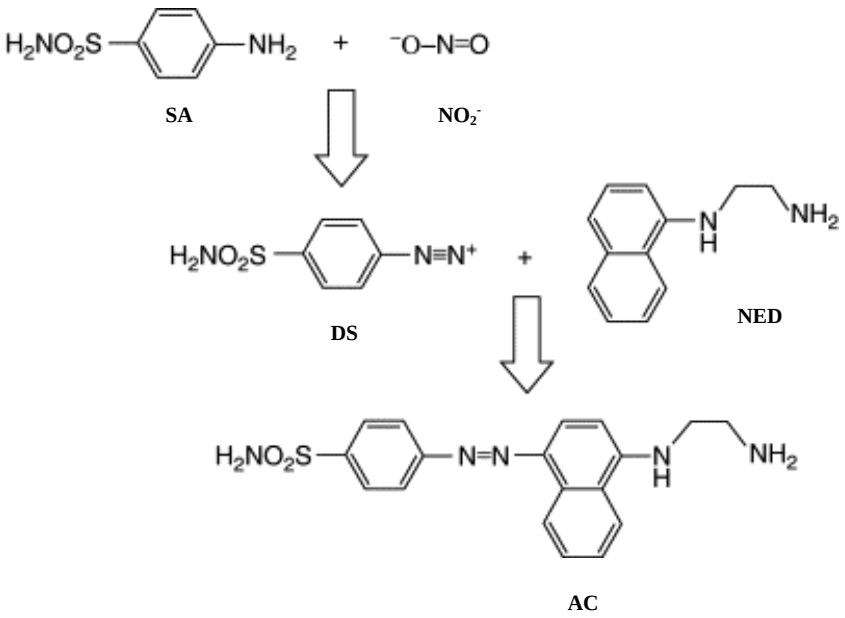

Figure 2.2: Nitrite conversion to azo compound with Griess reagent.

A volume of 80 µl SNP (5 mM; diluted in phosphate buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4; NO generator) solution was added to 20 µl of the peptide, spanning a final concentration range of 0.78 - 25 µM, and incubated for 1 hour at room temperature in the dark. Thereafter, 50 µl SA solution was added and incubated for a further 10 minutes. A volume of 50 µl NED was added and the absorbance was read at 570 nm. The concentration of NO in µM was determined using a standard sodium nitrite ($NaNO_2$) curve in PBS. Three independent experiments were performed and each experiment included triplicates for each compound at each concentration. NO scavenging was calculated as follows:

$$\% \text{ NO production} = \left(\frac{\text{Mean } \mu M NO_2 \text{ Sample} - \text{Mean } \mu M NO_2 \text{ PBS Control}}{\text{Mean } \mu M NO_2 \text{ SNP Control} - \text{Mean } \mu M NO_2 \text{ PBS Control}}\right) \text{ x } 100$$

$$\% \text{ NO scavenging activity} = 100 - \% \text{ NO production}$$

2.7 Determination of NO production by RAW 264.7 cells

LPS/IFN-γ-stimulated RAW 264.7 macrophage cells were used as *in vitro* model of inflammation. To determine the direct effect of peptides on NO production by the RAW 264.7 cells, without LPS/IFN-γ stimulation, a volume of 70 µl cells was plated in sterile 96-well cell culture microtiter plates (1 x 10^6 cells/ml). The cells were incubated with 20 µl PBS and 10 µl peptide (melittin and polymyxin B at 25 µM; Os and Os-C at 100 µM) and incubated for 24 hours.

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To determine the effect of peptides on NO production by RAW 264.7 cells with simultaneous addition of LPS and IFN-γ, a volume of 70 µl cells was plated (1 x 10^6 cells/ml). The cells were stimulated with 20 µl LPS/IFN-γ (0.1 µg/ml; 25 U/ml) and 10 µl peptide (melittin and polymyxin B at 0.098 - 25 µM; Os and Os-C at 0.098 - 100 µM) and incubated for 24 hours.

In a third experiment, the effect of pre-incubation of RAW 264.7 cells with Os and Os-C prior to LPS/IFN-γ stimulation was determined. The cells were pre-treated with the peptide (0.78 - 25 µM) for 90 minutes, followed by addition of LPS/IFN-γ (0.1 µg/ml; 25 U/ml) and incubated for 24 hours.

To measure NO production, an aliquot (50 µl) of the supernatant was removed and combined with 100 µl Griess reagent (SA and NED were diluted in 2.5% phosphoric acid) followed by incubation at room temperature for 10 minutes. The absorbance was measured with a microplate reader (BioTek plate reader, Analytical and Diagnostic Products, RSA) at 570 nm.

NO production by RAW 264.7 cells was determined using the Griess reagent (1:1mixture (v/v) of 1% SA and 0.1% NED in 2.5% phosphoric acid) with the same principle as described previously in section 2.6. The cell culture supernatants were assayed. NO production was measured using a standard $NaNO_2$ curve in medium. Three independent experiments were performed and each experiment included triplicates for each compound at each concentration. The percentage inhibition of NO was calculated as follows:

$$\%\ \text{Inhibition of NO} = 100 - \left(\frac{\text{Mean absorbance of Sample}}{\text{Mean absorbance of LPS stimulated cells}} \times 100\right)$$

2.8 RAW 264.7 cell viability assay

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay is a colorimetric assay which is used to measure the activity of metabolic enzymes (reductases) that reduce the insoluble yellow MTT crystals to soluble purple formazan crystals (**Figure 2.3**). The cell viability is directly proportional to the amount of purple crystals produced.

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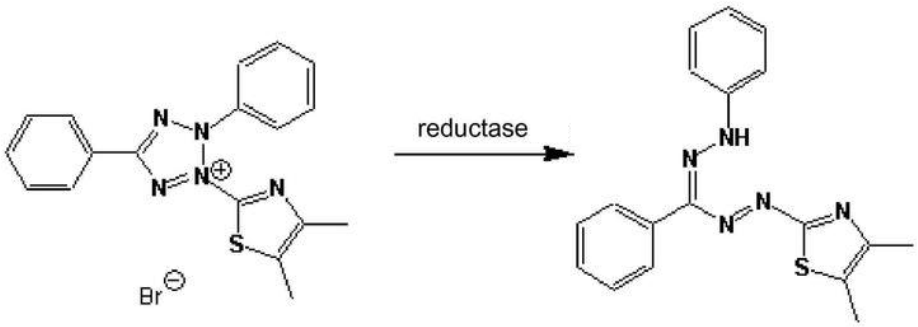

Figure 2.3: MTT metabolic reductase conversion to formazan crystals.

To evaluate the cytotoxicity of the peptides on RAW 264.7 macrophages when unstimulated (without LPS/IFN-γ) (melittin and polymyxin B at 25 µM; Os and Os-C at 100 µM), as well as stimulated with LPS/IFN-γ (melittin and polymyxin B at 0.098 - 25 µM; Os and Os-C at 0.098 - 100 µM), the MTT assay was performed as previously described (Mosmann, 1983). A volume of 5 µl of MTT (91 µg/ml) solution was added to each well and incubated for 3 hours at 37ºC and 5% CO_2. After the incubation, the medium containing MTT was removed and the plate was left at room temperature until completely dry. To dissolve the purple formazan crystals, 50 µl DMSO was added to each well. The plates were shaken for approximately 10 minutes until the crystals were dissolved. The absorbance of the solution in each well was measured using a microplate reader (BioTek plate reader, Analytical and Diagnostic Products, RSA) at a wavelength of 570 nm.

Untreated LPS-stimulated control cells were assigned a value of 100% viability and all the other conditions were compared to the control. Three independent experiments were performed and each experiment included triplicates for each compound at each concentration. The percentage of control of cell growth was calculated as follows:

$$\% \text{ Cell viability} = \left(\frac{\text{Mean absorbance of Sample}}{\text{Mean absorbance of Untreated}} \times 100 \right)$$

2.9 Measurement of TNF-α production by RAW 264.7 cells

To determine the effect of Os and Os-C on TNF-α production by RAW 264.7 cells, induced by LPS and IFN-γ, a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) was performed (Sigma-Aldrich; Germany). The TNF-α produced by the cells binds to TNF-α-specific capture antibodies coated on a 96-well plate. Biotinylated TNF-α-specific detection antibodies then binds

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to the antibody-bound TNF-α. Horseradish peroxidase (HRP) labelled-streptavidin then binds to the biotinylated secondary antibody. The peroxidase reacts with the substrate, 3,3'5,5'-tetramethylbenzidine (TMB), to produce a blue colour product. The reaction is stopped by the addition of an acidic stop reagent that turns the product yellow. The intensity of the colour product is directly proportional to the concentration of TNF-α present (**Figure 2.4**).

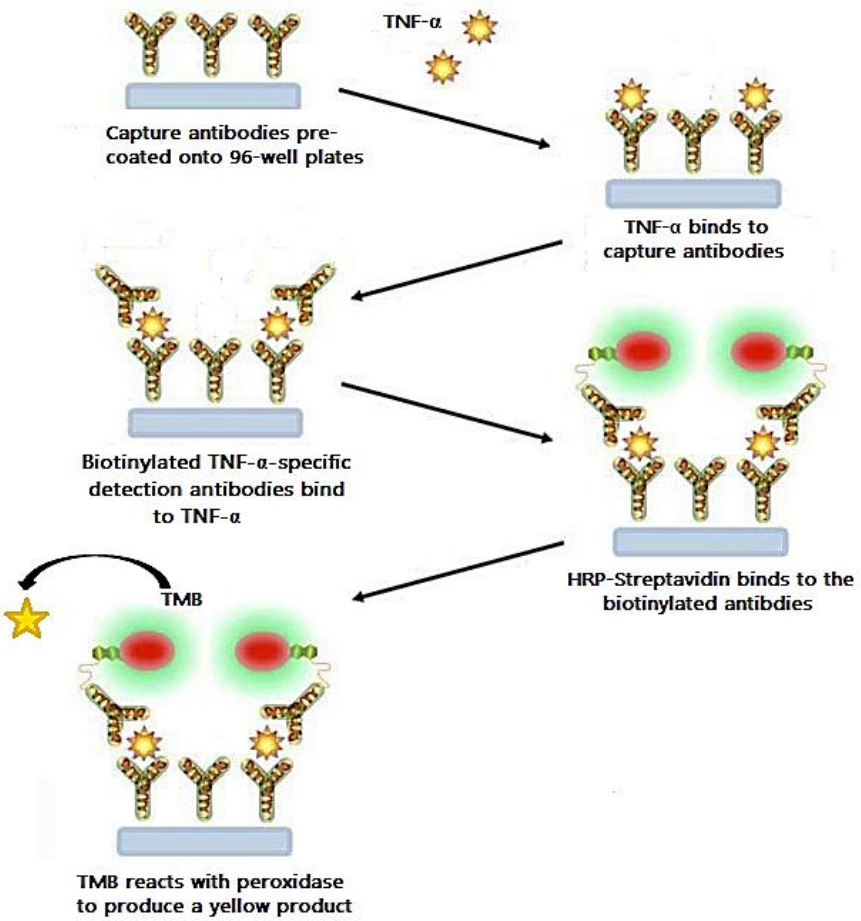

Figure 2.4: TNF-α ELISA assay principle. The relative amount of TNF-α (target protein) was determined using TNF-α-specific primary antibodies and secondary horseradish peroxidase (HRP)-conjugated antibodies. The HRP provides enzyme activity for the detection using TMB as substrate. (http://www.raybiotech.com/files/images/Sandwich-ELISA-Principle.jpg).

