University of Pretoria
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The role of cysteine residues on the bioactivity of the antimicrobial peptide Os derived from the tick *Ornithodoros savignyi*

Dissertation submitted in partial fulfilment for the degree Magister Scientiae with specialisation in Human Cell Biology

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Summary

Antimicrobial peptides (AMPs), small cationic molecules possessing a broad spectrum of bioactivities, have for long been considered promising multi-therapeutic candidates and antibiotic alternatives. High production costs and stability issues have however, limited the application of these molecules, emphasising the need for smaller, more stable, peptide analogues. Such synthetic analogues require structural and functional evaluation prior to their development and use.

Previously, the anti-endotoxin and anti-inflammatory potential of synthetic peptides, Os and its cysteine (Cys)-deficient analogue, Os-C, shown to exhibit more potent bactericidal activity than that of their parent peptide, tick defensin OsDef2, had been evaluated. Interestingly, Os demonstrated significantly greater anti-inflammatory activity compared with Os-C. To determine whether the Cys residues or a modified amino acid sequence was responsible for the differences observed between Os and Os-C, current research aimed to determine the cellular, antioxidant, and anti-inflammatory activity of the serine (Ser)-substituted Os analogue, Os(C→S). Selection of Ser was based on structural and chemical similarities between Ser and Cys residues.

Neither Os nor Os(C→S) elicited any significant toxic effect against mammalian L929 cells, human erythrocytes or human leukocytes. The cell-penetrating capacity of Os, compared with Os(C→S), although limited appeared to be associated with the presence of Cys residues, shown to be implicated in the overall hydrophobicity of the peptide. *In chemico,* Os and Os(C→S) were capable of inhibiting free radical production using both electron transfer (ET)- and hydrogen atom transfer (HAT)-based reactions. *In vitro,* however, the protective effect of Os(C→S) against 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH)-induced oxidative damage was largely negligible, implying some functional contribution of Cys residues to the antioxidant activity of Os. The *in chemico* nitric oxide (NO) scavenging potential of Os was significantly greater than that of Os(C→S), while *ex vivo,* extensive platelet activation was observed in whole blood (WB) exposed to both Os and Os(C→S), in the presence and absence of lipopolysaccharide (LPS), consistent with the inability of peptide to bind LPS.

The absence of significant antioxidant and anti-inflammatory activity by Os(C→S) correlates with the peptide's low hydrophobicity and associated limitation in it's ability to translocate across the cell membrane, reducing its intracellular availability and subsequent exertion of its protective effects. The thiol groups of sulphur-containing Cys residues present in Os therefore, play a significant role in the cell-penetrating and antioxidant activity of the peptide. Despite this, Os(C→S) may still be considered as an effective AMP owing to its potent antibacterial activity and lack of cytotoxicity in mammalian cells - emphasising the peptide's specificity towards bacterial cells.

Declaration of originality

I, René Stanley, declare that this dissertation submitted in partial fulfilment of the degree MSc Human Cell Biology, at the University of Pretoria, is my own work and has not previously been submitted at this or any other tertiary institution.

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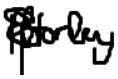
Signature:  **Date:** July 2018

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List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
3D	Three-dimensional
5-FAM	5-Carboxyfluorescein
A	
A. flavus	*Aspergillus flavus*
A. fumigates	*Aspergillus fumigates*
A. niger	*Aspergillus niger*
A. terreus	*Aspergillus terreus*
AA	Arachidonic acid
AAPH	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
AB	Alamar Blue
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ABTS$^{\bullet}$	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical
Ac	Acetyl
ADME	Absorption, distribution, metabolism and excretion
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
Ala	Alanine
AMP	Antimicrobial peptide
AMP*	Adenosine monophosphate
ANOVA	Analysis of Variance
Arg	Arginine
A-T	Adenine-thymine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU	Absorbance unit

AUC	Area under curve
B	
B. subtilis	*Bacillus subtilis*
C	
C. albicans	*Candida albicans*
C. krusei	*Candida krusei*
C. parapsilosis	*Candida parapsilosis*
Ca^{2+}	Calcium ion
CD	Cluster of differentiation
CD-40L	CD-40 ligand
CFU	Colony forming unit
cGMP	Cyclic guanosine monophosphate
Cit	Citrulline
cm^2	Square centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
CPP	Cell-penetrating peptide
CV	Crystal violet
CVD	Cardiovascular disease
Cys	Cysteine
Cys-SO_2H	Cys-sulphinic acid
Cys-SO_3H	Cys-sulphonic acid
Cys-SOH	Cys-sulphenic acid
D	
DAMP	Damage-associated molecular pattern
DAPI	4',6-Diamidino-2-phenylindole
DARC	Duffy antigen receptor for chemokines
DCF	Dichlorofluorescein
DCFH	Dichloro-dihydro-fluorescein
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
dddH_2O	Double distilled deionised water
DIC	Differential interference contrast
DMEM	Dulbecco’s Modified Essential Medium

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DTT	Dithiothreitol
E	
E. coli	*Escherichia coli*
ECACC	European Collection of Authenticated Cell Cultures
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ET	Electron transfer
EtOH	Ethanol
EU	Endotoxin units
F	
F. moniliforme	*Fusarium moniliforme*
F. oxysporum	*Fusarium oxysporum*
F. solani	*Fusarium solani*
F. verticilloides	*Fusarium verticilloides*
FBS	Foetal bovine serum
FEG	Field emission gun
G	
g	Gram
GAG	Glycosaminoglycan
GALA	Glutamate-alanine-leucine-alanine
GALT	Gut-associated lymphoid tissue
Glu	Glutamic acid/glutamate
Gly	Glycine
GP	Glycoprotein
GPL	Glycerophospholipid
GPx	Glutathione peroxidase
GSH	Glutathione
H	
H_2O_2	Hydrogen peroxide
HAT	Hydrogen atom transfer

Hb	Haemoglobin
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDP	Host defence peptides
His	Histidine
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
$HOCl^-$	Hypochlorous acid
$HOO^\bullet$	Hydroperoxide
HSA	Human serum albumin
HSV	Herpes simplex virus
I	
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
Ile	Isoleucine
INF	Influenza virus-derived peptide
isoPBS	Isotonic phosphate buffered saline
K	
K_2EDTA	Potassium ethylenediaminetetraacetic acid
$K_2S_2O_8$	Potassium persulphate
KCl	Potassium chloride
kDa	Kilodalton
KDO	2-keto-3-deoxyoctulosonic acid
L	
LAL	Limulus amoebocyte lysate
LB	Luria-Bertani
LBP	Lipopolysaccharide binding protein
Leu	Leucine
LFA	Leukocyte function-associated molecule
LPS	Lipopolysaccharide
LSM	Laser scanning microscope

LT	Leukotriene
LTA	Lipoteichoic acid
Lys	Lysine
M	
M. tuberculosis	*Mycobacterium tuberculosis*
Mal	MyD88 adaptor-like protein
MBC	Minimum bactericidal concentration
MCHC	Mean cellular haemoglobin concentration
MCP	Monocyte chemoattractant protein
MDR	Multidrug resistant
Met	Methionine
mg	Milligram
Mg^{2+}	Magnesium ion
MIC	Minimum inhibitory concentration
Min	Minimal
MIP	Macrophage inflammatory protein
ml	Millilitre
mM	Millimolar
mm	Millimetre
mmol	Millimole
mol	Mole
MSR	Macrophage scavenger receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD	Myeloid differentiation factor
N	
n.d.	Not determined
N_2O_3	Dinitrogen trioxide
Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaH_2PO_4 \cdot H_2O$	Sodium dihydrogen phosphate monohydrate
$NaNO_2$	Sodium nitrite

NAP	Neutrophil activating peptide
NED	*N*-naphthyl-ethylenediaminedihydrochloride
NET	Neutrophil extracellular trap
NF	Nuclear factor
NK	Natural killer
nm	Nanometre
NO	Nitric oxide
$NO^{\bullet}$	Nitroxyl
NO_2^-	Nitrite
$NO_2^{\bullet}$	Nitrogen dioxide
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NRF	National Research Foundation
NrTP	Nucleolar targeting peptide

O

O. savignyi	*Ornithodoros savignyi*
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide
OCS	Open canalicular system
OH^-	Hydroxyl ion
$OH^{\bullet}$	Hydroxyl ion
$ONOO^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
ONPG	ortho-nitrophenyl- β-D-galactopyranoside
ORAC	Oxygen radical absorbance capacity
Orn	Ornithine

P

P. aeruginosa	*Pseudomonas aeruginosa*
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDI	Protein disulphide isomerase
PEG	Polyethylene glycol
PF	Platelet factor
PG	Peptidoglycan
Phe	Phenylalanine
pI	Isoelectric point
PMN	Polymorphonuclear cell
p-NA	*p*-Nitroalinine
Pro	Proline
PRR	Pathogen recognition receptors
PS	Phosphatidylserine
PSGL	P-selectin glycoprotein ligand
PSIPRED	Protein Sequence Analysis Workbench
PTD	Protein transduction domain
R	
RANTES	Regulated upon activation normal T-cell expressed
RD	Radial diffusion
RDP	Research and Development Programme
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl
ROC	Republic of China
$ROO^{\bullet}$	Peroxyl
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
RPMI	Roswell Park Memorial Institute
RTI	Respiratory tract infection
RT-PCR	Real time-polymerase chain reaction
S	
S. aureus	*Staphylococcus aureus*
S/V	Surface area-volume ratio

SA	South Africa
SAA	Sulphanilamide
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
SEM	Scanning electron microscopy
Ser	Serine
siRNA	Small interfering ribonucleic acid
SM	Sphingomyelin
SMase	Sphingomyelinase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
sPL	Secretory phospholipase
T	
TB	Tuberculosis
TCR	T cell receptor
TEAC	Trolox equivalent antioxidant capacity
TEG	Thromboelastography
TF	Tissue factor
Thr	Threonine
TIR	Toll/interleukin-1 receptor-like domain
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-containing adaptor molecule
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Trp	Tryptophan
Tyr	Tyrosine
U	
U	Unit
USA	United States of America
UTI	Urinary tract infection
UV	Ultraviolet
V	

V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. vulnificus	*Vibrio vulnificus*
v/v	Volume per volume
Val	Valine
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
W	
w/v	Weight per volume
WB	Whole blood
WHO	World Health Organisation
X	
x *g*	Times gravity

Chapter 1: Introduction

Infectious diseases resulting from pathogenic host invasion are considered one of the major causes of mortality worldwide. The World Health Organisation (WHO) estimates that 23% of approximately 58 million deaths per year occur directly as a result of infectious disease (World Health Organisation, 2012). In South Africa, infectious diseases may be attributed to an estimated 40% of all deaths (Statistics South Africa, 2014).

Bacterial pathogens are a major target of drug development due to their continuous evolution into multidrug resistant (MDR) strains. Genetic mutation, induced by antibiotic abuse and negligence (Bantar *et al.*, 2003), and subsequent horizontal gene transfer is largely responsible for the evolution and spread of such MDR bacteria, enhancing their effects on both morbidity and mortality (Davies & Davies, 2010).

Numerous efforts aimed at alleviating this problem places considerable emphasis on Gram-positive bacteria, while the development of therapeutic agents that target Gram-negative bacteria, such as *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella* (Woodford & Livermore, 2009), has been largely neglected. Gram-negative bacteria possess an outer membrane that is capable of conferring antibiotic resistance; porins that enhance their membrane impermeability to antibiotics; as well as both endotoxins and exotoxins, making them arguably one of the most difficult organisms to treat (Hayat, 2013; Bush, 2015). This generates serious risks and challenges, particularly in the treatment of nosocomial infections. The development of therapeutic strategies that are capable of preventing the accumulation and spread of such microorganisms is, therefore, imperative to the combat of this global threat.

AMPs are promising therapeutic agents exhibiting antimicrobial potential against bacteria, fungi, viruses and protozoa. AMPs are generally non-specific molecules that directly interfere with microbial cell membranes, limiting their development of resistance (Wang *et al.,* 2016). Several AMPs are additionally capable of eliciting antioxidant and immunomodulatory effects (Hancock, 2001; Gordon & Romanowski, 2005; Marr *et al.,* 2006). Although research was initially focused on the antimicrobial activity of AMPs, more attention has recently been given to the multifunctional properties of these molecules (Pushpanathan *et al.,* 2013). Several such peptides have been shown in animals to inhibit LPS-induced inflammation, providing protection against oxidative damage associated with circulatory or lymphatic spread of pathogenic infection and septic shock

(Scott *et al.*, 2000). Protease susceptibility; insufficient knowledge of toxicity profiles and systemic effects; as well as high production cost have however, limited the direct use of AMPs as therapeutic agents. AMPs are therefore instead used as templates for the development of structural analogues having decreased size, increased activity and resistance to proteases (Giuliani *et al.,* 2008).

One such AMP, OsDef2, a defensin isolated from the haemolymph of the soft tick *Ornithodoros savignyi* (*O. savignyi*), previously served as a template from which two peptides, Os and Os-C, were derived. Studies performed by Prinsloo *et al.* (2013) and Malan *et al.* (2015) on Os and Os-C reported potent antibacterial activity against *E. coli* (American Type Culture Collection (ATCC) 700928), *P. aeruginosa* (ATCC 10145), *Bacillus subtilis* (*B. subtilis*, 13933) and *Staphylococcus aureus* (*S. aureus*, U3300) as well as significant free radical scavenging for both peptides, while the NO scavenging capacity of Os was found to be significantly greater than that of its Cys-deficient analogue - bringing into question the functional significance of Cys residues on the bioactivity of Os.

The thiol or sulfhydryl containing side chain of Cys residues present in peptides is considered ionisable and following deprotonation and subsequent formation of a negatively charged thiolate anion (Cys-S^-), exhibits enhanced reactivity (Poole, 2015). In the presence of hydrogen peroxide (H_2O_2), Cys-S^- undergoes oxidation generating Cys-sulphenic acid (Cys-SOH). Cys-SOH is unstable and reacts readily with thiols either forming inter- and intramolecular disulphide bonds or further oxidising to Cys-sulphinic acid (Cys-SO_2H) and Cys-sulphonic acid (Cys-SO_3H). Such redox sensitivity is characteristic of clustering of Cys residues to other Cys residues resulting in Cys-dimerisation and intramolecular disulphide bonds, generating cyclic peptides with modified biological activity, specificity and stability (Patil *et al.,* 2015). Oxidation of Cys residues at the N-terminus region of peptides or proteins as well as those in close proximity to lysine (Lys) residues has additionally been implicated in the generation of intramolecular sulphenamide, sulphinamide and sulphonamide (Wang *et al.,* 2004). Frequently, dissolution of peptides in acidic solvents or dithiothreitol (DTT) prevents unwanted oxidation, reducing disulphide bond formation and preserving the original thiols (Carter, 2015).

The aim of this study was, therefore, to determine the functional significance of Cys residues in the cellular, antioxidant and anti-inflammatory activity of Os, using the Ser-substituted analogue, Os(C→S). Ser has similarities in both the structural and chemical properties of Cys, i.e. polarity,

hydrogen bonding capacities and size; but does not interact to form disulphide bonds (Poole, 2015).

Chapter 2: Literature Review

The immune systems of all multicellular organisms are composed of an innate and, in vertebrates, an adaptive immune response. The innate immune response is responsible for the release of various host defence peptides (HDPs), including AMPs, which provide protection against invading pathogens, such as Gram-negative and Gram-positive bacteria, fungi and viruses (Campbell & Reece, 2005). Failure of the immune system to respond to host invasion and provide protection against subsequent disease and infection necessitates therapeutic intervention. Continuous use and abuse of such drugs, particularly antibiotics, has however, contributed to the ever-expanding global crisis of bacterial resistance, particularly within the agricultural and healthcare sectors. Such resistance to existing antibiotics has stimulated the research and development of novel therapeutic agents (Lazarev & Govorun, 2010). Recently, considerable emphasis has been placed on the development of AMPs, an extensive group of naturally occurring amino acid compounds isolated from the innate immune system of various vertebrate, invertebrate and plant species (Kindt *et al.*, 2007); several of which have already shown some success in clinical trials (Fox, 2013).

This chapter will review infection, inflammatory response and the ability of AMPs to target specific components and/or pathways associated with infection and inflammation.

2.1 Infection and immunity

Multicellular organisms rely largely on their immune systems, generating a broad range of cells and molecules for the recognition and elimination of foreign pathogens. In humans, such protection is tightly regulated by both the innate and adaptive immune systems (Kindt *et al.*, 2007).

The innate immune response is considered the first line of rapidly acting, non-pathogen specific defence (Kindt *et al.*, 2007). Innate immune systems are composed of three barriers, i.e. a physical barrier constituting the skin and mucous membranes; a chemical barrier produced by stomach acids and soluble antimicrobial molecules; and a cellular barrier composed of surface receptors responding to foreign stimulus. During an innate immune response, the rate of infection spread at the site of tissue damage is reduced following the accumulation of immune cells and chemical mediators (Madigan *et al.*, 2003).

Adaptive immune responses on the other hand, target particular foreign agents, often reacting over an extended period of time, i.e. several days, following initial exposure to the pathogen (Silverthorn, 2007). Adaptive immunity is directed by dendritic cells, localising antigens at the site of infection and presenting them to T cells within lymphoid tissue (Kindt *et al.*, 2007).

2.2 Infection and inflammatory response

Foreign pathogenic or toxic exposure following tissue damage is responsible for the induction of a non-specific pro-inflammatory reaction, stimulating a host defence inflammatory response and initiating tissue repair (Kindt *et al.*, 2007). Inflammatory response is an essential reaction which occurs within all living organisms capable of wound healing, producing localised redness, oedema, pain and heat.

Inflammation may be classified as either acute, or chronic according to the duration of the inflammatory response. Acute inflammation occurs when an inflammatory stimulus is removed resulting in immediate cessation of the response. Chronic inflammation, on the other hand, is a pathological condition resulting from the continuous stimulation and induction of inflammation and tissue damage, characterised by the persistence of mononuclear immune cells, including macrophages, monocytes and leukocytes (Vetriselvan *et al.*, 2013). During inflammation, cytokines and mediators, including chemokines, bradykinin, thrombin, plasmin and anaphylatoxins, are released by both innate and adaptive immune systems inducing an inflammatory response (Kindt *et al.*, 2007) and subsequent initiation of angiogenesis and fibrosis (Vetriselvan *et al.*, 2013). If left untreated, tissue damage, initially repaired by inflammatory response, may accumulate into organ damage and ultimately, septic shock (Qureshi & Rajah, 2008).

AMPs contribute considerably to controlling microbial proliferation and host cell signalling in response to injury. AMPs are predominantly cationic and act against microbes via simple electrostatic interactions with the anionic phospholipids of microbial cell membranes resulting in membrane disruption or destruction and ultimately cell death (Table 2.1). The development of microbial resistance is, therefore, less prevalent compared to conventional antibiotics.

Table 2.1: Microorganisms effectively inhibited *in vitro* using AMPs.

Microorganism	Clinical effects	Reference
Acid-fast bacteria		
Mycobacterium tuberculosis (*M. tuberculosis*)	Tuberculosis (TB): chronic cough with bloodied sputum, fever and weight loss	(Kang *et al.,* 2017)
Gram-positive bacteria		
S. aureus	Skin infections e.g. impetigo, acute infective endocarditis, sepsis, necrotising pneumonia, meningitis, osteomyelitis, toxinoses	(Omardien *et al.,* 2016; Kang *et al.,* 2017)
Staphylococcus epidermidis	Infections of implanted prostheses and catheters	(Kang *et al.,* 2017)
Enterococcus faecalis	Bacterial endocarditis, biliary tract infection, urinary tract infection (UTI)	(Kang *et al.,* 2017)
Gram-negative bacteria		
E. coli	Diarrhoea, UTI, infantile meningitis, hospital-acquired pneumonia and sepsis	(Kang *et al.,* 2017)
P. aeruginosa	Pneumonia, UTI, corneal infection, endocarditis, osteomyelitis, burn wound infection, sepsis, malignant external otitis	(Kang *et al.,* 2017)
Aeromonas hydrophila	Gastroenteritis, wound infection (cellulitis), sepsis, ocular and respiratory tract infection (RTI), UTI	(Kang *et al.,* 2017)
Vibrio species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*)	Gastroenteritis, sepsis	(Kang *et al.,* 2017)
Salmonella typhimurium	Salmonellosis with gastroenteritis, parathyroid fever, osteomyelitis, sepsis	(Kang *et al.,* 2017)
Fungi		
Candida species (*C. albicans*, *C. krusei*, *C. parapsilosis*)	Candidiasis e.g. oropharyngeal, vulvovaginal, subpreputial, fungaemia, invasive	(Hegedüs & Marx, 2013; Kang *et al.,* 2017)
Aspergillus species (*A. terreus*, *A. niger*, *A. flavus*, *A. fumigates*)	Aspergilloses e.g. allergic bronchopulmonary, acute invasive, disseminated invasive, aspergilloma	(Rautenbach *et al.,* 2016)
Fusarium species (*F. solani*, *F. verticilloides*, *F. oxysporum*, *F. moniliforme*)	Onychomycosis, keratomycosis, neutropenia, fungaemia	(Rautenbach *et al.,* 2016)

Viruses		
Human immunodeficiency virus (HIV)-1	HIV and acquired immune deficiency syndrome (AIDS)	(Carriel-Gomes *et al.,* 2007)
Herpes simplex virus (HSV)-1 and -2	Oral and genital herpes	(Carriel-Gomes *et al.,* 2007)
Human cytomegalovirus (HCMV)	HCMV	(Carriel-Gomes *et al.,* 2007)
Respiratory syncytial virus	RTI	(Carriel-Gomes *et al.,* 2007)
Hepatitis B and C virus (HBV and HCV)	Hepatitis	(Carriel-Gomes *et al.,* 2007)
Rotavirus	Gastroenteritis	(Carriel-Gomes *et al.,* 2007)
Human adenovirus	Upper RTI	(Carriel-Gomes *et al.,* 2007)
Parasites		
Leishmania	Leishmaniasis	(Bahar & Ren, 2013)

Considerable emphasis has been focused on the development of AMPs as potential immunomodulators, the effects of which relies on several mechanisms, including direct antimicrobial activity, preventing infections; the binding and inactivation of bacterial toxins, such as LPS, reducing pro-inflammatory response; and the influence on cellular behaviour, enhancing cellular migration and proliferation (Mangoni *et al.,* 2016). The *in vivo* contribution of AMPs to inflammatory responses additionally depends on their capacity to induce the production of pro-inflammatory cytokines, enhancing phagocytosis (Méndez-Samperio, 2013). Several AMPs are currently in commercial development for the treatment of inflammatory diseases (Table 2.2).

Table 2.2: AMPs in clinical trial phases for the treatment of inflammatory diseases (Mahlapuu *et al.*, 2016; Safder & Islam, 2017)

AMP	Description	Indication	Phase	Outcome of clinical trial
Pexiganan (MSI-78)	Analogue of magainin (skin of the African clawed frog)	Diabetic foot ulceritis	Phase III	Phase III trials show no advantage over existing therapies.
Orniganan	Derived from indolicidin (bovine)	Acne	Phase II/III	Phase II trials showed considerable efficacy. Phase III trials unsuccessful.
hLF1-11	Derived from lactoferricin (human)	Bacteraemia and fungal infections in immunocompromised haematopoetic stem cell transplant recipients	Phase I/II	Positive phase I trials. Under phase II trials.
Iseganan (IB-367)	Derived from protegrin-1 (porcine leukocytes)	Oral mucositis in patients receiving radiotherapy	Phase III	Phase III trials show no advantage over existing therapies.
PAC-113	Derived from histatin-3 (human saliva)	Oral candidiasis in HIV sero-positive patients	Phase II	Phase IIb trials showed positive results with increased endpoint efficacy

2.3 Gram-negative bacteria, LPS and inflammation

The bacterial cell membrane, comprising both an inner cell membrane and outer cell wall, is designed to provide protection from otherwise damaging environmental stresses (Silhavy *et al.*, 2010). Gram-negative bacterial cell walls possess extensive outer and cytosolic membranes, anchored to each other via periplasmic lipoproteins and separated by a thin layer of peptidoglycan (PG) (Cohen, 2002; Madigan *et al.*, 2003). The outer membrane bilayer is composed largely of outer, negatively charged LPS and inner phospholipids. LPS alters membrane permeability, limiting the movement of toxic and pathogenic substance into the cell (Li *et al.*, 2012) and inducing the production and subsequent release of pro-inflammatory cytokines, i.e. tumour necrosis factor-α (TNF-α) and interferons (IFNs), in host macrophages (Park *et al.*, 2011). Entry of essential nutrients is permitted by porins, forming transmembrane diffusion channels (Li *et al.*, 2012).

LPS is composed of a lipid A component, consisting of a biphosphorylated D-glucosamine disaccharide backbone with between six and seven fatty acyl chains per molecule; and a

hydrophilic polysaccharide component, formed by a core and O-specific polysaccharide, or antigen (Ding *et al.*, 2003; Hitchcock *et al.*, 1986). The core polysaccharide consists of 2-keto-3-deoxyoctulosonic acid (KDO), among others, that interacts with the phosphorylated β1.6 linked D-glucosamine of lipid A. The antigen, consisting of a chain of three to eight repetitive oligosaccharides, is strain-specific and capable of serological identification of different bacterial strains (Madigan *et al.*, 2003). Lipid A is considered the active component of LPS, responsible for expressing endotoxic activity. The negative charges associated with the core oligosaccharide and phosphate group of LPS is linked to the affinity of LPS for cations (Sun & Shang, 2015).

The tolerance of bacteria towards drugs is correlated with the thickness of the LPS layer which is capable of preventing the import of toxic molecules, enhancing the survival of bacteria. The permeability barrier of LPS is thought to be stabilised by LPS-associated cations, including magnesium ion (Mg^{2+}), through salt bridges that function in the neutralisation of repulsive forces permitting the association between adjacent LPS molecules. This provides bacteria protection from various host-defence hydrophobic molecules and permits the adhesion of bacteria to the surface of host cells, initiating an innate immune response (Sun & Shang, 2015).

Bacterial LPS is released during cell division, cell death or antibiotic treatment of infection (Rosenfeld *et al.*, 2006). When released into the bloodstream (Youn *et al.,* 2011), LPS binds to LPS-binding protein (LBP) forming a complex that interacts with a primary receptor, cluster of differentiation (CD)-14 (Li *et al.*, 2012). CD-14 may either be soluble, present in tissue fluid, or bound to the surface of macrophages (Cohen, 2002). The complex initiates toll-like receptor (TLR) signalling, by binding to transmembrane protein, TLR-4 (Cohen, 2002), ultimately activating the nuclear factor (NF)-κB pathway. The pathway activates the transcription of genes encoding inflammatory cytokines, adhesion molecules and other innate immune response effectors (Kindt *et al.*, 2007).

Vascular modification plays an essential role in inflammation (Figure 2.1). Histamine, prostaglandins and NO induce vasodilation, increasing blood flow and recruiting circulating leukocytes while inflammatory mediators, such as histamine and leukotrienes, influence endothelial cells, increasing vascular permeability and permitting the departure of plasma proteins and leukocytes from circulation. Cytokines, including TNF and interleukin (IL), promote leukocyte extravasation by increasing the concentrations of leukocyte adhesion molecules on endothelial cells. Activated immune cells remove foreign molecules and host debris via phagocytosis, further

secreting cytokines that modify the lymphocyte-mediated adaptive immune response (Newton & Dixit, 2012).

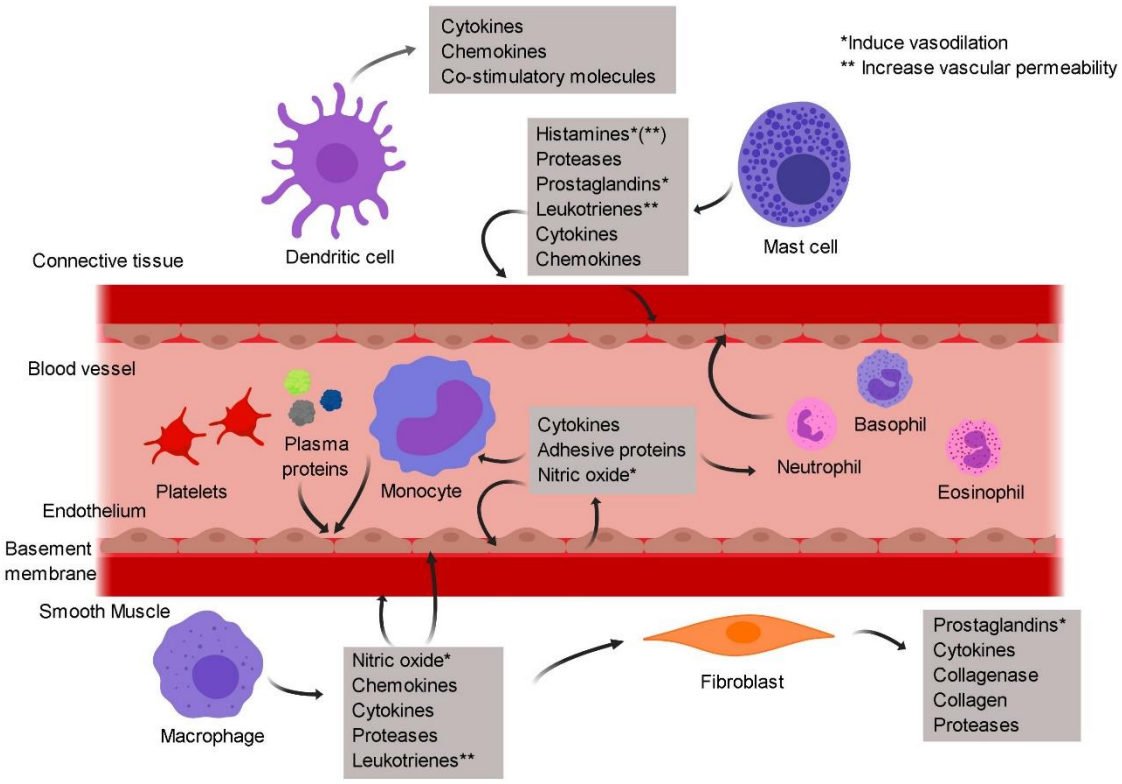


Figure 2.1: Cells and mediators associated with inflammation. Plasma protein-derived molecules and cells mediate inflammation in response to tissue damage or pathogenic invasion by stimulating vascular modification and leukocyte migration and activation (Newton & Dixit, 2012).

Several AMPs are capable of modulating this response by antagonising LPS. Such LPS-neutralising peptides primarily interact with the phosphate head groups of the lipid A component of LPS. Peptides capable of binding LPS typically have the motif of BBXB (B: any basic amino acid; X: any hydrophobic amino acid) or BZB (B: any basic amino acid; Z: any aromatic ring-containing amino acid) (Youn *et al.,* 2011) as observed in melittin (GIGAVLKVLTTGLPALISWIKRKRQQ), with overlapping BBXB and BZB motifs, and polymyxin B, containing 6 basic amino acid residues of α, γ-diaminobutyric acid, a hydrophobic leucine (Leu) residue and aromatic phenylalanine (Phe) residue (Paulus & Gray, 1964).

2.3.1 The role of macrophages in infection and inflammation

Macrophages are essential mediators of the progression of tissue inflammation. Macrophages are classified into two distinct activation states according to their unique reaction to different stimuli. M1 macrophages, or classically activated macrophages, are involved in microbial defence and tumour regression while M2 macrophages, or alternatively activated macrophages, function in parasitic infections, tissue modelling, immunoregulation, allergies and tumour progression (Chanput *et al.,* 2013).

During bacterial infection, macrophage scavenger receptors (MSRs) bind LPS, stimulating the secretion of pro-inflammatory cytokines and mediators, including TNF-α, IL and NO; and initiating an inflammatory response (Kindt *et al.*, 2007). Activated macrophages subsequently increase their phagocytic activity, readily degrading invading pathogens in available phagosomes (Cohen, 2002). Overstimulation of macrophages may however, result in unregulated expression and secretion of pro-inflammatory cytokines and mediators, contributing to the pathogenesis of septic shock (Rosenfeld *et al.*, 2006), particularly in the case of systemic infection (Vodovotz *et al.*, 1994).

Macrophage activity is regulated by inflammatory mediators - reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Syahida *et al.*, 2006). ROS is a normal by-product of metabolism, functioning as a secondary messenger in inflammatory signal transduction by stimulating the increased expression of both cytokines and chemokines (Guzik *et al.*, 2003). The toxic effect of NO is mediated by the product of its interaction, peroxynitrite ($ONOO^-$), with superoxide ($O_2^{\bullet -}$) radicals. Overproduction of free radicals stimulates oxidative stress and subsequent inflammatory response resulting in cell membrane, deoxyribonucleic acid (DNA) and protein oxidation-induced tissue damage (Hsu & Wen, 2002).

2.3.2 The role of neutrophils in infection and inflammation

Neutrophils, immune cells constituting between 40 and 70% of leukocyte fractions, are considered the primary non-specific host defence mechanism against invading microorganisms. The activation of neutrophils by bacterial products, cytokines or chemokines, such as TNF-α, IL-8 and IFN-γ, plays an essential role in the regulation of inflammatory response. During neutrophil activation, intracellular granules mobilise to the plasma membrane, increasing the expression of cell-surface receptors. The subsequent migration of neutrophils from the circulation to the site of

inflammation is largely regulated by interactions with the vascular endothelium (Wright *et al.,* 2010).

In the presence of LPS, neutrophil activation is regulated by the simultaneous down-regulation of L-selectin and up-regulation of CD-11b/-18 on the cell surface. L-selectin is a transmembrane, constitutive monomeric protein expressed on the surface of neutrophils (Soler-Rodriguez *et al.,* 2000) that permits the interaction between ligands on the surface of endothelial cells, e.g. E- and P-selectin, and P-selectin glycoprotein ligand (PSGL)-1 (Wright *et al.,* 2010). CD-11b/-18 on the other hand, is a transmembrane, constitutive dimeric glycoprotein (GP) formed by the noncovalent association of one α- and one β-subunit. Both L-selectin and CD-11b/-18 are essential for the mediation of neutrophil adhesion to the vascular endothelium, permitting the migration of neutrophils from the blood into infected tissue during early inflammation (Soler-Rodriguez *et al.,* 2000).

The migration of neutrophils from the circulation to site of inflammation occurs along a chemotactic gradient, through the junctions of adjacent endothelial cells and via surface ligands including intercellular adhesion molecule (ICAM)-2, platelet endothelial cell adhesion molecule-1 and proteins of the junctional adhesion molecule family (Wright *et al.,* 2010). At the site of infection, membrane receptors recognise and bind opsonised bacteria resulting in the formation of pseudopodia, phagocytosis of the pathogen and subsequent destruction within the intracellular phagosome (Böhmer *et al.,* 1992; Nagano *et al.,* 2002; Wright *et al.,* 2010). The secretion of enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, during phagocytosis does however, promote the production of toxic ROS (Boots *et al.,* 2012) and susbequent oxidative burst, often damaging host tissue and inappropriately activating neutrophils to release tissue-damaging molecules, such as proteases, or pro-inflammatory chemoattractants and cytokines (Wright *et al.,* 2010).

2.3.3 The role of platelets in infection and inflammation

Platelets have recently been identified as key effectors in systemic inflammatory processes, both initiating local and systemic inflammatory reactions and participating in inflammation associated with tissue injury (Ding *et al.,* 2014).

Platelets are anucleate, discoid cells approximately 2-3 µm in diameter. Between 150 and 400 x 10^9 platelets per litre of WB are estimated to circulate throughout the average human body,

persisting for up to 10 days (Thon & Italiano, 2012; Thomas & Storey, 2015). Three major types of storage granules are present within platelets, including α-granules, dense granules and lysosomes. α-Granules play a significant role in innate immunity where they either function in the modulation of platelet adhesion factor expression for leukocyte interaction or by releasing cytokines for leukocyte modification. Chemokines including CXCL1, platelet factor (PF)-4, CXCL4, CXCL5, CXCL7, IL-8, CXCL8, CXCL12, macrophage inflammatory protein (MIP)-1α or CCL3, regulated upon activation normal T-cell expressed (RANTES) or CCL5 are major constituents of α-granules functioning in the up-regulation of leukocyte pro-inflammatory processes such as leukocyte movement, vascular migration, phagocytosis and the generation of ROS (Thomas & Storey, 2015).

PF-4 possesses a broad spectrum of activities associated with innate immunity, including chemotaxis in both monocytes and neutrophils. PF-4 is responsible for the release of neutrophil granules and subsequent endothelial adhesion, and mediates the production of L-selectin and leukocyte function-associated molecule (LFA)-1. In monocytes alone, PF-4 prevents apoptosis, induces differentiation into macrophages for phagocytosis and stimulates the production of ROS. Additionally, PF-4 stimulates the release of cytokines, including CXCL8, CXCL3, IL-1α, IL-1β, IL-6, IL-19, TNF-α, CCL2, CCL3 and CCL22, which combined with RANTES promotes monocyte arrest and the up-regulation of E-selectin expression (Thomas & Storey, 2015).

Platelets are capable of expressing numerous receptors, common to immune cells, including members of the TLR family. These receptors are capable of recognising both microbial-derived molecules and molecules released by the host by damage-associated molecular patterns (DAMPs) (Ding *et al.,* 2014). The release of DAMPs resulting from tissue damage and cell death, particularly necrosis, is responsible for the initiation of an inflammatory response (Thomas & Storey, 2015). TLR-2, TLR-3, TLR-4 and TLR-9 contribute considerably to inflammatory responses associated with haemorrhagic shock and other associated sterile injury (Ding *et al.,* 2014).

TLRs transmit warning signals to the innate immune system, mediating inflammatory events capable of recruiting and activating cells of the adaptive immune system in response to invading pathogens. Numerous studies have identified TLR-4 (Figure 2.2) on platelets, where it functions in LPS-induced thrombocytopenia, cytokine production, and platelet accumulation in the liver and lungs. In other cell types, both platelet activation and LPS-induced TLR-4 signalling is associated

with increased thrombotic risk (Wu *et al.,* 2015) and contributes significantly to the pathogenesis of organ injury following haemorrhagic shock (Ding *et al.,* 2014). Since TLR-4 expression increases with advancing megakaryocyte maturation, platelets are thought to acquire their expression of TLR-4 from megakaryocyte membranes during proplatelet formation (Wu *et al.,* 2015).

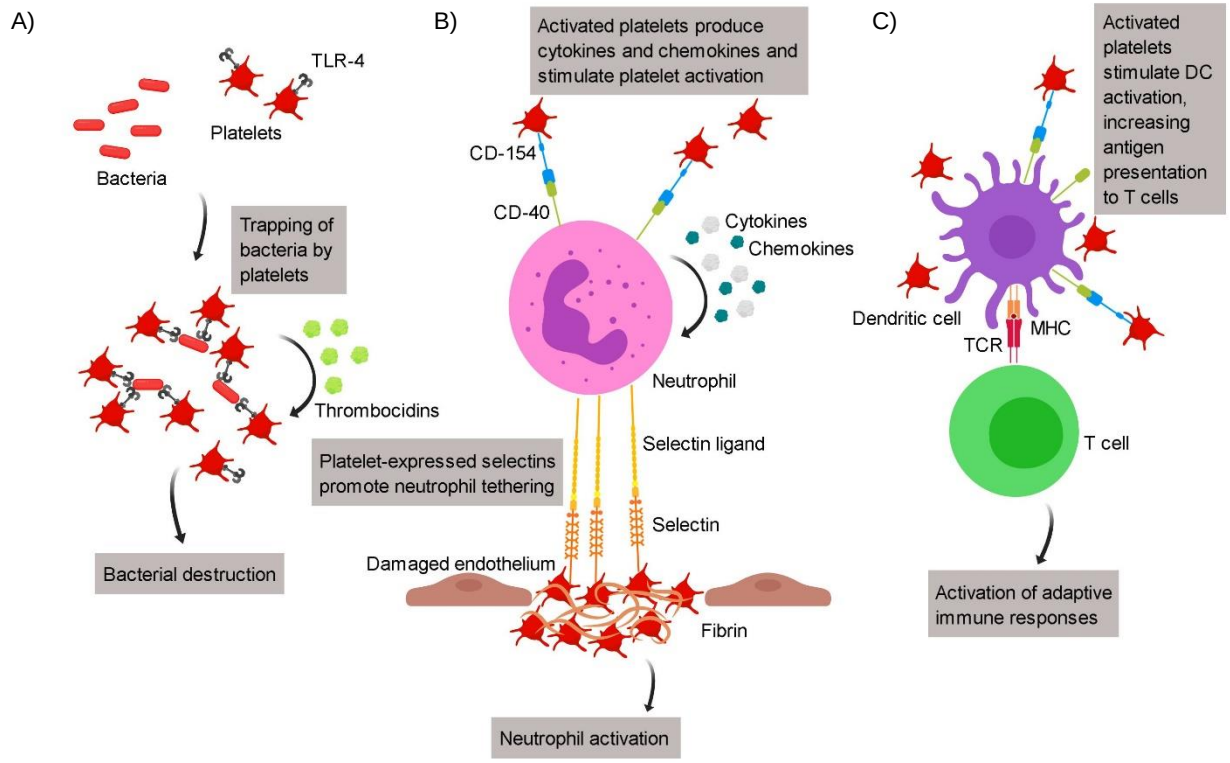


Figure 2.2: Examples of how activated platelets can mediate cell-to-cell interactions and affect innate immune responses. A) Platelet TLR-4 expression enables the binding of activated platelets to bacteria, directly killing the bacteria by the production of thrombocidins or via aggregation and bacterial entrapment for subsequent phagocytosis. B) Interaction of platelets with leukocytes, promoting neutrophil tethering and activation through the selective expression of selectins, CD-40 ligand (CD-40L) and inflammatory cytokines and chemokines. C) Platelet-induced stimulation of monocyte and dendritic cell activation via CD-40-CD-40L interactions resulting in increased antigen presentation to T cells and enhancement of adaptive immune responses (Semple *et al.,* 2011). CD: Cluster of differentiaion; DC: Dendritic cell; MHC: Major histocompatibility complex; TCR: T cell receptor; TLR: Toll-like receptor.

The contribution of TLR-4 to platelet activation and associated functional modifications was demonstrated by Ding *et al.* (2014) using TLR-4 knockout mice. The selective deletion of TLR-4 from platelets was shown to inhibit the increase in circulating IL-6, promoting platelet segregation and organ injury. Additionally, the absence of TLR-4 contributed extensively to the impairment of platelet function and the deposition of platelets in the liver and lung following haemorrhage.

Circulation of platelets through the vasculature of inflamed tissue exposes them to numerous soluble inflammatory mediators including lipid mediators, e.g. platelet-activating factor (PAF); cytokines, e.g. IFN-γ and IL-2; and chemokines, e.g. CXCL12 and CCL22, released by activated leukocytes, endothelial cells and perivascular cells (Figure 2.3). These mediators interact with receptors on platelets, initiating an activation response characterised by the degranulation of dense granules and α-granules, the release of platelet products and the mobilisation and activation of platelet adhesion molecules. Oxidative stress accompanying inflammatory signalling further stimulates the activation of phospholipase A2 and the production of PAF and other arachidonic acid metabolites, contributing to the induction of platelet activation (Stokes & Granger, 2012).

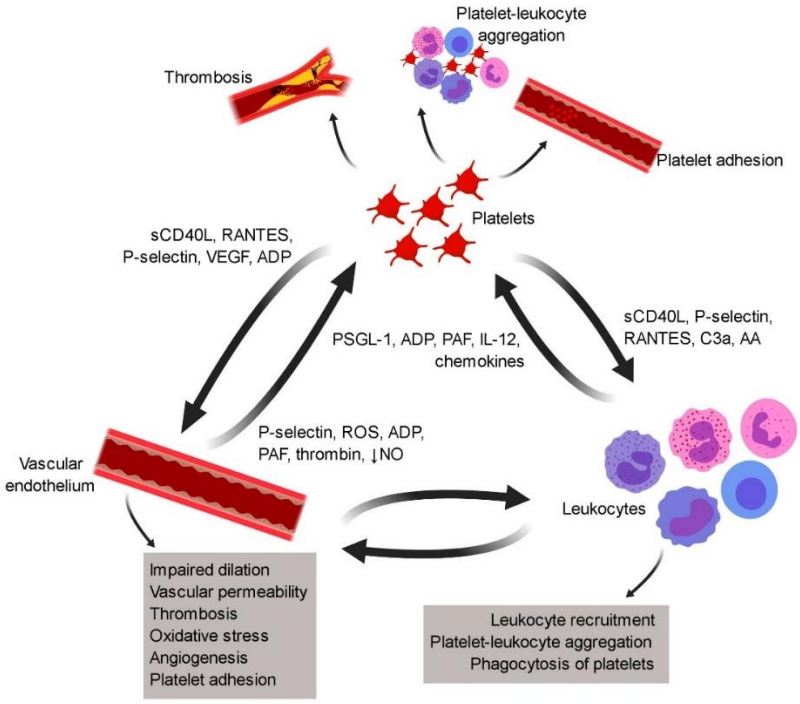


Figure 2.3: The interaction between platelets, vascular endothelium and leukocytes in response to inflammatory stimuli. Arrows indicate the direction of communication, demonstrating both the physical interactions (via adhesion molecules, e.g. P-selectin) and soluble factors (e.g. chemokines and cytokines) that function in the regulation of autocrine and paracrine pathway mediated cell-to-cell communication. Pathological changes resulting from platelet interference are indicated below the endothelial cells and leukocytes and above the platelets (Stokes & Granger, 2012). AA: Arachidonic acid; ADP: Adenosine diphosphate; IFN: Interferon; PAF: Platelet-activating factor; IL: Interleukin; LTB_4: Leukotriene B_4; PSGL: P-selectin glycoprotein ligand; RANTES: regulated upon activation, normal T cell expressed; ROS: Reactive oxygen species; sCD40L: Soluble CD40-ligand; VEGF: vascular endothelial growth factor.

Engagement of platelet-expressed chemokine receptors, CXCR4 and CCR4, with their respective chemokine ligands, CXCL12 and CCL22, induces the expression of P-selectin and subsequent release of several compliment chemokines and additional granular components from platelets into the extracellular fluid. Cytokines function by promoting degranulation of either dense granules, as is the case for IFN-γ, or α-granules. The degranulation of dense- and α-granules is further promoted by the production of adenosine diphosphate (ADP) and subsequent stimulation of platelet $P2Y_1$ and $P2Y_{12}$ G-protein coupled receptors (Thomas & Storey, 2015), induced both by the accumulation of immune cells in inflamed tissue and rapid conversion of adenosine triphosphate (ATP) via lymphocyte-expressed ecto-ATPase. Platelet function is additionally modified by the release of microparticles from the membranes of endothelial cells, following cytokine activation (Stokes & Granger, 2012).

Activated platelets typically bind to the walls of inflamed microvessels, either directly by attaching to endothelial cells, or indirectly via attachment to already adherent leukocytes. The absence of platelet adhesion is attributed to inhibitory mechanisms associated with NO, prostacyclin and adenosine – normally produced within the vascular endothelium. Reduced capillary perfusion or the inhibition of ectonucleotidase CD-39, expressed on the surface of endothelial cells and circulating immune cells, stimulates the hydrolysis of ATP to ADP. ATP hydrolysis promotes platelet adhesion and aggregation, and subsequent conversion of ADP to adenosine monophosphate (AMP*) and ultimately adenosine. The formation of adenosine from AMP* inhibits platelet adhesion and aggregation. Oxidative stress and pro-inflammatory cytokines, i.e. TNF-α, downregulates the expression of CD-39, promoting the accumulation of ADP and subsequent inhibition of adenosine production. Excess production of superoxide in inflamed tissue induces NO inactivation and further reduces the expression of endogenous CD-39. The combined depletion of adenosine and NO, and the accumulation of inflammatory mediators functioning in platelet activation, results in the increased expression of adhesion molecules, enabling the interaction of platelets with endothelial cells and circulating blood cells (Stokes & Granger, 2012).

Numerous adhesion GP-ligand pairs have been implicated in inflammation-mediated platelet and endothelial cell adhesion, including P-selectin (endothelial cells or platelets)-PSGL-1 (endothelial cells or platelets), GP-Ib (platelet)-von Willebrand factor (vWF; endothelial cells), and GP-IIb/-IIIa (platelets)-fibrinogen ICAM-1 (endothelial cells) interactions (Stokes & Granger, 2012). The close proximity of adherent platelets and endothelial cells enables the deposition of large quantities of chemokines, cytokines and growth factors, including vascular endothelial growth factor (VEGF),

inducing signal transduction pathways that assist in the regulation of endothelial cell metabolic, adhesive and proliferative properties (Stokes & Granger, 2012).

During platelet activation and aggregation, exposed collagen interacts with platelet GP-Ib/-V/-IX complex in the presence of vWF. Exposed collagen subsequently binds to platelet membrane receptors: GP-Ia/-IIb and GP-VI, inducing platelet activation. Mediators released by activated platelets, including serotonin, ephinephrine and thrombin, further activate platelet G-protein coupled receptors and increase calcium (Ca^{2+}) levels within the cytosol. Ca^{2+} induces changes in platelet morphology, such as the formation of pseudopodia measuring up to 5 µm in length (Thon & Italiano, 2012); as well as integrin activation for stable adhesion (Thomas & Storey, 2015).

Following platelet activation, platelets release more than 300 biologically active proteins and small molecules, capable of influencing the function of both the vascular wall and circulating immune cells. Numerous such molecules, including growth factors, cytokines, chemokines, angiogenic factors, ATP/ADP and coagulation factors are preformed and stored in dense bodies or α-granules for subsequent release during platelet activation. Other bioactive molecules either undergo synthesis by platelets, e.g. thromboxane, ROS and IL-1β; or are released from the surface of activated cells, e.g. CD-40L. CD-40L is a membrane GP, belonging to the TNF family, expressed on platelets and other circulating cells. Co-localisation of $CD\text{-}40L^{+}$ platelets and endothelial cells results in endothelial cell activation, increasing the expression of endothelial adhesion molecules, including ICAM-1; the production of neutrophil chemoattractant IL-8 and the adhesion between leukocyte and endothelial cells (Stokes & Granger, 2012). This response and subsequent enhancement of platelet-endothelial cell interaction, is however, dependent on both the interaction of platelet CD-40L with endothelial cell CD-40 as well as the secretion of the chemokine, RANTES (Stokes & Granger, 2012). Platelets are considered the primary source of RANTES, a member of the CC-chemokine family. RANTES functions in the induction of leukocyte chemotaxis, promoting leukocyte activation and adhesion through an oligomerisation-dependent interaction with glycosaminoglycans (GAGs) on the surface of endothelial cells. This interaction ensures that a high concentration of blood cell-derived RANTES is localised to the endothelial cell surface of damaged or inflamed tissue (Stokes & Granger, 2012).

2.3.4 The role of erythrocytes in infection and inflammation

Erythrocytes structure and function is largely dependent on the lipid composition of the plasma membrane, frequently modified during sepsis. Erythrocyte membranes are predominantly composed of phospholipids, with glycerophospholipid (GPL), phosphatidylcholine (PC) and sphingomyelin (SM) forming the major constituents of the outer membrane of the lipid bilayer. The catalysis of GPLs into lysophospholipids and free fatty acids; and SM into ceramide and choline by secretory phospholipase (sPL)-A_2 and sphingomyelinase (SMase), respectively, has recently been implicated in the pathology of inflammation (Dinkla *et al.,* 2016).

Systemic inflammation, a pathological coagulation potential, is frequently associated with the increased expression of pro-inflammatory cytokines, IL-1β, IL-6 and IL-8 in circulating blood. Such pro-inflammatory cytokines are capable of inducing the activation of the coagulation system, functioning in the production of blood clotting factors, and subsequent down-regulation of several anticoagulant pathways (Bester & Pretorius, 2016). Inflammatory response, both chronic and acute, is additionally associated with the increased expression of fibrinogen, a high molecular weight soluble plasma protein, functioning in haemostasis and thrombosis (Spencer *et al.,* 2007).

Erythrocytes are capable of interacting with intravascular pro-inflammatory chemokines via the cell surface receptor, duffy antigen receptor for chemokines (DARC) (Almeida *et al.,* 2012). DARC functions as a receptor for monocyte chemotactic protein (MCP)-1 (or CCL2), PF-4, or CXCL4; IL-8, or CXCL8; and neutrophil activating peptide (NAP)-2 (or CXCL7) associated with the induction of pro-inflammatory and procoagulant responses (Østerud *et al.,* 2015). Further intervention of erythrocytes in the inflammatory response is associated with the recruitment of leukocytes to the endothelium and subsequent interaction with fibroblasts, increasing the expression and secretion of IL-8 necessary for leukocyte signalling (Almeida *et al.,* 2012).

The involvement of erythrocytes in the mediation of various pro-inflammatory and procoagulant processes was demostrated by Østerud *et al.* (2015) who investigated the effect of erythrocytes on the LPS-induced production pro-inflammatory mediators, including tissue factor (TF) and MCP-1. Erythrocytes were shown to enhance LPS-induced TF-a in human monocytes, both in the presence and absence of Fragmin-anticoagulated plasma. Additionally, subcompartmentalisation of MCP-1 produced by monocytes in response to LPS was shown to be mediated by erythrocytes, increasing monocyte TF protein.

2.3.5 The role of host defence peptides in infection and inflammation

Cationic HDPs, or AMPs, act locally at the site of infection resulting in pathogenic membrane disruption, generation of DNA, ribonucleic acid (RNA) and protein synthesis inhibitory effects, as well as activation of lytic antimicrobial enzymes (Kindt *et al.*, 2007). During acute inflammation, these AMPs function by direct lysis of bacterial membranes, neutralisation of LPS and subsequent regulation of macrophage response to LPS stimuli (Kindt *et al.*, 2007). Mast cells, neutrophils and polymorphonuclear leukocytes (PMNs) are stimulated by AMPs to increase blood permeability, chemotaxis and antigen presentation, respectively. AMPs further facilitate the adhesion between PMN and phagocytes, and the inhibition of fibrinolysis resulting in the reduction of bacterial spread (Kindt *et al.*, 2007). Wound healing is subsequently induced by the promotion of fibroblastic chemotaxis and growth and inhibition of proteases, limiting tissue damage and stimulating apoptosis of pathogen-infected host cells (Kindt *et al.*, 2007).

Chronic inflammation and adaptive immune responses are initiated after prolonged bacterial infection, not improved by acute inflammatory responses (Hancock & Diamond, 2000). During chronic inflammation, AMPs act as monocytic chemotaxins by recruiting T-cells. This leads to enhanced chemokine production and proliferation of T-helper cells. Macrophage LPS-signalling that typically reduces cytokine production and activates specific gene expression in macrophages, is further suppressed by AMPs. AMPs also contribute to apoptosis and lymphocyte activation in macrophages, potentially eliminating all pathogen-infected cells (Hancock & Diamond, 2000).

2.4 Antimicrobial peptides

AMPs are an important component of the innate immune system of all organisms, including *Homo sapiens*. AMPs have been identified in numerous different species, including invertebrates, plants and mammals, as novel scaffolds for the development of therapeutic agents (Table 2.3). Bioactive AMPs are small cationic molecules, common throughout both animal and plant kingdoms (Fogaca *et al.*, 2004). The amphipathic properties of AMPs are critical for membrane interaction and internalisation (Falanga *et al.,* 2015), allowing them to be both aqueous-soluble and lipid-penetrable (Izadpanah & Gallo, 2005). Most AMPs are of similar size, often approximately 10 kDa, or less than 50 amino acids (Seo *et al.*, 2012). AMPs differ in their amino acid sequence, composition and mode of action, depending on their host derivatives (Fogaca *et al.*, 2004). In the three-dimensional (3D) conformation of AMPs, positively charged amino acids, particularly Lys

and arginine (Arg) residues, are typically positioned on one side of the structure, while a significant proportion of hydrophobic residues, including glycine (Gly), Phe and tryptophan (Trp), are positioned on the opposite side (Seo *et al.*, 2012). AMPs destroy bacteria through a wide range of mechanisms, frequently via membrane permeabilisation and intracellular targeting, resulting in autolytic enzyme stimulation as well as inhibition of DNA, protein, cell wall and membrane synthesis (Cudic & Otvos, 2002; Jensen *et al.,* 2006).

Table 2.3: Different types of AMPs isolated from various sources (Pushpanathan *et al.*, 2013).

Source	Examples of AMPs
Insects	Ceratoxin, stomoxyn, spinigerenin, thanatin, helopmicin, sapecin, defensin A, smD1, gallerimycin, termicin, royalisin, pyrrhocoricin, melittin
Amphibians	Distinctin, japonicin, nigrocin, brevinin-20a, maximin-1
Echinoderms	Strongylocins, centrocins, betathymosins, filamin A
Crustaceans	Callinectin, astacidin 2, armadillidin, homarin, scygonadin, penaeidin, arasin, stylicin, haemocyanin-derived peptides
Plants	Thionins, plant defensins, lipid transfer proteins
Mammals	Defensin, protegrin, lactoferricin
Bacteria	Bacilomycin, syringomycin, syringostatins, syringotoxins, nikkomycins
Fungi	Echinocadins, aculeacins, mulundocandins, aureobasidin, helioferins
Fish	Pardaxins, misgurin, peurocidins, chrysophsin, parasin

AMPs are structurally classified as α-helical, β-sheet - stabilised by two to three disulphide bonds, extended or looped/disordered peptides (Seo *et al.*, 2012). Amphipathic α-helical and β-sheet AMPs are considered the most abundant in nature (Sun & Shang, 2015). These structural conformations as well as their dynamic ability to cluster cationic and hydrophobic residues, arranging them into discrete molecular segments, are largely responsible for the amphipathic property observed across all AMPs (Zasloff, 2002). The relationship between AMP structure and activity is fundamental for synthetic peptide modelling in which 3D rendered structures are used as scaffolds to generate novel AMPs with optimised amino acid sequences, allowing enhanced activity and stability, as well as reduced toxicity and cost. Novel drug design, development and modification are important for pharmaceutical application (Seo *et al.*, 2012).

2.4.1 The mechanism of antibacterial action of antimicrobial peptides

Naturally occurring AMPs offer a broad range of antimicrobial activities, exploiting the dynamic nature of their structural conformations to prevent pathogenic spread and subsequent peptide resistance (Jensen *et al.*, 2006; Yeung *et al.*, 2011).

Despite variations in structures and amino acid sequences, most AMPs exhibit amphipathic structures with hydrophobic surfaces comprising non-polar amino acids and a hydrophilic face composed of polar, positively charged amino acids (Sun & Shang, 2015). Many such AMPs have been found to be effective against MDR bacteria and have low susceptibility for developing resistance (Seo *et al.*, 2012). This low propensity for AMP resistance is owing to their distinguished mode of action (Seo *et al.*, 2012). AMPs either act directly on microbial membranes as a result of their amphipathic characteristics, or due to their cationic properties, interact selectively with the negatively charged surface of microbial membranes, resulting in their accumulation (Seo *et al.*, 2012). Consequently, AMPs undergo conformational changes promoting the formation of peptide-lipid specific interactions, peptide translocation across membranes, intracellular targeting and membranolysis. AMPs are therefore capable of selectively interacting with numerous components of both Gram-positive and Gram-negative bacteria, distinguished according to several distinct characteristics of the cell envelope (Cowan & Talaro, 2014).

Gram-positive bacterial cell walls are composed of between 50-90% PG, contain teichoic acids (absent in Gram-negative bacteria), are approximately 20-30 nm thick and are generally smooth with 70-80% murein. Gram-negative bacterial cell walls on the other hand, are composed of only 10% PG, are surrounded by an outer membrane containing hydrophobic transmembrane channels, absent in Gram-positive bacteria; are approximately 8-12 nm thick, usually irregular and contain only between 10-20% murein. Additionally, Gram-negative bacterial cell walls have high lipid content, usually 20-30%, and are rich in LPS. Gram-negative bacteria are primarily endotoxic, displaying considerably more resistance to antibiotics, whereas Gram-positive bacteria are predominantly exotoxic (Figure 2.4) (Cowan & Talaro, 2014).

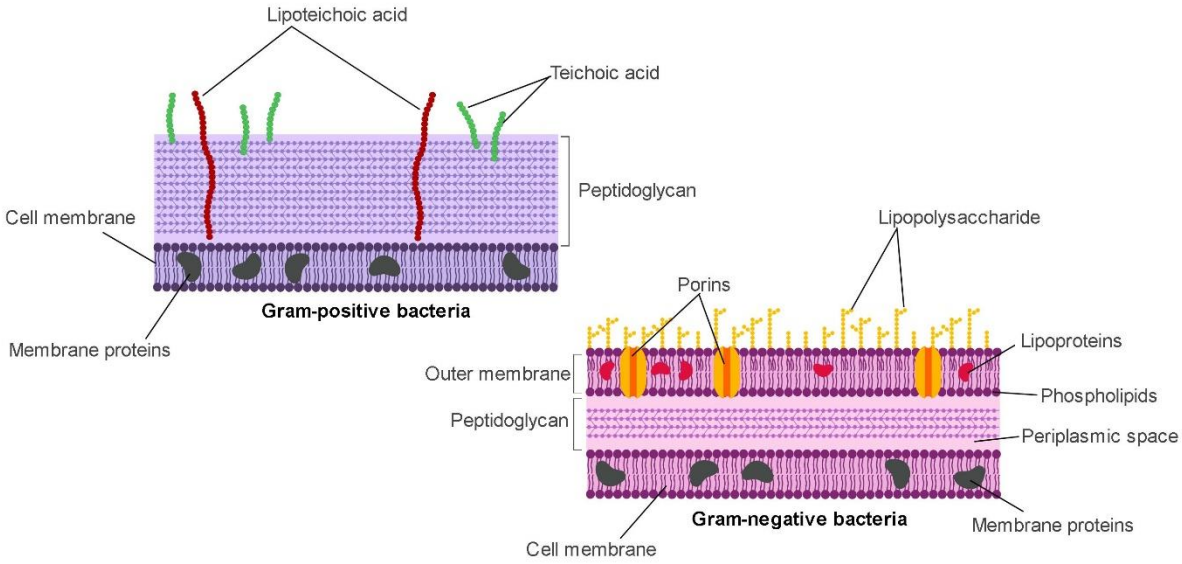


Figure 2.4: The major differences in composition of Gram-positive and Gram-negative cell envelopes (Cowan & Talaro, 2014).

Lantibiotics are AMPs which contain lanthionine, thioester amino acids produced by Gram-positive bacteria that function as bacteriocins. These proteinaceous toxins interfere with membrane-associated transglycosylation, inhibiting the biosynthesis of PG. Since PG is a major constituent of Gram-positive bacterial cell envelopes, inhibition of its biosynthesis is an ideal mechanism for inhibiting the growth and replication of bacterial cells (Brogden, 2005; Park *et al.*, 2011). These peptides have additionally been shown to be effective against drug resistant Gram-positive bacteria.

The cell envelopes of Gram-negative bacteria is composed of only a thin layer of PG and a thick outer membrane containing LPS which provides additional negative charges that act as a physical barrier against various antibacterial agents, stimulates bacterial growth and maintains cellular viability (Park *et al.*, 2011; Rosenfeld & Shai, 2006). During infection, LPS stimulates the release of pro-inflammatory cytokines, i.e. TNF-α, IL-1 and IL-6, by host macrophages. AMPs neutralise LPS by interacting directly with LPS molecules, which functions as pathogen-associated molecular patterns (PAMPs), preventing their accessibility by pattern recognition receptors (PRRs). Neutralisation of LPS subsequently inhibits LPS-induced release of pro-inflammatory cytokines, preventing phagocytic activation. LPS neutralisation is additionally capable of inhibiting the growth of MDR bacteria (Rosenfeld & Shai, 2006).

Bacterial plasma membranes, or inner membranes, are bilayers composed of an outer layer of lipids with numerous negatively charged phospholipid head groups. Cationic AMPs are thought to primarily target this distinct, bacteria-specific characteristic (Zasloff, 2002; Park *et al.*, 2011) by electrostatically interacting with the negatively charged phospholipid head groups (Zasloff, 2002). Following the binding of AMPs to the bacterial plasma membrane, AMPs may exert their antibacterial activity by translocating across the membrane and inhibiting essential cellular processes, including protein, nucleic acid and cell wall synthesis, as well as enzymatic activity (Pushpanathan *et al.*, 2013).

AMPs are also thought to possess an extensive array of intracellular bacterial targets (Li *et al.*, 2012). Following their uptake into bacterial cells, AMPs may alter the formation of cytoplasmic membrane septae, inhibit the synthesis of proteins and nucleic acids, activate autolysin, and inhibit essential enzymatic activity (Brogden, 2005). AMPs are also able to interfere with cytokinesis by inducing cell filamentation (Li *et al.*, 2012). Additionally, some anti-biofilm AMPs have been identified which act by either moving into the extracellular polymeric substance of biofilms, via pore formation, or decreasing bacterial cell-surface adhesion, promoting biofilm dispersion (Park *et al.*, 2011). These AMPs frequently synergise in antibiotic-combined therapies (Park *et al.*, 2011). and may further serve as vaccine adjuvants, enhancing antigenic immune responses (Yeung *et al.*, 2011).

2.4.2 The cytotoxic potential of antimicrobial peptides

The development of AMPs as therapeutic agents relies on the optimisation of their antimicrobial activity, enhancing their bacterial cell specificity and selectivity while reducing their toxic and haemolytic effects on mammalian cells and erythrocytes, respectively. Toxicity profiling of AMPs is, therefore, essential to assess the relative effects of AMPs against eukaryotic hosts.