To study the effects of Os and Os-C with simultaneous addition of LPS/IFN-γ on TNF-α production on RAW 264.7 cells, a volume of 70 µl cells was plated in sterile 96-well microtiter plates (1 x 10^6 cells/ml). The cells were stimulated with 20 µl LPS and IFN-γ (0.1 µg/ml; 25 U/ml) and 10 µl peptide (1.56 - 100 µM) simultaneously and incubated for 24 hours. Supernatants were collected for TNF-α determination. The assay was performed according to the protocol recommended by the manufacturer. A standard curve for TNF-α was prepared with various concentrations of TNF-α standard (0.188 – 3 ng/ml). A volume of 100 µl supernatant or TNF-α

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standard was added into appropriate wells of the antibody-coated ELISA 96-plate, covered and incubated for 2.5 hours at room temperature while gently shaking. The solution was discarded and washed 4 times with 300 µl washing buffer. With each wash step all the liquid was completely removed by blotting against a clean paper towel. This was followed by the addition of 100 µl biotinylated detection antibody and incubated for 1 hour at room temperature while gently shaking. After the incubation step, the solution was discarded and washed 4 times with 300 µl washing buffer as explained previously. To each well 100 µl HRP-Streptavidin was added, covered and incubated for 45 min at room temperature while gently shaking. After the incubation step, the solution was discarded and washed 4 times with 300 µl washing buffer as explained previously. Thereafter, 100 µl ELISA colorimetric TMB substrate was added to each well, incubated for 30 min at room temperature while gently shaking in the dark. To terminate the reaction, 50 µl of stop solution was added to each well. The absorbance of the solution in each well was measured with a microplate reader (BioTek plate reader, Analytical and Diagnostic Products, RSA) at a wavelength of 450 nm. The nanograms per millilitre (ng/ml) values of TNF-α were determined using the standard TNF-α curve. Two independent experiments were performed and each experiment included triplicates for each compound at each concentration. The percentage inhibition of TNF-α production was calculated as follows:

$$\%\ \text{TNF}\alpha\ \text{production} = \left(\frac{\text{Mean TNF}\alpha\ \text{Sample} - \text{Mean TNF}\alpha\ \text{Blank}}{\text{Mean TNF}\alpha\ \text{Control} - \text{Mean TNF}\alpha\ \text{Blank}}\right) \text{x } 100$$

$$\%\ \text{Inhibition of TNF}\alpha\ \text{production} = 100 - (\%\ \text{TNF}\alpha\ \text{production})$$

2.10 Cell-based oxidative damage assay

To investigate whether the peptides are able to neutralize the potential oxidative damage induced by 2,2'-azobios(2-amidinopropane) dihydrochloride (AAPH) in RAW 264.7 cells, the method of Wang and Joseph, (1999) was followed with some modifications. Dichlorofluorescein diacetate (DCFH-DA) spontaneously crosses the plasma membrane and is cleaved by cytosolic esterases to generate intracellular DCFH, which is then oxidized by free radicals to DCF that is highly fluorescent. In **Figure 2.5**, the chemical structure of the fluorescent dye and the process that causes it to fluoresce is shown.

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A volume of 100 µl RAW 264.7 cells was plated in sterile 96-well microtiter plates (1 x 10^6 cells/ml) and incubated for 24 hours. The cells were then incubated with 50 µl DCFH-DA (25 µM) for 45 min, after which the cells were washed with sterile PBS.

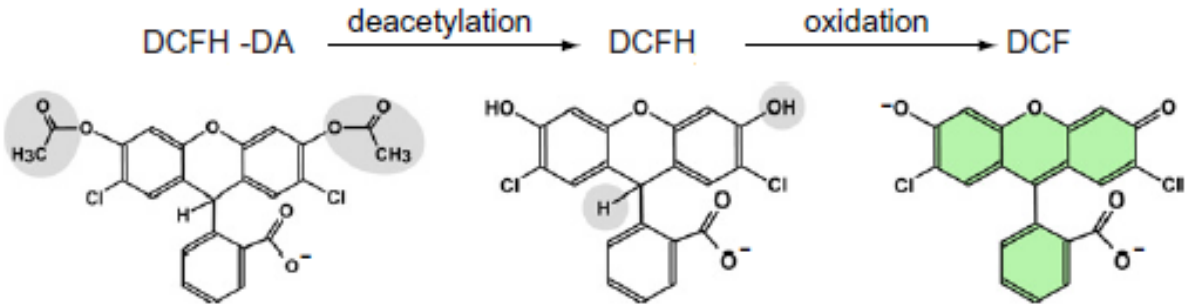

Figure 2.5 Chemical structures of DCFH-DA and derivatives at physiological pH.

The cells were exposed to 50 µl peptide (0.39 - 100 µM) and 50 µl AAPH (2 mg/ml). The fluorescence was measured with a FLUOstar OPTIMA plate reader (BMG labtechnologies, Germany), using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. A negative control (0% damage) made up with the same volume of water and PBS was included as well as a positive control (100% damage) was made up of water and AAPH.

The percentage protection against AAPH damage was calculated as follows:

$$\text{slope} = \frac{\text{Ft}_0 - \text{Ft}_{60}}{\text{t}_0 - \text{t}_{60}}$$

$$\%\ \text{Oxidative damage} = \left(\frac{\text{slope}_{(\text{Sample})} - \text{slope}_{(\text{PBS})}}{\text{slope}_{(\text{AAPH})} - \text{slope}_{(\text{PBS})}}\right) x\ 100$$

$$\%\ \text{Protection against oxidative damage} = 100 - \%\ \text{Oxidative damage}$$

Where Ft is the fluorescence values measured every 2 minutes after incubation of RAW 264.7 cells with peptide and AAPH for 60 minutes.

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2.11 Data analysis

All experiments were obtained from three independent experiments in duplicate or triplicate as indicated in each section and are presented as mean ± SEM. GraphPad Prism version 4.0 software was used to generate the graphs and to perform statistical analyses. Statistical analysis was performed using one-way ANOVA to obtain statistical significance with $p \leq 0.05$.

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CHAPTER 3: RESULTS

3.1 Binding of peptides to LPS

Anti-endotoxin activity is the ability of a compound to bind LPS and as a result neutralize LPS-induced inflammatory responses. Binding of Os and Os-C to LPS (from *E.coli* 0111:B4) was determined by using a quantitative chromogenic LAL assay, which measures their efficacy to inhibit the LPS-induced activation of LAL enzyme, compared to the untreated control (**Figure 3.1a**). Melittin (linear AMP) and polymyxin B (cyclic lipopeptide) were used as positive controls as both possess the ability to bind LPS (Srivastava, *et al.*, 2012; Morrison, *et al.*, 1976). The peptides were tested at a concentration range of 0.098 – 25 µM and showed inhibition of the LPS-induced activation of LAL enzyme in a dose-dependent manner. At 25µM, melittin and polymyxin B inhibited the LAL enzyme by 88% and 84%, respectively, whereas at this concentration inhibition by Os and Os-C was only 19% and 28%, respectively.

The dosage effect obtained for melittin and polymyxin B was compared to that of Os and Os-C (**Figure 3.1b**). Analysis showed that there was a significant difference between the LPS binding activity of melittin compared to the binding activity of Os and Os-C. Likewise, the LPS binding activity of polymyxin B was significantly higher than the effect obtained by Os and Os-C. The dosage effect of both Os and Os-C was compared and analysis showed that there was a significant difference between the activity obtained between Os and Os-C (**Table 3.1**).

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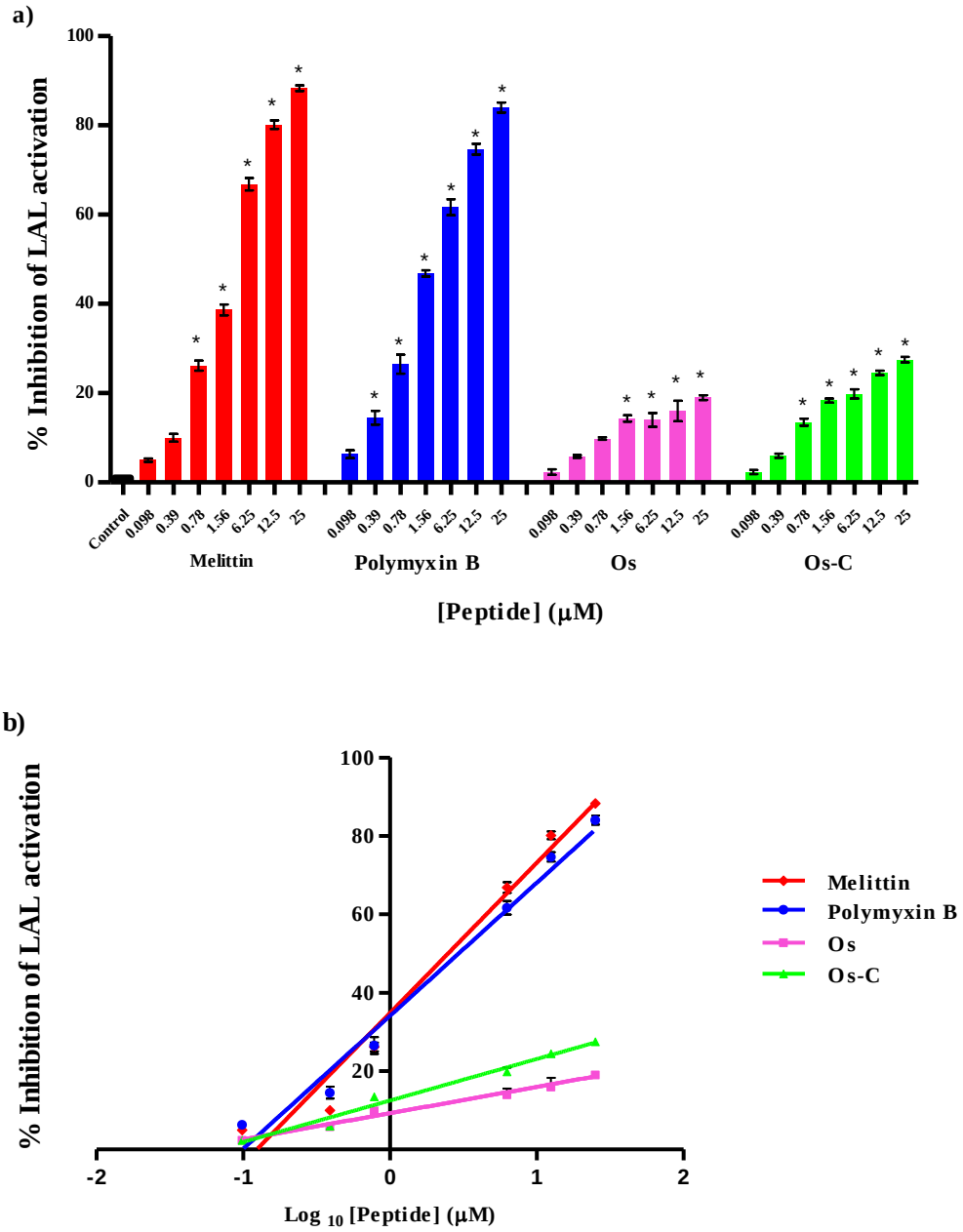

Figure 3.1 The ability of Os and Os-C to bind LPS. a) *In vitro* inhibition of LPS-induced activation of LAL enzyme by melittin, polymyxin B, Os and Os-C. Data is expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant difference between the inhibition of LAL activity by the peptides at their respective concentrations compared to the untreated control (without peptide); *p≤0.05. **b) Comparison of dosage effect of poylmyxin B and melittin to Os and Os-C.**

3.2 Effect of LPS on the bactericidal activity of Os and Os-C

To determine whether LPS affects the bactericidal activity of the peptides against *E. coli* (**Figure 3.2**), various concentrations of LPS (1.56 – 50 µg/ml) were incubated with both peptides at their respective MBCs (0.76 µM Os and 1.74 µM Os-C) and compared to the killing effects exerted by

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the peptides alone. Pre-treatment of the peptides with LPS reduced the bactericidal activity of Os and Os-C. This was shown by the increase in the viability of *E.coli* as the LPS concentration increased, indicating that free LPS binds to Os and Os-C, reducing their active concentration.

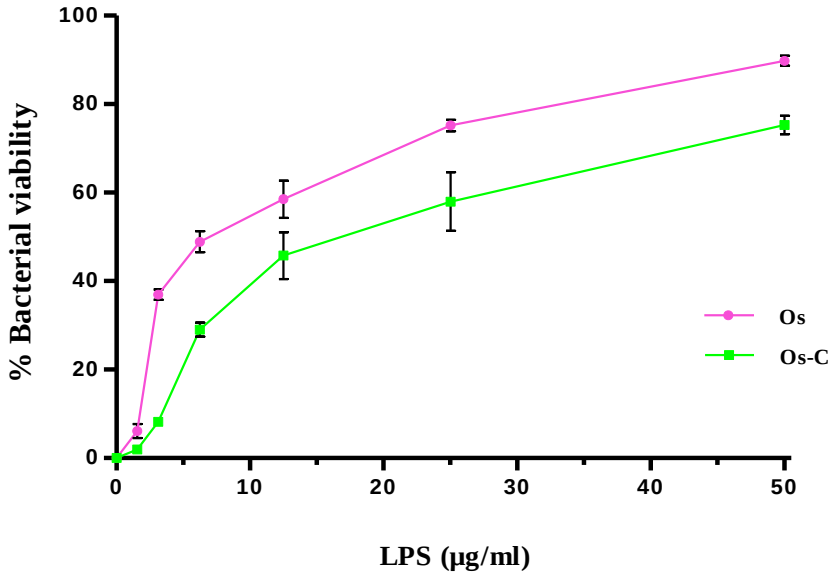

Figure 3.2: The effect of LPS on the bactericidal activity of Os and Os-C against *E.coli*. Data is expressed as the means ± SEM of three independent experiments in triplicate.