At high concentrations, numerous AMPs have been shown to exert some level of haemolysis or cytotoxicity towards mammalian cells (Bacalum & Radu, 2015; Edwards *et al.,* 2016), limiting their therapeutic development. Such cytotoxicity may be a product of either necrosis, associated with a loss of membrane integrity, cell lysis and subsequent extracellular leakage of the cytoplasmic content; or induced apoptosis, resulting in DNA fragmentation, the inhibition of cell division and ultimate cessation of cell growth (Laverty & Gilmore, 2014). Several strategies have been explored to exploit the antimicrobial properties of AMPs while reducing their cytotoxic effects,

including a prodrug strategy which involves the reversible inactivation of AMPs, conjugated to prodrug moieties, until desirable conditions are achieved. This strategy was applied to melittin, an otherwise highly toxic AMP, reducing the peptides *in vitro* cytotoxicity and permitting its application in a mouse model of cancer for tumour reduction (Forde *et al.,* 2016). No drug is, however, considered completely free from toxicity, instead a balance exists between the need for treatment and the level of toxicity produced at therapeutic doses.

2.4.3 Antimicrobial peptides with cell-penetrating properties

Conventional antibiotic regimes are frequently unable to alleviate recurrent infections associated with the localised cellular internalisation of pathogenic microbes (Bahnsen *et al.,* 2015). Several strategies, such as microinjection, electroporation, liposomes and viral vectors previously employed to combat such ineffective applications, have themselves been limited by poor efficiency, specificity, bioavailability and enhanced toxicity (Henriques *et al.,* 2006). The treatment and eradication of intracellular infections by AMPs possessing additional cell-penetrating properties has been shown to have several advantages over these strategies, including enhanced bioavailability, improved clinical efficacy and reduced adverse side-effects, although with limited cellular specificity.

Cellular uptake of AMPs may be distinguished according to their energy dependency. The non-receptor-mediated membrane-lytic bactericidal activity of AMPs (Le *et al.,* 2017) is commonly energy-independent and occurs via one of three mechanisms, i.e. the barrel-stave model, carpet model or toroidal pore model; while energy-dependent uptake of AMPs is thought to occur through the endocytic process of macropinocytosis (Figure 2.5). During macropinocytosis the plasma membrane invaginates, along with the peptide, and forms macropinosomes which subsequently release the peptide into the cytoplasm for intracellular targeting and exertion of their antimicrobial effects (Pushpanathan *et al.,* 2013).

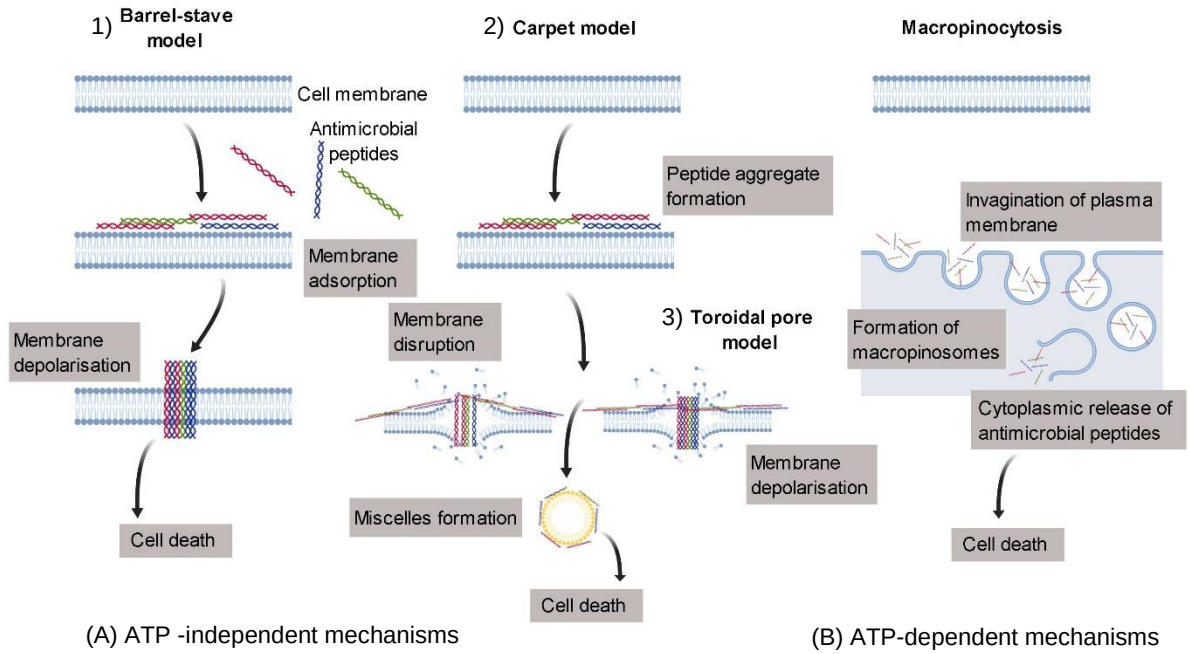


Figure 2.5: Mechanisms of action of AMPs. A) ATP-independent mechanisms: 1) barrel-stave model, 2) carpet model and 3) toroidal pore model; and B) ATP-dependent mechanisms (Pushpanathan *et al.*, 2013). AMP: Adenosine monophosphate; ATP: Adenosine triphosphate.

Cell-penetrating peptides (CPPs), or protein transduction domains (PTDs), are short generally non-bioactive peptide sequences composed of between 8 and 30 amino acid residues that are capable of facilitating the intracellular translocation of large therapeutic molecules, including nucleic acids, proteins and synthetic drugs. Since the isolation of several natural CPPs, numerous derived and synthetic CPPs have been generated for improved uptake and increased cellular and intra-cellular specificity. Such CPPs have been considered for several applications, including skin diseases, myocardial infarction, ischemia, pain, splicing correction, HIV vaccinations and several types of cancer (Dinca *et al.,* 2016).

CPPs are commonly classified into cationic, amphipathic and membranotropic groups, according to both their chemical and physical properties. Cationic CPPs are a class of peptides possessing similar structural characteristics to cationic AMPs. Both cationic AMPs and CPPs possess short amino acid chain lengths, have a net positive charge necessary for interacting with negatively charged phospholipid membranes, and are composed of a significant number of non-polar amino acid residues that promote peptide insertion within the hydrophobic core of the membrane bilayer

(Bahnsen *et al.,* 2015). Small sequence modifications are capable of altering the predominant biological properties of highly cationic peptides from either cell-penetrating to antimicrobial or antimicrobial to cell-penetrating (Falanga *et al.,* 2015). Peptides considered both antimicrobial and cell-penetrating (Table 2.4) are frequently used for therapeutic development, alone or as drug delivery vectors.

Table 2.4: Membrane interacting properties of several common CPPs and AMPs (Splith & Neundorf, 2011).

	Structure	Suggested mechanism
AMPs		
Pyrrhocoricin	Random coil/reverse turns at termini	Receptor-mediated
Lactoferricin	β-Loop	Direct penetration/pore formation
Bac7	Hybrid of PPII helix and α-helix	Receptor-mediated/pore formation
PG-1	Cationic, amphipathic antiparallel β-sheet	Endocytosis/pore formation
LL-37	α-Helical	Pore formation
Buforin II	Extended amphipathic, α-helical	Direct penetration
CPPs		
sC18	Amphipathic, α-helical	Endocytosis
Tat (48-60)	Random coil/PPII helix	Direct penetration/pore formation
Penetratin	Amphipathic, α-helical/β-sheet (at high concentrations)	Direct penetration/pore formation
pVEC	Amphipathic, β-sheet	Direct penetration/transporter-mediated
Pep-1	Amphipathic, α-helical	Direct penetration/pore formation
Transportan (TP10)	Amphipathic, α-helical	Endocytosis/direct penetration
MAP	Amphipathic, helical	Multiple mechanisms

Critical to the success of CPPs as drug delivery vectors is their ability to penetrate biological membranes to achieve their intracellular fate or, more crucially, the fate of their molecular cargo (Cleal *et al.,* 2013). CPP-mediated cellular uptake typically occurs either via one of four distinct mechanisms of endocytosis, i.e. caveolae, clathrin-mediated, macropinocytosis and phagocytosis (Figure 2.6) or by direct energy-independent mechanisms. The mechanism of uptake depends largely on the characteristics of peptide/cell interaction (Falanga *et al.,* 2015), extracellular concentration, cell or tissue target, temperature, size and type of cargo, i.e. drugs, proteins, plasmid DNA, small interfering RNA (siRNA), nanoparticles and bacteriophages (Rodrigues *et al.,* 2013); and peptide sequence (Padari *et al.,* 2005; Duchardt *et al.,* 2007; Vivès *et al.,* 2008). Low temperatures, in particular, inhibit CPP internalisation via endocytic pathways, consequently inducing a direct mechanism of cellular translocation (Jiao *et al.,* 2009).

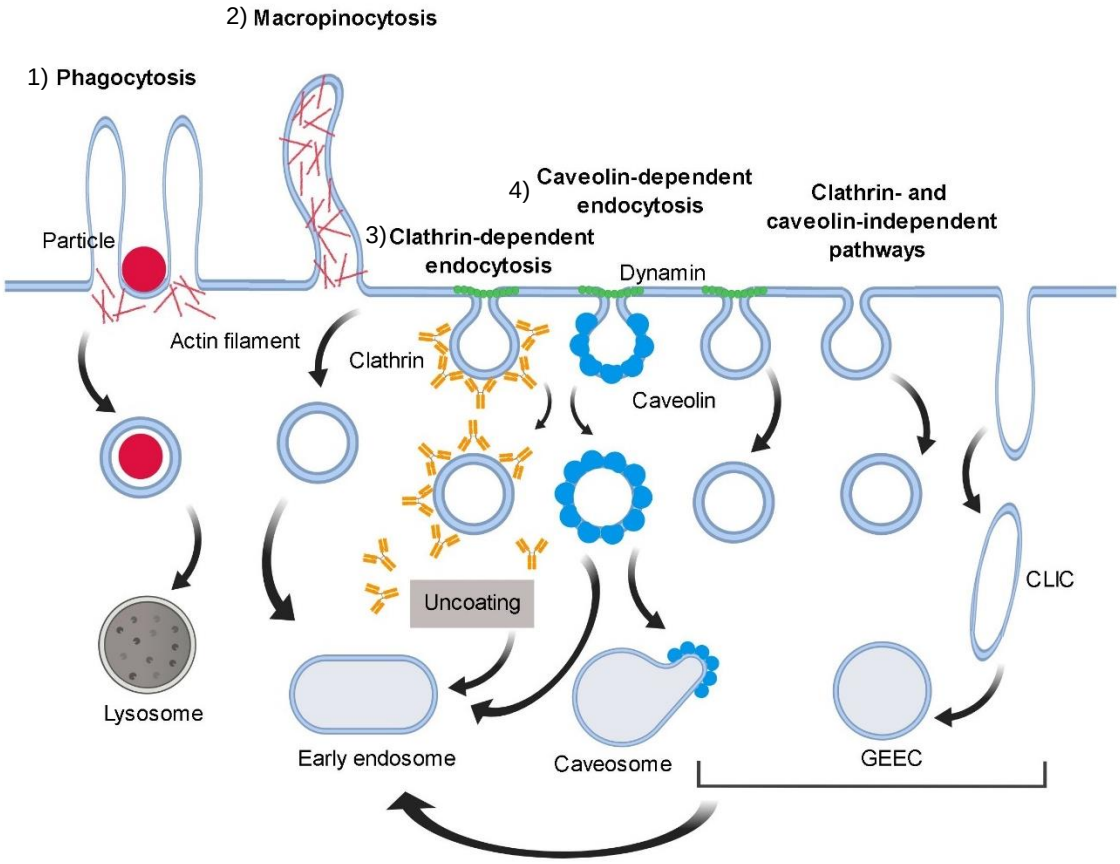


Figure 2.6: Mechanisms of endocytosis. 1) Phagocytosis, 2) macropinocytosis, 3) clathrin-coated vesicles and 4) caveolae (Mayor & Pagano, 2007). CLIC: Clathrin- and dynaminindependent carriers; GEEC: Glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments.

Clathrin-mediated endocytosis is the most well-characterised mechanism involving the uptake of molecules from clathrin cage invaginations at the interface of the cell membrane, pinching off to form clathrin-coated vesicles for subsequent cellular internalisation (Cleal *et al.,* 2013; Tang *et al.,* 2015). Following internalisation, vesicles lose their clathrin coat (Cleal *et al.,* 2013) and undergo fusion with early endosomes for subsequent sorting and recycling. Early endosomes often return back to the plasma membrane, or in late endosomes fuse with lysosomes for degradation of the internalised molecules and cell membrane proteins. Caveolae, or lipid raft-dependent endocytosis, on the other hand, relies primarily on cholesterol-rich membrane domains for plasma membrane invagination and subsequent internalisation of viruses, bacteria, sphingolipids, endothelin, growth hormone and IL (Cleal *et al.,* 2013; Tang *et al.,* 2015).

Phagocytosis involves the cellular engulfment of large macromolecules, usually more than 0.5 µm in size (Cardelli, 2001; Jutras & Desjardins, 2005; Tang *et al.,* 2015). The phagocytic process is confined to specific cell types, including macrophages, neutrophils and dendritic cells and is initiated by cell-surface receptor recognition of specific ligands. The recognition of ligands by cell-surface receptors further initiates a complex of distinct signal transduction pathways that stimulates cytoskeletal rearrangement and membrane remodelling and invagination for subsequent molecular engulfment. Complete engulfment of exogenous molecules by membrane remodelling induces the intracellular release of membrane-bound phagosomes that undergo intricate maturation processes characterised by sequential fusion with aging endosomes and ultimately, lysosomes for degradation. Mature phagosomes additionally acquire hydrolytic enzymes that contribute to the digestion and degradation of their internalised content (Jutras & Desjardins, 2005). Phagocytosis plays a critical role in tissue remodelling, ensuring the elimination of cellular debris and apoptotic bodies, and in innate immunity, activating host defences against invading pathogens (Henneke & Golenbock, 2004).

The fluid internalisation process of macropinocytosis occurs under considerably restrictive conditions, within a limited number of cell types, including dendritic cells, generating large vesicles usually greater than 1 µm in diameter (Cardelli, 2001). Macropinocytosis has additionally been found to be prominent in cells that are incapable of undergoing phagocytosis and those responding to growth factor stimulation. Following the addition of growth factors, substantial volumes of extracellular material are engulfed in enlarged, extracellular macropinosomes, rapidly increasing the uptake of markers associated with fluid phase endocytosis and inducing considerable membrane reorganisation or ruffling. The subsequent formation of enclosed and internalised macropinosomes is largely thought to be associated with the fusion of protruding, unruffled sections of the plasma membrane. Macropinocytosis contributes significantly to antigenic presentation by the immune system and is frequently exploited by numerous pathogens for cellular invasion and evasion of immune surveillance (Kerr & Teasdale, 2009). Both phagocytosis and macropinocytosis are actin-dependent processes (Cardelli, 2001), common in their production of large vesicles, morphological heterogeniety and independence of clathrin mediation (Jones, 2007).

The fate of CPPs and their conjugates undergoing endocytic processes of internalisation depends largely on each molecules' ability to escape the endosomal vesicle, allowing cytoplasmic access and subsequent intracellular targeting (Henriques *et al.,* 2006), prior to lysosomal delivery for

degradation. Such endosomolysis may be induced by the addition of pH-responsive elements, such as the short peptide derived from the influenza virus-derived peptide (INF), haemagglutinin, as well as the synthetic peptide, glutamate (Glu)-alanine (Ala)-Leu-Ala (GALA), both of which are active in the acidic environment of endosomes without disrupting the membranes of other organelles. Several CPPs alone are however capable of acidifying the endosomal environment themselves, facilitating their own escape prior to lysosomal delivery and degradation (Dinca *et al.,* 2016).

2.4.4 Antimicrobial peptides as immunomodulatory peptides

Recent research has shown that cationic HDPs, or AMPs, are capable of both direct antimicrobial killing and innate immune modulation (Figure 2.7), a property that enhances their potential as anti-infective therapeutic agents. Innate immunity is rapidly acting, relatively non-specific and involves the packaging of effector molecules, reducing the development of resistance. Activation of the innate immune response occurs following the interaction of conserved bacterial molecules, such as LPS, with host PRRs (Hancock & Sahl, 2006).

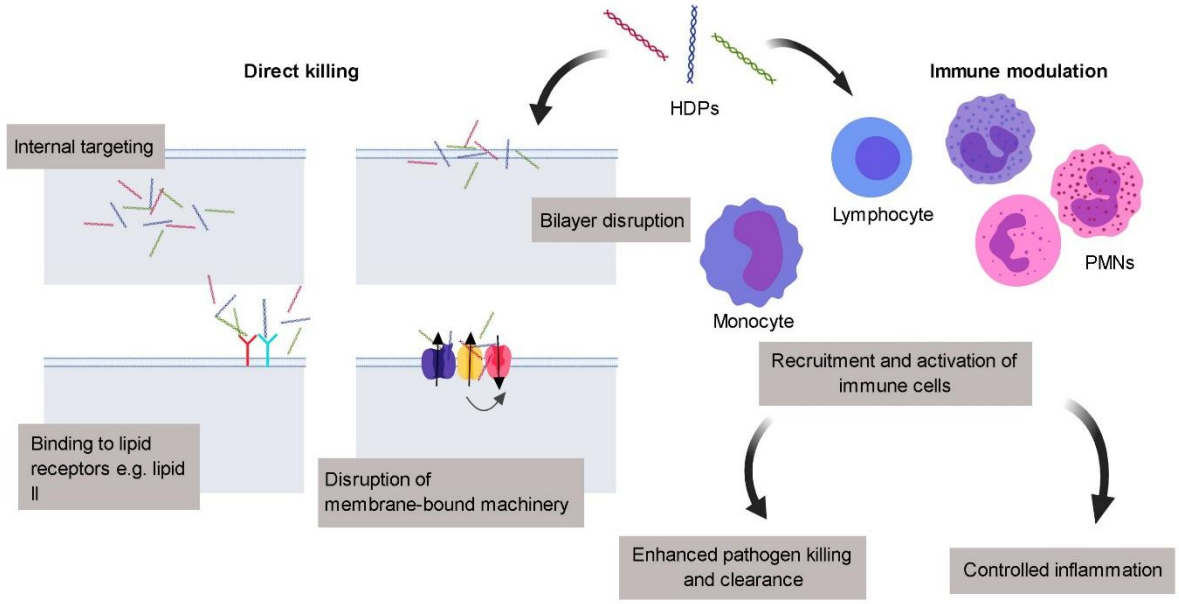


Figure 2.7: Biological role of HDPs (Ulm *et al.,* 2012). ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; HDP: Host defence peptide; PMNs: Polymorphonuclear cells.

Cationic AMPs shown to possess modulatory activity, modify host inflammatory responses by enhancing pro-inflammatory activities, including leukocytic chemotaxis, histamine release and

cytokine induction, while subsequently providing necessary toxic and lethal protection from such responses that may otherwise cause sepsis (Brown & Hancock, 2006). HDPs balance inflammatory responses by stimulating the expression of anti-inflammatory genes, regulating apoptosis in epithelial cells and neutrophils as well as promoting angiogenesis and wound healing (Yeung *et al.*, 2011). Some peptides have even been shown to bind DNA and RNA (Brandenburg *et al.,* 2012).

Immunomodulatory peptides are capable of targeting multiple intracellular processes, signalling pathways and receptors, depending on the cell type and amino acid composition of the peptide. The human cathelicidin peptide, LL-37, for example, exhibits a broad range of immunomodulatory properties and is known to suppress pro-inflammatory cytokine production and subsequent macrophage activation in response to bacterial LPS and lipoteichoic acid (LTA); up-regulate the production of chemokines and chemokine receptors; and promote angiogenesis and wound healing. LL-37 has additionally been shown to indirectly stimulate the P2X(7) receptor and transactivate the epidermal growth factor receptor in human embryonic kidney cells and epithelial cells, respectively. When biotinylated, LL-37 has been found to actively internalise and accumulate within the perinuclear region of epithelial cells via endocytosis where it is available to bind to intracellular targets. Such properties have been observed in other peptides, including defensins and other synthetic peptide derivatives. Little is, however, known about the structure and sequence requirements responsible for the immunomodulatory properties of HDPs, likely owing to variations in target specificity, cellular interaction and uptake between peptides (Haney & Hancock, 2013).

2.4.5 AMPs in clinical development

The research and development of AMPs has grown extensively in recent years, with more than 5500 sequences having been discovered, characterised or synthesised to date (Speck *et al.*, 2014). These sequences are either synthesised *de novo* from naturally occurring templates; isolated from microbes, vertebrates, invertebrates and plants; or synthetically engineered from combinatorial libraries (Rathinakumar *et al.*, 2009). AMPs exhibit considerable structural diversity and possess non-specific, rapid membrane permeabilisation mechanisms (Aoki & Ueda, 2013) that contribute to the combat of microbial resistance.

AMPs have diverse therapeutic potential and may either be developed as single antimicrobial agents or into multi-therapeutic drugs, having additional immunomodulatory properties that produce a synergetic effect. Peptides exhibiting immunomodulatory properties may enhance the innate immune system of the host or neutralise endotoxins, including LPS, preventing sepsis and ultimate septic shock (Rozek *et al.*, 2003). Despite current clinical evaluation of numerous peptides (Table 2.5), most AMPs have not yet been approved for introduction into the consumer market due to several limitations.

Table 2.5: Application of peptides in clinical trial or development (Fox, 2013).

Peptide	Indication
Magainin peptide/pexiganan acetate	Diabetic foot ulcers
Omiganan	Rosacea
Novexatin	Fungal infections of the toenails
Arenicin	Multiresistant Gram-positive bacterial strains
Avidocin and purocin	Narrow spectrum antibiotic for food safety
Lytixar (LTX-109)	MSA*
OP-145	Chronic bacterial middle ear infection
NVB302`	*Clostridium difficile*
MU1140	Gram-positive bacteria
IMX924	Gram-positive and Gram-negative bacteria

*MSA: Methicillin-resistant *S. aureus*

2.4.6 Limitations of AMPs

Regardless of their complex mechanisms and broad range of activities, the instability of peptides under physiological conditions as well as possible cytotoxicity resulting from their hydrophobicity, particularly those which are membrane-acting, provide considerable limitations to their therapeutic development (Yeung *et al.*, 2011; Hancock & Sahl, 2006). An additional limitation of AMPs is protease lability, causing enzymes such as trypsin, to target basic residues within the peptide sequence (Hancock & Sahl, 2006). Several methods have however, been employed to counteract these limitations, including the replacement of L-amino acids with D-amino acid stereoisomers, resulting in preotease-resistance, as well as the attachment of polyethylene glycol (PEG), extending the half-life in serum and reducing proteolytic degradation (Park *et al.*, 2011).

High manufacturing costs additionally hinder the therapeutic development of AMPs (Varkey *et al.*, 2006). To overcome the cost of peptide synthesis, shorter peptide fragments are frequently generated by the omission of specific amino acids. Although the omission of amino acids may

modify the therapeutic potential of the peptides, peptides derived from the carboxyl-terminal region of defensins (Varkey *et al.*, 2006) have been shown to maintain their antimicrobial activity. Modification of peptide structure may additionally improve peptide sensitivity to changing environmental conditions, such as pH, serum, salts and proteases, while enhancing their therapeutic effects (Rozek *et al.*, 2003). Despite such limitations and associated efforts to counteract them (Table 2.6), the development of AMPs as therapeutic alternatives continues to expand.

Table 2.6: Design and development strategies for AMPs (Hancock and Sahl, 2006).

Limitations	Essential requirements	Strategies
Screening	Large variety of peptides	Peptide arrays Natural lead molecules Mimetic approaches Non-natural amino acids Screen both antimicrobial and anti-inflammatory activities
Toxicity	Understand the mechanisms of toxicity	Animal models Assessment of subtle toxicities Toxicogenomics
Pharmacology	Improved *in vivo* half-life	Peptidomimetics Modified and/or D-amino acids Novel compositions Dosage regulation of immunomodulating peptides
Cost	Reduced cost of therapeutics	Shorter analogues Recombinant manufacturing processes Natural sources Local and small dosage administration
Efficacy	Improved activities in model infections	Realistic animal models of disease *In vitro* assays should mimic *in vivo* physiological conditions Development of *in vitro* methods for effective prediction of immunomodulaton

2.4.7 Defensins

Defensins are genetically distinct members of a large family of AMPs found in numerous plant, vertebrate and invertebrate species, that contribute to the microbial action of granulocytes, mucosal host defence within the small intestines and epithelial host defence in the skin and

surrounding epithelium (Ganz, 2003). Defensins isolated from invertebrates in particular, such as insects, scorpions, mussels and ticks, are usually either transcribed and released into the fat body or haemolymph following septic injury, or expressed in the haemolymph and later released during threats to the immune system (Hoffmann & Hetru, 1992).

Defensins are classified into three distinct subfamilies, i.e. α-defensins, β-defensins and θ-defensins, all of which contain a characteristic β-sheet fold and six evolutionary conserved Cys residues differentially paired according to their particular subfamily (Nakajima *et al.*, 2003). Both α- and β-defensins are composed of a characteristic triple-stranded β-sheet and 'defensin' fold (Figure 2.8).

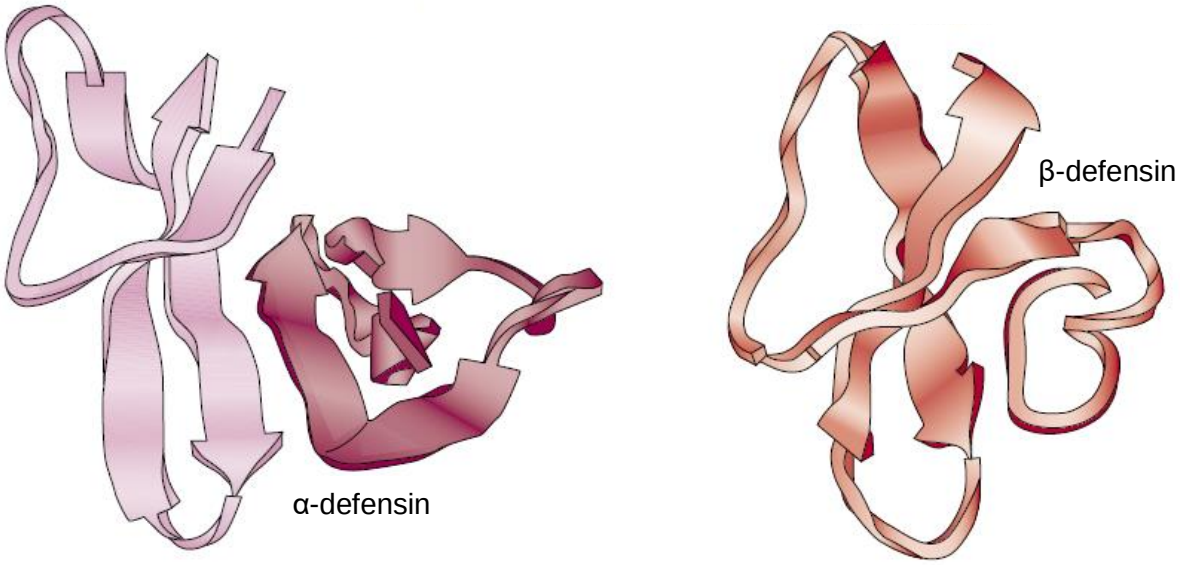


Figure 2.8: Illustration of the structure of α- and β-defensin showing the characteristic triple stranded β-sheets, indicated by flat ribbons and arrows, and 'defensin' folds (Ganz, 2003).

Defensins are initially synthesised as inactive prepeptides that subsequently undergo proteolytic processing for activation (Jensen *et al.*, 2006). These peptides are stored in leukocyte granules which upon phagocytic microbial ingestion, fuse with intracellular vacuoles releasing high concentrations of the microorganism-targeting defensins (Ganz, 2003). During inflammation, defensins typically recruit neutrophils and monocytes to the site of inflammation and induce mast cell activation, increasing prostaglandin D2 production and intracellular Ca^{2+} mobilisation. Defensins are also capable of inducing the production of chemokines, including MCP-1, MIP-3α and IP-10 (Brandenburg *et al.,* 2012). In the small intestine, specialised host defence cells, i.e.

Paneth cells, store high concentrations of defensins for subsequent release into intestinal crypts, via secretory granules (Ganz, 2003; Jensen *et al.*, 2006). Defensins are also produced by numerous secretory and barrier epithelial cells, either constitutively or in response to microbial infection (Ganz, 2003).

Since defensins exhibit a broad spectrum of activities, improved understanding of their structure-activity relationship, with regard to both pathogen specificity and undesirable side effects, is essential for the rational design of novel therapeutic AMPs (Koehbach, 2017).

2.4.7.1 Tick defensins

Ticks, responsible for the transmission of numerous arthropod-borne diseases, possess memory-deficient non-specific innate immune systems that have the ability to recognise molecules characteristic of pathogenic invasion, including LPS, PGs and glycans, present on microbial surfaces (Chrudimská *et al.*, 2010). Recognition of these molecules induces the activation of intrinsic humoral and cellular innate immune systems, resulting in AMP synthesis for subsequent antibiotic activity (Chrudimská *et al.*, 2010). Such defence mechanisms present in ticks have directed the focus of current research into these peptides, commonly isolated from the salivary glands, ovaries, haemolymph and midgut within the tick (Figure 2.9), for the development of antimicrobial therapeutic agents.

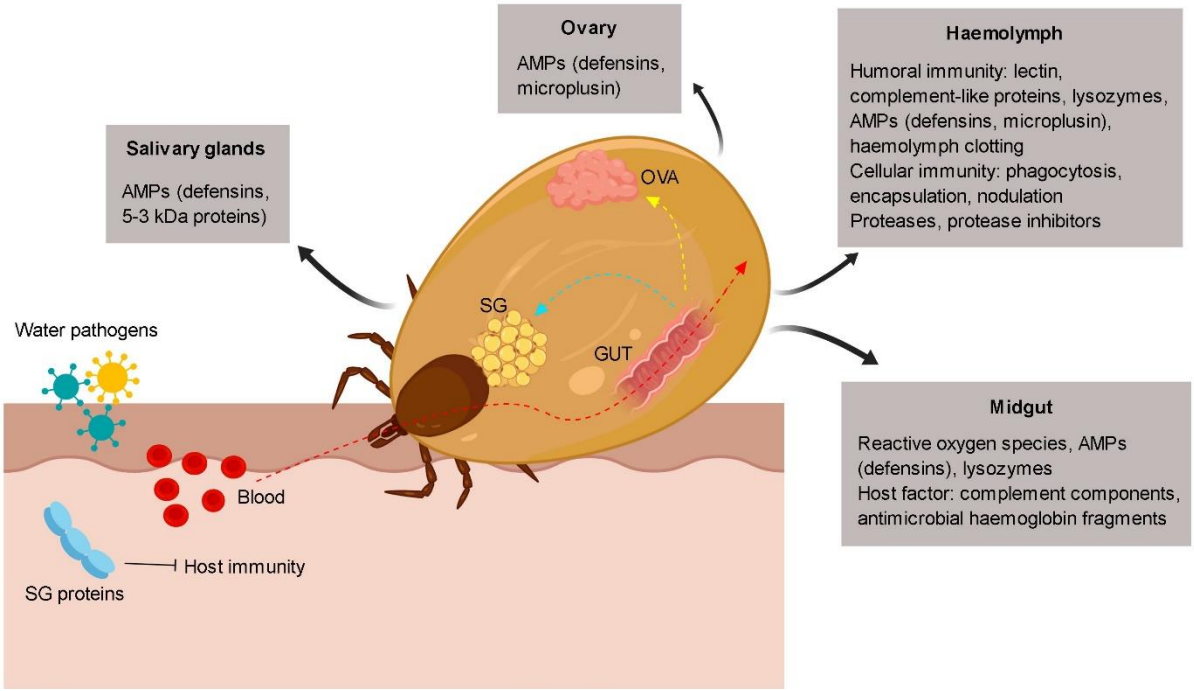


Figure 2.9: Site of molecules constituting potential barriers within the immune system of ticks (Hajdušek *et al.*, 2013). AMPs: Antimicrobial peptides; GUT: Midgut; OVA: Ovary; SG: Salivary gland.

2.5 Problem statement

Infectious diseases resulting from pathogenic host invasion are considered one of the major causes of mortality worldwide. Since bacterial pathogens, in particular, are subject to continuous evolution into MDR strains, with limited treatment options, they have become a major target for drug development. AMPs possess numerous attractive properties for such therapeutic development including their broad-spectrum activity against both Gram-positive and Gram-negative bacteria, low incidence of bacterial resistance, specific mode of action involving pore formation, low toxicity towards eukaryotic cells and stability in a wide range of pH and temperature. AMPs are additionally, capable of synergising with conventional antibiotics and neutralising endotoxins, and have recently been recognised for their role in human immunity (Chung & Khanum, 2017).