There was no significant difference between the growth control (only bacteria) and the control that consisted of bacteria and LPS, indicating that the free LPS added had no effect on the bacterial growth in this experiment. This finding confirms the affinity of the peptides for LPS as observed in the LPS binding assay (**Figure 3.1**).

3.3 NO scavenging activity of peptides

In a reaction where SNP in an aqueous environment spontaneously forms NO, the ability of each peptide to scavenge NO was measured as decrease in NO quantified with the Griess reagent. The peptides were tested at a concentration range of 0.78 - 25 µM. The stable, pink-purple azo compound that formed was measured and the percentage NO scavenged was determined (**Figure 3.3**). In **Figure 3.3a**, the percentage NO scavenging activity obtained by GSH, melittin, Os and Os-C was compared to the untreated control (without peptide). The peptide, Os scavenged NO and followed the same trend as GSH (known NO scavenger), whereas the scavenging capability of Os-C and melittin were insignificant. The tripeptide, GSH, is known to bind NO (Asahi, *et al.*,

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1995; Khansari, *et al.*, 2009). Analysis showed that there was no significant differences between the NO scavenging activity of GSH and Os (**Figure 3.3b**).

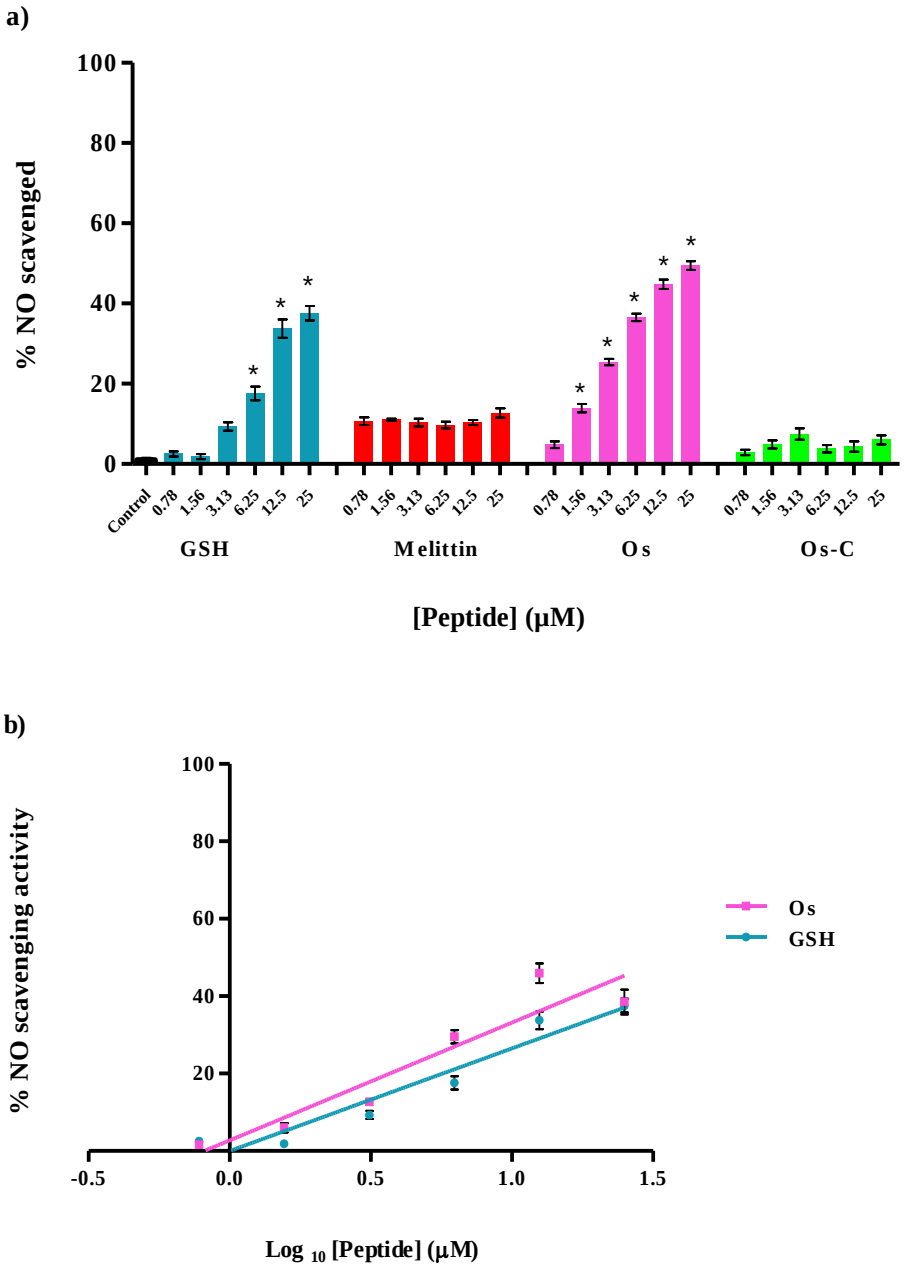

Figure 3.3: NO scavenging capacity of Os and Os-C. a) Percentage NO scavenging of GSH, melittin, Os and Os-C. Data is expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant difference between the scavenging ability of the peptides at their respective concentrations compared to the untreated control; *$p \leq 0.05$. **b) Comparison of dosage effect of GSH and Os.**

3.4 Effects of peptides on NO production and cell viability of RAW 264.7 cells

NO is produced as a result of inflammatory reactions and has been associated with septic shock (Lin, *et al.*, 2008). Low concentrations of LPS induce NO production by RAW 264.7 macrophage

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cells. The ability of Os and Os-C to inhibit NO production either by binding LPS and/or NO scavenging released NO was determined after 24 hours incubation. To optimize the NO assay it was necessary to determine the resting NO production by the RAW 264.7 cells, the effect of Os and Os-C on NO production in the absence of LPS/IFN-γ and whether exposure of cells to LPS/IFN-γ increased NO production.

The initial peptide control concentration, used to evaluate the effect of Os and Os-C on NO production by the RAW 264.7 cells in the absence of LPS/IFN-γ, was 25 µM for all the peptides (melittin, polymyxin B, Os and Os-C). Due to the low cytotoxicity of Os and Os-C the effect of 100 µM Os and Os-C was also evaluated (**Figure 3.4**).

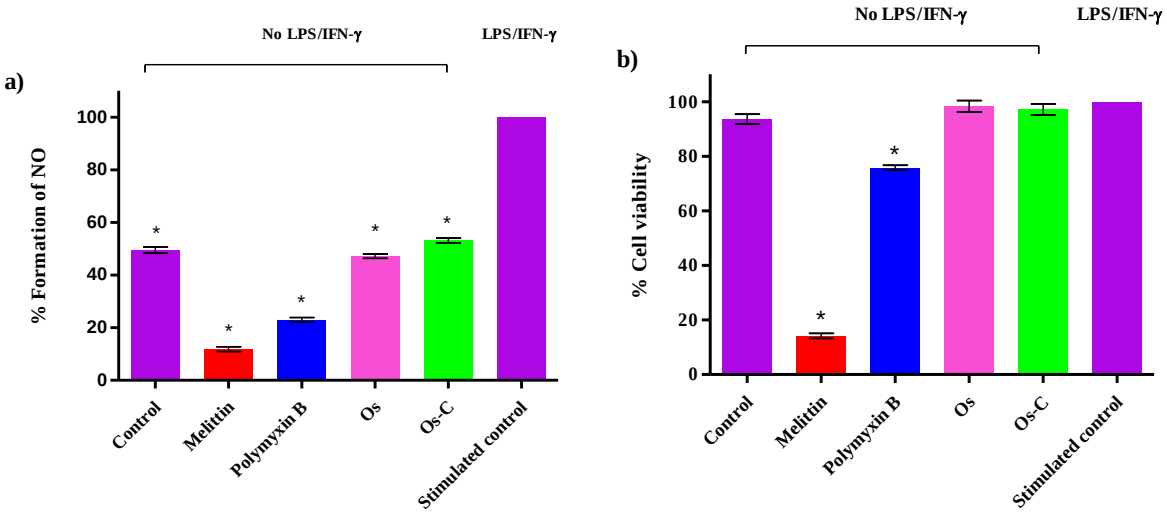

Figure 3.4: NO production by RAW 264.7 cells and cell viability. Control represents the untreated cells (without LPS/IFN-γ; without peptide) and stimulated control represents the cells stimulated with LPS/IFN-γ (0.1 µg/ml; 25 U/ml) (without peptide). Peptide concentrations corresponding to the highest concentration tested with only cells (melittin and polymyxin B (25 µM); Os and Os-C (100 µM)) were used. Data is expressed as the means ± SEM of five independent experiments in triplicate. **a) The percentage NO formed by RAW 264.7 cells.** NO accumulation in the culture supernatant after 24 hours exposure. Statistical analysis was performed and showed significant difference between the amount of NO formed with LPS/IFN-γ stimulation compared to the untreated cells (control) and cells treated with each peptides at their highest concentration; *$p \leq 0.05$. **b) The percentage cell viability of RAW 264.7 cells.** Statistical analysis was performed and showed significant difference between the cell viability of the control, stimulated control, and cells treated with Os and Os-C, repectively, compared to the cells treated with melittin and polymyxin B; *$p \leq 0.05$.

The amount of NO produced by RAW 264.7 macrophage cells was measured indirectly by the quantification of the nitrite concentration. The LPS/IFN-γ stimulated RAW 264.7 cells showed significant production in NO as compared to the untreated (non LPS/IFN-γ-stimulated; control) cells (**Figure 3.4a**), indicating that an inflammatory response in RAW 264.7 cells was achieved.

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Compared to stimulated cells, significantly lower amounts of NO were produced by unstimulated cells that were non-treated or treated with melittin, polymyxin B, Os and Os-C. In the presence of melittin and polymyxin B the level of NO in these cells was lower than that of the untreated cells, whereas in the presence of Os and Os-C, the level of NO was similar to the NO produced by the untreated (control) cells. Reduced NO production by the unstimulated RAW 264.7 cells was seen as inhibition of NO production by melittin and polymyxin B (**Figure 3.4a**).

To determine if the reduction in NO production was due to peptide action or cell death, the viability of the cells was determined with the MTT assay. Cell viability was assessed following the removal of supernatants used for NO determination. MTT was added to the cells, incubated for 3 hours and the formed dye was solubilized by DMSO. There was no significant difference between the cell viability of the untreated cells or LPS/IFN-γ stimulated cells (**Figure 3.4b**), confirming that the RAW 264.7 cells were viable. Melittin and polymyxin B at 25 µM were cytotoxic to unstimulated RAW 264.7 cells, whereas Os and Os-C at 100 µM displayed no cytotoxicity. It is also important to note that in the absence of LPS/IFN-γ Os and Os-C did not have a pro-inflammatory effect.

After determining the amount of NO produced by the peptides on their own, the ability of these peptides to inhibit intracellular NO formation stimulated by LPS/IFN-γ was then investigated. Two experimental strategies were designed to evaluate this effect. The first strategy was to expose the RAW 264.7 cells simultaneously to LPS/IFN-γ and the peptides for 24 hours to measure the direct consequence of peptide binding to LPS. The second strategy was to perform a 90 min pre-incubation of the cells with peptides alone, followed by LPS/IFN-γ stimulation and determination of NO produced after 24 hours to ascertain whether the peptide can interact with medium components or cells (either extracellular or intracellular interaction).

Simultaneous exposure of RAW 264.7 cells with peptides at a concentration range (melittin and polymyxin B: 0.098 - 25 µM; Os and Os-C: 0.098 - 100 µM) and a constant amount of LPS/IFN-γ (0.1 µg/ml; 25 U/ml) indicated that melittin, polymyxin B, Os and Os-C neutralized LPS-induced NO production in a dose-dependent manner (**Figure 3.5a)**. Although Os and Os-C were less effective, melittin (1.56 - 25 µM) and polymyxin B (25 µM) compared to the LPS/IFN-γ control caused significant cell death. In contrast, Os and Os-C showed no toxicity (**Figure 3.5b)**.

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Polymyxin B and melittin are known to inhibit NO production as a result of strong affinity for LPS (Srivastava, *et al.*, 2012; Morrison and Jacobs, 1976). The dosage effect obtained for melittin and polymyxin B was compared to that of Os and Os-C (**Figure 3.5c**). Analysis showed that there was a significant difference between the inhibition of NO production activity of melittin compared to inhibition activity of Os and Os-C. Similarly, the inhibition of NO production activity of polymyxin B was significantly higher than the effect obtained by Os and Os-C. The dosage effect of both Os and Os-C was compared and analysis showed that there was no significant difference between the activity obtained between Os and Os-C (**Table 3.1**).

Pre-incubation of RAW 264.7 cells with peptide followed by addition of LPS/IFN-γ indicated that all four peptides inhibited NO production in a dose-dependent manner (**Figure 3.6a**). Interestingly at 0.098 - 1.56 µM inhibition of NO production was greater than simultaneous exposure (**Figure 3.5a**). The inhibition of NO production by Os and Os-C showed that there was no significant difference between each consecutive concentration, however, there was an increase in the mean values. The cell viability was also determined (**Figure 3.6b**). Pre-incubating melittin and polymyxin B for 90 minutes indicated that compared to the LPS/IFN-γ control, significant cell death occurred at even lower concentrations for both peptides (0.098 µM) when compared to simultaneous exposure, whereas Os and Os-C were not cytotoxic towards the cells compared to the LPS control (LPS/IFN-γ only).

The dosage effect obtained for melittin and polymyxin B was compared to that of Os and Os-C (**Figure 3.6c**). Analysis showed that there was a significant difference between the inhibition of NO production activity of melittin compared to inhibition activity of Os and Os-C. Similarly, the inhibition of NO production activity of polymyxin B was significantly higher than the effect obtained by Os and Os-C. The dosage effect of both Os and Os-C was compared and analysis showed that there was a significant difference between the activity obtained between Os and Os-C (**Table 3.1**).

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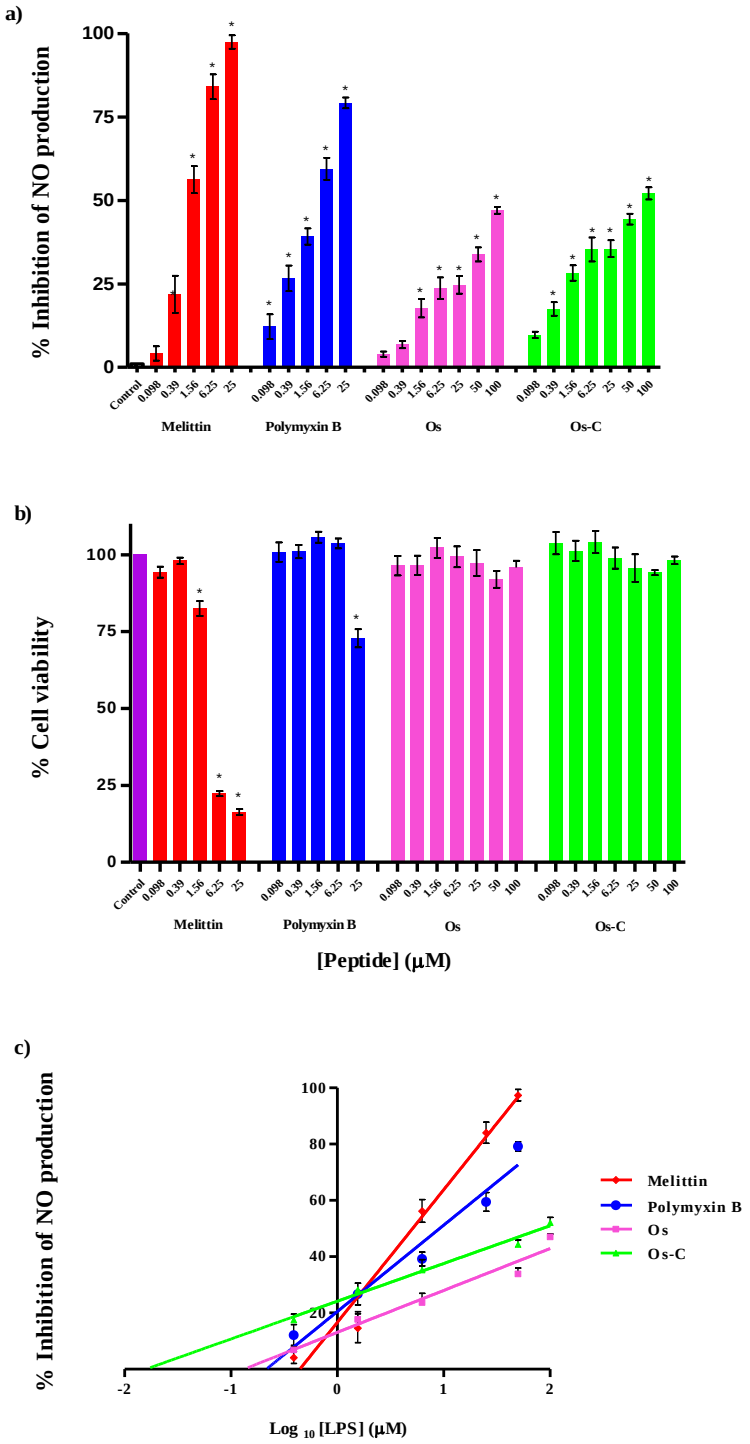

Figure 3.5: Effect of simultaneous incubation of RAW 264.7 cells with LPS/IFN-γ and increasing concentrations of peptides. Control represents the RAW 264.7 cells stimulated with LPS/IFN-γ (0.1 µg/ml; 25 U/ml) (without peptide). Data is expressed as the means ± SEM of three independent experiments in triplicate. **a) Percentage inhibition of NO production.** Statistical analysis was performed and showed significant difference between the inhibitory effects of the peptides at their respective concentrations compared to the control; *p≤0.05. **b) The percentage cell viability of RAW 264.7 cells.** Statistical analysis was performed and showed significant difference between the cell viability of the control and cells treated with Os and Os-C, repectively, compared to the cells treated with melittin and polymyxin B; *p≤0.05. **c) Comparison of dosage effect of poylmyxin B and melittin to Os and Os-C.**

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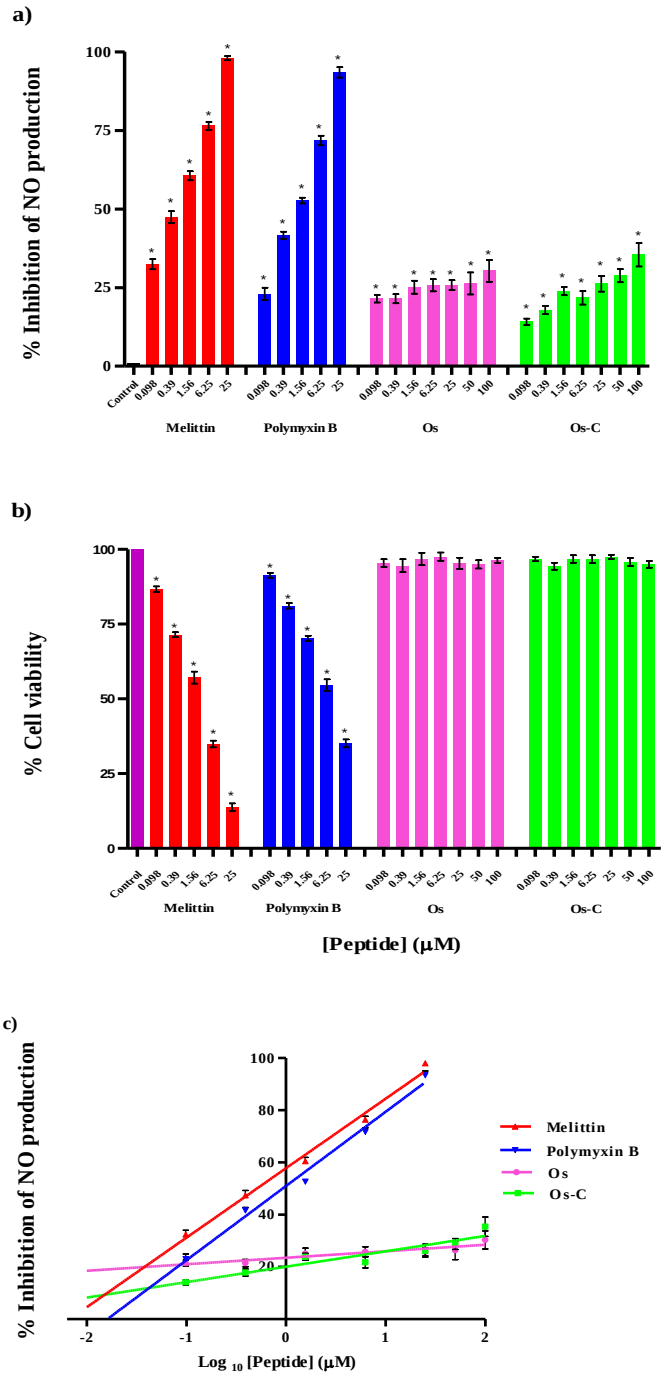

Figure 3.6: Effect of 90 min pre-incubation of RAW 264.7 cells with increasing concentrations of peptides followed by LPS/IFN-γ stimulation. Control represents the RAW 264.7 cells stimulated with LPS/IFN-γ (0.1 µg/ml; 25 U/ml) (without peptide). Data is expressed as the means ± SEM of three independent experiments in triplicate**. a) Percentage inhibition of NO production.** Statistical analysis was performed and showed significant difference between the inhibitory effects of the peptides at their respective concentrations compared to the control; *p≤0.05. **b) The percentage cell viability of RAW 264.7 cells.** Statistical analysis was performed and showed significant difference between the cell viability of the control and cells treated with Os and Os-C, repectively, compared to the cells treated with melittin and polymyxin B; *p≤0.05. **c) Comparison of dosage effect of poylmyxin B and melittin to Os and Os-C.**

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The results obtained with the different experimental strategies were compared between the two peptides. This analysis will identify the greatest effect between simultaneous and pre-incubation of each peptide, as well as to identify other possible mechanisms. The results indicated that the simultaneous incubation of the peptides, Os and Os-C, with LPS/IFN-γ (**Figure 3.7a; Figure 3.7b; Table 3.1**) had an enhanced effect on the inhibition of LPS-induced NO production and/or NO scavenging at higher concentrations (6.25 – 100 µM) when compared to the results obtained when cells were pre-incubated with peptide.

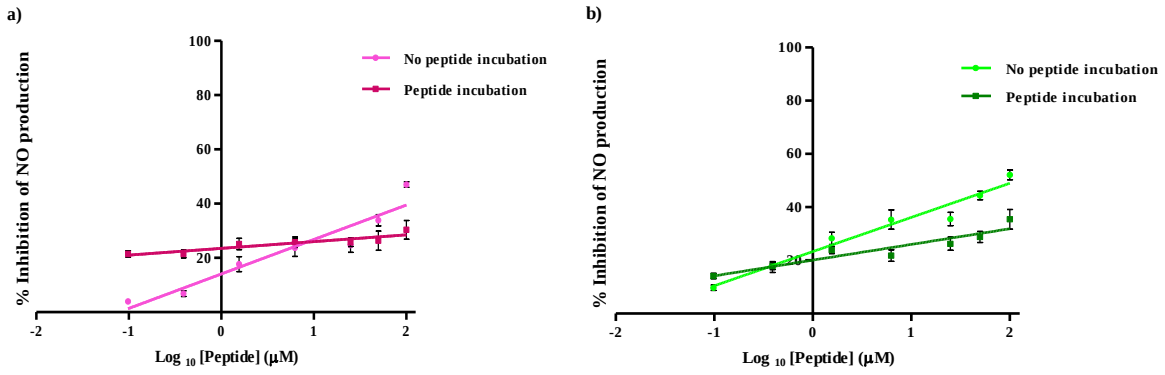

Figure 3.7: Comparison of the effect of simultaneous and 90 minute pre-incubation strategies of Os and Os-C. a) The effect of **s**imultaneously incubated cells with LPS/IFN-γ and Os (light pink) compared to the effect of 90 minute pre-incubated cells with Os before LPS/IFN-γ addition (dark pink). b) The effect of **s**imultaneously incubated cells with LPS/IFN-γ and Os-C (light green) compared to the effect of 90 minute pre-incubated cells with Os-C before LPS/IFN-γ addition (dark green).