2.6 Background to study

Synthetic peptides, Os and Os-C, derived from the carboxyl-terminal region of OsDef2, a defensin identified from the midgut of soft tick *O. savignyi* were previously evaluated by Prinsloo *et al.* (2013) and Malan *et al.* (2015). Prinsloo *et al.* (2013) investigated the cytotoxic effects, as well as

the antibacterial and antioxidant activities of Os and Os-C. Both peptides displayed potent antibacterial activity against Gram-positive and Gram-negative bacteria, while the parent peptide, OsDef2, proved potent to Gram-negative bacteria only. Neither Os, nor Os-C exhibited any significant cytotoxic effects on mammalian cells and both peptides displayed considerable antioxidant activity. Malan *et al.* (2015) further evaluated the anti-inflammatory potential of Os and Os-C and found that the NO scavenging activity of Os was significantly greater than that of known NO scavenger, glutathione (GSH). No significant scavenging activity was, however, detected for Os-C. The Cys residues, absent in Os-C, were therefore thought to be largely accountable for the anti-inflammatory activity reported in Os since sulphur-containing compounds, particularly the thiol groups of amino acids, react with NO to form S-nitrosothiols. Despite the observation that Cys residues contributed directly to the NO scavenging capacity of Os, the omission of Cys residues in Os-C modified the amino acid sequence, shortening the peptide and possibly affecting its activity. Therefore, to determine whether the Cys residues or a modified amino acid sequence was responsible for the differences in the NO scavenging capacity observed between Os and Os-C, a novel peptide was synthesised in which Cys residues were replaced with Ser residues, forming Os(C→S), ensuring that the number of residues within amino acid sequence of Os was retained (Table 2.7). Additionally, the substitution of Cys residues with Ser residues removed free thiol groups or disulphide bonds, preventing folding or mixed disulphide pairing that would otherwise affect peptide function (Furman *et al.,* 2015). Selection of Ser was based on similarities in both the structure and chemistry of Cys residues.

Table 2.7: The amino acid sequences and properties of tick defensin, OsDef2, and derived peptides.

Peptide	Source	Amino acid sequence	pI*	Net charge (pH 7)**	Hydrophobicity <H>**	MIC of *E. coli* (µM)***	MBC of *E. coli* (µM)***	Reference
OsDef2	Defensin isoform 2 from *O. savignyi*	GYGCPFNQYQCHSCHKG IRGYKGGYCKGAFKQTC KCY	9.20	6	0.371	>28.7	-	(Prinsloo *et al.,* 2013)
Os	Carboxyl-terminal of OsDef2	KGIRGYKGGYCKGAFKQ TCKCY	9.67	6	0.249	>48.8	0.76	(Prinsloo *et al.,* 2013)
Os-C	Carboxyl-terminal of OsDef2, with three Cys residues omitted	KGIRGYKGGYKGAFKQT KY	10.22	6	0.045	>55.8	1.74	(Prinsloo *et al.,* 2013)
Os(C→S)	Carboxyl-terminal of OsDef2, with three Cys residues substituted with three Ser residues	KGIRGYKGGYSKGAFKQ TSKSY	10.22	6	0.034	10	3.11	(Minnaar, 2015 unpublished)

*pI: isoelectric point. Data obtained from the ProtParam tool of the ExPASy server, available at [https://web.expasy.org/cgi-bin/protparam/protparam]; accessed on 13 April 2018.

**Data obtained from HeliQuest server, available at [http://heliquest.ipmc.cnrs.fr]; accessed on 02 February 2018.

***MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. MIC and MBC was determined by Prinsloo *et al.* (2013) using the micro-dilution broth assay, in Luria-Bertani (LB) broth, and the colony forming unit (CFU) assay, and by Minnaar (2015, unpublished) using the radial diffusion (RD) assay and CFU assay, respectively. The CFU assay eliminates the effect of additional salts, proteins and other broth constituents that otherwise influence the inhibitory activity of peptides as observed by Prinsloo *et al.* (2013) .

2.7 Aim

The aim of this investigation was to evaluate the contribution of Cys residues to the cytotoxic potential, cellular localisation, antioxidant activity and anti-inflammatory capacity of Os, using a Ser-substituted analogue, Os(C→S). Additionally, the eligibility of Os(C→S) for therapeutic development was determined.

2.8 Objectives

The specific objectives of this investigation was to determine whether, compared with Os:

1. Os(C→S) affects the density and viability of L929 cells.
2. Os(C→S) induces membranolysis of human erythrocytes.
3. Os(C→S) is able to penetrate the cell membrane of human peripheral blood mononuclear cells (PBMCs) and PMNs without inducing any significant cytotoxicity.
4. Os(C→S) provides protection against free-radical production and subsequent *in vitro* oxidative damage in L929 cells.
5. Os(C→S) is capable of scavenging NO and eliciting an *ex vivo* anti-inflammatory effect in human platelets.
6. Os(C→S) binds LPS.

These objectives were achieved using the experimental strategy presented in Figure 2.10.

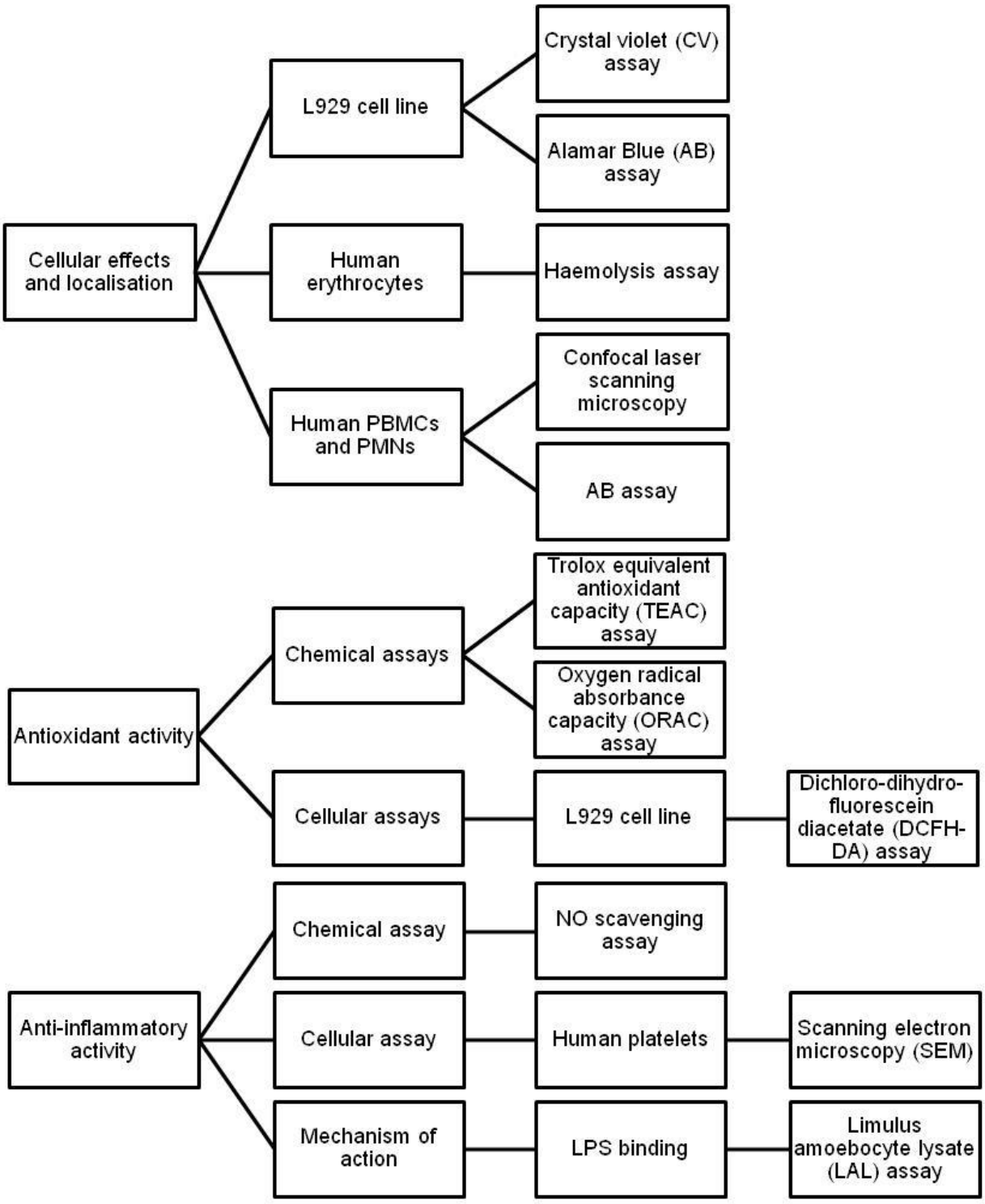


Figure 2.10: Methods used to evaluate the cellular, antioxidant and anti-inflammatory activity of Os(C→S) to determine the functional contribution of Cys residues in the defensin-derived peptide, Os.

Chapter 3: Cellular effects and localisation of Os(C→S) compared with Os

3.1 Introduction

A primary limitation of the pharmacological application of AMPs is their ability to cross the membranes of different cell types, without exerting direct membranolytic effects, for targeted drug delivery (Mäe & Langel, 2006; Cardozo *et al.,* 2007). Cationic AMPs possessing additional cell-penetrating properties, therefore, hold considerable pharmaceutical potential and may alone, or as a combination therapy, provide a suitable alternative treatment for persistent, residual intracellular infections (Bahnsen *et al.,* 2015), latent reservoirs of infections frequently associated with HIV and TB, immune deficiencies and various cancers. Since the emergence of several systemic delivery strategies, including microinjection, electroporation and liposome- and viral-based vectors (Copolovici *et al.,* 2014), such cell-penetrating AMPs have been increasingly favoured for therapeutic development (Dinca *et al.,* 2016), although limited by poor efficiency, bioavailability, specificity and high toxicity (Copolovici *et al.,* 2014). As drug delivery vectors, cell-penetrating AMPs exploit several properties of CPPs as well as unique features of the target microenvironment, mediating the non-invasive import of cargo molecules, including peptides, proteins up to 120 kDa in size, oligonucleotides, plasmids, peptide nucleic acids and nanoparticles (Fischer *et al.,* 2004).

AMPs provide protection both directly, by interacting with invading microbes, and indirectly, through several alternative mechanisms including maintaining normal gut homeostasis and modulating host inflammatory responses (Wang *et al.,* 2016). This multifunctionality of cationic AMPs provides a major advantage for the rapid recovery of superficial and systemic infections. AMPs introduced systemically, themselves or by novel drug delivery vectors (Maher & McClean, 2006), are however, likely to interact with erythrocytes and immune cells - the latter often activated by the presence of infective agents. Immune cell activation frequently results in the modification of cell membrane properties which further modifies host cell response to AMPs and subsequent manifestation of their cytotoxic effects (Pacor *et al.,* 2002). Such effects include direct interaction with metabolites and induction of mitochondrial translocation and disruption, resulting in the up-regulation of apoptosis (Maher & McClean, 2006).

Previously the cellular effects of Os, an AMP derived from the carboxyl terminal region of tick defensin, OsDef2, was evaluated by Prinsloo *et al.* (2013) and Malan *et al.* (2015). No significant haemolytic effect in human erythrocytes or reduction in the viability of SC-1, Caco-2 and RAW 264.7 cells was observed, even at a concentration (100 µM) several fold greater than the MBCs of Os i.e. 0.77 µM against *B. subtilis* (13933), 6.10 µM against *S. aureus* (U3300) and 1.52 µM against *E. coli* (ATCC 700928). Against PBMCs and PMNs, Taute (2017) only observed toxic effects at 50 and 100 µM Os. Additionally, Taute (2017) reported that Os was capable of binding DNA, but required peptide uptake for the regulation of eukaryotic cellular function.

To evaluate the contribution of Cys residues to the observed cellular effects of Os, the cytotoxic and membranolytic effects of Os(C→S) was evaluated against murine fibroblasts and human erythrocytes, respectively. Additionally, the cell-penetrating capacity of Os and Os(C→S) compared with penetratin, was determined in human PBMCs and PMNs, representing the major cell types associated with the topical and systemic application of AMPs.

The specific objectives outlined in this chapter were:

1. To determine the cytotoxic potential of Os(C→S), compared with Os, in the L929 cell line.
2. To evaluate the membranolytic effect of Os(C→S), compared with Os, in human erythrocytes.
3. To determine the membrane permeability, intracellular localisation and cytotoxicity of Os(C→S), compared with Os, in human PBMCs and PMNs.

3.2 Materials

3.2.1 Equipment and Reagents

All reagents used were of analytical grade, and double distilled deionised water (dddH_2O; Millipore system Q, Millipore, United States of America (USA)) was used for all experiments.

Foetal bovine serum (FBS), antibiotic solution (containing 10 000 U/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B), Dulbecco's Modified Essential Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, CV, AB, formaldehyde, acetic acid, formic acid, dimethyl sulphoxide (DMSO), ethanol (EtOH), trypsin, poly-L-lysine, 4',6-diamidino-2-phenylindole (DAPI), sodium dodecyl sulphate (SDS), sodium chloride (NaCl), potassium chloride

(KCl), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \bullet H_2O$) and disodium hydrogen phosphate (Na_2HPO_4,) were obtained from SIgma-Aldrich (Johannesburg, South Africa (SA)).

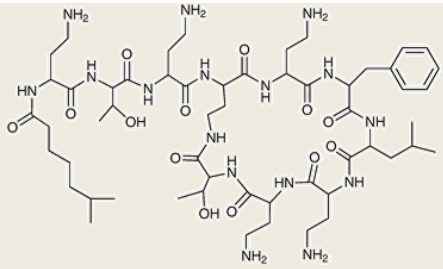
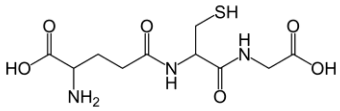
Control peptides, polymyxin B, melittin and GSH were purchased from Sigma-Aldrich (Johannesburg, SA). Penetratin and the OsDef2 derivatives, Os and Os(C→S), were synthesised using FlexPeptide™ technology by GenScript (New Jersey, USA). Reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry was used by the supplier to determine the purity and molecular mass of each peptide, respectively. Peptide concentration was determined using the molar extinction coefficient of chromophoric amino acid residues: Tyrosine (Tyr; 1200 AU/mmol/ml) and Trp (5560 AU/mmol/ml), and the Beer-Lambert equation:

$$c = \frac{A \times df \times M_r}{n(\varepsilon\, Tyr) + n(\varepsilon Trp)}$$

where c is the peptide concentration (mg/ml); A is the absorbance of the peptide measured at 280 nm; df is the dilution factor; M_r is the relative molar mass of the peptide (g/mol) and ε is the respective extinction coefficient (AU/mmol/ml).

All peptides were made up to a 1 mM stock solution in sterile $dddH_2O$, and stored at -20°C as aliquots of 60 µl in polypropylene tubes, until needed. Dilutions from the stock solutions were prepared in sterile $dddH_2O$, as required. Table 3.1 shows the amino acid sequences and properties of all peptides used in this study, including the controls.

Table 3.1: Amino acid sequences, physiochemical properties and sources of peptides.

Peptide	Amino acid sequence	Net charge (pH 7)*	Hydrophobicity <H>*	MW (g/mol)	Source
Experimental peptides					
Os	KGIRGYKGGYCKGAFKQTCKCY	+6	0.249	2459.92	Carboxyl terminal region of OsDef2
Os(C→S)	KGIRGYKGGYSKGAFKQTSKSY	+6	0.034	2411.72	Carboxyl terminal region of OsDef2 (Cys substituted with Ser)
Control peptides					
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	+5	0.511	2846.48	Venom of *Apis mellifera*, Western honey bee
Polymyxin B		+5	-	1385.61	Strains of *Bacillus polymyxa*
GSH		-1	-	307.32	-
Penetratin	RQIKIWFQNRRMKWKK	+7	0.193	2245.73	Antennapedia homeoprotein
Reference peptide					
Os-C	KGIRGYKGGYKGAFKQTKY	+6	0.045	2150.51	Carboxyl-terminal of OsDef2, with three Cys residues omitted

*Data obtained from HeliQuest server, available at [http://heliquest.ipmc.cnrs.fr]; accessed on 02 February 2018.

All media, reagents, enzyme solutions and buffers were sterile-filtered through a Millex 0.2 µm filter. Glassware was sterilised using a vertical steriliser (model number: STA-400/STA-410; Taiwan, ROC) at 121°C for approximately 20 minutes.

Microtiter plates: 96- and 24-well, 25 cm^2 and 75 cm^2 tissue culture flasks, 15 ml and 50 ml centrifuge tubes, 5 ml and 10 ml serological pipettes and pipette tips (10 µl, 200 µl, and 1000 µl),

coverslips and 600 µl and 1500 µl Eppendorf tubes were obtained from either Greiner Bio-One supplied by LASEC (Johannesburg, SA) or Thermo Fischer Scientific (Johannesburg, SA).

3.2.2 Cell lines and blood cells

The L929 cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC) supplied by Sigma-Aldrich (Johannesburg, SA). L929 cells are an adherent fibroblast cell line isolated from areolar and adipose tissue and derived from the species *Mus musculus* (mouse). Erythrocytes and leukocytes were separated from the venous blood of healthy, consenting donors (Health Sciences Research Ethics Committee number: 417/2017).

3.3 Methods

3.3.1 L929 cell culture

The L929 cell line was cultured in DMEM, supplemented with 10% (v/v) FBS, 2 mM glutamine and 1% antibiotic solution (DMEM/FBS), and stored at 37°C in 5% atmospheric carbon dioxide (CO_2). Once the cell culture reached 70% confluence, the cells were detached enzymatically, using a 5% (w/v) trypsin solution. The resulting suspension was transferred into a 15 ml tube and centrifuged at 200 x gravity (*g*) for 2 minutes using a HERMLE Z 300 centrifuge (Labortechnik GmbH, Wehingen, Germany). The cells were then resuspended in fresh DMEM/FBS and plated at the desired concentration in a sterile 96-well microtiter plate. Wells containing only DMEM/FBS were included as controls.

3.3.2 Cell density: Crystal violet assay

The relative density of adherent cells may be efficiently and economically quantified using CV, otherwise known as Gentian violet, a basic triphenylmethane dye capable of ionising in solution to form positively charged chromophores. The positively charged chromophores bind to negatively charged cellular components, including DNA and protein, of both viable and non-viable cells. Non-viable cells typically lose their adherence capacity and are eliminated during a washing procedure following staining. The remaining bound dye is subsequently solubilised and the resulting density is quantified as a percentage relative to the control, measured spectrophotometrically, where cell density is directly proportional to the colour intensity of the solution. Non-specific staining associated with CV does however, limit the resolution between

viable and persistent nonviable-but-still-intact cells, over estimating cell density (Rampersad, 2012).

The cytotoxic effect of positive controls, melittin, GSH and polymyxin B, and synthetic peptides, Os and Os(C→S) was evaluated on the fibroblastic L929 cell line. Once confluent, cells were collected and resuspended at a seeding density of 4×10^4 cells/ml DMEM/FBS in a sterile 96-well microtiter plate and incubated at 37ºC and 5% CO_2 for 24 hours. Melittin, polymyxin B, GSH, Os and Os(C→S) were serially diluted and a volume of 10 µl of each dilution was added to each well containing 90 µl media to obtain a final concentration range of 1.56 - 100 µM. The cells were incubated at 37°C for a further 24 hours. Following exposure, 10 µl of a 20% (v/v) formaldehyde fixative solution was added to each well and incubated at 37°C for 20 minutes. The formaldehyde was then removed and the plate was washed under running water and allowed to dry. The cells were stained using approximately 200 µl of a 0.1% (w/v) CV dye prepared in formic acid (200 mM; pH 3) for 30 minutes. After staining, excess dye was removed and the plate was washed under running water and allowed to dry. The cells were assessed microscopically, using an Olympus IX71 (Olympus Corporation, Tokyo, Japan) inverted light microscope and images were obtained using a ZEISS AxioVision Rel. 4.8.2 camera. A volume of 50 µl of a 10% acetic acid solution was then added for 30 minutes to solubilise the bound dye. The absorbance was measured using an Emax Plus microplate reader (Molecular Devices, California, USA) at 630 nm. Cellular density, in the presence of peptide, was expressed as a percentage relative to the untreated control, i.e. 100% cell density, using the following equation:

$$\%\ Cell\ density = \left(\frac{absorbance_{sample}}{absorbance_{untreated}} \times 100\right)$$

3.3.3 Cell viability: Alamar Blue assay

AB is a water soluble indicator molecule capable of indirectly quantifying mitochondrial and cytosolic enzyme activity (Hamid *et al.,* 2004) according to the compound's own reduction. The reduction of AB from the oxidised, blue resazurin form to the reduced, red resofurin form occurs as a result of the reduction of oxygen (O_2) by cytochrome oxidase during cellular respiration (Mo *et al.,* 2008), the extent of which relies on metabolic pathways influenced by the reducing capacity of individual cells (Zachari *et al.,* 2014). This limits the assays ability to distinguish between active and quiescent cells, often over estimating cell viability (Rampersad, 2012). Additionally, the AB

assay is frequently subjected to interference from reducing compounds, such as GSH, and molecules with intrinsic fluorescence characteristics. Extended incubation of AB with cells may, additionally, induce toxic effects or enhance the effects produced by the experimental compounds to which the cells are exposed (Niles *et al.,* 2009). Using both the CV and AB assay has the advantage of distinguishing between the loss of cell density and associated loss of cell viability due to necrosis or metabolic inactivation.

The AB assay was performed as described by Collins & Franzblau (1997) with modification. Serial dilutions of melittin, GSH, polymyxin B, Os and Os(C→S) were prepared and a volume of 10 µl of each dilution was added to 80 µl cells, at a seeding density of 4×10^4 cells/ml DMEM/FBS, obtaining a final concentration range of 1.56 - 100 µM. The cells were then incubated at 37°C for 6 hours. A volume of 10 µl of a 1 mM (0.1 mM final concentration) AB solution was added and the cells were incubated at 37°C for a further 18 hours – increasing the detectable range and resulting sensitivity of the assay (Voytik-Harbin *et al.,* 1998). Fluorescence was measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), at an excitation wavelength of 520 nm and an emission wavelength of 590 nm, for 1 minute. Cell viability in the presence of peptides was expressed as a percentage relative to the untreated control, i.e. 100% cell viability, using the following equation:

$$\%\ Cell\ viability = \left(\frac{fluorescence_{sample}}{fluorescence_{untreated}} \times 100\right)$$

The intrinsic capacity of peptides to spontaneously reduce resazurin to resafurin, in the absence of cells, was not assessed.

3.3.4 Blood collection

To obtain erythrocytes and leukocytes, a volume of 10 ml venous blood was collected from healthy, consenting donors by a trained phlebotomist (Health Sciences Research Ethics Committee number: 417/2017) using a sterile needle inserted into labelled vacutainers (LASEC, Johannesburg, SA) containing 3.2% sodium citrate and 1.8 mg/ml potassium ethylenediaminetetraacetic acid (K_2EDTA), respectively. The tubes were labelled using a laboratory-based labelling system i.e. Cell type: Researcher's name: Date of collection.

3.3.5 Erythrocyte cell membrane effects: Haemolysis assay

The haemolysis assay is considered a direct indicator of toxicity, evaluating the release of haemoglobin (Hb) resulting from toxin-induced membranolysis and subsequent erythrocyte destruction (Zohra & Fawzia, 2014). The advantage of the haemolysis assay is largely due to its biological relevance as well as the critical role of erythrocytes in antioxidant protection within the blood. Erythrocytes as a cell model additionally, reduces the confounding contribution of cellular signalling and is not affected by mitochondrial production of ROS, instead generating cellular energy through the pathway of glycolysis - providing improved indications of the protective capacity and systemic use of test compounds compared to other cell types (Honzel *et al.*, 2008; Kabanova *et al.*, 2009). The sources from which blood is obtained as well as its storage life do, however, limit the reproducibility of the method (Blasa, *et al.*, 2007).

Erythrocytes were isolated by centrifugation of WB at 400 x *g* for 3 minutes, using a HERMLE Z 300 centrifuge (Labortechnik GmbH, Wehingen, Germany), and washed with 10 mM isotonic phosphate buffered saline (isoPBS; 0.137 M NaCl, 3 mM KCl, 1.9 mM $NaH_2PO_4 \bullet H_2O$, 8.1 mM Na_2HPO_4, pH 7.4). Erythrocytes were then diluted in isoPBS to a 50% stock solution and stored at 4˚C. The haemolysis assay was performed as described by Nakajima *et al.* (2003).

Stored erythrocyte stock solution was allowed to warm to room temperature and washed with isoPBS. Melittin, Os and Os(C→S) were serially diluted to a final concentration range of 1.56 - 100 µM. A volume of 90 µl of a 5 % erythrocyte working solution, diluted in isoPBS, was exposed to 10 µl prepared peptides in separate tubes and incubated for 30 minutes at 37°C. The negative control was exposed to isoPBS only and taken as 0% haemolysis. The positive control was exposed to 2% SDS, inducing haemolysis, and was taken as 100% haemolysis. The erythrocytes were pelleted by centrifugation, the supernatant was collected and transferred to a 96-well microtiter plate, and the absorbance of the supernatant was measured using an Emax Plus microplate reader (Molecular Devices, California, USA) at 570 nm. Haemolytic activity, in the presence of peptide, was expressed as a percentage relative to the untreated control and the positive control exposed to 2% SDS, using the following equation:

$$\%\, Haemolysis = \left(\frac{Absorbance_{sample} - Absorbance_{0\%}}{Absorbance_{100\%} - Absorbance_{0\%}} \right) \times 100$$

3.3.6 Cellular localisation: Confocal laser scanning microscopy

The labelling of peptides with fluorescent probes permits the visualisation of membrane permeabilisation and intracellular localisation using confocal laser scanning microscopy. To determine whether Os and Os(C→S) are capable of penetrating the membranes of PBMCs and PMNs for subsequent internalisation, each leukocyte population was exposed to 5-carboxyfluorescein (5-FAM) N-terminus-labelled peptide, synthesised by GenScript (New Jersey, USA). Peptide concentration was selected in accordance with recommendations by Cardozo *et al.* (2007) who suggested that successful *in vitro* application of CPPs should occur at a concentration less than or equal to 10 µM – ensuring adequate cellular internalisation and limited toxic effects. Since neither Os nor Os(C→S) exhibited any significant cytotoxic effects at high concentrations, the concentration of each peptide was doubled in attempt to maximise their potential cell-penetrating capacity.

Leukocytes were isolated by layering WB onto Histopaque 1077 and 1119 and centrifuging the resulting suspension at 500 x *g* for 30 minutes using a HERMLE Z 300 centrifuge (Labortechnik GmbH, Wehingen, Germany). The PBMCs, or agranulocytes, consisting of lymphocytes and monocytes; and PMNs, or granulocytes, consisting of basophils, neutrophils and eosinophils (Figure 3.1 A and B, respectively), were collected into separate tubes and washed twice with RPMI medium supplemented with 10% FBS and 1% antibiotics (RPMI/FBS). Following each wash, the cells were centrifuged at 250 x *g* for 10 minutes. The pelleted leukocytes were treated with 4 ml dddH_2O for 30 seconds, haemolysing any remaining erythrocytes. A volume of 4 ml of 1.8% saline solution was then immediately added, restoring physiological isotonic conditions. Following haemolysis, the cell suspension was centrifuged, resuspended in RPMI/FBS medium and used immediately.

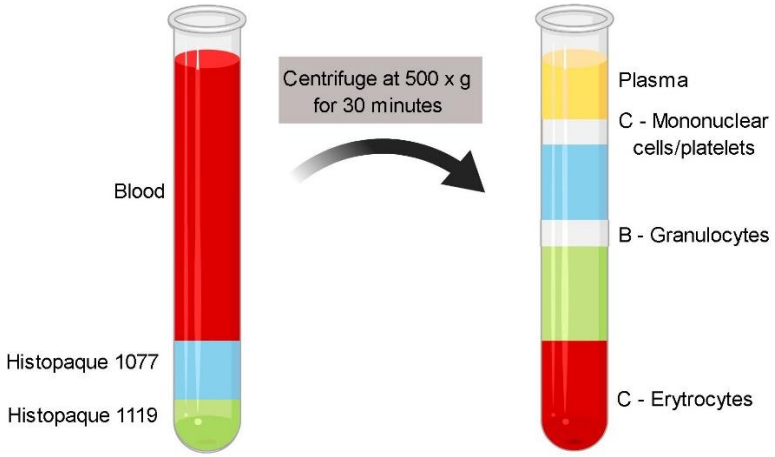


Figure 3.1: Layers of cells after centrifugal separation with Histopaque 1077 and 1119 (Sigma-Aldrich, 2010).

A volume of 180 µl cells at a density of 1 × 10^6 cells/ml was incubated with 20 µl fluorescently labelled penetratin (100 µM), Os (200 µM) and Os(C→S) (200 µM), to a final concentration of 10 µM, 20 µM and 20 µM, respectively; at 37°C and 5% CO_2 for 30 minutes, as described by Guterstam *et al.* (2009) with modification. The suspension was then centrifuged at 500 x *g* for 2 minutes. Following centrifugation, the supernatant was removed and a volume of 400 µl of 10 µg/ml DAPI in isoPBS was added to each cell pellet and incubated at 37°C and 5% CO_2 for 30 minutes. The cells were then washed twice with isoPBS, centrifuging at 500 x *g* for 2 minutes between washes. A volume of 5 µl of the resulting pellet was added to 10 mm diameter coverslips, mounted onto glass slides using clear nail polish. Cells were observed with a Zeiss laser scanning microscope (LSM) 880 with Airyscan (Carl Zeiss NTS GmbH, Oberkochen, Germany) at an excitation wavelength of 359 nm and emission wavelength of 461 nm for DAPI, and 490 nm and 520 nm for 5-FAM. Images were obtained using a ZEISS Axiocam 506 mono camera and viewed using the ZEN imaging software.

3.3.7 Statistical analysis

Unless otherwise stated, all experiments were performed in triplicate, and repeated three times, resulting in nine data points. All data was presented as the mean ± standard error of the mean (SE). Multiple comparisons were tested with Analysis of Variance (ANOVA) and the Tukey nonparametric test to test for significant differences using a confidence interval of 95%. Linear regression analysis confirmed whether a trend existed between the dependent and independent

variables. Statistical analysis was performed using GraphPad Prism version 6.01 (California, USA).

3.4 Results

Cytotoxicity is an essential parameter for determining the feasibility of compounds as therapeutic agents. Since preliminary data obtained by Minaar (2015) showed that Os and Os(C→S) capable of killing Gram-negative bacteria, with an MBC of 10 and 37 µM against *E. coli* (ATCC 7009268), respectively; toxicity profiling was essential to establish the specificity of peptides towards bacterial cells. The cytotoxicity of Os and Os(C→S), compared with positive control, melittin was, therefore, evaluated on mammalian L929 cells using the CV and AB assays. Polymyxin B and GSH were additionally included as controls to determine whether the density and/or viability of L929 cells would be compromised by their utilisation in the cellular antioxidant activity assay, the DCFH-DA assay (Chapter 4, Section 4.4.2)

3.4.1 Os and Os(C→S) does not alter the density of L929 cells

To determine the effect of peptides on cell density, mammalian L929 cells were evaluated using the CV assay, following 24 hours exposure to melittin, GSH, polymyxin B, Os and Os(C→S). The CV assay indirectly quantifies a reduction in cell density, associated with the detachment of non-viable cells, as a proportional reduction in the amount of bound dye - representative of the number of remaining, adherent cells.

Quantitative analysis of the cytotoxic effects of melittin against murine L929 cells, following the extraction of bound CV, demonstrated an average reduction of 50.27% in cell density and relative increase in cytotoxicity at concentrations ranging from 12.5 - 100 µM (Figure 3.2). Cells exposed to antioxidant and anti-inflammatory controls, GSH and polymyxin B, did not show any significant reduction in cell density, compared to the untreated control, even at the highest concentration of 100 µM. Cells exposed to Os and Os(C→S) similarly, did not exhibit any significant loss of cell density, compared to the untreated control.

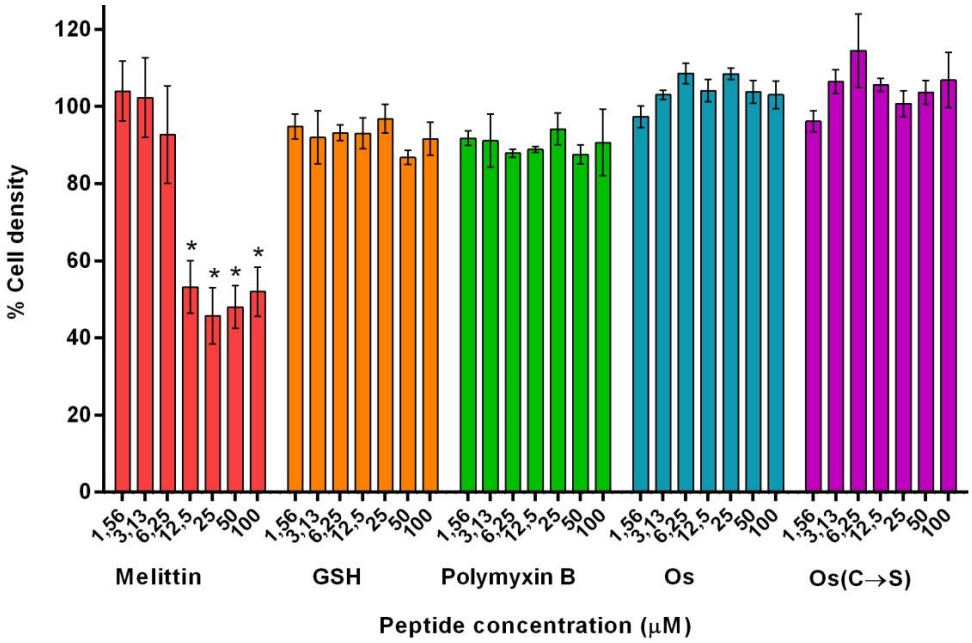


Figure 3.2: The effect of melittin, GSH, polymixin B, Os and Os(C→S) on the density of L929 cells evaluated using the CV assay after 24 hours exposure. Data is expressed as a mean ± SE of three independent triplicate experiments. An asterisk (*) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (100% density).

Light microscopy images, representing the effects of peptides on mammalian cells as a function of staining intensity, revealed comparable cell densities between the untreated control and cell exposed to 1.56 µM melittin (Figure 3.3 B). At 100 µM melittin, a significant reduction in staining intensity and consequently cell density was observed (Figure 3.3 C). Altered fibroblast morphology was additionally observed in cells exposed to 100 µM melittin, with adherent fibroblasts appearing as round and shrunken cellular structures. In contrast, no major difference in cell density or cell morphology was observed in cells exposed to 1.56 and 100 µM Os (Figure 3.3 D and E) or Os(C→S) (Figure 3.3 F and G), both of which appeared similar to the untreated control.

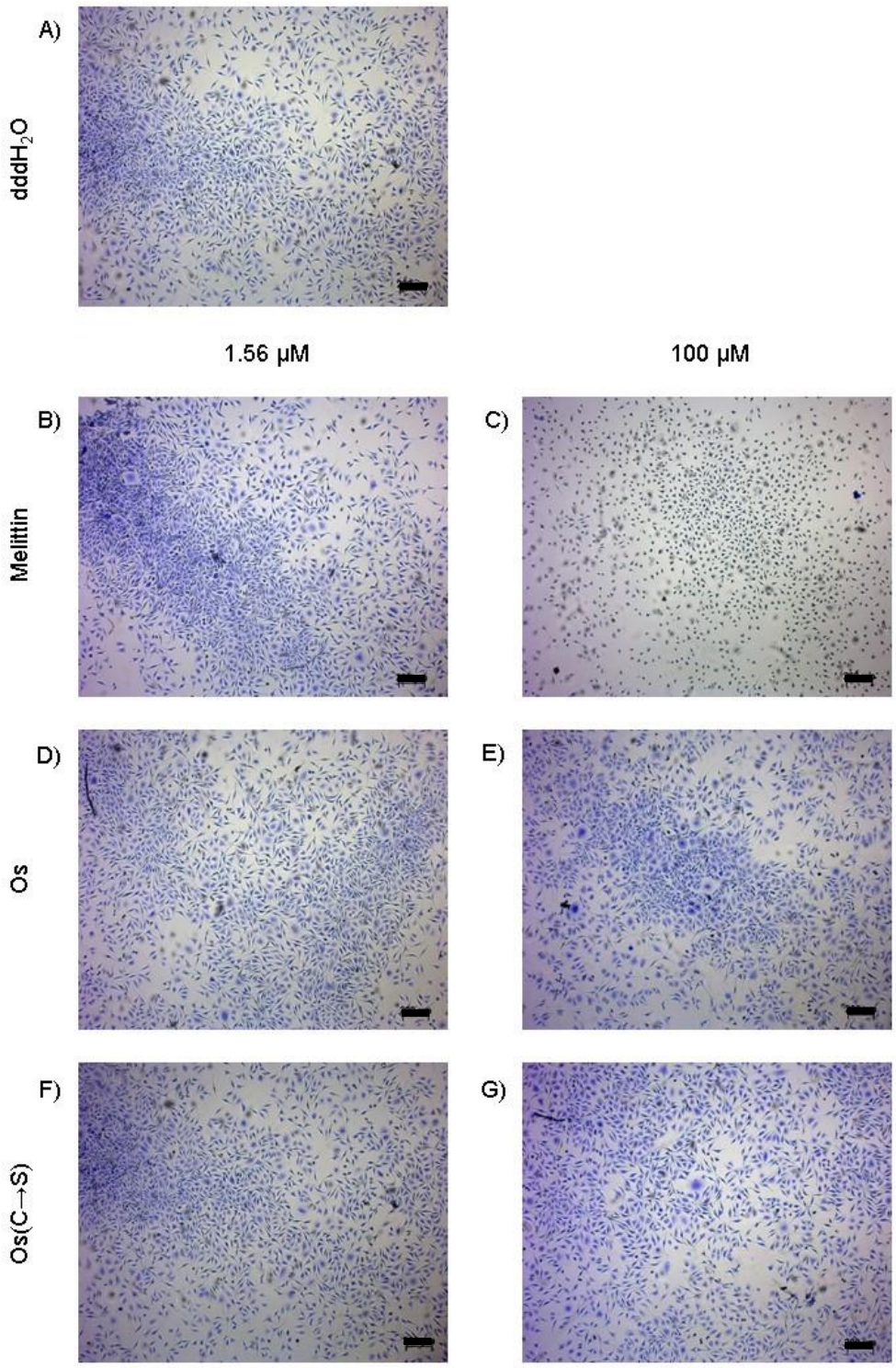


Figure 3.3: Light microscope images of L929 cells showing CV staining of (A) the untreated control, containing $dddH_2O$, and following 24 hours exposure to the lowest (1.56 µM) and highest (100 µM) concentrations of (B and C) melittin, (D and E) Os and (F and G) Os(C→S). Images are representative of three independent triplicate experiments. Scale bars = 200 µM.

3.4.2 Os and Os(C→S) does not alter the viability of L929 cells

To determine the effect of peptides on cell viability, L929 cells were exposed to increasing concentrations of melittin, GSH, polymyxin B, Os and Os(C→S) and the resulting effect evaluated using the AB assay. A reduction of resazurin to resofurin by mitochondrial and cytosolic reductases, generating a blue to red colour change, is proportional to cellular metabolic activity and relative cell viability.

A dose-dependent reduction in cellular viability was observed for melittin at concentrations ranging from 3.13 - 100 µM. Cells exposed to increasing concentrations of antioxidant and anti-inflammatory controls, GSH and polymyxin B, did not exhibit any significant reduction in viability compared to the untreated control. Similarly, cells treated with Os and Os(C→S), at all tested concentrations, did not induce any significant loss of cellular viability (Figure 3.4). A high correlation between the AB assay and CV staining was observed for the lytic peptide, melittin, with R^2 = 0.966, indicative of a strong association between the loss of cell viability and reduction in cell density (Figure 3.5).

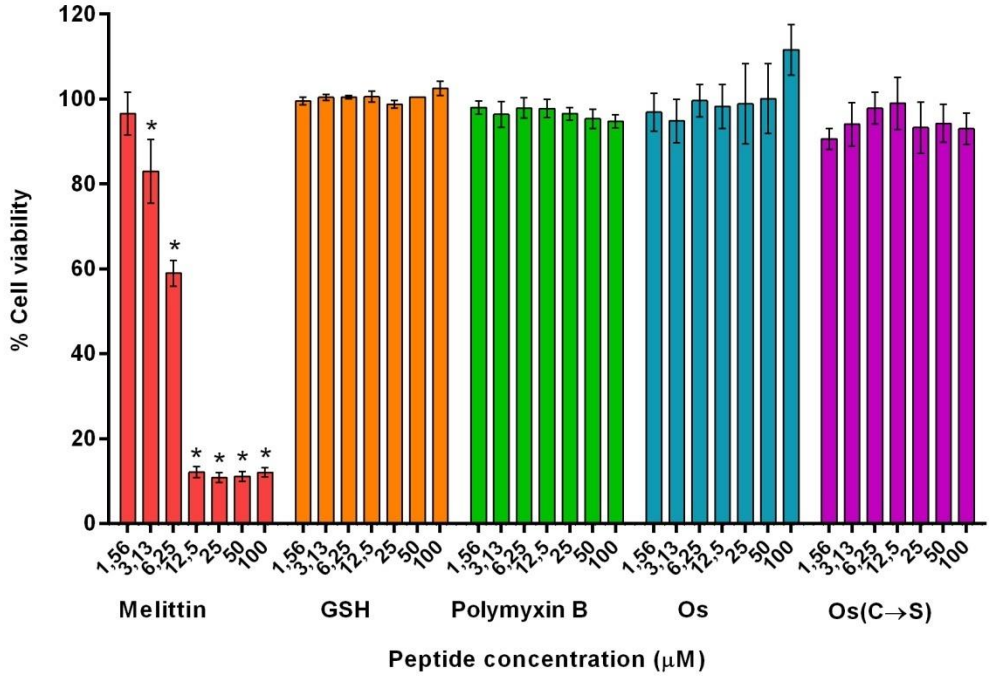


Figure 3.4: The effect of melittin, GSH, polymixin B, Os and Os(C→S) on the viability of L929 cells evaluated using the AB assay after 24 hours exposure. Data is expressed as a mean ± SE of three independent triplicate experiments. An asterisk (*) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (100% viability).

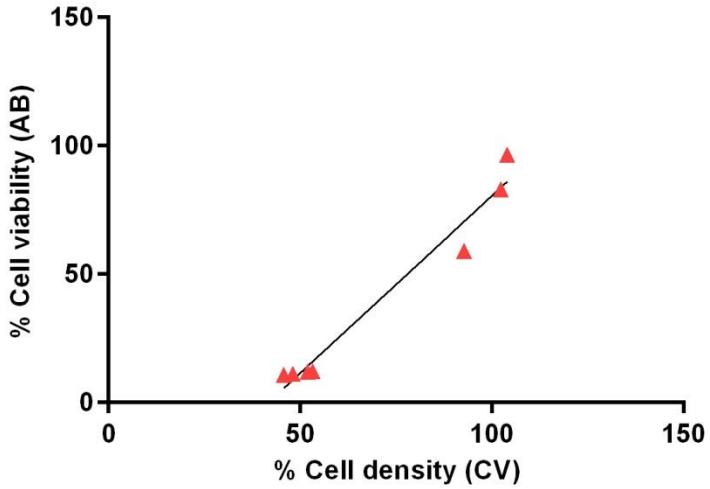


Figure 3.5: Correlation between the percentage cell density and cell viability of L929 cells exposed to increasing concentrations of melittin, as determined by the CV and AB assay, respectively. Equation obtained from linear regression analysis was $y=1.381x-57.5$ ($R^2=0.966$).

3.4.3 Os and Os(C→S) do not induce membranolysis in human erythrocytes

Using isolated human erythrocytes, the cytotoxicity of Os and Os(C→S), compared to positive control melittin, was determined. Significant membranolysis was observed in erythrocytes exposed to increasing concentrations of melittin. Melittin produced a perfect dosage-related increase in haemolytic activity at concentrations ranging from 1.56 – 6.25 µM, with R^2=1.00 (Figure 3.6 B). At concentrations ranging from 6.25 - 100 µM, melittin induced complete erythrocyte lysis comparable to the ionic detergent, SDS. Os and Os(C→S) did not display any significant haemolytic effects, compared to the untreated control, even at the highest tested concentration of 100 µM (Figure 3.6 A).

The observed membrane-damaging or haemolytic effects of peptides was further investigated with SEM (Chapter 5, Section 5.4.2, Figure 5.6).

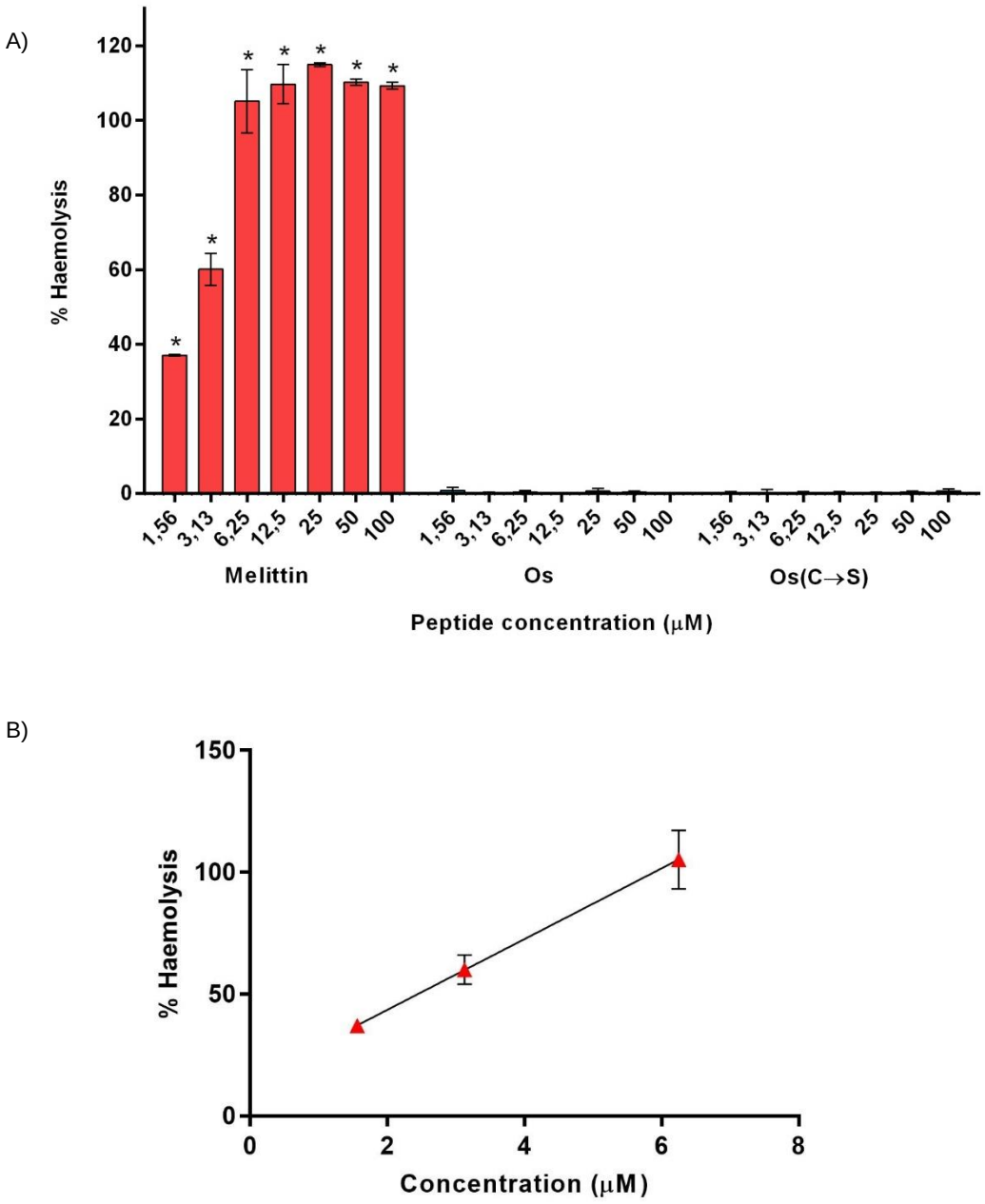


Figure 3.6: The haemolytic effect of Os and Os(C→S) against human erythrocytes after 30 minutes exposure. (A) Percentage haemolysis induced by melittin, Os and Os(C→S). (B) Linear dose-response obtained for melittin. Data is expressed as a mean ± SE of two independent duplicate experiments. An asterisk (*) indicates significant differences ($p \leq 0.05$) relative to the control containing isoPBS only (0% haemolysis). Equation obtained from linear regression analysis for melittin was $y=14.51x+14.54$ ($R^2=1.00$). No dosage effect was observed for Os or Os(C→S).

3.4.4 Ability of Os and Os(C→S) to penetrate leukocytes is less than penetratin

To evaluate the cell-penetrating properties of Os(C→S), compared with Os and penetratin, an established CPP, PBMCs and PMNs were exposed to fluorescently labelled peptides and the resulting membrane translocation and cellular internalisation of each peptide was assessed using confocal laser scanning microscopy. Artefacts of cellular uptake and intracellular distribution of peptides induced by fixation was avoided by performing all experiments in living cells.

All peptides appeared to be capable of translocating into both PBMCs and PMNs, although with variation in fluorescence intensity reflecting differences in internalisation and subsequent intracellular distribution. In PBMCs, penetratin, a 16 residue long PTD derived from the Antennapedia homeodomain of the *Drosophila*, appeared to be distributed between both the nuclear and cytoplasmic compartments of the cells (Figure 3.7 C and D). Os appeared to preferentially accumulate on the surface of the cell membrane. Occasional internalisation, via endocytic vesicle formation, with subsequent perinuclear localisation of Os was observed (Figure 3.7 E and F). Os(C→S) similarly appeared to enter the cells via endocytic vesicles, although to a lesser degree than Os and with only slight cytoplasmic accumulation (Figure 3.7 G and H).

The uptake and internalisation of peptides by PMNs differed substantially from that observed for PBMCs. Penetratin appeared to be confined to endocytic vesicles of PMNs, with sparse and limited cytoplasmic distribution (Figure 3.7 K and L). The fluorescent intensity of penetratin in these cells was, additionally, significantly lower than that observed in PBMCs. Endocytic vesicles containing Os remained predominantly confined to the internal border of the cell membrane with weak extracellular leakage of entrapped fluorescent dye (Figure 3.7 M and N). Os(C→S) similarly appeared to enter the cells via endocytic vesicles, although with limited internalisation and cytoplasmic localisation (Figure 3.7 O and P).

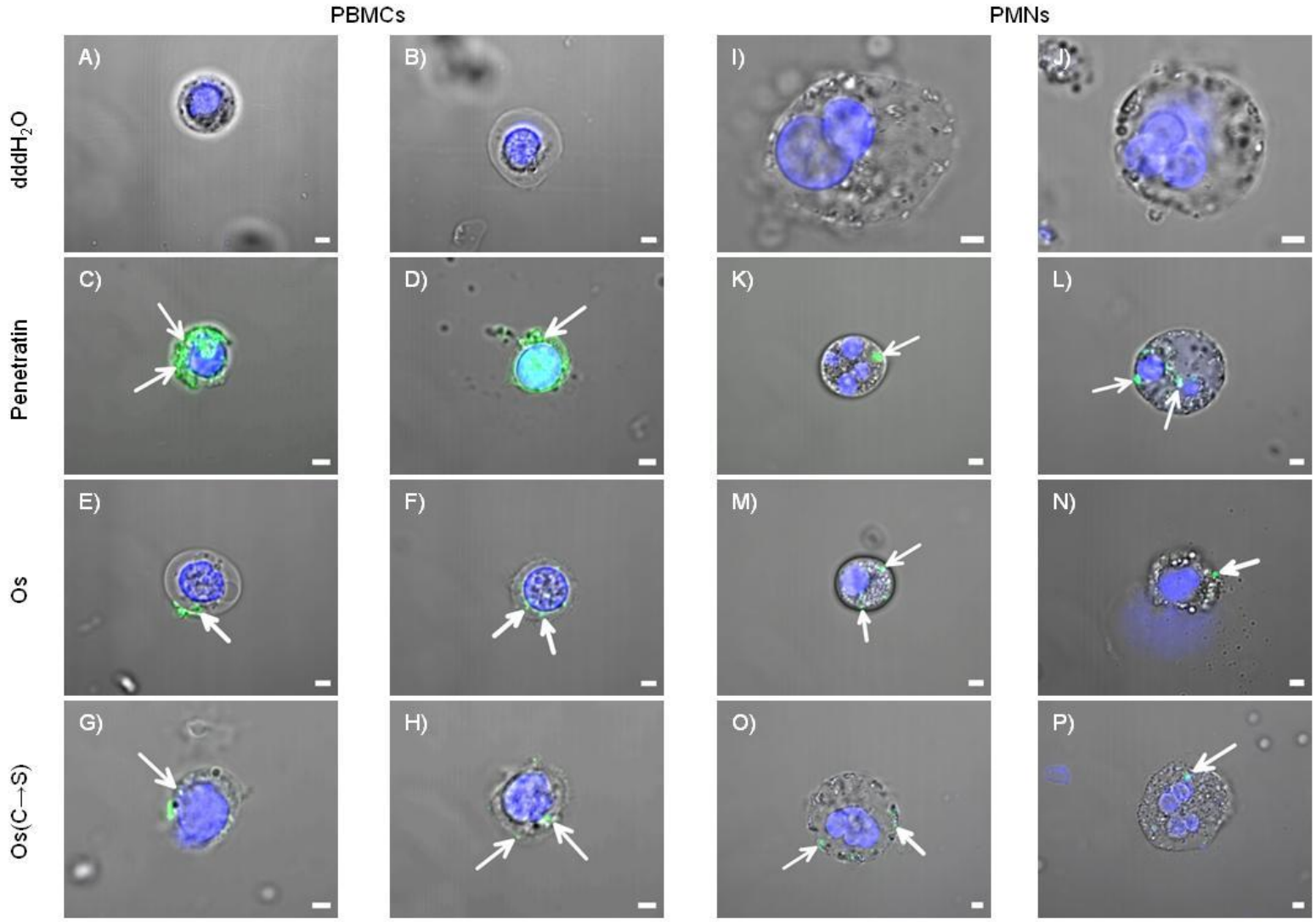


Figure 3.7: Confocal fluorescence microscope images of PBMCs and PMNs exposed to (A, B, I and J) dddH$_2$O and 5-FAM labelled (C, D, K and L) penetratin (10 µM), (E, F, M and N) Os (20 µM) and (G, H, O and P) Os(C→S) (20 µM), counterstained with DAPI. Images are representative of three independent duplicate experiments. Arrows indicate peptide clusters associated with endocytic vesicles**.** Scale bars = 2 µM.

Using differential interference contrast (DIC) it was possible to distinguish the predominant cell types present within the PBMC and PMN fractions. The large, spherical nucleus with a thin cytoplasmic rim in cells constituting the majority of PBMCs was characteristic of lymphocytes, while the appearance of multilobulated nuclei accompanied by cytoplasmic granules in the PMN fraction was typical of neutrophils. No obvious morphological characteristics of apoptosis were observed in either cell type.

To confirm the absence of cytotoxicity in leukocytes following 30 minutes exposure to melittin (0.16 - 5 µM), Os (1.56 - 50 µM) and Os(C→S) (1.56 - 50 µM), the viability of PBMCs and PMNs was evaluated using the AB assay as described in Section 3.3.3.

A significant reduction in the viability of PBMCs was observed at the highest concentration of melittin (5 µM). Melittin also reduced cell viability by 6.2 and 6.9% at 0.16 and 0.31 µM respectively, and increased cell viability by 5.8% at 2.5 µM, although significant, compared to the untreated control these differences were considered negligible. A similar, significant but negligible reduction in the viability of PBMCs was observed at all tested concentrations of Os and Os(C→S), with the exception of 12.5 and 25 µM Os (Figure 3.8 A). In PMNs, melittin reduced cell viability at all tested concentrations. At 5 µM melittin, the viability of PMNs was 68.3%, considerably less than the untreated control. A significant but negligible reduction in the viability of PMNs was observed at all tested concentrations of Os and Os(C→S), with the exception of 12.5 and 25 µM Os (Figure 3.8 B). Neither Os nor Os(C→S) therefore, exhibited any cytotoxic effects at their MBCs against *E. coli* (ATCC 700928) - 1.52 and 3.11 µM, respectively.

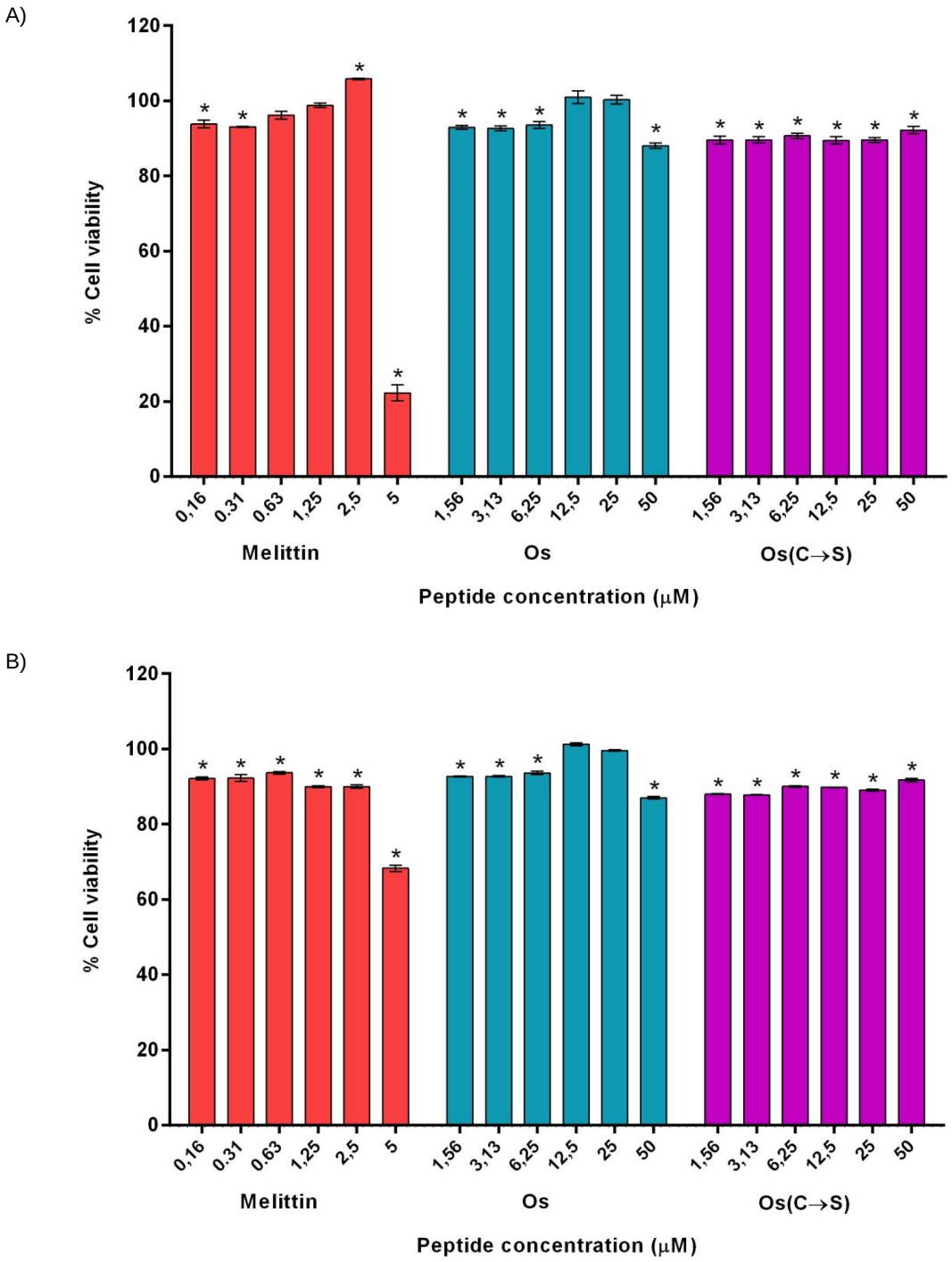


Figure 3.8: The effect of melittin, Os and Os(C→S) on the viability of human leukocytes evaluated using the AB assay after 30 minutes exposure. (A) Percentage viability of PBMCs. (B) Percentage viability of PMNs. Data is expressed as a mean ± SE of two independent triplicate experiments. An asterisk (*) indicates significant differences ($p \leq 0.05$) relative to the untreated control (100% viability).

3.5 Discussion

The therapeutic development of AMPs, typically active against a broad spectrum of microorganisms associated with numerous superficial and systemic infections, places considerable emphasis on their target specificity. In diabetic ulcers for example, eradication of infection using AMPs should not influence the viability and functioning of fibroblasts and epithelial cells responsible for wound healing and subsequent closure. Similarly, in the treatment of both primary and secondary systemic infections the viability and functioning of haemocytes, including erythrocytes and leukocytes, should not be compromised, especially where activation of the immune system is capable of initiating adverse inflammatory responses.

To establish the effect of Os and Os(C→S) on mammalian cells, L929 murine embryonic fibroblast cells were exposed to increasing concentrations of the respective peptides and evaluated using the CV and AB assays. A significant reduction in cell density (Figure 3.2) and cell viability (Figure 3.4), as well as associated changes in the distribution and morphology of L929 cells (Figure 3.3 B and C), was observed following exposure to all but the lowest measured concentrations of melittin, i.e. 1.56 and 3.13 µM. Additional controls GSH and polymyxin B, as well as Os and Os(C→S), did not induce any significant changes in cell density, cell viability or cell morphology even at each peptides highest measured concentration of 100 µM.

Results obtained following CV staining were comparable to data obtained by Prinsloo *et al.* (2013) and preliminary results described by Minnaar (2015, unpublished). Prinsloo *et al.* (2013) evaluated the cytotoxic effects of Os, Os-C and melittin on SC-1 (embryonic, *Mus musculus*, fibroblast) and Caco-2 (adult, *Homo sapiens*, epithelial, colorectal adenocarcinoma) cells using the CV assay and found that at concentrations of between 0.82 - 52 µM melittin produced an approximate 2.5- and 1.25-fold reduction in cell density in SC-1 and Caco-2 cells, respectively. At concentrations ranging from 0.78 - 49 µM for Os and 0.88 - 55.8 µM for Os-C, these peptides did not induce any significant changes in cell density in either SC-1 or Caco-2 cells. Preliminary results obtained by Minnaar (2015, unpublished), who evaluated the cytotoxicity of Os, Os(C→S) and melittin on murine SC-1 using the CV assay, similarly showed an approximate 2.5-fold reduction in the density of SC-1 cells at concentrations of melittin ranging from 6.25 - 100 µM. Neither Os, nor Os(C→S) exhibited any significant reduction in cell density, even at the highest tested concentration (100 µM).

Data obtained by Malan *et al.* (2015), who evaluated the effects of Os, Os-C, melittin and polymyxin B on the viability of RAW 264.7 (adult, *Mus musculus*, monocyte/macrophage, Abelson murine leukaemia virus-transformed) cells using the 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, was consistent with the results obtained from the AB assay. The colorimetric MTT assay quantifies the activity of metabolic reductases, in metabolically active cells, by the reduction of water-soluble yellow MTT tetrazolium salts to insoluble purple formazan crystals by mitochondrial succinate dehydrogenase, mediated by nicotinamide adenine dinucleotide (NADH)/NADPH both outside the mitochondria and within the cell (Fotakis & Timbrell, 2005). Since formazan crystals are impermeable to cell membranes (Weyermann *et al.*, 2005), the resulting amount of purple formazan crystals produced is directly proportional to cell viability.

Malan *et al.* (2015) found that both melittin and polymyxin B exhibited significant cytotoxic effects at concentrations from 1.56 and 25 µM, respectively. Os and Os-C did not induce any significant reduction in cell viability at any of the tested concentrations, including the highest concentration of 100 µM. The variation between the toxicity profiles of polymyxin B, described in the present study and by Malan *et al.* (2015), may be owing in part to the physiological and biochemical dissimilarities associated with the L929 and RAW 264.7 cell lines, i.e. RAW 264.7 murine macrophages are representative of cells involved in inflammatory responses while L929 fibroblasts are characteristic of cells implicated in wound healing (Petrochenko *et al.,* 2015).

The predominant cell type in the blood is erythrocytes and is usually the first cell type used for the evaluation of systemic toxicity. Mature erythrocytes are characteristically anucleate and devoid of both ribosomes and mitochondria, are unable to perform mitotic divisions, oxidative phosphorylation or protein synthesis (Lux & Palek, 1995), making them ideal models of typical lipid bilayers (Honzel *et al.*, 2008). The lipid bilayer of erythrocytes, separating the intracellular content from the external environment, is composed primarily of phospholipids and cholesterol, along with transmembrane proteins embedded within it (Fairbanks, *et al.*, 1971). Disruption of this lipid bilayer results in cytoplasmic leakage and subsequent Hb release. This may be effectively quantified to ascertain the direct toxic effect of a compound. In cell lines, this membranolytic effect results from the indirect activation of several pathways, disrupting processes of metabolism, conjugation and excretion.