3.5 Effects of peptides on TNF-α production by RAW 264.7 cells

The overstimulation of macrophages by LPS has been shown to cause an increase in TNF-α production during inflammation and to participate in the onset of sepsis or septic shock when not contained at the site of infection (Kindt, *et al.*, 2007; Strivastava, *et. al.*, 2012). To determine whether Os and Os-C affects the production of TNF-α induced by LPS/IFN-γ, the relative amount of TNF-α produced by the RAW 264.7 cells, was measured using a mouse TNF-α ELISA kit.

All the peptides tested showed inhibition of the LPS/IFN-γ-induced activation of TNF-α in a dose-dependent manner. Os and Os-C showed lesser activity compared to the control, polymyxin B (**Figure 3.8**). The dosage effect of both Os and Os-C was compared and analysis showed that there was no significant difference between the activity obtained between Os and Os-C (**Table 3.1**).

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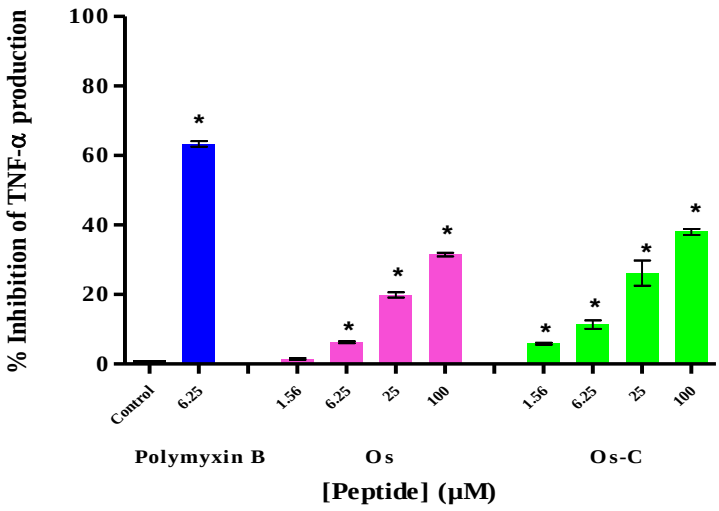

Figure 3.8: The effect of Os and Os-C on TNF-α production. Percentage inhibition of TNF-α production when simultaneously incubating RAW 264.7 cells with increasing concentrations of peptide and LPS/IFN-γ. Control represents the RAW 264.7 cells stimulated with LPS/IFN-γ (0.1 µg/ml; 25 U/ml) (without peptide). Data is expressed as means ± SEM of two independent experiments in duplicate. Statistical analysis was performed and showed significant difference between the inhibitory effects of the peptides at their respective concentrations compared to the untreated LPS simulated cells;*$p \leq 0.05$.

3.6 The ability of Os and Os-C to protect against oxidative damage of RAW 264.7 cells

Free radicals are frequently detected at regions of inflammation, and the overproduction of ROS may cause damage to surrounding tissue components (Choi, *et al.*, 2013). ROS can interact with NO to form RNS. Os has been shown to scavenge NO which implies RNS formation may be reduced. The ability of Os and Os-C to reduce free radical formation was determined. Peptides (0.39 - 100 µM) and AAPH (2 mg/ml) were added to DCFH internalized RAW 264.7 cells and the protection provided by the peptides was determined by measuring the fluorescence obtained due to the presence of free radicals. GSH, a known antioxidant peptide, was used as positive control and compared to Os and Os-C.

The results in **Figure 3.9a** indicated that both Os and Os-C protected RAW 264.7 cells against AAPH-induced oxidative damage. At 100 µM, Os was more effective showing 71% protection, whereas Os-C provided 40% protection. GSH at the same concentration protected RAW 264.7 cells by 79%. Melittin and polymyxin B did not show a dosage dependent increase in antioxidant activity (results not shown).

The dosage effect obtained for GSH was compared to that of Os and Os-C (**Figure 3.9b**). Analysis showed that there was no significant difference between the protection provided against oxidative

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damage to the RAW 264.7 cells by GSH and Os. Whereas there was a significant difference between the protection provided by GSH compared to the effect attained by Os-C. The results showed that GSH and Os are strong antioxidant peptides, whereas Os-C was less effective in reducing AAPH oxidative damage. The dosage effect of both Os and Os-C was compared and analysis showed that there was a significant difference between the activity obtained between Os and Os-C (**Table 3.1**).

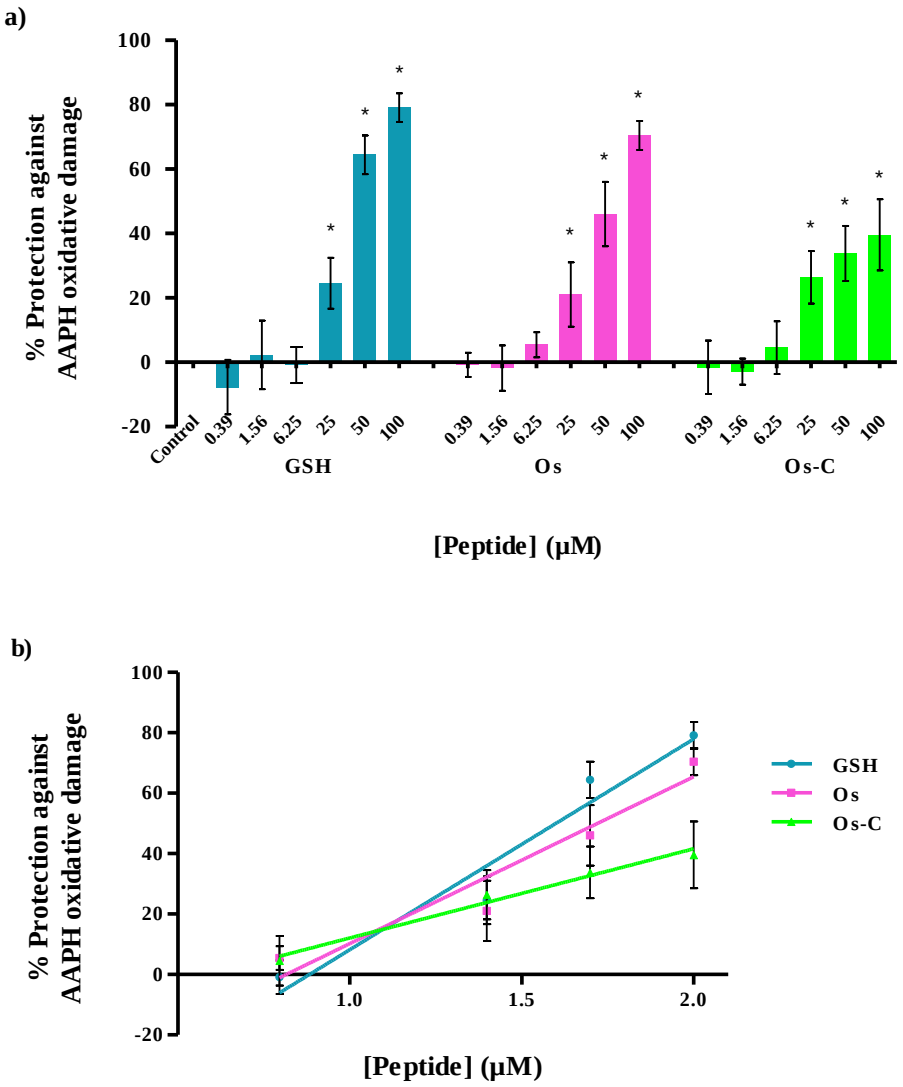

Figure 3.9: The ability of Os and Os-C provides protection against ROS in RAW 264.7 cells. Control represents the RAW 264.7 cells without AAPH and peptide The RAW 264.7 cells were incubated with various concentrations of each peptide and AAPH (2 mg/ml). **a) Percentage protection against AAPH oxidative damage provided by GSH, Os and Os-C.** Data is expressed as means ± SEM of four independent experiments in duplicate. Statistical analysis was performed and each peptide concentration was compared to the control; *$p \leq 0.05$. **b) Comparison of dosage effect of Os and Os-C to GSH.**

To complete the results section, the dosage effects of Os and Os-C related to the various anti-inflammatory properties, are tabulated in **Table 3.1.**

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Table 3.1: Summary of the compared dosage effect between Os and Os-C

	Experiments performed	Gradient		Fold difference		Differences between Os and Os-C ($p \leq 0.05$)	Activity
		Os	Os-C	Os	Os-C		
LPS	• LAL assay	6.70	10.58	1.00	1.58	√	• Os and Os-C bind LPS
NO	• NO scavenging assay (SNP)	30.43	0.9713	31.33	1.00	√	• Os scavenges NO; Os-C inactive
	• RAW 264.7 cells (LPS stimulated) • **Simultaneous incubation**	14.90	13.42	1.11	1.00	X	• Os and Os-C inhibit NO production
	• **90 min pre-incubation (peptide)**	2.49	5.90	1.00	2.37	√	• Os and Os-C inhibit NO production to a lesser extent; Indicates possible intracellular targets
TNF- α	• TNF-α ELISA kit	17.22	18.50	1.00	1.07	X	• Os and Os-C inhibits TNF-α production
ROS/RNS	• DCFH-DA assay	55.24	29.53	1.87	1.00	√	• Os and Os-C provides protection against oxidative damage

*Analysis of the dosage effect between Os and Os-C.
X Indicates no significant difference between Os and Os-C.
√ Indicates a significant difference between Os and Os-C (*$p \leq 0.05$).

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CHAPTER 4: DISCUSSION

LPS, as an endotoxin, is a very potent soluble molecule that binds to macrophages and promotes the synthesis and secretion of pro-inflammatory cytokines and other inflammatory mediators (Dinarello, 1997). Despite the fact that bacterial infections are treated with antibiotics, the direct killing of pathogens promotes the release of LPS, promoting endotoxinaemia, and does not address the problem of sepsis (Hancock, 2007). Therefore, the search of molecules with high affinity towards LPS and blocking LPS-induced activation of macrophages in early stages of inflammation, is crucial (Jerala and Porro, 2000).

Cationic host-defence AMPs contain, in addition to their antimicrobial activity, additional types of activities such as anti-cancer, anti-inflammatory and antioxidant activities, which make them promising candidates for the development of new therapeutic drugs (Gordon and Romanowski, 2005; Hancock, 2001; Marr, *et al.*, 2006; Yi, *et al.*, 2014). According to Yeung, *et al.* (2011), the advantages of multifunctional peptides are that the wound is freed of infection and inflammation, as well as associated free radicals.

Ticks are vectors of severe diseases, caused by viruses and bacteria in mammals, and are capable of transferring blood-borne pathogens without being affected themselves (Chrudimská, *et al.*, 2011; Sonenshine and Hynes, 2008). Ticks rely only on innate immune defence for protection against microbes by producing a variety of AMPs, like defensins (Chrudimská, *et al.*, 2010). These tick defensins could potentially function as templates for the development of multifunctional peptides. In a study done by Prinsloo, *et al.* (2013), the structural characteristics and bactericidal properties of Os and Os-C derived from the carboxy-terminal fragments of defensin isoform, OsDef2, were investigated. As a result of the antimicrobial activity these peptides showed against Gram-positive bacteria and Gram-negative bacteria, non-toxicity towards mammalian cells and potent antioxidant activity, it was of interest to determine whether Os and Os-C also possess anti-inflammatory properties, as well as antioxidant activity in an *in vitro* cell-based model.

The peptides, Os and Os-C examined in this study are positively charged (+6) at physiological pH (**Table 2.1**). Os and Os-C are short (19-22 residues) peptides containing both hydrophobic and hydrophilic amino acids. Melittin and polymyxin B were used as controls due to the fact that they

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are well known for their anti-endotoxic activity. Melittin, polymyxin B and Os-C lack cysteine residues. Melittin, is a cationic (+5), linear 26-residue amphipathic, α-helical peptide isolated from honey bee (*Apis mellifera*) venom (Vogel and Jähnig, 1986). Due to its strong amphipathic surface activity, the peptide was used as a model to determine peptide-lipid interactions (Dempsey, 1990). Melittin has been shown to spontaneously associate with natural and artificial membranes due to the presence of hydrophobic amino acids (Raghuraman and Chattopadhyay, 2007). The amphipathic nature of melittin enhances its antimicrobial activity as well as its cytotoxic and hemolytic characteristics (Kuchinka and Seeling, 1989; Pérez-Paya, *et al*., 1994; Yokum, *et al.*, 2001; Zhang, *et al*., 1999). Polymyxin B is derived from *Bacillus polymyxa* strains and is a natural cationic (+5) cyclic lipopeptide that consists of a ten amino acids of which seven amino acids form a cyclic structure and a terminal hydrophobic, eight or nine carbon fatty acid, 6-methyl octanoic and 6-methyl heptanoic acid, tail (Morrison and Jacobs, 1976; Vogel and Jähnig, 1986). Polymyxin B has also been reported numerous times to show cytotoxicity towards eukaryotic cells which limits its use to topical applications (Hancock, 1997; Tsuzuki, *et al.*, 2001). GSH, a tripeptide, was used as control because it is a known NO scavenger and possesses antioxidant activity. The secondary structures of Os and Os-C obtained from circular dichroism (CD) spectroscopy by Prinsloo, *et al.* (2013) indicated that Os is random coiled with some α-helical properties, whereas Os-C is random coiled with some β-strand properties when prepared in SDS (membrane-mimicking environment).