The membranolytic effects of Os and Os(C→S), compared to positive control melittin, was evaluated in human erythrocytes. Melittin produced a dosage-related increase in haemolysis between concentrations ranging from 1.56 - 6.25 µM with complete erythrocyte lysis occurring at 6.25 - 100 µM. No significant haemolytic effects were observed for Os and Os(C→S), even at the highest tested concentration (Figure 3.6). The haemolytic and membrane damaging effect of melittin, following 30 minutes exposure, was supported by visible morphological alternations to the cell surface and subsequent echinocyte formation, observed with SEM (Chapter 5, Section 5.5.2). Os and Os(C→S) on the other hand, did not exhibit any notable structural characteristic associated with membranolysis.

Data obtained by Prinsloo *et al.* (2013), who similarly investigated the ability of Os, Os-C and melittin to induce haemolysis in human erythrocytes, showed that neither Os nor Os-C exhibited any haemolytic effects at any of the tested concentrations, even at concentrations 16 times greater than the MBC against *P. aeruginosa* (ATCC 10145) (ranging from 0.38 - 6.5 µM). In contrast, a dose-related increase in haemolytic activity was observed for melittin at concentrations ranging from 2.5 - 100 µM.

Hydrophobicity and amphipathicity are critical parameters for membrane-targeting peptides. Chen *et al.* (2007) investigated the role of hydrophobicity on the mechanism of action of α-helical AMPs by systemically reducing the hydrophobicity of the synthetic peptide, $V13K_L$. The study showed that peptide hydrophobicity dramatically modifies biological activity in both prokaryotic and eukaryotic cells. In eukaryotic cells, peptides with higher hydrophobicity tend to penetrate deeper into the hydrophobic core of cells, inducing stronger cytotoxic effects by the pore- or channel-forming barrel-stave mechanism of cellular uptake. This is consistent with the high hydrophobicity (0.511) of melittin (Table 3.1) and observed cytotoxic and membranolytic effects on L929 cells and human erythrocytes, respectively. The low hydrophobicity (0.034) of Os(C→S) prevented sufficient binding of the peptide to the plasma membrane and subsequent exertion of its biological responses.

Since the MBC of melittin (0.17 µM) against *E. coli* (ATCC 700928) paralleled the concentrations required to induce haemolysis in human erythrocytes, melittin cannot be considered as a suitable candidate for therapeutic application in infectious disease (Maher & McClean, 2006). Several strategies have been explored to exploit the antimicrobial properties of such highly toxic AMPs while simultaneously reducing their cytotoxic effects, including a prodrug strategy which involves

the reversible inactivation of AMPs, conjugated to prodrug moieties, until desirable conditions are achieved. This strategy was previously applied to melittin reducing its *in vitro* cytotoxicity and permitting its application in a mouse model of cancer for tumour reduction (Forde *et al.,* 2016).

AMPs are capable of responding to infection both directly and indirectly by acting on microbial and host targets, respectively, providing an advantage over conventional antibiotic therapies (Augustyniak *et al.,* 2012). The direct effects of AMPs occur if the peptide is released at the site of microbial invasion, gaining direct access to the target microorganism; while indirect immunomodulatory effects are associated with the interaction between peptides and G-coupled receptors of immune cells. AMPs contribute to host immunomodulation either by exerting dual or opposing effects on particular cellular processes; acting in concert with other inflammatory mediators or intrinsic antimicrobials; or by various receptors or non-receptor mechanisms. AMPs may however, act preferentially on microbes through electrostatic interaction between positively charged peptides and negatively charged microbial membranes or destabilisation and disorganisation of the phospholipid membrane bilayer resulting in disruption of membrane integrity, membrane permeabilisation and cell death (Augustyniak *et al.,* 2012). Membrane permeabilisation and cellular internalisation is a prerequisite for the successful development of such AMPs, alone or as drug delivery vectors.

Penetratin, a cationic molecule composed of 16 amino acids derived from the Antennapedia homeodomain - an essential transcription factor of *Drosophila* development, is a class of CPPs capable of translocating across the cell membrane for intracellular localisation (Bárány-Wallje *et al.,* 2005). Penetratin is considered the most common positive control for the evaluation of peptide uptake in eukaryotic cells (Stalmans *et al.,* 2013). Previously, Taute (2017) identified Os as a possible CPP candidate.

Following the incubation of PBMCs and PMNs with 5-FAM-labelled peptides, intracellular fluorescence was observed for both Os (Figure 3.7 E, F, M and N) and Os(C→S) (Figure 3.7 G, H, O and P), although significantly less than the control, penetratin (Figure 3.7 C, D, K and L). The additional appearance of endocytic vesicles in cells exposed to penetratin was indicative of its mechanism of cellular uptake, shown to involve the endosomal pathway, facilitated by the binding of penetratin to GAGs present on the cell surface (Fischer *et al.,* 2004; Dupont *et al.,* 2011). Os appeared to penetrate cells more readily than Os(C→S) despite the presence of adjacent, unstained cells and extracellular fluorescence. No significant reduction in viability was

observed in cells exposed to Os and Os(C→S) (Figure 3.8 A and B). The reduction in uptake and cellular internalisation of Os(C→S) is likely owing to the absence of Cys residues, known to be implicated in the peptides hydrophobicity and associated affinity with mammalian cell membranes. These results are consistent with that obtained by Rodrigues *et al.* (2013) who investigated the potential of CPPs belonging to the family of nucleolar targeting peptides (NrTPs) as *in vivo* drug delivery vectors. Since the Cys residues present in NrTP6 were capable of dimerisation, a Ser-substituted analogue was synthesised for precaution. Despite the minimal change in peptide structure resulting from Ser-substitution, both the rate of uptake and subsequent internalisation of the peptide in PBMCs was significantly reduced.

Despite the presence of Cys residues in Os conferring a greater hydrophobicity (0.249) to the peptide than that associated with penetratin (0.193), penetratin contains two more Arg residues within its peptide sequence, compared with Os. Arg amino acid residues are known to enhance the cell-penetrating properties of peptides. Wender *et al.* (2000) compared the cellular uptake of short oligomers of Arg, Lys, ornithine (Orn) and histidine (His). Differences between cell surface binding and internalisation was additionally assessed using sodium azide as an inhibitor of energy-dependent cellular uptake. Deletion of one Arg residue from either the N- or C-terminus of the oligomer resulted in a significant loss of intracellular localisation. Deletion of Lys, on the other hand, from the C-terminus reduced cellular internalisation by approximately two-thirds, whereas no significant reduction was observed following the deletion of a Lys residue from the N-terminus. This suggests that Arg residues contribute more significantly to cellular uptake than Lys. The presence of Arg does, however, increase the toxicity of peptides towards mammalian cells (Li *et al.,* 2017).

The observed differences in uptake and subsequent internalisation of peptides within the PBMC population and between PBMCs and PMNs may be attributed to differences associated with each cell type. PBMCs, or agranulocytes, possess two primary cell populations, i.e. lymphocytes and monocytes, both of which are involved in immune system adaptation towards invading microbes and subsequent combating of infection (Rodrigues *et al.,* 2013). Distinct lymphocyte subsets perform various unique functions within the immune system - helper T cells function in the regulation of other immune cells; cytotoxic T and natural killer (NK) cells induce lysis in virus-infected cells, tumour cells, and allografts; and B cells are responsible for antibody secretion. Monocytes on the other hand, are involved in phagocytosis, antigen presentation and cytokine production and are considered the precursors from which macrophages and dendritic cells are

derived. In pathology, PBMCs may be implicated both directly, as in the case of leukaemia and HIV-1 infection, and indirectly, contributing to part of the immune system response.

Resting memory CD-4+ T cells are considered the predominant reservoir for latent HIV infections. Despite this, monocytes; non-CD-4+ T cells; tissue macrophages in the lungs; adipose tissue, gut-associated lymphoid tissue (GALT); semen; genital tract; bone marrow and central nervous system (CNS) must be considered in the design of HIV eradication strategies (Sacha & Ndhlovu, 2016). Similarly, infection with *M. tuberculosis* metabolically alters macrophages which acquire a phenotype characterised by the accumulation of lipid bodies that function as a source of nutrients and secure a niche for persistent bacteria (Singh *et al.,* 2012). Such latently infected cells are capable of escaping viral immune response and persist for long periods of time. The susceptibility of specific cell populations as well as their life span, proliferative capacity, and their ability to produce infectious viruses following physiological and immunological control is critical for the determination of the contribution of such cells to viral persistence (Alexaki *et al.,* 2008). The selective cellular uptake and capacity of peptides to act as intracellular drug reservoirs, therefore, holds considerable potential in the development of therapeutic agents possessing intrinsic biocompatibility and biodegradability and capable of long-term release for sustained antimicrobial effects.

PMNs, or granulocytes, comprising neutrophils, basophils and eosinophils, are considered key effectors of the inflammatory response, inducing substantial antimicrobial mechanisms that are responsible for both the elimination of pathogens and removal of necrotic tissue (Draskovic-Pavlovic *et al.,* 2012). Granulocytes function in the production of chemotactic signals, such as chemokines: MCP-1, MIP-1α, MIP-1β, MIP-3α; azurocidin; cathepsin G and α-defensin that attract monocytes and dendritic cells; and are additionally capable of inducing macrophage differentiation, towards either a pro- or anti-inflammatory state; influencing B-cell differentiation and regulating immunoglobulin production. Granulocytes are therefore critical to the specificity of the immune response (Draskovic-Pavlovic *et al.,* 2012).

Since Os exhibited no significant cytotoxicity and a greater uptake efficiency in human primary immune cells, compared with Os(C→S), the peptide, both alone and as a vector for the translocation of other therapeutic molecules, has significant potential in therapeutic application, particularly in the sub-cellular delivery of anticancer drugs and siRNAs (Rodrigues *et al.,* 2013). This therapeutic potential of Os is further enhanced by the peptides high antioxidant capacity

(Chapter 4, Section 4.4.2, Figure 4.7), providing possible applications against the adverse effects of infection-induced oxidative stress.

3.6 Conclusion

Neither Os nor Os(C→S) exhibited any cytotoxic or membranolytic effects in the L929 cell line, human erythrocytes or human leukocytes. Cellular internalisation of peptides appeared more apparent in PBMCs than in PMNs. The uptake efficacy of Os and Os(C→S) was low compared with known CPP, penetratin, and was associated with endocytic vesicle formation.

Chapter 4: Antioxidant effects of Os(C→S) compared with Os

4.1 Introduction

Increased oxidation, or oxidative stress, resulting in the overproduction of free radicals within the body has been implicated in the progression of several diseases, including diabetes mellitus, neurodegeneration, cardiomyopathy and cancer (Lesgards *et al.*, 2011). The endogenous formation and overproduction of free radicals resulting in cellular morphological and functional changes, additionally induces a cascade of reactions within, for example, phospholipid membranes, further stimulating free radical formation (Gupta-Elera *et al.*, 2011). Endogenous and exogenous free radicals are commonly produced during normal physiological processes, disease and toxicity, and following exposure to ultraviolet (UV) radiation or pro-oxidants, respectively (Irshad & Chaudhuri, 2002; Pérez *et al.*, 2009; Zeraik *et al.*, 2011). Free radicals are highly reactive, short half-life, molecules capable of removing electrons from other molecules, stimulating further production of free radicals (Farber, 1994). Reactive species are considered hormetic - exhibiting stimulatory or beneficial effects following low dosage exposure, necessary for normal physiological function, while high dosage exposure induces inhibitory or toxic effects, often promoting the destruction of macromolecules, including lipids, DNA and proteins (Pérez *et al.*, 2009). During normal physiological processes, low concentrations of reactive species function in the regulation of cell growth, modulation of the innate immune system and as signalling molecules, initiating communications between the mitochondria and other cellular compartments (Vaziri, 2008; Ristow & Schmeisser, 2011). Macromolecular destruction following exposure to high concentrations of reactive species on the other hand, induces large-scale tissue damage, mutagenesis, carcinogenesis and senescence (Pérez *et al.*, 2009; Kedare & Singh, 2011).

Antioxidants are capable of counteracting the damage induced by the formation of free radicals. Saturation of the endogenous antioxidant systems of an organism following excessive radical production does however, prevent successful reduction and repair of oxidative damage (Irshad & Chaudhuri, 2002; Cassanova *et al.*, 2008). The reduction in oxidative damage therefore relies on the introduction of exogenous antioxidants within an organism. Antioxidants are typically classified into two groups, i.e. primary or chain-breaking antioxidants and secondary or preventative antioxidants. Primary antioxidants are capable of reacting with lipid radicals, such as alkylperoxyl radical, converting them to more stable products. Secondary antioxidants reduce the rate of lipid

autoxidation by either binding metal ions, scavenging O_2, decomposing hydroperoxide ($HOO^{\bullet}$) to non-radical species, absorbing UV radiation or deactivating singlet O_2 (Maleki *et al.,* 2015).

Numerous AMPs have been shown to be capable of inhibiting the formation of free radicals (Udenigwe & Aluko, 2012). Several mechanisms influencing the antioxidant properties of AMPs have been suggested, including metal ion chelation, ROS scavenging, UV absorption and the dual inhibition of enzymatic and non-enzymatic peroxidation of lipids and essential fatty acids (Udenigwe & Aluko, 2012). His, proline (Pro), Cys, methionine (Met) and aromatic amino acids, in particular, are major contributors to the antioxidant activity of AMPs. Trp, Tyr and Phe function predominantly in UV absorption (Scott, 1997) while His amino acid residues are capable of chelating metal ions, further participating in HAT and ET reactions via their imidazole group (Chan & Decker, 1994), resulting in both the quenching of active O_2 and scavenging of hydroxyl radicals ($OH^{\bullet}$). The addition of hydrophobic amino acid residues, such as Pro and Leu, to the N-terminal region of a His-His dipeptide is associated with an increase in antioxidant activity, attributed to the enhanced capacity of the peptide to interact with hydrophobic cellular targets (Chen *et al.,* 1998).

Sulphur-containing amino acids, including Cys and Met, are especially sensitive to oxidation and have been shown to possess potent antioxidant activity against the effects of $O_2^{\bullet-}$, H_2O_2 and hypochlorous acid ($HOCl^-$) (Iwao *et al.,* 2012). $O_2^{\bullet-}$ and H_2O_2 are considered to be initiators of other ROS by generating $OH^{\bullet}$ following the reaction of H_2O_2 with metal ions and $HOCl^-$ by stimulated PBMCs at sites of inflammation, contributing significantly to disease progression. In GSH, Cys residues contribute to the tripeptide's low redox potential that influences the redox state of cells (Pereira *et al.,* 2013), providing them with redox buffering properties (Abegg *et al.,* 2012).

The antioxidant effects of Os was previously evaluated by Prinsloo *et al.* (2013), Malan *et al.* (2015) and preliminarily by Minnaar (2015, unpublished). Significant HAT- and ET- based antioxidant activity was observed for Os, determined using the DCFH-DA assay on RAW 264.7 cells as well as the ORAC and TEAC assay, respectively. To further evaluate the contribution of Cys residues to the observed antioxidant activity of Os, the chemical and cellular antioxidant activity of Os(C→S) was investigated and compared with Os.

The specific objectives outlined in this chapter were:

1. To determine the antioxidant activity of Os(C→S), compared with Os, using the TEAC and ORAC assays.
2. To determine the protective effects of Os(C→S), compared with Os, on AAPH-induced oxidative damage in the L929 cell line.
3. To determine whether chemical antioxidant activity is correlated with cellular antioxidant protection.

4.2 Materials

4.2.1 Reagents

In addition to the reagents described in Chapter 3, Section 3.2.1, AAPH, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium peroxodisulfate ($K_2S_2O_8$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), fluorescein and DCFH-DA, obtained from Sigma-Aldrich (Johannesburg, SA), were used.

4.2.2 Cell lines

The L929 cell line was used for all antioxidant studies and was sourced and maintained as described in Chapter 3, Section 3.2.2.

4.3 Methods

Antioxidant assays may either be based on single ET, observed as a colour reaction following the reduction of an oxidant, or HAT, in which competition for free radicals occurs between an antioxidant and substrate, or probe (Huang *et al.*, 2005).

Reactions based on ET, involve two components (Huang *et al.*, 2005), i.e. an antioxidant (AOH) and the oxidant probe (M). During the reaction, the probe undergoes reduction, oxidising the antioxidant, and resulting in a colour change that is directly proportional to the concentration of the antioxidant:

$$\mathbf{M\ (n) + e^-\ (from\ AOH)} \longrightarrow \mathbf{AOH^{\bullet+} + M\ (n-1)}$$

The gradient of a curve generated by known antioxidants or standards, including Trolox, gallic acid or catechin, represents the antioxidant reducing capacity which is reported as equivalence. In the absence of O_2 radicals, antioxidant capacity is assumed to be equivalent to reducing capacity (Huang *et al.*, 2005).

Reactions based on HAT, such as the ORAC and DCFH-DA assays (Tabart *et al.*, 2009), specifically quantify the antioxidant capacity of radical chain breaking antioxidants (Huang *et al.*, 2005). The fluorometric ORAC and DCFH-DA assays similarly employ AAPH generating peroxyl radicals ($ROO^{\bullet}$) from which the protective capacity of antioxidants towards an indicator molecule, (i.e. fluorescein and dichloro-dihydro-fluorescein (DCFH), respectively) can be quantified:

$$ROO^{\cdot} + AOH \longrightarrow HOO^{\bullet} + AO^{\cdot}$$

$$ROO^{\cdot} + LH \longrightarrow HOO^{\bullet} + L^{\cdot}$$

Frequently, several assays are employed and combined for more accurate quantification of antioxidant capacity, compensating for the shortcomings and underestimation associated with each. ET reactions, for example, are pH dependent resulting either in the suppression of antioxidant reducing capacity following protonation of the antioxidant in acidic conditions (Huang *et al.*, 2005). The TEAC assay is often combined with the ORAC assay as well as the cellular DCFH-DA assay, providing more relevant results. Assays based on HAT are, however, considered more biologically relevant having the advantage of a constant rate of free radical production and increased sensitivity, compared to ET-based assays.

The antioxidant capacity of Os(C→S), compared with Os, was therefore, chemically determined using both the TEAC and the ORAC assay, and further evaluated using the cellular DCFH-DA in the L929 cell line.

4.3.1 Chemical antioxidant assays

4.3.1.1 Trolox equivalent antioxidant capacity assay

The TEAC assay was performed as described by Awika *et al.* (2003) with modification. Radical anions are generated by $ROO^{\bullet}$-induced oxidation of ABTS, resulting in the formation of an intensely coloured product. ABTS radicals ($ABTS^{\bullet}$) are reduced in the presence of antioxidants undergoing oxidation, reducing the colour intensity of the product (Figure 4.1) (Prior *et al.*, 2005).

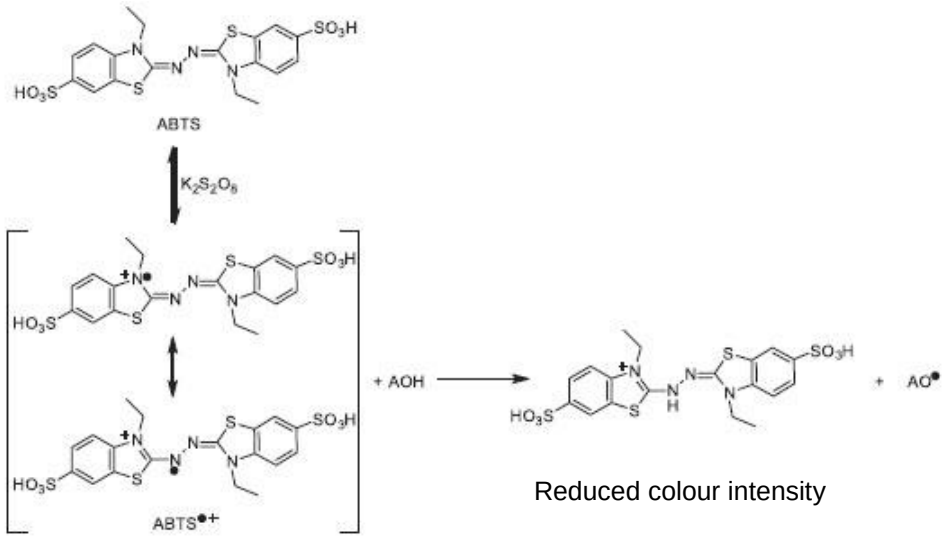


Figure 4.1: The generation and reduction of ABTS• anions in the presence of antioxidants (de Oliveira *et al.*, 2014).

ABTS stock solution (1:1 (v/v) of 3 mM $K_2S_2O_8$ solution and 8 mM ABTS) was prepared and incubated at room temperature in the dark for a minimum of 12 hours. The solution was used within 16 hours of preparation. A 0.1 M phosphate buffer, pH 7.4, was used to dilute the ABTS stock solution to a final working solution of 0.26 mM ABTS. GSH, Os and Os(C→S) were serially diluted to a concentration range of 1.56 - 100 µM. A volume of 290 µl of ABTS working solution was added to 10 µl of peptide and incubated at room temperature for 15 minutes. The absorbance was measured using an Emax Plus microplate reader (Molecular Devices, California, USA) at 650 nm. The results were compared to a standard curve of Trolox at a linear concentration range of 0 - 1000 µM. The linear dose-response equations were additionally used to determine and compare the µM TE/µM peptide.

4.3.1.2 Oxygen radical absorbance capacity assay

The ORAC assay was performed as described by Ou *et al.* (2001) with modification. The assay quantifies the antioxidant capacity of compounds based on HAT, during which antioxidants and substrates compete for ROO• produced by thermal decomposition of azo-compounds (Zulueta *et al.*, 2009). The measured reduction in the fluorescence of fluorescein is considered a function of structural and conformational changes resulting from ROO•-induced oxidative damage. Antioxidants, providing protection against such oxidative damage, inhibit the reduction in fluorescence (Figure 4.2) (Zulueta *et al.*, 2009; Ou *et al.*, 2001). The ORAC assay therefore

quantifies the capability of antioxidants to provide protection against oxidative damage and subsequent loss of fluorescence, by evaluating the area under a fluorescence decay curve (AUC) and comparing it to a suitable control and a reference antioxidant standard (Ou *et al.*, 2001; Cao *et al.,* 1993).

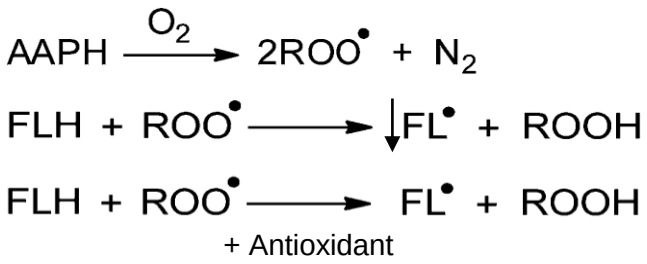


Figure 4.2: The reduction of fluorescein (FL) in the presence of ROO•, generated by the oxidation of AAPH. Antioxidants compete with fluorescein, inhibiting the reduction in fluorescence and subsequent oxidative damage (Dorta *et al.*, 2015).

A volume of 25 µl AAPH at a concentration of 0.11 µM was added to 10 µl peptide (GSH; Os and Os(C→S) serially diluted to 1.56 – 100 µM) and 165 µl fluorescein, diluted to 0.139 nM in 10 mM sodium phosphate (NaP) buffer (3.87 mM Na_2HPO_4, 6.12 mM NaH_2PO_4•H_2O, pH 7.4). The fluorescence was then measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, every minute for 2 hours at 37 °C. The results were compared to a standard curve of Trolox, with a linear concentration range of 0 - 1000 µM. The linear dose-response equations were additionally used to determine and compare the µM TE/µM peptide.

4.3.2 Cellular antioxidant assay

To determine whether the Os(C→S) is capable of neutralising oxidative damage induced by AAPH in L929 cells, the method of Wang & Joseph (1999) was used with modification. DCFH-DA spontaneously cross the plasma membrane and is cleaved by cytosolic esterases generating intracellular DCFH. Intracellular DCFH is oxidised to highly fluorescent dichlorofluorescein (DCF)

by free radicals (Figure 4.3). Due to the use of AAPH for the generation of ROO•, the DCFH-DA assay is considered the physiologically equivalent of the ORAC assay.

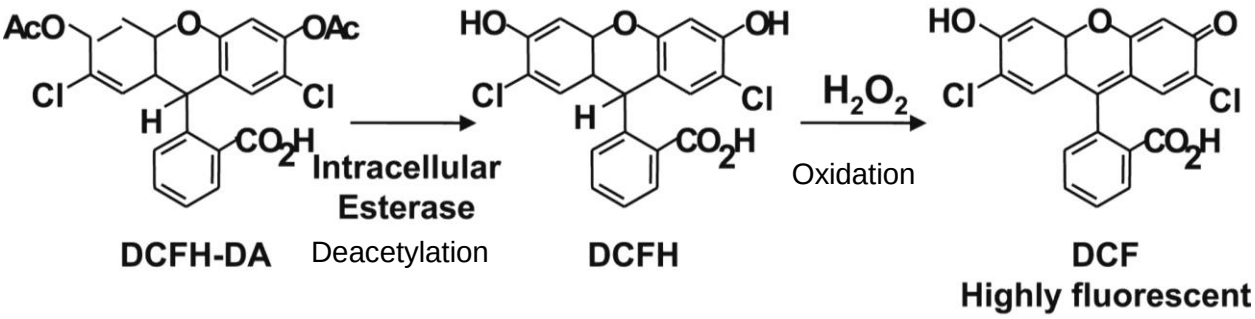


Figure 4.3: Cleavage of DCFH-DA by cytosolic esterases and subsequent oxidation in the presence of free radicals (Dikalov *et al.*, 2007).

L929 cells (100 µl; final concentration of 1 × 10^6 cells/ml) were plated in a sterile 96-well microtiter plate and incubated for 24 hours at 37°C and 5% CO_2. A volume of 50 µl of a 25 µM DCFH-DA solution was added and incubated for 45 minutes at 37°C and 5% CO_2. The cells were then washed using sterile 0.1 M PBS (0.081 M Na_2HPO_4, 0.019 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, pH 7.4) and simultaneously exposed to 40 µl of GSH, Os and Os(C→S) at a concentration range of 1.56 – 100 µM, and 40 µl of PBS or AAPH (0.1 mg/ml; 0.5 mg/ml final concentration). Fluorescence was measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, for 1 hour. The percentage protection of the peptides against AAPH-induced oxidative damage was calculated according to the AUC and expressed relative to the untreated control using the equation below. The linear dose-response equations were additionally used to determine and compare the % radical production/µM peptide.

$$\%\, Radical\ production = \left(\frac{AUC_{sample} - AUC_{PBS}}{AUC_{AAPH} - AUC_{PBS}}\right) \times 100$$

4.3.3 Statistical analysis

Statistical analysis was performed as described in Chapter 3, Section 3.3.7.

4.4 Results

4.4.1 Chemical antioxidant assays

4.4.1.1 Os and Os(C→S) effectively scavenges ABTS•

Evaluation of the antioxidant activity of GSH, Os and Os(C→S), using the ET-based, TEAC assay, revealed no significant free radical scavenging by GSH, compared to the untreated control. Comparable dosage effects were observed for both Os and Os(C→S) where increasing concentrations of each peptide was associated with increasing antioxidant activity. Additionally, the antioxidant activity obtained for Os and Os(C→S) was significantly higher than that of GSH, particularly at concentrations ranging from 12.5 to 100 µM (Figure 4.4 A). Linear regression analysis was performed showing no significant difference between Os and Os(C→S) (R^2=0.989 and 0.98, respectively), The molar ratio determined for Os was, however, 1.16-fold greater than that of Os(C→S), with 6.43 and 5.55 µM TE/µM peptide, respectively. The antioxidant activity of Os was however on average 1.15-fold greater than that of Os(C→S), contrary to that observed with the ORAC assay (Figure 4.4 B).

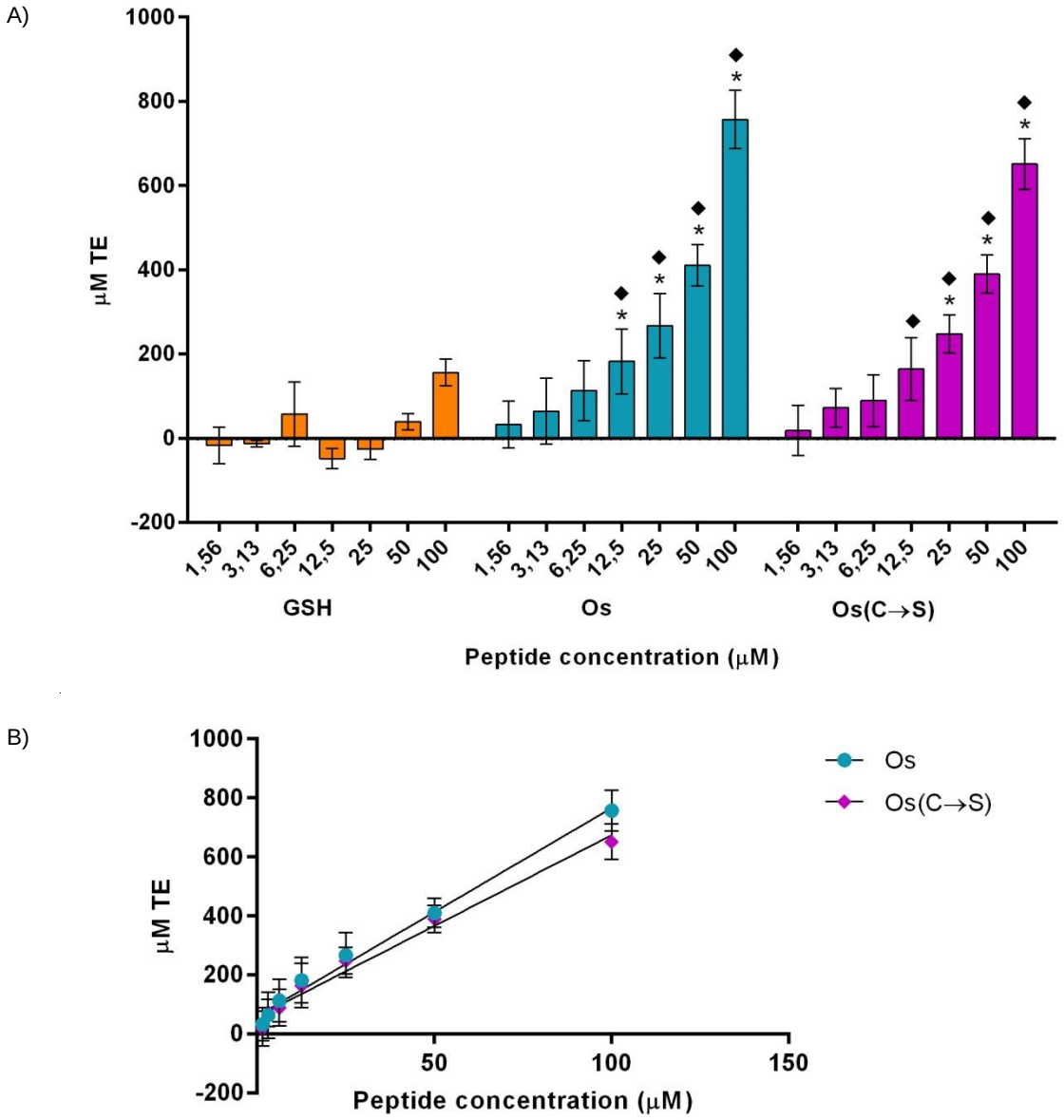


Figure 4.4: The antioxidant activity of Os and Os(C→S) determined using the TEAC assay. (A) µM TE of GSH, Os and Os(C→S). (B) Comparison of the linear dose-response between Os and Os(C→S). Data is expressed as a mean ± SE of three independent triplicate experiments. An asterisks (*) and solid diamond (◆) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (0 µM TE), GSH and Os, respectively. Equations obtained from linear regression analysis for Os was $y=7.048x-61.49$ ($R^2=0.8441$) and Os(C→S) was $y=6.148x-59.36$ ($R^2=0.85$). No significant difference ($p \leq 0.05$) was observed between the slopes and intercepts. No dosage effect was observed for GSH.

4.4.1.2 Os and Os(C→S) effectively scavenge peroxyl radicals

The radical chain breaking capacity of Os and Os(C→S), compared to known antioxidant, GSH, was further evaluated using the ORAC assay. Compared to GSH, Os and Os(C→S) exhibited significantly higher antioxidant activity at concentrations ranging from 12.5 - 100 µM. Additionally, both Os and Os(C→S) produced comparable dose-responses, with increasing antioxidant capacity associated with increasing concentrations. At 50 and 100 µM, the antioxidant activity of Os(C→S) was however, significantly greater than that produced by Os. The presence of Ser residues, therefore, appeared to enhance the activity of Os(C→S) in HAT-based antioxidation (Figure 4.5 A). Linear regression analysis was performed showing greater molar ratios for both Os and Os(C→S) (R^2=0.989 and 0.965 respectively) compared to the positive control, GSH. The molar ratio determined for Os(C→S) was however, 1.32-fold greater than that of Os, with 6.88 and 9.14 µM TE/µM peptide, respectively (Figure 4.5 B). A high correlation between the TEAC and ORAC assay was observed for Os and Os(C→S), with R^2=0.9827 and 0.9841, respectively, indicative of the capacity of both peptides to scavenge free radicals using HAT- and ET-based mechanisms (Figure 4.6 A and B). Additionally the slope of the linear dose-response for Os and Os(C→S) obtained for both the TEAC and ORAC assay were comparable, further emphasising both peptides nondiscriminant capacity to partake in HAT- and ET-based reactions.

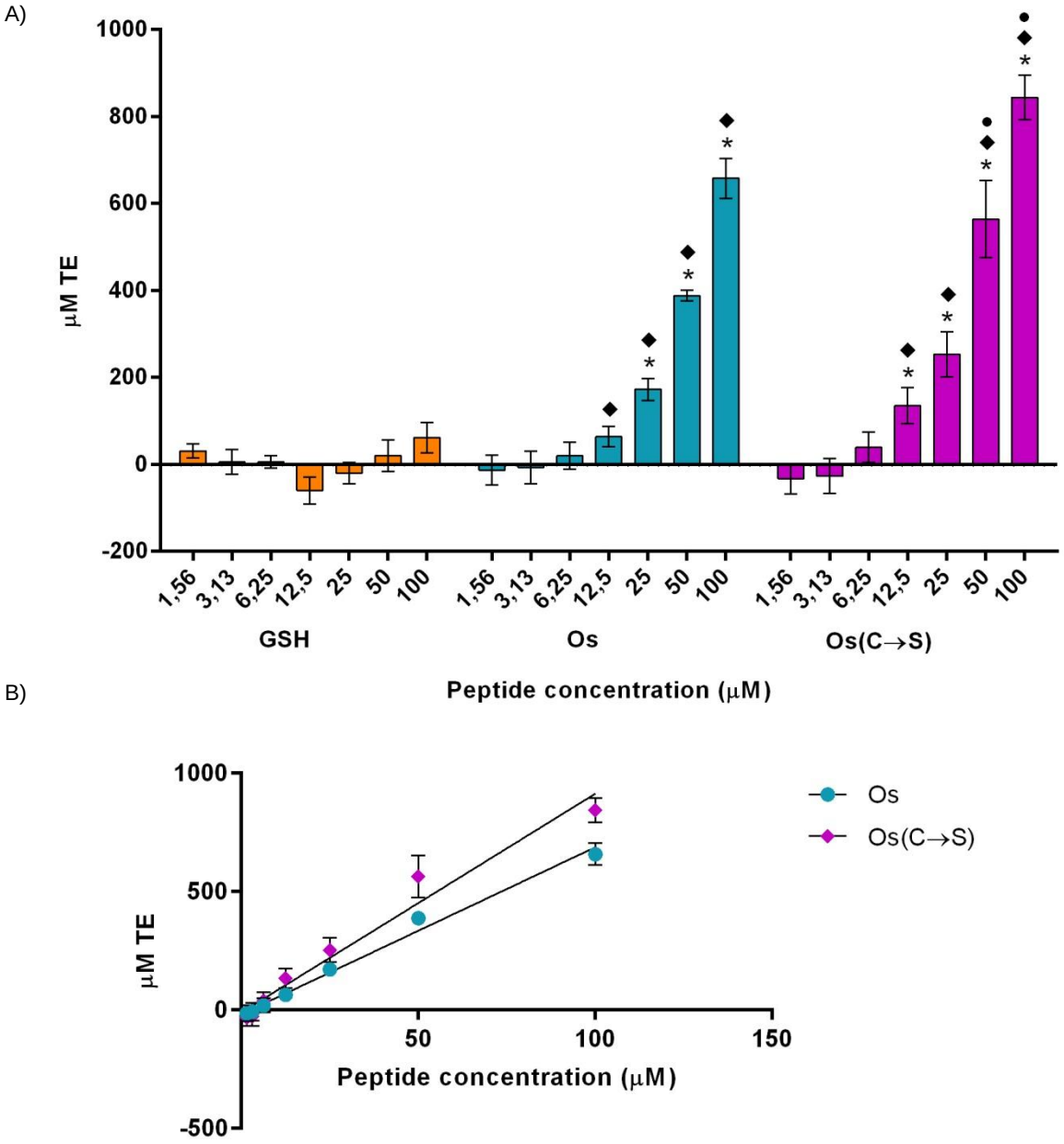


Figure 4.5: The antioxidant activity of Os and Os(C→S) determined using the ORAC assay. (A) µM TE of Os, Os(C→S) and GSH. (B) Comparison of the linear dose-response between Os and Os(C→S). Data is expressed as a mean ± SE of three independent triplicate experiments. An asterisks (*), solid diamond (◆) and solid circle (●) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (0 µM TE), GSH and Os, respectively. Equations obtained from linear regression analysis for Os was y=7.057x-18.03 (R^2=0.9532) and Os(C→S) was y=9.241x-10.27 (R^2=0.9225). Slopes of Os and Os(C→S) differed significantly ($p \leq 0.05$). No dosage effect was observed for GSH.

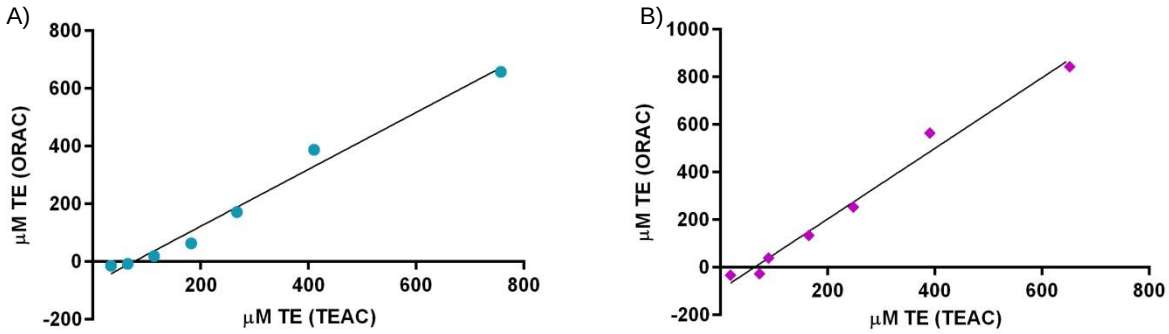


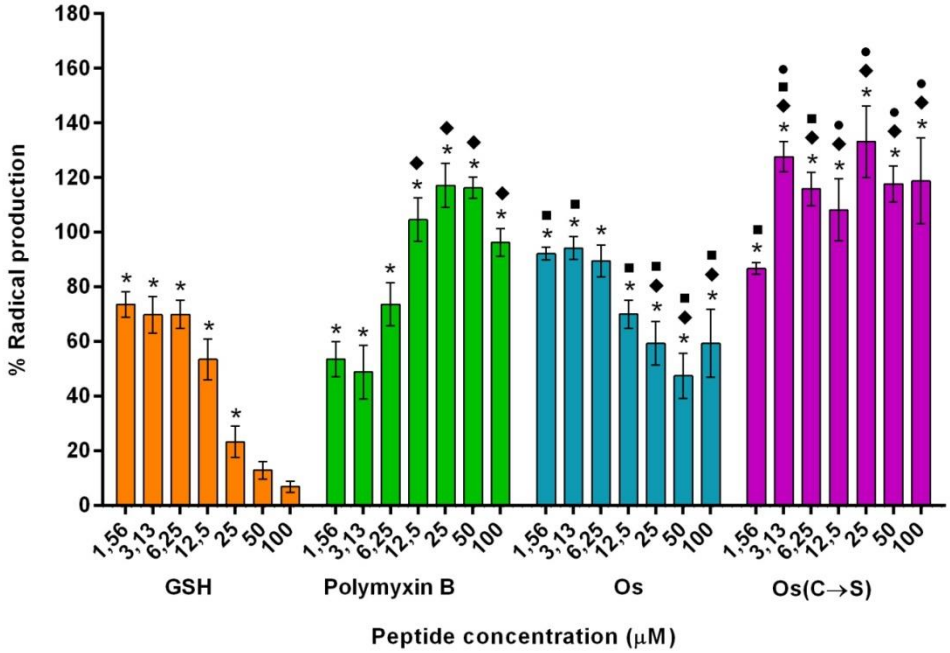
Figure 4.6: Correlation between the antioxidant activity of Os and Os(C→S), as determined by the TEAC and ORAC assay. (A) µM TE of Os. (B) µM TE of Os(C→S). Equations obtained from linear regression analysis for Os was y=0.9868x-74.7 (R^2=0.9827) and Os(C→S) was y=1.484x-93.12 (R^2=0.9841).

4.4.2 Os(C→S) lacks cellular antioxidant activity

The ability of peptides to reduce the formation of free radicals, implicated in oxidative damage, was evaluated using the DCFH-DA assay. The change in fluorescence as a result of intracellular cleavage of DCFH-DA was determined following AAPH-induced production of $ROO^{\bullet}$. Known antioxidant, GSH, was used as a positive control and compared to Os and Os(C→S). At concentrations ranging from 3.13 - 100 µM, both GSH and Os provided protection against AAPH-induced oxidative damage in L929 cells, although with varying degrees of efficiency. Between 25 - 100 µM, Os exhibited a significant reduction in its protective effect against AAPH-induced oxidative damage, compared to GSH. At the highest concentration, the protective effect of GSH was approximately 8.59-fold greater than that of Os. At 1.56 - 6.25 µM, polymyxin B exhibited significant radical scavenging while at concentrations greater than 6.25 µM, the antioxidant activity of polymyxin B was lost, instead exhibiting a pro-oxidant effect. Os(C→S) similarly produced a significant increase in radical production or pro-oxidant effect at concentrations ranging from 3.13 - 100 µM. Polymyxin B and Os(C→S) is therefore likely to interact synergistically with AAPH, stimulating the formation of free radicals (Figure 4.7 A). Comparison of the dose-response obtained for GSH and Os, at a concentration range of 3.13 - 50 µM, demonstrated no significant difference between the protective effects of each peptide (Figure 4.7 B). The molar ratio determined for GSH was however, 11.96-fold greater than that of Os, with -0.61 and -0.051 % radical production/µM peptide, respectively. At concentrations ranging from 1.56 - 50 µM, a high correlation between the TEAC and DCFH-DA assay as well as the ORAC and DCFH-DA assay was observed for Os, with R^2=0.9476 and 0.8649 respectively (Figure 4.8 A and B). At the same concentrations, the correlation between the TEAC and DCFH-DA assay

as well as the ORAC and DCFH-DA assay for GSH and Os(C→S) was largely negligible, with R^2=0.006763 and 0.01787 for GSH, and 0.2010 and 0.5406 for Os(C→S), respectively (not shown).

A)



B)

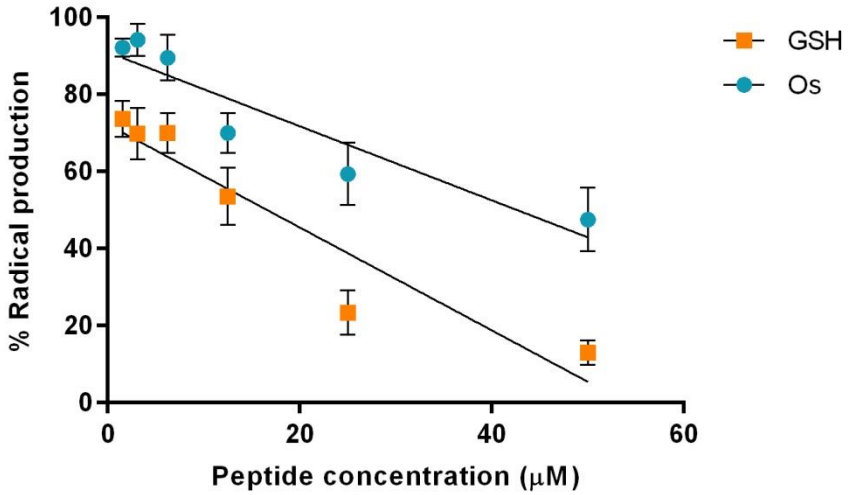


Figure 4.7: The protective effects of Os and Os(C→S) against AAPH-induced oxidative damage in L929 cells. (A) Percentage radical production in the presence of AAPH-induced oxidative damage. (B) Comparison of the linear dose-response obtained for GSH and Os. Data is expressed as a mean ± SE of two independent duplicate experiments. An asterisks (*), solid diamond (◆), solid square (■) and solid circle (●) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (0% radical production), GSH, polymyxin B and Os, respectively. Equations obtained from linear regression analysis for GSH was y=-1.335x+72.22 (R^2=0.7998) and Os was y=-0.961x+91.01 (R^2=0.7361). Intercepts of GSH and Os differed significantly ($p \leq 0.05$). No dosage effect was observed for Os(C→S).

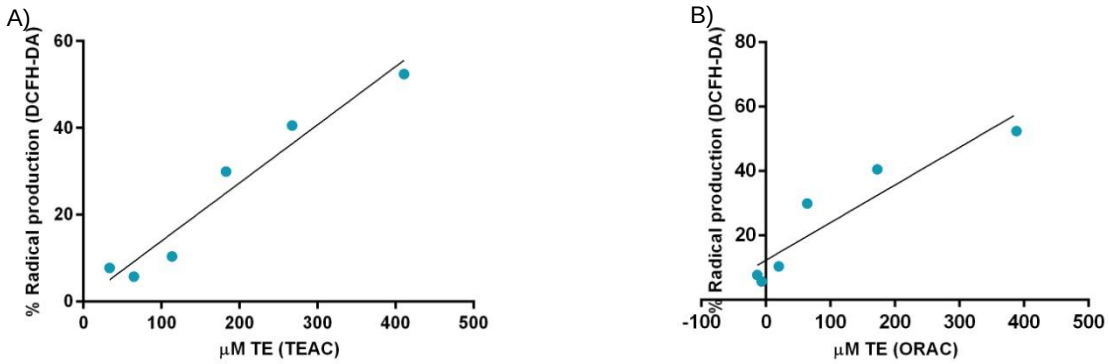


Figure 4.8: Correlation between the antioxidant activity of Os as determined by the TEAC, ORAC and DCFH-DA assay. (A) Percentage radical production and µM TE (TEAC) of Os. (B) Percentage radical production and µM TE (ORAC) of Os. Equations obtained from linear regression analysis for Os (DCFH-DA/TEAC) was y=0.134x+0.567 (R^2=0.9476) and Os (DCFH-DA/ORAC) was y=0.1168x+12.36 (R^2=0.8649).

4.5 Discussion

Oxidative stress, the oxidative modification of cellular macromolecules, including lipids, proteins, carbohydrates and nucleic acids (Vertuani *et al.,* 2004) is generated by the production of free radicals resulting in oxidative damage. Oxidative stress is largely associated with aging and chronic diseases, including Alzheimer's disease, cardiovascular disease (CVD), cancer, diabetes (Noguchi *et al.,* 2000; Simon *et al.,* 2000; Milner, 2000; Viuda-Martos *et al.,* 2008) and inflammation (Reuter *et al.,* 2010). The accumulation of free radicals and overproduction of ROS associated with inflammation promotes localised tissue damage (Choi *et al.,* 2013). ROS are capable of reacting with NO resulting in the formation of RNS. In the presence of NO scavengers however, the production of RNS should be largely impaired following the reduced availability of NO. Since Os was shown to function in NO scavenging (Chapter 5, Section 5.4.1, Figure 5.4) it was expected that the production of RNS from NO and ROS, in the presence of Os, should be significantly reduced.

ROS are free radicals derived from molecular O_2 (Buetnner & Jurkiewicz, 1996; Turrens, 2003). The high reactivity of molecular O_2 is formed following the gain of energy in a single unpaired electron and subsequent change in spin. These unstable electrons are capable of reacting with electron pairs of other molecules, particularly those associated with double bonds. Different types of ROS are distinguished according to their life span: $OH^{\bullet}$, alkoxyl ($RO^{\bullet}$) and $ROO^{\bullet}$ radicals are considerably short-lived; while nitroxyl ($NO^{\bullet}$) and $O_2^{\bullet-}$ radicals and non radicals, i.e. H_2O_2, organic $HOO^{\bullet}$, and $HOCl^{-}$ have medium life expectancies (Buetnner & Jurkiewicz, 1996; Turrens, 2003).

Antioxidants are heterogeneous, organic substances that act as radical scavengers breaking down radical chain reactions and counteracting the effects oxidative damage (Huang *et al.,* 2005). Antioxidants mediate their effect at concentrations lower than that of the oxidisable substrate, preventing its oxidation (Pérez *et al.,* 2006). Several groups of antioxidants can be defined according to their nature, enzymatic or non-enzymatic; and structure, phenolic acids or flavonoids (Vertuani *et al.,* 2004). Their mode of action, chemical origin, site of action and mechanism of action further subdivide these groups.

The antioxidant capacity of Os(C→S), compared with Os, was evaluated using both the ET-based TEAC assay and the HAT-based ORAC assay. Compared to the TEAC assay, the ORAC assay has the advantage of employing biologically relevant free radicals, i.e. $ROO^{\bullet}$, mimicking the

physiological conditions in which ROO• are generated during the oxidation of fatty acids (Zulueta *et al.*, 2009). The ORAC assay is additionally advantageous due to its standardisation across various laboratories as well as its ability to quantify both time and intensity of an antioxidant reaction (Zulueta *et al.*, 2009).

Chemically, both Os and Os(C→S) exhibited a comparable dose-dependent increase in antioxidant activity, significantly greater than known antioxidant, GSH. With the TEAC assay the molar ratio determined for Os was 1.16-fold greater than that of Os(C→S) (Figure 4.4). Results obtained from the TEAC assay did however, differ from findings of Costa *et al.* (2015) who evaluated the effects of 3,4-dihydroxyphenylalanine (DOPA)-melanin derivatives, chemically modified through oxidation with Cys, Ser and threonine (Thr), on the antioxidant activity of L-DOPA. Using the ET-based DPPH assay, Ser-DOPA exhibited enhanced antioxidant activity with half-maximal effective concentration of 1.02 µg/ml compared to 7.69 µg/ml produced by Cys-DOPA. Such single-Cys variants of peptides are typically considered inactive but have been shown to retain some degree of activity. A single-Cys variant of protein disulphide isomerase (PDI) for example, is capable of forming intermolecular disulphide bonds with its substrate. The subsequent disulphide-thiol exchange reaction results in the formation of intramolecular disulphide bonds within the substrate - reducing but not completely inhibiting its activity (Tyuryaeva *et al.,* 2017). The differences between the results obtained from this study and the present study may therefore, be attributed to physiochemical and structural differences between each compound as well as each compounds preferential mechanism of free radical scavenging, i.e. ET or HAT-based. Additionally, detection of the reduction of ABTS• to ABTS and DPPH to 1,1-diphenyl-2-picryl hydrazine in the presence of antioxidants with the TEAC and DPPH assay, respectively, may differ between assays (Floegel *et al.,* 2011).

Preliminary results obtained by Minnaar (2015, unpublished), who evaluated the antioxidant activity of Os and Os(C→S) at a concentration range of 3.13 - 50 µM, using the ORAC assay found that at the highest concentration there was no significant difference between the antioxidant activity of Os and Os(C→S), comparable to that observed in the present study. The molar ratio determined for Os(C→S) in the present study was however, 1.32-fold greater than that of Os (Figure 4.5). Although not statistically different, improved ROO• scavenging by Os(C→S), compared with Os, was observed.

Since drug delivery in the body is influenced by numerous complex combinations of factors, including tissue barriers, metabolic activity, transport, drug binding and partitioning to the intra- and extracellular compartments (Vellonen *et al.,* 2014); cell culture models should, to some degree, mimic the physical and biochemical absorption, distribution, metabolism and excetion (ADME) of molecules. The use of cell culture models in antioxidant research is essential to understand and appropriately predict the mechanisms of action of compounds in the promotion of human health (Gu *et al.,* 2012). The antioxidant capacity of Os(C→S), compared with Os, was further evaluated *in vitro* using the DCFH-DA assay. DCFH-DA is exceptionally sensitive and used extensively as a probe for the detection of intracellular H_2O_2 and associated oxidative stress (Eruslanov & Kusmartsev, 2010). The probe is cell-permeable, undergoing intracellular hydrolysis to the DCFH carboxylate anion and subsequent two-electron oxidation resulting in the formation of a fluorescent product, DCF. DCFH-DA is however limited by its inability to react directly with H_2O_2; its sensitivity to oxidation by several one-electron oxidising species, including $OH^{\bullet}$, nitrogen dioxide ($NO_2^{\bullet}$), compounds formed from peroxidase or heme interaction with H_2O_2, $HOCl^-$ and reactive species generated from the decomposition of $ONOO^-$/peroxynitrous acid (ONOOH); redox recycling following the reaction of $DCF^{\bullet}$ with O_2 forming $O_2^{\bullet}$; its reaction with cytochrome C during apoptosis, increasing the formation of $DCF^{\bullet}$; and redox metals that promote the oxidation of DCFH in the presence of H_2O_2 (Kalyanaraman *et al.,* 2012).

Following AAPH-induced oxidative damage in L929 cells, both GSH and Os significantly reduced radical production. The molar ratio determined for GSH was 11.96-fold greater than that of Os (Figure 4.7). These results were comparable to preliminary results obtained by Minnaar (2015, unpublished) and that obtained by Malan *et al.* (2015).

Malan *et al.* (2015) similarly evaluated the capacity of peptides, melittin, GSH, polymyxin B, Os and Os-C to reduce free radical formation following AAPH-induced oxidative damage in the murine RAW 264.7 cell line. All peptides exhibited a dose-dependent increase in antioxidant activity, with the exception of polymyxin B and melittin. Both Os and Os-C were capable of eliciting considerable protective effects against AAPH-induced oxidative damage. The protective effect of Os-C was, however, 1.78-fold less than that of Os. No significant difference was observed between the protective effects of GSH and Os.

Differences between the findings of Malan *et al.* (2015) and the present study may be associated with the physiological and biochemical properties of L929 fibroblasts and RAW 264.7 macrophage

cells, attributed to each cell types' respective role in extracellular matrix (ECM) synthesis and immunity. Similar trends observed between peptides are however, suggestive of the functional role of Cys residues in the antioxidant and protective effects of Os, *in vitro* - confirmed by the absence of Cys residues and observed pro-oxidant effect of polymyxin B and Os(C→S).

The antioxidant activity of peptides is commonly associated with molecular weight - peptides exhibiting the best initial and sustained antioxidant activity typically range from 400 - 600 Da (Zou *et al.,* 2016), structurally unique linkage type of peptides - demonstrated in tripeptides containing Tyr or Trp residues at the C-terminus and valine (Val) or Leu at the N-terminus (Gu *et al.,* 2012) as well as several specific amino acids including Cys, Met, Phe, Tyr, His, Pro and Trp (Elias *et al.*, 2008, Shen *et al.*, 2010 and Yang *et al.*, 2009). These amino acids possess phenolic side chains and hydroxyl ions (OH^-), in the case of Tyr, and thiol groups, in the case of Cys, permitting the donation of an electron to a radical species for subsequent stabilisation (Zou *et al.,* 2016). The contribution of Cys residues to the antioxidant activity of human serum albumin (HSA) for example, was estimated by Bourdon *et al.* (2005) to be between 40 - 80%. Since more of such amino acids are present in Os, including three Cys, one Phe and one Tyr, compared with Os(C→S) which contains only one Phe and one Tyr, the *in vitro* results are consistent with Os having a higher antioxidant capacity.

The absence of significant cellular internalisation (Chapter 3, Section 3.4.4) and greater hydrophobicity of Os (0.249), compared with Os-C (0.045) and Os(C→S) (0.034), may, additionally, have permitted hydrophobic interactions with the membrane lipid bilayer of the L929 fibroblasts resulting in extracellular accumulation of Os and subsequent exertion of the peptides' radical scavenging activity (Zou *et al.,* 2016). The reduction in the protective effects of Os against AAPH-induced oxidative damage and observed increase in radical production at high concentrations of GSH and Os may be attributed to the capacity of free radicals to selectively oxidise Cys sulfhydryl groups forming disulphide bonds (Wu *et al.,* 2017). These peptides may be considered redox-sensitive, existing as thiolate anions at neutral pH as a result of a reduction in pK_a by charge interactions with adjacent amino acids, enhancing their vulnerability to oxidation (Cumming *et al.,* 2004).

4.6 Conclusion

In chemico, both Os and Os(C→S) exhibited significant antioxidant activity - scavenging free radicals and preventing AAPH-induced oxidative damage via ET- and HAT-based reactions, respectively. The *in vitro* antioxidant capacity of Os(C→S) was, however, largely negligible, implying a significant functional contribution of Cys residues and associated hydrophobicity to the intracellular radical scavenging capacity of Os.

Chapter 5: Anti-inflammatory effects of Os(C→S) compared with Os

5.1 Introduction

NO is essential for normal physiological functioning, such as smooth muscle relaxation (Irshad & Chaudhuri, 2002), and is formed enzymatically following the conversion of L-Arg to L-citrulline (Cit) in the presence of nitric oxide synthase (NOS). Cellular and tissue exposure to inducers of inflammation, such as *E. coli*-derived LPS induces, inducible nitric oxide synthestase (iNOS), increasing NO levels. Although NO has an important physiological function, excessive NO production initiates a toxic reaction with ROS, forming $ONOO^-$ and ONOOH that causes cellular oxidative damage (Sheikh *et al.*, 2010; Zhu *et al.*, 2011). Overproduction of these RNS induces nitrosative stress, a condition associated with an imbalance in the presence of antioxidants and nitrosants: $NO_2^\bullet$ and dinitrogen trioxide (N_2O_3) (Irshad & Chaudhuri, 2002). Such stress-induced damage to macromolecules initiates a toxic cellular response, often observed as apoptosis, lipid peroxidation, DNA degradation, inflammation or protein modification.

Host infection by invading microbes is capable of stimulating systemic inflammation, innate immunity and complement pathways as well as the activation of haemostasis. During infection, thrombin production is induced through either bacteria initiation with endothelial invasion and platelet activation; bacterial polyphosphate initiation; endothelial cell expression of TF, vascular cell recruitment and activation by thrombin, cytokines and microparticles; or fibrin network, neutrophil extracellular traps (NETs) and histones. Such immunohaemostasis functions in pathogenic arrest, prevention of tissue invasion and concentration of antimicrobial cells and peptides to the site of infection (Delabranche *et al.,* 2017).

NETs function in the support of histones and other granular enzymes, and are capable of trapping pathogens and blood cells, including platelets, in their networks. NETs are essential for both host defence, through innate immunity, and other procoagulant mechanisms, such as coagulation factor assembly; enzymatic inhibition of TF pathway inhibitor and thrombomodulin; direct recruitment and activation of platelets by histones (Delabranche *et al.,* 2017).

Platelets are considered primary mediators of haemostasis. Recently however, the role of platelets in inflammatory response has been increasingly recognised (Stokes & Granger, 2012; Thomas & Storey, 2015). The association between haemostasis and inflammation has been

largely attributed to the capacity of activated platelets to readily bind and interact with endothelial cells and leukocytes (Stokes & Granger, 2012). Platelets are capable of expressing numerous receptors common to immune cells, such as leukocytes, lymphocytes and megakaryocytes; most notably the transmembrane GP TLR-4. Platelets expressing TLR-4 have the capacity to recognise bacterial proteins, including LPS, and upon activation function as inflammatory mediators, surrounding and isolating the infectious stimuli while simultaneously modulating pro-inflammatory cytokine release (Ding *et al.,* 2014). LPS plays a significant role in inducing platelet activation and enhancing the sensitivity of platelets to aggregation. Cytokines produced in response to LPS, such as NO, IL-1, IL-3 and IL-6, function in the regulation of host responses to infection, immunity and inflammation (Wu *et al.,* 2015).

AMPs contribute considerably to controlling microbial proliferation and host cell signalling in response to injury. AMPs are predominantly cationic and act against microbes via simple electrostatic interactions with the anionic phospholipids of microbial cell membranes, resulting in membrane disruption or destruction and ultimately cell death. The development of microbial resistance to AMPs is therefore less prevalent compared to conventional antibiotics directed at individual, enzymatic targets. AMPs have been identified as LPS neutralising agents, immune cell chemoattractants and inducers of cellular proliferation. The effect of such immunomodulatory AMPs relies on several mechanisms, including direct antimicrobial activity, preventing infections by binding and inactivating bacterial toxins, reducing pro-inflammatory response and altering cellular behaviour by enhancing cellular migration and proliferation (Mangoni *et al.,* 2016). The *in vivo* contribution of AMPs to inflammatory responses additionally depends on their capacity to induce the production of pro-inflammatory cytokines, enhancing phagocytosis (Méndez-Samperio, 2013).

The anti-inflammatory and LPS-binding capacity of Os was previously evaluated by Malan *et al.* (2015) and preliminarily by Minnaar (2015, unpublished). Os was found to scavenge NO *in chemico* and reduced NO levels in LPS/IFN-γ stimulated RAW 264.7 macrophages. Additionally, Os demonstrated considerable LPS-binding, although significantly less than that observed for known LPS-binding peptides: melittin and polymyxin B. To further evaluate the contribution of Cys residues to the observed anti-inflammatory and LPS-binding capacity of Os, the chemical NO scavenging capacity, *ex vivo* LPS-induced inhibition of platelet activation and LPS-binding potential of Os(C→S) was investigated and compared with Os.

The specific objectives outlined in this chapter are:

1. To determine the NO scavenging capacity of Os(C→S), compared with Os, using the sodium nitroprusside (SNP) assay.
2. To determine the effects of Os(C→S), compared with Os, on LPS-stimulated platelets and exposed erythrocytes.
3. To evaluate the role of direct NO scavenging and LPS binding on the observed effects.

5.2 Materials

5.2.1 Reagents

In addition to the reagents described in Chapter 3, Section 3.2.1, sulphanilamide (SA), *N*-naphthyl-ethylenediaminedihydrochloride (NED), phosphoric acid, SNP, sodium nitrite ($NaNO_2$), LPS, glutaraldehyde, osmium tetraoxide (OsO_4) and hexamethyldisilazane (HMDS), obtained from Sigma-Aldrich (Johannesburg, SA), were used.

5.2.2 Blood cells

Erythrocytes and platelets were collected from the venous blood of healthy, consenting male donors as described in Chapter 3, Section 3.2.2 (Health Sciences Research Ethics Committee number: 417/2017).

5.3 Methods

5.3.1 Chemical NO scavenging capacity assay

The spectrophotometric chemical NO scavenging assay, making use of a modified Griess reagent, was used to determine the NO scavenging capacity of Os(C→S). The assay indirectly quantifies the amount of NO present by using both its short half-life and readied conversion to nitrate (NO_3^-) and nitrite (NO_2^-). NO_2^- reacts with SAA, a diazotizing agent, producing diazonium salt in the presence of acidic medium. Interaction with the coupling reagent, NED, then forms a stable, dark pink azo compound (Figure 5.1) (Giustarini *et al.*, 2008).

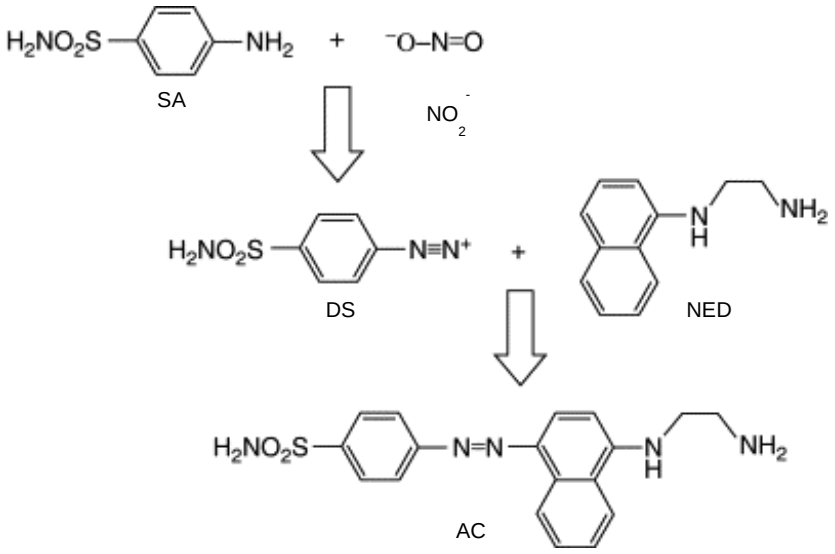


Figure 5.1: The interaction of NED with a diazonium salt (DS) product, formed by the interaction between sulfanilamide and NO_2^-, resulting in a pink azo compound (AC) (Rodeo, 2013).