LPS is recognized by extracellular endotoxin receptor proteins that bind LPS and presents the endotoxin to the CD14 receptors that form part of the initial steps involved in macrophages activation. Examples of these extracellular endotoxin receptor proteins are LBP, MD-2 and BPI (section 1.3.1 and section 1.5), which are able to bind and neutralize LPS (Reyes, *et al.*, 2002; Mancek, *et al.*, 2002; Gray, *et al.*, 1995). A conserved LPS interacting region has been identified within these proteins that may serve as another reason to why Os and Os-C are able to bind LPS and inhibit pro-inflammatory responses. The LPS interacting region consists of a tri-peptide, H/A-B-H, sequence motif (H = hydrophobic amino acid; A = aromatic amino acid; B = basic amino acid). For example, Bhunia, *et al*. (2008) synthesized a 12-mer peptide, called YW12D, with a primary amino acid sequence, YVKLWRMIKFIR, which consists of three non-overlapping tri-peptide sequence patterns, VKL, WRM and IKF that follows the just mentioned sequence motif. The results indicated that YW12D possessed anti-endotoxin activity through binding LPS, inhibiting NF-κB induction (section 1.3.1) and displayed almost no haemolytic activity. The H/A-

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B-H sequence motif is present in both Os, GIRGYKGGYCKGAFKQTCKCY and Os-C, GIRGYKGGYKGAFKQTKY, and may contribute to the anti-endotoxin activity of these peptides.

Many AMPs that possess antibacterial activity are also able to bind LPS and supress the pro-inflammatory cytokines and NO production by macrophages (Jerala and Porro, 2000). Firstly, the ability of Os and Os-C to bind LPS was evaluated (**Figure 4.1A**). The results indicated that both Os and Os-C possess the ability to directly bind LPS by means of inhibiting the LAL enzyme activity in a dose-dependent manner (**Figure 3.1**). The inhibitory activities of Os and Os-C are indistinguishable, although each being significantly less than the inhibitory activities of the LAL enzyme obtained by melittin and polymyxin B.

Since the electrostatic and hydrophobic interactions between peptides and LPS is necessary to ensure the binding of the peptides, it is important for peptides to contain both hydrophilic and hydrophobic amino acids to facilitate these interactions (Iwagaki, *et al.*, 2000). It was expected that both Os and Os-C would be able to form this initial interaction with LPS as a result of their highly basic (+6) nature (due to the presence Arg and Lys residues) of these peptides, binding to the negatively charged diphosphoryl head groups of LPS (Bhunia, *et al.*, 2007). This is followed by the stabilization of the resulting molecular complex through strong hydrophobic interactions between the hydrophobic amino acids (Ala, Gly, Ile, Phe) and fatty acid tail of LPS (Bhunia, *et al.*, 2007; David, *et al.*, 1992; Morrison and Jacobs, 1976; Rustici, *et al.*, 1993; Tsubery, *et al.*, 2001).

Some primary sequences of disulphide containing cationic AMPs were analysed by Yount and Yeaman (2004) for the conserved γ-core motif by multiple sequence alignments. Peptides containing the γ-core usually possess antimicrobial activity. The γ-core contains a conserved $GXCX_nC$ or $CXGX_nC$ motif (G = Gly; X = any amino acid; C = Cys; n = any number of amino acids). Interestingly, some peptide sequences that correspond to the γ-core, are also able to bind LPS. For example, Trachyplesin I, KWCFRVCYRGICYRRCR, a 17-mer peptide isolated from the horseshoe crab, *Tachypleus tridentatus*, possessed in addition to its antimicrobial avtivity, anti-endotoxin activity (Iwanaga, *et al.*, 1998). The γ-core motif, $CXGX_nC$, is present in Os, GIRGYKGGYCKGAFKQTCKCY, and may contribute to the LPS binding capacity of the peptide.

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Interestingly, the dosage effect obtained by Os-C, peptide from which the Cys residues have been deleted, is higher than the dosage effect of Os, suggesting that deletion of the Cys residues enhance the binding of the peptide to LPS. This is in contrast to the results obtained by Ramamoorthy, *et al.* (2006), which indicated that the deletion of 4 cysteine residues from Tachyplesin I did not affect the affinity of the peptide (CDT) towards LPS.

The higher effect of Os-C could rather be attributed to the hydrophobic interactions involved in the binding of peptides to LPS. Bhunia, *et al.* (2007) demonstrated that the hydrophobic interactions between melittin and LPS plays a dominant role in the affinity of the peptide for LPS. Melittin, in this current study, had a remarkably higher dosage effect on the inhibition the LAL enzyme, as compared to Os and Os-C, which can be linked to the higher percentage of hydrophobic amino acids of melittin (58%) compared to Os (36%) and Os-C (42%). This indicates that hydrophobicity is a major contributing factor for LPS binding.

LPS has been shown to inhibit or influence the bactericidal activity of AMPs. The CFU assay was used as an indirect method of confirming LPS-binding to Os and Os-C. The CFU method is an established protocol in our laboratory used to determine the MBC values of peptides against various microorganisms. Both peptides have been reported by Prinsloo *et al.* (2013) to possess bactericidal activity towards Gram negative bacteria at micromolar concentrations. The effect of LPS on the bactericidal activity of the peptides towards *E. coli* was determined by incubating the peptides at their respective MBCs, Os (1.875 µg/ml; 0.762 µM) and Os-C (3.75 µg/ml; 1.743 µM), with increasing LPS concentrations (1.56 – 50 µM). The results indicated that the presence of free LPS did affect the antibacterial activity of both Os and Os-C (**Figure 3.2**). The growth control consisting of *E.coli* and free LPS showed no increase from the expected amount colonies that formed, indicating that the free LPS had no effect on the microbial growth. The finding indicated that free LPS binds to both Os and Os-C and reduced their bactericidal activity, which also confirms the affinity of the peptides for LPS as observed in the binding LAL assay. This is in good correlation with a previous study by Lin, *et al.* (2008), which showed that the bactericidal activity of the peptide, CM4, isolated from the Chinese silk worm, *Bombys mori*, was reduced with the increase of free LPS concentrations.

Several cationic AMPs bind preferentially to negatively charged cell components such as LPS (Jerala and Porro, 2004). The binding affinity of Os and Os-C towards LPS serves as correlation

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of the antibacterial activity towards Gram-negative bacteria obtained by Prinsloo, *et al.* (2013). For direct killing of Gram-negative bacteria to occur, cationic AMPs must be attracted towards the negatively charged outer membrane, which is mainly composed of LPS, and then attach to the bacteria in order to penetrate the outer membrane through the self-promoted uptake pathway (Ding, *et al.*, 2003; Hancock, 1997). The cationic AMPs compete with cations, such as Ca^{2+} and Mg^{2+}, and interact with LPS. The displacement of the cations cause the destabilization of the outer membrane and subsequently allows the passage of the AMPs through transient defects (Dathe and Wieprecht, 1999; Hancock, 1997; Jerala and Porro, 2004). As a result the AMPs are able to reach the cytoplasmic membrane of the bacteria where antibacterial activity is believed to take place (Jerala and Porro, 2004).

NO is an ever-present free-radical moiety that plays a pivotal role in the regulation of inflammation upon macrophage activation as explained in section 1.3.1 and 1.3.2 (Balakrishnan, *et al.*, 2009; Guzik, *et al.*, 2003). It is important to note that excessive NO production, by iNOS, that is present in various cell types, such as macrophages, triggers disadvantageous cellular responses associated with production of systemic inflammatory signals, participation in the occurrence of systemic inflammation as well as in cellular and tissue damage (Guzik, *et al.*, 2003; Lever and Mackenzie, 2007; Udenigwe, *et al.*, 2009; Wadsworth *et al.*, 2001). The NO scavenging activity of peptides can lower the toxic effect of NO, preventing thus damage to the host tissue (Udenigwe, *et al.*, 2009) (**Figure 4.1B**). NO is a highly reactive molecule and interacts with the thiol groups of GSH, a known NO scavenger, to form nitrosothiols (Khansari, *et al.*, 2009; Tracey, *et al.*, 1988). In this study GSH, caused a dose-dependent decrease of the amount NO generated from the decomposition of SNP *in vitro*. Similarly, Os scavenged NO and followed the same trend as GSH, whereas the scavenging capability of Os-C and melittin were insignificant (**Figure 3.3**). The NO scavenging ability of Os, which contains three Cys residues, is in agreement with the results obtained by GSH. Additionally, the almost non-existing NO scavenging activity of Os-C and melittin could be due to the fact that neither contains Cys residues.

NO and TNF-α are key mediators of pro-inflammatory responses and are involved in the onset of septic shock as a result of unregulated stimulation of the macrophages by LPS (Choi, *et al.*, 2013; Tracey, *et al.*, 1988). The ability of the peptides to counteract the excessive formation of NO and pro-inflammatory cytokines is essential for the prevention of these potentially harmful conditions (Fessler, *et al.*, 2002; Gough, *et al.*, 1996; Hirata, *et al.*, 1994; Kim, *et al.*, 2004; Park, *et al.*, 2004;

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Rosenfeld, *et al*., 2006). Having established that both Os and Os-C bind to LPS, it was necessary to ascertain whether such binding would result in inhibition of pro-inflammatory responses such as NO and TNF-α production, which usually occurs as a result of LPS stimulation of RAW 264.7 macrophage cells.

In this study, it was necessary to establish that an induced inflammatory response in the RAW 264.7 cells was achieved, before studying the effect of Os and Os-C on this *in vitro* inflammatory model. To assess this, the NO levels present in the cell culture supernatant after incubating for 24 hours with and without LPS (different LPS concentrations), was measured with Griess reagent. The initial pitfall was to successfully obtain a significant increase in NO levels of the LPS-stimulated cells compared to the untreated (non LPS-stimulated) cells. After a series of optimization steps that included troubleshooting of: a) dissolving LPS in different solvents and combinations thereof, b) exposing cells to different LPS concentrations, c) using heat killed *E.coli*, d) adding IFN-γ, e) performing experiments with different cell concentrations, f) supplementing the culture medium, g) conducting experiments with different exposure and incubation times, as well as h) using different concentrations and solvents of the Griess reagent, an inflammatory response when stimulating the RAW 264.7 cells was achieved.

Stimulating the RAW 264.7 cells with LPS/IFN-γ showed a significant increase in NO levels in the cell culture supernatant after 24 hours of incubation compared with the untreated (non LPS/ IFN-γ-stimulated) cells (**Figure 3.4a**). To support this, studies indicated the synergism between LPS and IFN-γ was more successful in supplying a more favourable and sustainable source of NO due to the enhancement of iNOS transcription in RAW 264.7 macrophage cells (Vodovotz, *et al.*, 1993; Xie, *et al.*, 1994). The peptide controls used in this assay consisted of RAW 264.7 cells and peptide without LPS/IFN-γ. Both Os and Os-C (100 µM) did not cause an increase in NO production in untreated (non LPS/IFN-γ-stimulated) cells, whereas a significant reduction in NO production was obtained for melittin and polymyxin B (25 µM). The substantial reduction of NO by melittin and polymyxin B was due to cell death measured as loss in cell viability. (**Figure 3.4b**).

The results obtained by the different experimental strategies (simultaneous (**Figure 4.1C**) and pre-incubated (**Figure 4.1D**)) with Os and Os-C, respectively, indicated that both peptides attenuated LPS-induced production of NO (**Figure 3.5a; Figure 3.6a**). Although the inhibition of NO production by Os and Os-C is lower when compared to melittin and polymyxin B (**Figure 3.5c;**

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Figure 3.6c), it is important to note that the high percentage of NO inhibition is in part due to the toxicity of melittin and polymyxin B (**Figure 3.5b; Figure 3.6b**). The dosage effects obtained for the simultaneous and pre-incubation of each peptide (Os, **Figure 3.7a**; Os-C **Figure 3.7b**) were compared to ascertain which incubation strategy is more effective in inhibiting LPS-induced NO production, as well as to identify other possible mechanisms. The simultaneous incubation of the peptides, Os and Os-C, with LPS/IFN-γ had an enhanced effect on the inhibition of LPS-induced NO production and/or NO scavenging when compared to the results obtained with cells pre-incubated with peptides. These experiments provided an initial indication that Os and Os-C may have intracellular targets of NO production.

Having established that both Os and Os-C bind to LPS, as well as inhibit the NO response in RAW 264.7 cells, it was furthermore necessary to determine whether such binding would result in inhibition of the pro-inflammatory TNF-α response that usually occurs as a result of LPS stimulation. The simultaneous incubation of LPS/IFN-γ and peptides to RAW 264.7 cells strategy was used to measure the amount of TNF-α secreted into the supernatant. The simultaneous incubation strategy was chosen as it had in the previous study provided a greater effect on the inhibition of NO production by the RAW 264.7 cells. Only one concentration of the control compound, polymyxin B was used is this assay. The reasons for this was that polymyxin B is a known inhibitor of LPS-induced macrophage production of TNF-α and as depicted in the **Figure 3.5b**, polymyxin B did not reduce the cell viability of the RAW 264.7 cells at 1.56 μM (Tsubery, *et al.*, 2001). When compared to the LPS/IFN-γ control, both Os and Os-C attenuated, in a dose-dependent manner, the LPS-induced production of TNF-α (**Figure 3.8**).

Melittin had a significantly higher binding activity for LPS and inhibiting activity towards NO production compared to Os and Os-C. A study done by Srivastava, *et al.* (2012) demonstrated the importance of its leucine zipper motif in the binding of the peptide to LPS and neutralization of LPS-induced inflammatory responses in macrophages. The replacement of Leu with Ala decreased the ability of the analogues to bind LPS. The difference in LPS binding by Os and Os-C compared to melittin might be due to the presence of the leucine zipper as both Os and Os-C do not contain leucine-residues (Srivastava, *et al.*, 2012).

With the simultaneous incubation strategy, LPS/IFN-γ and peptides were added to the extracellular environment of the RAW 264.7 cells and a dosage effect was obtained in the attenuation of the

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LPS-induced inflammatory responses. Interestingly, the gradual increase of inhibition of NO and TNF-α production up to 100 µM (the maximum concentration tested) might be as a result of the direct interaction of Os and Os-C, respectively, with LPS in solution. A possible mechanism by which Os and Os-C can prevent LPS-induced inflammatory responses in RAW 264.7 cells is that both peptides are able to bind to LPS aggregates and cause the dissociation thereof, rendering LPS unavailable to the binding sites of CD14 receptors present on the membrane of RAW 264.7 cells (Aurell Wistrom, 1998; Hailman, *et al.*, 1994; Yu and Wright, 1996). LL-37 and melittin dissociate LPS aggregates and bind to LPS and subsequently prevent the binding of LPS to LBP, thus inhibiting LPS-induced NO and TNF-α responses by the macrophages (Rosenfeld, *et. al.*, 2006; Scott, *et al.*, 2000; Srivastava, *et al.*, 2012). The reduced percentage of NO measured in the supernatant might also be as a result of the NO scavenging activity of Os.

With the pre-incubation strategy, the attenuated effect of the LPS-induced NO response by Os and Os-C was significantly less. This might be an indication that when Os and Os-C are pre-incubated with RAW 264.7 cells, the peptides may have other targets. A possible explanation is that both Os and Os-C might be able to bind directly to the outer membrane of the RAW 264.7 cells as a result of their affinity towards phospholipids. This could reduce the availability of the peptides in the extracellular environment to bind LPS. After the 90 minute incubation period, upon LPS/IFN-γ stimulation, unbound Os and Os-C were available to bind LPS and attenuated the LPS-induced NO response. Alternatively, both Os and Os-C may be internalized and interact with intracellular targets involved in the regulation of protein-protein and/or protein DNA interactions or interfere with signalling pathways. Some cationic AMPs have been shown to translocate, in a non-destructive mannner, across eukaryotic cell membranes (Henriques, *et al.*, 2006). Penetratin, a 16-mer peptide derived from the homeodomain of the *Drosophila* transcription factor, Antennapedia, displayed no cytotoxicity towards L929 fibroblasts, as well as RAW 264.7 macrophages at 50 µM. The peptide was shown to effect the NF-κB complex that resulted in the induction of pro-inflammatory responses in both cell lines (Derossi, *et al.*, 1998; Letoha, *et al.*, 2006). This suggests the crucial role of LPS-Os and LPS-Os-C interactions for neutralizing the pro-inflammatory responses in macrophages and support the possibility of both peptides having other targets, hence either on the cellular membrane or within the cytosol or nucleus (Lindgen, *et al.*, 2000).

In this study, the MTT assay revealed that Os and Os-C (100 µM) had no effect on the cell viability of the RAW 264.7 cells when the cells were incubated with only peptide, simultaneously with

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peptide and LPS/IFN-γ and pre-incubated for 90 minutes with peptide before LPS/IFN-γ stimulation (**Figure 3.4b; Figure 3.5b; Figure 3.6b**). This demonstrated that the inhibition of the NO response by Os and Os-C was not attributed to the direct cytotoxic effect of these peptides. In comparison, a significant reduction in cell viability was obtained for melittin and polymyxin B at 1.56 µM and 25 µM, respectively, with simultaneous incubation with peptide and LPS/IFN-γ. A greater toxic effect was obtained for melittin and polymyxin B (0.098 µM) with the 90 minute pre-incubation strategy. This implies that when melittin and polymyxin B binds LPS, the concentration of free peptide is reduced and consequently the peptides are less toxic. Indirectly, this also confirms binding between the peptides and LPS.

Both melittin and polymyxin B have been reported to show toxicity towards zwitterionic mammalian membranes (Asahi, *et al.*, 1995; Asthana, *et al.*, 2004). The results from the MTT assay are also in good correlation with the results obtained by Prinsloo, *et al.* (2013) where Os and Os-C showed no haemolysis of erythrocytes at 100 µM and no toxicity towards the eukaryotic cell lines, SC-1 and Caco-2, in the presence of Os (48.8 µM) and Os-C (55.8 µM), respectively. Melittin (2.5 – 100 µM) caused a dose-dependent increase of haemolysis in human erythrocytes and caused a decrease of approximately 60% and 20% of SC-1 and Caco-2 cell number, respectively, at a concentration of 42.4 µM. Os and Os-C can thus be considered for further investigation as potential anti-endotoxic therapeutic agents.

The difference in selectivity of the peptides towards microbial and mammalian cells can be as a result of the differences in membrane composition. It has been shown that cationic AMPs have a higher affinity towards negatively charged membranes, for example, that of Gram-negative bacterial cell membranes which are rich in acidic phospholipids (phosphatidylglycerol, cardiolipin or phosphatidylserine) and polyanionic LPS that provide an overall negative charge (Yeaman and Yount, 2003). As a result of the electrostatic interactions between the positively charged peptide and the negatively charged bacterial membrane these AMPs have affinity towards bacterial cells. Mammalian cytoplasmic membranes are rich in zwitterionic phospholipids (phosphatidyl-ethanolamine, phosphaditylcholine or sphingomyelin) that provide an overall neutral charge, resulting in the low affinity of the AMPs (Yeaman and Yount, 2003). Both polymyxin B and melittin are known to interact with both, polar and non-polar regions of phospholipids that contributes towards it non-selective toxicity towards negatively charged bacterial and zwitterionic mammalian membranes (Asthana, *et al.*, 2004).

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The helicity of cationic AMPs has been shown to contribute to the cytotoxicity of the peptides toward mammalian cells. In several studies, the substitution and/or insertion of helical favouring amino acids (Met, Ala, Leu, Lys) to enhance the antimicrobial activity of a peptide correlated with enhanced haemolytic and cytotoxic activities of the peptides (Blondelle and Houghten, 1991; Blondelle, *et al.*, 1995; Dathe and Wieprecht, 1999). The leucine zipper motif of melittin (mentioned above) plays a vital role in its toxicity towards mammalian cells. The disruption of this motif changes the α-helical structure in both aqueous and zwitterionic lipid environments, which resulted in the disruption of the permeability mechanism of melittin on zwitterionic membranes (Asthana, *et al.*, 2004; Zang *et al*., 1999).

Os-C in SDS (membrane-mimicking solvent) lacks α-helical properties as indicated by CD spectrometry results (Prinsloo, *et al.*, 2013). The secondary structure constituents of Os-C was predicted to be 21% β-sheet and 79% coiled, explaining the selectivity of Os-C for micro-organisms over mammalian cells. In contrast, because Os has a predicted secondary structure consisting of 32 % α-helix and 68% coiled regions in SDS, it would be expected to contain some haemolytic and cytotoxic activities. A possible explanation why Os is non-toxic might be that Os may adopt another secondary structure in a lipid environment. For example, peptidyl-glycylleucine-carboxyamide (PGLa), a frog skin peptide, transitions into a disordered conformation when exposed to zwitterionic membranes but adopts an α-helical structure in the presence of negatively charged membranes (Latal, *et al.*, 1997). Similarly, the CD results obtained by Matsuzaki, *et al.* (1989) showed that magainins displayed α-helical transition only when interacting with anionic membranes. It is thus possible that Os and Os-C adopts different (other than an α-helical) conformations when exposed to mammalian cell membranes which permits the peptides to not display toxicity towards the particular cell type.

There is also a great interest towards potential therapeutic peptides to possess antioxidant properties and would contribute to the overall concept of multifunctional biopeptides (**Figure 4.1E**). Inflammatory tissue injury has been shown to be mediated by overproduction of reactive toxic mediators, such as ROS and RNS (Choi, *et al.*, 2013 and Syahida, *et al.*, 2006). Oxidizing agents are produced by phagocytic leukocytes, such as macrophages, that invade the tissue at the site of infection. Substantial amounts of reactive metabolites promote cytotoxicity towards the infected tissues and initiate and/or increase the inflammatory response (Conner, *et al.*, 1996 and Lu and Wahl, 2005).

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Both Os and Os-C exhibited a dose-dependent increase in antioxidant activity, providing cellular protection against free radicals (**Figure 3.9a**). Os displayed equally potent activity when compared to GSH, whereas Os-C was found to possess less antioxidant activity (**Figure 3.9b**). The results obtained were in good agreement with the results attained by Prinsloo, *et al.* (2013) who showed that both Os and Os-C possess antioxidant properties towards erythrocyte cells and the same activity was obtained in a chemical-based assay.

The antioxidant activity of numerous peptides has been shown to be attributed to the radical scavenging properties of the peptide (Sarmadi and Ismail, 2010). Several amino acids contribute to the antioxidant activity of peptides, such as Cys, Met, Phe, Tyr, His, Pro and Trp (Elias, *et al.*, 2008, Shen, *et al.*, 2010 and Yang, *et al.*, 2009). In various studies, replacing the above mentioned amino acids of known antioxidant peptides with glycine have shown to reduce their antioxidant activity (Yang, *et al.*, 2009). The sequences of Os and Os-C contains some of these antioxidant amino acids. Os contains more antioxidant amino acids (three Cys, one Phe and one Tyr) than Os-C (one Phe and one Tyr), allowing Os to possess a higher antioxidant activity. This is attributed to the phenolic side chains, as well as the hydroxyl (Tyr) and thiol (Cys) groups of these amino acids that enable them to donate an electron to the radical species and as a result stabilizes them.

In a study by Madhyastha and Vatsala (2010), the antioxidant activity of the cysteine-rich cyanopeptide β2 isolated from *Spirulina fussiformis*, was attributed to the presence of the six cysteine residues present in the peptide. This suggests that the presence of the three cysteine residues in Os may contribute to the higher antioxidant activity observed compared to Os-C. Although the reducing agent, DTT, was added to the peptides prior to lyophilisation, no additional DTT was added when stock solutions were prepared. It is expected that in the absence of additional DTT, Os Cys residues may undergo oxidation. However, some of the Os might have remained in its reduced form. As previously explained in section 3.3, Os possess NO scavenging activity and may be due to the ability of Os to form nitrosothiols when exposed to NO. This confirms that the Cys residues are also responsible for the observed activity. Additionally, BIOPEP analysis showed that the dipeptide sequence, IR, which is associated with antioxidant activity is present in both Os and Os-C.

The cell-based assays were performed in the presence of medium (sDMEM) and buffer (NaP and PBS). Since the cell culture medium contains both salts and serum these components could

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influence the results. The study by Prinsloo, *et al.* (2013) showed that in the presence of monovalent ion (Na^+), both Os and Os-C retained their bactericidal activity. It is unlikely that it affected the ability of Os and Os-C to bind LPS and inhibit the LPS-induced inflammatory responses, as well as their cytotoxicity. Furthermore, cations (Ca^{2+} and Mg^{2+}) might be competing for the cationic-binding sites present on LPS. According to Hancock (1997) cationic peptides have three orders of magnitude higher affinity for LPS than divalent cations, which would result in the displacement of the cations present in solution. The negatively charged protein in serum, namely albumin, present in the cell culture medium may bind to the positively charged peptides as it has been reported to bind to various molecules and drugs (Bowdish, *et al.*, 2005; Varshney, *et al.*, 2010). The interaction with albumin is an important area of research in the understanding of cellular effects. Considering the binding property of albumin it is possible that polymyxin B (+5), melittin (+5), Os (+6) and Os-C (+6) could have bound to albumin that would result in decrease of LPS binding, inhibitory activity towards LPS-induced inflammatory responses, as well as cytotoxicity of these peptides. However, melittin and polymyxin B were still shown to successfully possess these activities under the same conditions. Another confirmation of this is that Prinsloo, *et al.* (2013) determined that serum albumin did not affect the bactericidal activity of Os.

The potential anti-inflammatory properties of Os and Os-C, as well as possible mode of action is schematically represented in Figure 4.1.

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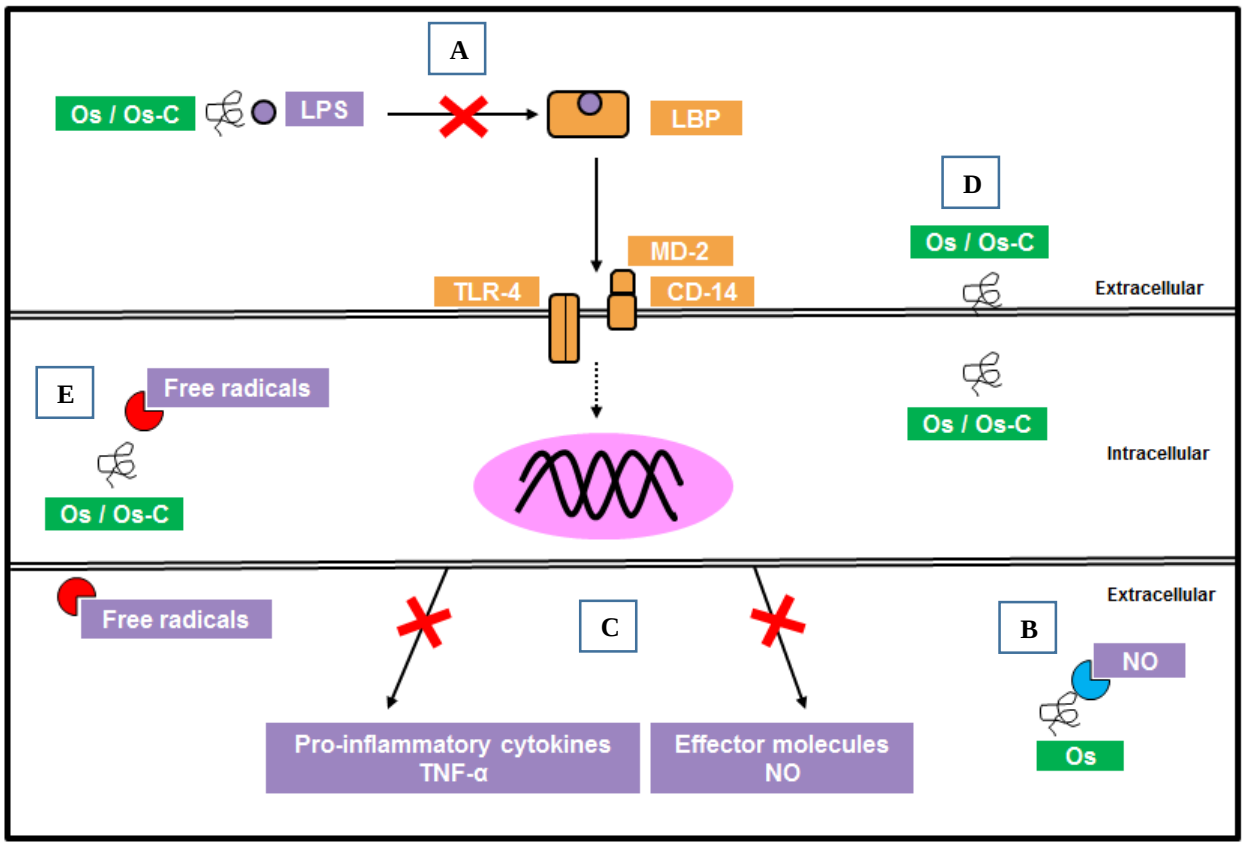

Figure 4.1: Schematic representation of the anti-inflammatory and antioxidant activities of Os and Os-C. **A**) Os and Os-C binds LPS. **B**) Os has in addition NO scavenging activity. **C**) Simultaneous incubation of RAW 264.7 cells with Os and Os-C, respectively with LPS/IFN-γ showed a reduction in NO and TNF-α production. **D**) Pre-incubation of RAW 264.7 cells with Os and Os-C, respectively for 90 minutes, followed by LPS/IFN-γ stimulation showed lower activity towards NO production, but might indicate possibly other modes of actions, such as entering the RAW 264.7 cells or binding to target proteins present on the cellular membrane. **E**) Both Os and Os-C provides protection against ROS in unstimulated RAW 264.7 cells.

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CHAPTER 5: CONCLUSSION AND FUTURE PROSPECTS

Multifunctional peptides show potential to treat bacterial infections and prevent inflammation, as well as oxidative damage associated with these conditions (Sawa, *et al.*, 1998). OsDef2, derived from the tick *O. savignyi*, was used as a template for the design of shorter peptides. Os was derived from the carboxy-terminal of OsDef2 and Os-C is an analogue of Os, where the three Cys residues were omitted. Both Os and Os-C have strong antibacterial activity against Gram-positive bacteria and Gram-negative bacteria (Prinsloo, *et al.*, 2013). It would be of great value if both Os and Os-C possess the ability to bind LPS and as a result neutralize LPS-induced inflammatory responses.

The results in this study has shown that Os and Os-C have the ability to bind LPS. The effect of LPS binding was further researched by investigating the effect of LPS on the bactericidal activity of both peptides against *E.coli*. The result indicated that LPS reduced bactericidal activity. This served as an indirect indication that both peptides possess the ability to bind LPS. It has been shown that AMPs adopt predominant secondary structures, especially α-helices, in the presence of LPS. Further studies can be undertaken to determine the ability of LPS to interact, bind and oligomerize in the presence of LPS (Rosenfeld, *et al.*, 2008; Srivastava, *et al.*, 2012). To determine the secondary structures of Os and Os-C in the presence of LPS, CD spectroscopy, can be performed. To further elucidate the ability of both peptides to bind LPS, it is important to investigate whether the peptides can disaggregate LPS aggregates in an aqueous environment (Skoog, *et al.*, 2004). According to Rosenfeld, *et al.* (2006) a correlation exists between the abilities of AMPs to disaggregate LPS aggregates and to inhibit LPS-induced pro-inflammatory responses.

Both Os and Os-C inhibited NO and TNF-α production by RAW 264.7 cells when incubated simultaneously with LPS/IFN-γ. In addition, Os was shown to possess NO scavenging activity. More in-depth studies are required to study the effect of both peptides on the suppression of LPS-induced expression of NO and TNF-α. It would be of great value to determine the effect of these peptides on the LPS-induced expression of iNOS and TNF-α. LPS-induced activation of the NF-κB protein complex is known to control the transcription of pro-inflammatory genes (section 1.3.1). NF-κB is activated as a result of phosphorylation of the IκB protein, which is normally bound to NF-κB (rendering it inactive), and translocated into the nucleus. It is expected that the

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majority of the p65 subunit would remain in the cytosol in the presence of Os and Os-C. Additionally, the MAPK signal transduction pathway plays a critical role in the modulation of LPS-induced activation of macrophages and results in the production of pro-inflammatory cytokine production. To evaluate the potential differences in NF-κB activation as a result of Os and Os-C, the phosphorylation of p38, ERK1/2 and JNK kinases can be studied. These results will indicate whether Os and Os-C can prevent LPS to induce MAPK pathways and subsequently NF-κB translocation.

The exposure of RAW 264.7 cells for 90 minutes with Os and Os-C, respectively, before stimulating the RAW 264.7 cells with LPS/IFN-γ exposed a possible different mechanism of action. The dosage effect obtained with pre-incubation was significantly lower than the dosage effect obtained with simultaneous incubation, which might indicate that Os and Os-C may possibly bind directly to the outer membrane of the RAW 264.7 cells or are able to cross the plasma membrane of the RAW 264.7 cells in a non-destructive manner. It is thus important to determine whether the peptides are able to enter these cells. This can be achieved using fluorescently labelled peptides, the localisation of which can be determined with fluorescence confocal microscopy.

Os and Os-C showed no cytotoxic effect towards RAW 264.7 cells, as was also found in previous cytotoxic and haemolytic studies by Prinsloo, *et al.* (2013). Bio-activity related to anti-inflammatory effects of Os and Os-C indicated that salts and proteins in serum do not cause a loss of activity and this activity will be retained in a physiological environment. Both Os and Os-C exhibited antioxidant activity and can be viewed as providing cellular protection against free radicals in the RAW 264.7 cells. As indicated in the results, large SEM values were obtained with analysis of the data. This might be due to the semi-adherence of the RAW 264.7 cells to the 96-well plates. As the methodology required several washing steps many unattached cells may be lost. Better results could be obtained by determining the *in vitro* antioxidant activity of both peptides with a LPS responsive adherent cell line, such as a promonocytic cell line, THP-1, that can be differentiated into adherent monocytes with retinoic acid, 1,25-dihydroxycholecalciferol and IFN-γ (Azofeifa, *et al.*, 2013).

This study has identified that Os and Os-C have potential anti-inflammatory properties, based on their ability to bind LPS and inhibit LPS-induced pro-inflammatory responses, as well as potential wound healing properties because of the antioxidant and NO scavenging properties. Both peptides

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have been shown to be not cytotoxic. Additional effects that can be investigated include direct cellular effects such as the ability of the peptides to promote cell migration and consequently wound closure. *In vitro* wound healing models can be used to determine these aspects. An easy, well developed and cost effective method is the scratch assay used to study cell migration and proliferation (Liang, *et al.*, 2007). Determination of the cellular hydroxyproline content in this model study can be used to determine the effect of Os and Os-C on collagen synthesis. In addition the effect of these peptides on wound contraction can be evaluated with the fibroblast wound contraction assay. Wound healing is a complex process and due to several restricting factors and limitations of the *in vitro* assays, it is necessary to study the effect of Os and Os-C with *in vivo* models (Gottrup, *et al.*, 2000).

Due to the inhibition of NO and TNF-α production by Os and Os-C in the *in vitro* model of inflammation used in this study, it is important to determine whether these effects are also obtained in an LPS-treated inflammatory mouse model. LPS injected intradermally into the ears of mice will induce inflammation in the skin. After injection, different concentrations of Os and Os-C, respectively, mixed with a fatty ointment can be applied to the surface of ears. After treatment blood can be collected and analysed for changes in the serum levels of NO and TNF-α as described in this study as well as changes in the levels of other pro-inflammatory markers. Following euthanizing the mice, the ears can be removed and histological analysis with immunohistochemistry can be used to determine the distribution of inflammatory cells such as macrophages (Kim, *et al.*, 2011; Lee, *et al.*, 2014). Furthermore, the effects of Os and Os-C on gene expression of inflammatory markers such as iNOS and TNF-α in this tissue can be determined by means of immunoblotting and RT-PCR analysis (Lee, *et al.*, 2014).

The ability of Os and Os-C to reduce inflammation and bacterial infection in wounds is a further important area of research and this includes the effect of Os and Os-C on wound specific bacteria as well as antibiotic resistant strains. Biofilm bacterial infections within wounds can persist for long periods due to their lower susceptibility to the immune defence system of the host (Percival and Bowler, 2004) and the effect of Os and Os-C on biofilm formation is another important aspect for investigation.

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CHAPTER 6: REFERENCES

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