A 5 mM SNP solution, diluted in 0.1 M PBS, was incubated in the light for 1.5 hours (Floryszak-Wieczorek *et al.*, 2006). Following incubation, a volume of 80 µl SNP was added to 20 µl GSH, polymyxin B, Os and Os(C→S) at a final concentration of 1.56 – 100 µM, and incubated at room temperature, in the dark, for 1 hour. A blank was included containing the same volume of dddH_2O with no reagents. A volume of 100 µl Griess reagent (1:1 (v/v) of 1% SAA and 0.1% NED in 2.5% phosphoric acid) was subsequently added and incubated in the dark for a further 10 minutes. The absorbance was measured using an Emax Plus microplate reader (Molecular Devices, California, USA) at 570 nm. The concentration (µM) of NO produced was calculated using a standard curve of $NaNO_2$, in PBS, at a linear concentration range of 0 - 0.1 mM. The amount of NO scavenged in the presence of peptide was expressed as a percentage relative to the control containing all reagents excluding peptide, i.e. 0% scavenged, using the equation below. The linear dose-response equations were additionally used to determine and compare the % NO scavenging/µM peptide.

$$\%\ NO\ production = \left(\frac{sample - Blank}{control - Blank} \times 100\right)$$

$$\%\ NO\ scavenged = 100 - \%\ NO\ production$$

5.3.2 LPS-stimulated platelet activation and erythrocyte deformability

WB contains approximately 5 x 10^{12} erythrocytes/L and 3 x 10^{11} platelets/L (Invitrogen, 2008) - constituting between 40 and 45% of the total blood volume (American Society of Haematology, 2018). Due to the high erythrocyte content in WB, evaluation of the effects of LPS, alone and in combination with peptide, provided an opportunity to further evaluate the effects of peptides on erythrocyte morphology - relating to the observations in Chapter 3, Section 3.4.3

To determine the indirect anti-inflammatory effects of peptides, the morphology of human platelets and erythrocytes following LPS-induced TLR-4 signalling was evaluated with SEM. A volume of 5 µl LPS (*E. coli* O111:B4; 500 ng/ml) was incubated with 5 µl of 500 µM melittin, polymyxin B, Os and Os(C→S), and incubated at room temperature for 10 minutes to permit possible binding. The resulting LPS-peptide solution was then added to 40 µl WB, to a final concentration of 50 ng/ml LPS and 50 µM peptide; vortexed, and incubated at room temperature for a further 30 minutes. Following incubation, 5 µl of each cell suspension was smeared onto clean, 10 mm diameter coverslips and allowed to dry at room temperature for between 10 and 20 minutes. The coverslips were then transferred to 24-well Cellstar polystyrene plates (Greiner Bio-One GmbH, Kremsmünster, Austria) and washed in 0.075 M NaP buffer (0.046 M Na_2HPO_4, 0.029 M NaH_2PO_4•H_2O, pH 7.4) for 20 minutes at room temperature while shaking. A 2.5% glutaraldehyde and formaldehyde fixative solution in NaP buffer was added to each coverslip and incubated for 30 minutes at room temperature. Following fixation, coverslips were washed three times in NaP buffer for 5 minutes. OsO_4 was then added for 30 minutes for secondary fixation. The coverslips were washed again three times using 0.075 M NaP buffer for 5 minutes. For dehydration, a series of EtOH dilutions: 30%, 50%, 70%, 90% and three changes of 100% was added to each coverslip for 5 minutes. A 50% solution of HMDS, in 100% EtOH, was then added and incubated for 30 minutes. Approximately 1 to 2 drops of 100% HMDS was added to the surface of each coverslip and allowed to dry overnight. Coverslips were then mounted with carbon tape onto aluminium stubs, and coated three times with carbon. Resulting images were obtained with a Zeiss Ultra plus field emission gun (FEG) SEM (Oberkochen, Germany).

5.3.3 Bacterial LPS binding - Limulus amoebocyte lysate assay

To determine whether Os and Os(C→S) are capable of binding to LPS, a chromogenic limulus amoebocyte lysate (LAL) kit (Lin *et al.*, 2008) was obtained from Pierce Biotechnology (Rockford,

USA). LPS activates the proteolytic activity of factor C which catalyses the cleavage of acetyl (Ac)-Isoleucine (Ile)-Ala-Arg-*p*-nitroaniline resulting in the release of *p*-nitroaniline (*p*-NA) and the formation of a yellow coloured product. A reduction in absorbance of the coloured product is inversely proportional to the inhibition of LAL activation (Figure 5.2).

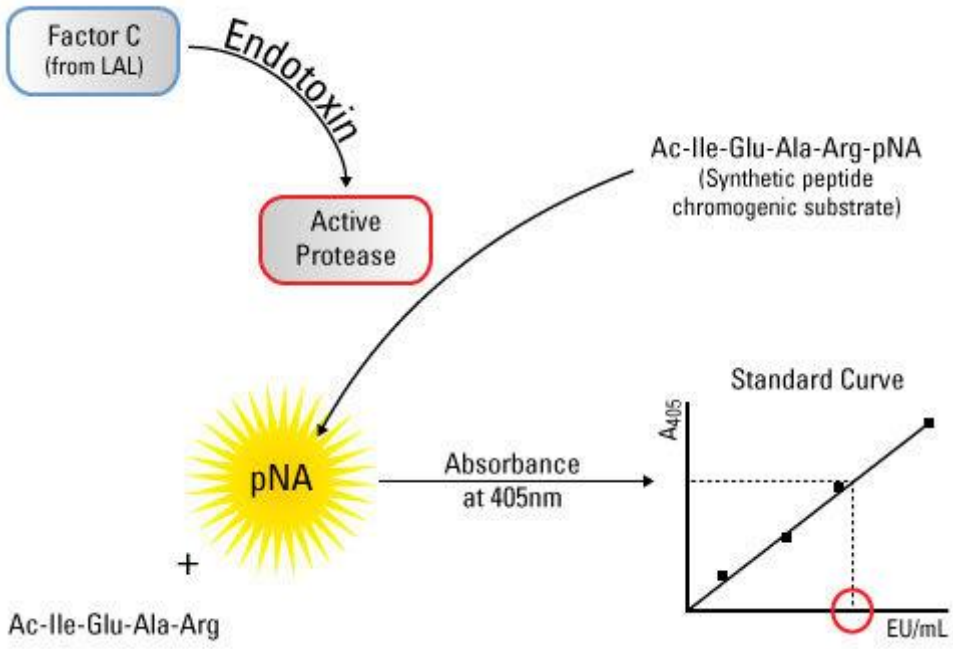


Figure 5.2: The catalysed cleavage of Ac-Ile-Ala-Arg-*p*-nitroaniline producing a yellow coloured product (Thermo Fisher Scientific, 2016).

LPS neutralisation was performed according to recommended protocol with modification. Stock solutions of melittin, polymyxin B, Os and Os(C→S) was prepared in the pyrogenic water supplied by the manufacturer. In a pyrogenic sterile 96-well microtitre plate, 10 µl of serial concentrations of peptide (1.56 - 25 µM) was incubated with 10 µl of LPS (1 EU/ml; *E. coli* O111:B4) at 37°C for 5 minutes. The resulting mixture was added to 10 µl of the LAL reagent, provided by the manufacturer, and incubated at 37°C for a further 5 minutes. A volume of 20 µl of the LAL chromogenic substrate solution was then added and the solution was incubated for 10 minutes, allowing the development of the coloured product. A 25% acetic acid solution was added to terminate the reaction and the absorbance was measured using an Emax Plus microplate reader (Molecular Devices, California, USA) at 405 nm. The inhibition of LAL activation in the presence of peptide was expressed as a percentage relative to the control containing all reagents excluding peptide, i.e. 0% inhibition, using the following equation:

$$\%\ Inhibition\ of\ LAL\ activation = 100 - \left(\frac{Mean\ absorbance_{sample}}{Mean\ absorbance_{untreated\ control}} \times 100 \right)$$

5.3.4 Statistical analysis

Statistical analysis was performed as described in Chapter 3, Section 3.3.7.

5.4 Results

5.4.1 Os(C→S) does not scavenge NO effectively

The NO scavenging ability of Os and Os(C→S), compared to controls: GSH and polymyxin B, was evaluated following the spontaneous formation of NO from SNP, a transition metal NO complex, and subsequent production of a stable, dark-pink azo compound (Giustarini *et al.*, 2008).

Following initial experimentation, NO production from SNP was largely negligible, producing inconsistent and unreliable results. Subsequent optimisation experiments found that incubation time, temperature, pH, salinity nor SNP concentration, influenced NO production significantly (results not shown), stimulating further investigation.

According to Wang *et al.* (2002) SNP is a photosensitive compound capable of spontaneous degradation, accelerated in the presence of O_2. Floryszak-Wieczorek *et al.* (2006) observed a maximum NO concentration following exposure to light, at an intensity of 8880 lux, for two hours after which concentration of released NO was gradually reduced. NO emission from SNP was additionally shown to be completely inhibited in the dark. Consequently, NO production from SNP was evaluated following exposure to varying light and dark conditions as well as combinations thereof. Both natural, window-filtered light and a white fluorescent lamp was used as sources of luminescence. Direct, natural light has an average intensity of 20000 lux (LiquiSearch, 2016). This intensity is however halved by the filtering action of windows, eliminating short UV wavelengths. The resultant intensity of light used therefore approximated 10000 lux, comparable to that used by Floryszak-Wieczorek *et al.* (2006).

Incubation of SNP in indirect, natural light for 1.5 hours, dramatically enhanced the NO scavenging capacity of known antioxidant, GSH (Figure 5.3). These results were consistent with descriptions by Wang *et al.* (2002) and subsequent findings by Floryszak-Wieczorek *et al.* (2006). The time required to produce sufficient NO from SNP did, however, differ considerably from that described by Floryszak-Wieczorek *et al.* (2006) likely owing to the difference in intensity produced by natural light compared to that produced by a fluorescent lamp.

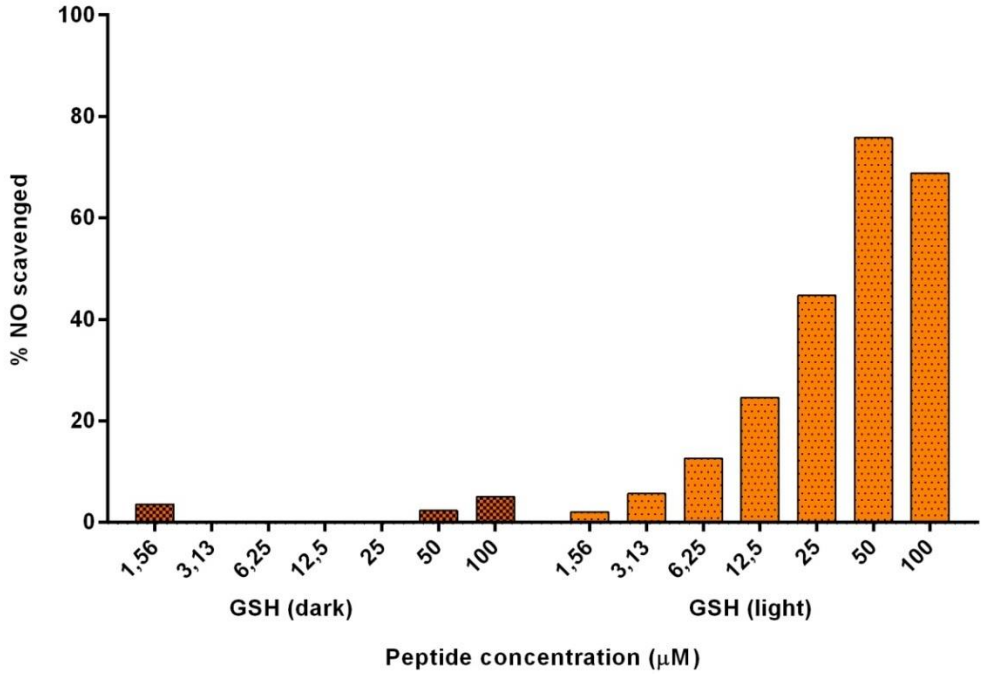


Figure 5.3: Optimisation of the SNP assay. NO scavenging following the production of NO from SNP incubated in the dark, at 37°C, for 1 hour (left) and in the light, at room temperature, for 1.5 hours (right). GSH, a known NO scavenger, was used as the experimental compound. Data is representative of a single experiment without repeats.

Following optimisation, the GSH, polymyxin B, Os and Os(C→S) were serially diluted to a concentration range of 1.56 - 100 µM and the NO scavenging capacity of each was compared to a negative control containing dddH_2O only. The respective NO scavenging capacity of GSH, polymyxin B, Os and Os(C→S) differed significantly from the negative control. At the highest concentration of 100 µM, GSH, polymyxin B, Os and Os(C→S) reduced NO production by 68.38%, 25.13%, 50.86% and 19.95%, respectively. The NO scavenging capacity of GSH at 100 µM was however, 1.048-fold less than that observed at 50 µM likely indicative of a threshold effect. Although insignificant, a similar trend was observed for Os (Figure 5.4 A). A linear dose-response with a high degree of correlation was found for all peptides. At concentrations ranging from 1.56 - 50 µM, the molar ratio of GSH was similar to that of Os, with a % NO scavenged/µM peptide of 1.46 and 1.62, respectively (Figure 5.4 B). Similarly, no significant difference between the molar ratios of polymyxin B and Os(C→S) at concentrations ranging from 1.56 - 100 µM was observed, each with a % NO scavenged/µM peptide of 0.25 and 0.21, respectively (Figure 5.4 C). The NO scavenging activity of polymyxin B and Os(C→S) was 6.48- and 7.71-fold less than than that of Os, respectively.

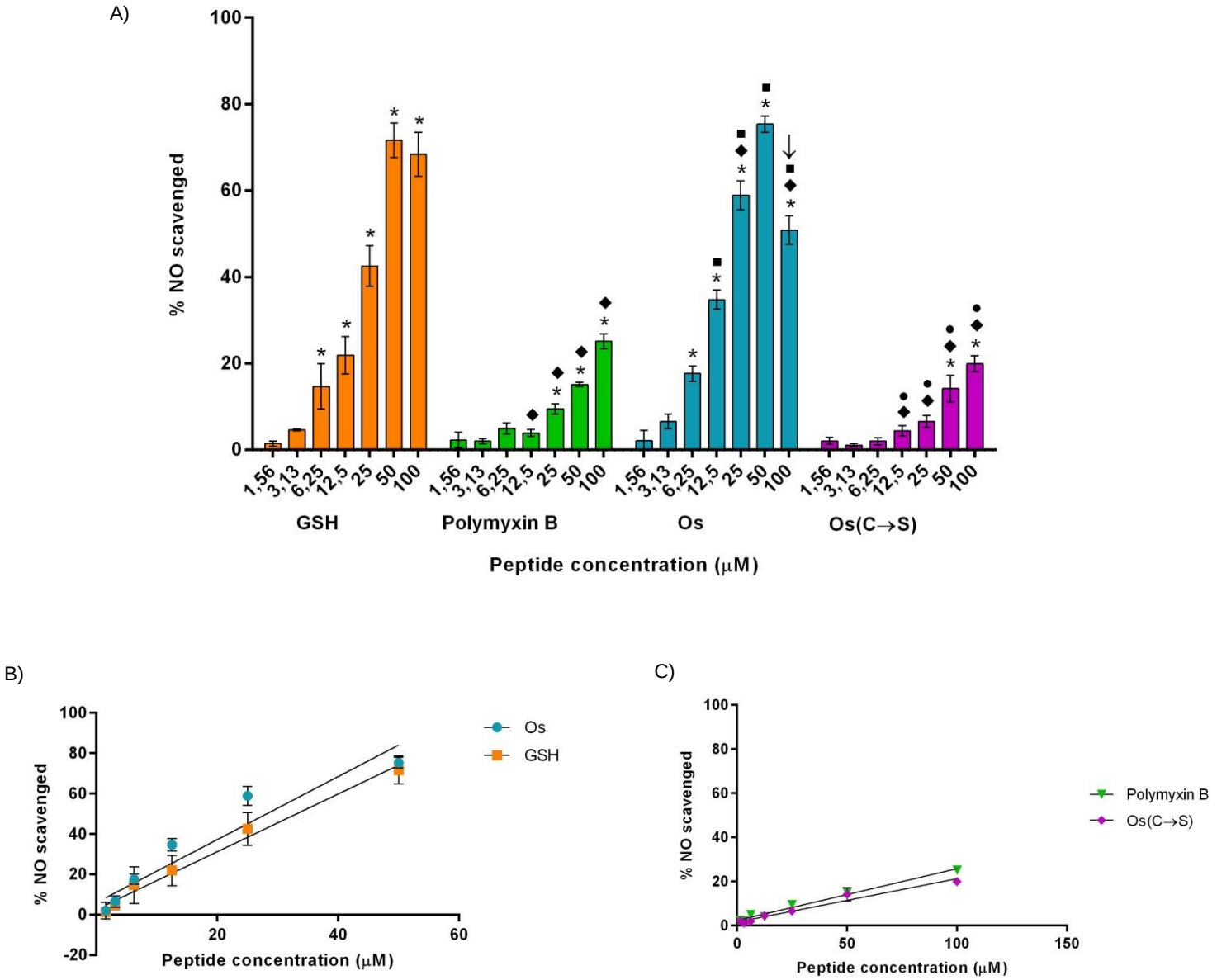


Figure 5.4: The NO scavenging capacity of GSH, polymyxin B, Os and Os(C→S). (A) Percentage NO scavenging of peptides: GSH, polymyxin B, Os and Os(C→S). (B) Comparison of the linear dose-response between GSH and Os. (C) Comparison of the linear dose-response between polymyxin B and Os(C→S). Data is expressed as a mean ± SE of three independent triplicate experiments. An asterisks (*), solid diamond (◆), solid square (■) and solid circle (●) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (0% NO scavenging), GSH, polymyxin B and Os, respectively. The down arrow (↓) indicates a significant reduction in the NO scavenging activity of Os at 100 μM. Equations obtained from linear regression analysis for GSH was $y=1.429x+2.715$ ($R^2=0.9407$); Os was $y=1.56x+6.082$ ($R^2=0.9031$); polymyxin B was $y=0.2349x+1.2345$ ($R^2=0.9362$); and Os(C→S) was $y=0.1967x+1.619$ ($R^2=0.8661$). Intercepts of polymyxin B and Os(C→S) differed significantly ($p \leq 0.05$).

5.4.2 Os and Os(C→S) does not inhibit LPS-stimulated platelet activation or erythrocyte deformability

To determine the effects of peptides Os, Os(C→S), polymyxin B and melittin on LPS-induced platelet activation, WB was exposed to peptides alone and in combination with a low concentration of LPS. Following incubation, the resulting morphological changes were assessed using SEM.

Control platelets exhibited a typical, spherical structure with no or one pseudopodium slightly extending from the platelet body (Figure 5.5 A and B). Such minor activation is expected as a result of contact activation during sample preparation. A few small pores associated with the open canalicular system (OCS) were, additionally, evident on each platelet with no observed platelet interactions. In the presence of LPS, pores of the OCS appeared enlarged with several visible pseudopodia extending from each platelet body - typical of platelets exposed to LPS. Sparse distribution of platelets with occasional membrane spreading, adhesion and blebbing, characteristic of platelet activation, was additionally observed (Figure 5.5 K and L).

Alone, both melittin (Figure 5.5 C and D) and polymyxin B (Figure 5.5 E and F) exhibited enlarged OCS, enhanced pseudopodia elongation and membrane spreading. Melittin additionally, appeared to induce membrane blebbing while significant platelet interaction was observed in platelets exposed to polymyxin B. Platelets exposed to Os (Figure 5.5 G and H) and Os(C→S) (Figure 5.5 I and J) exhibited considerable platelet spreading accompanied by the appearance of pseudopodia. At a concentration of 50 µM and incubation time of 30 minutes, all peptides therefore, induced significant platelet activation, associated with the absence of NO.

Co-incubation of peptides with LPS, prior to platelet exposure, was thought to be capable of demonstrating either the limited interaction between each compound - permitting the effects of both peptide and LPS on platelet morphology; or LPS binding. Following LPS binding and saturation, the compound in excess of the other will be available to exert its effect on platelet morphology - either enhancing or inhibiting platelet activation, or producing an additive effect.

Platelets exposed to melittin and LPS in combination (Figure 5.5 M and N) stimulated pseudopodia elongation and pore formation similar to that observed in platelets exposed to LPS alone. Additionally, membrane blebbing observed in platelets exposed to melittin alone was

significantly reduced in the presence of LPS. Since melittin possesses strong surface amphipathicity, permitting a strong interaction with LPS (Bhunia *et al.*, 2007), the observed effects on platelet morphology is likely due to melittin saturation with LPS and the subsequent interaction of free/unbound LPS with platelets - generating morphological characteristics comparable to that observed in platelets exposed to LPS alone.

Polymyxin B and LPS in combination (Figure 5.5 O and P), appeared to produce several pores associated with the OCS; pseudopodia, with increased length and frequency; and membrane spreading, comparable to the observed effects of LPS alone. Platelets exposed to polymyxin B and LPS additionally, did not display any enhanced platelet effects as observed in platelets exposed to polymyxin B alone. Similar to melittin, polymyxin B is capable of binding LPS (Mitaka & Tomita, 2011; Domingues *et al.*, 2012). Binding and saturation of polymyxin B with LPS therefore, reduced the expected additivity or synergy between peptide and LPS, permitting the interaction between free/unbound LPS and platelets and subsequent effects on platelet morphology.

Platelets exposed to Os (Figure 5.5 Q and R) and Os(C→S) (Figure 5.5 S and T) in the presence of LPS exhibited platelet spreading and pseudopodia formation similar to that observed in platelets exposed to Os and Os(C→S) alone. These results are consistent with limited LPS-binding and peptide availability.

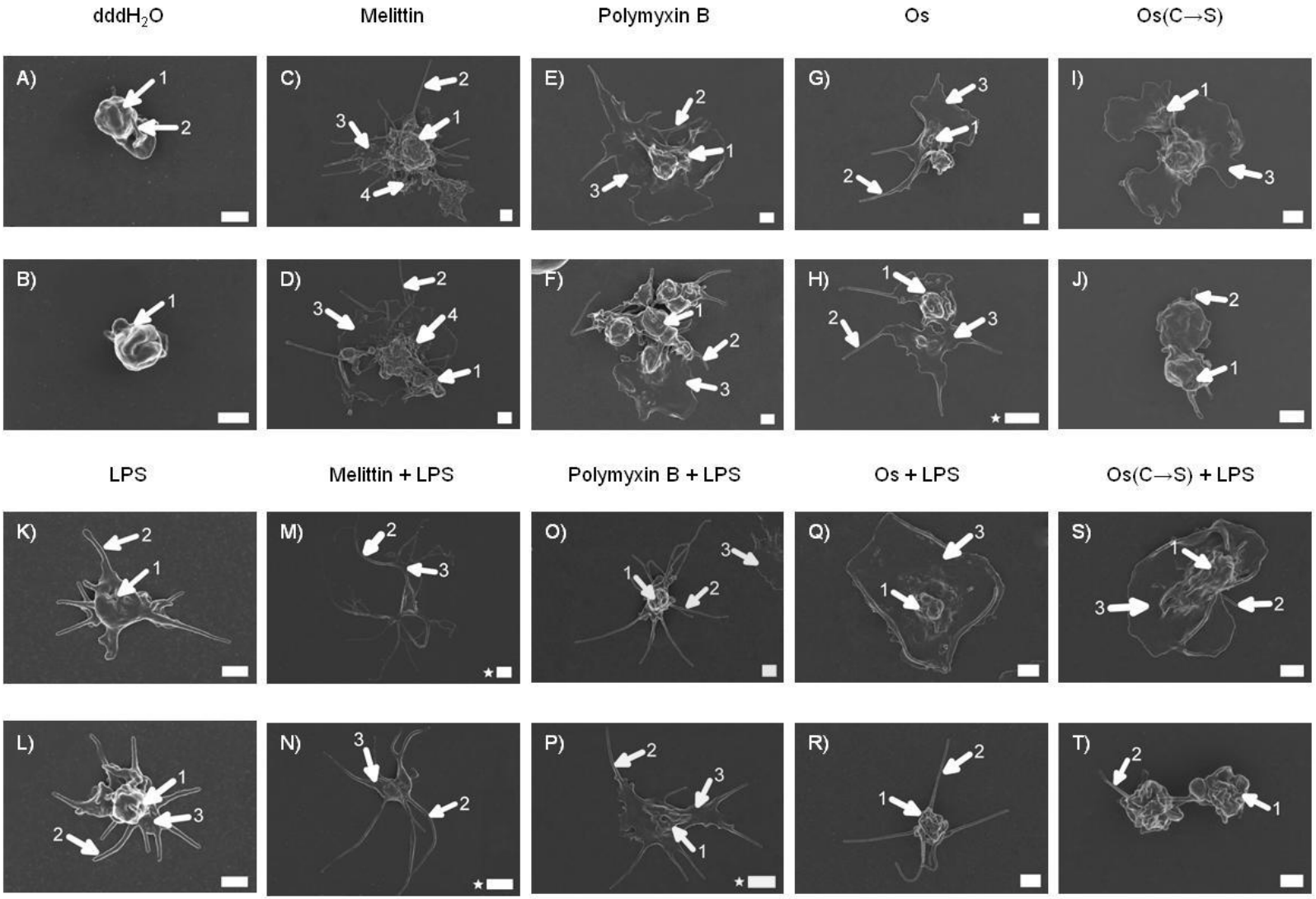


Figure 5.5: SEM images of human platelets treated with 50 µM (C, D, M and N) melittin, (E, F, O and P) polymyxin B, (G, H, O and R) Os and (I, J, S and T) Os(C→S), alone or in combination with 50 ng/ml LPS. Images are representative of two independent duplicate experiments. Extended morphology of individual platelets indicate (1) pores associated with the OCS, (2) pseudopodia, (3) platelet spreading and (4) membrane blebbing. Scale bars = 1 µm. Scale bars* = 2 µm.

The *ex vivo* effects of peptides, both alone and in combination with bacterial LPS, on ultrastructure morphology of erythrocytes, was further evaluated using SEM.

Control erythrocytes, exhibited a typical, biconcave structure, representative of normocytes and discocytes, with minimal eryptosis (Figure 5.6 A and B). Such minor morphological defects are expected as a result of sample preparation. In the presence of LPS, several types of poikilocytes were observed, including spherocytes, spherical cells with increased Hb content; echinocytes, cells possessing numerous evenly distributed spicules of comparable size and knizocytes, cells having two or more concavities, typically triconcave, with a thin linear region containing Hb (Kadiyam, 2015). Knizocytes were however, the predominant poikilocyte associated with LPS (Figure 5.6 K and L).

Exposure of erythrocytes to peptides, in the absence of LPS, did not induce any significant morphological modifications to erythrocyte membranes with the exception of melittin which exhibited extensive echinocyte formation (Figure 5.6 C and D). Sparse distribution of spherocytes and knizocytes was observed in erythrocytes exposed to polymyxin B (Figure 5.6 E and F), while erythrocytes exposed to Os (Figure 5.6 G and H) and Os(C→S) (Figure 5.6 I and J) exhibited considerable knizocyte formation.

Co-stimulation of peptides with LPS exhibited morphological modifications comparable to those observed in the absence of LPS. Extensive echinocyte formation was observed in erythrocytes exposed to melittin and LPS (Figure 5.6 M and N), comparable to that observed following exposure to melittin alone. The appearance of sparsely distributed spherocytes and knizocytes was characteristic of polymyxin B and LPS exposure (Figure 5.6 O and P) while erythrocytes exposed to Os (Figure 5.6 Q and R) and Os(C→S) (Figure 5.6 S and T), in combination with LPS, exhibited significant knizocyte formation. No significant difference in knizocyte formation was therefore, observed in erythrocytes exposed to peptide, both in the presence and absence of LPS.

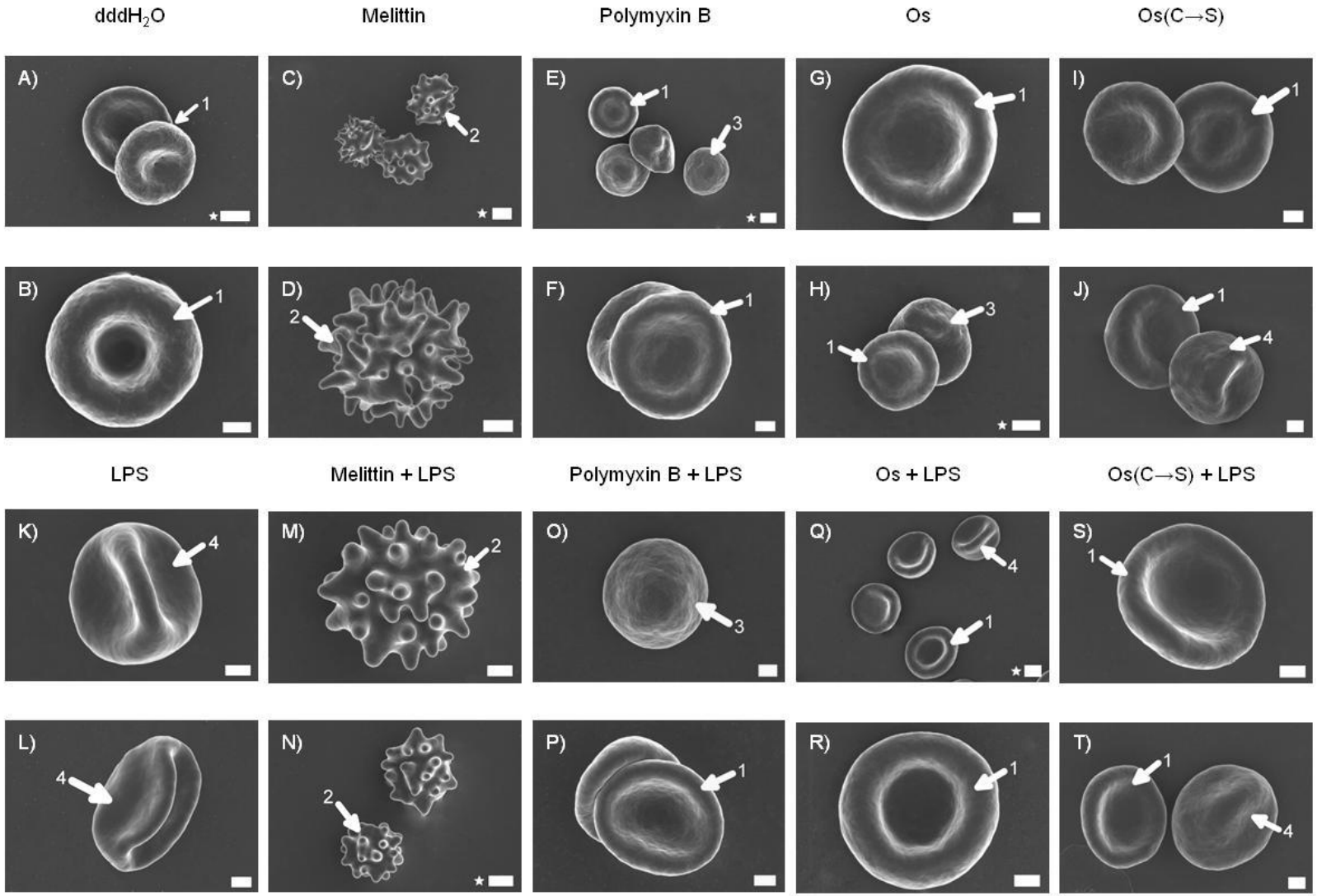


Figure 5.6: SEM images of human erythrocytes treated with 50 µM (C, D, M and N) melittin, (E, F, O and P) polymyxin B, (G, H, O and R) Os and (I, J, S and T) Os(C→S), alone or in combination with 50 ng/ml LPS. Images are representative of two independent duplicate experiments. Extended morphology of individual erythrocytes indicate (1) normocytes/discocytes, (2) echinocytes, (3) spherocytes and (4) knizocytes. Scale bars = 1 µm. Scale bars* = 2 µm.

5.4.3 Os and Os(C→S) have limited LPS-binding capacity

The ability of peptides, polymyxin B, Os, and Os(C→S) to bind LPS (from *E. coli* O111:B4), neutralising LPS-induced inflammatory responses, was evaluated using the chromogenic LAL assay - quantifying the inhibitory efficacy of each peptide following LPS-induced activation of the LAL enzyme.

Polymyxin B, Os and Os(C→S) were evaluated at a concentration range of 0.78 – 25 µM. All peptides were capable of inhibiting LPS-induced activation of LAL enzyme, although independent of dosage and with varying degrees of efficiency. At concentrations ranging from 1.56 – 25 µM, the inhibitory effect of polymyxin B was significantly greater than that of Os and Os(C→S), with the exception of Os(C→S) at 6.25 µM. A plateau effect was additionally observed between 1.56 and 25 µM for both Os and Os(C→S). At 25 µM, polymyxin B inhibited LAL enzyme activation by 27.06% while Os and Os(C→S) only bound 4.84% and 13.94% LPS, respectively, 5.59- and 1.94-fold less than polymyxin B (Figure 5.7). The LPS-binding capacity of Os(C→S) was greater than that observed for Os.

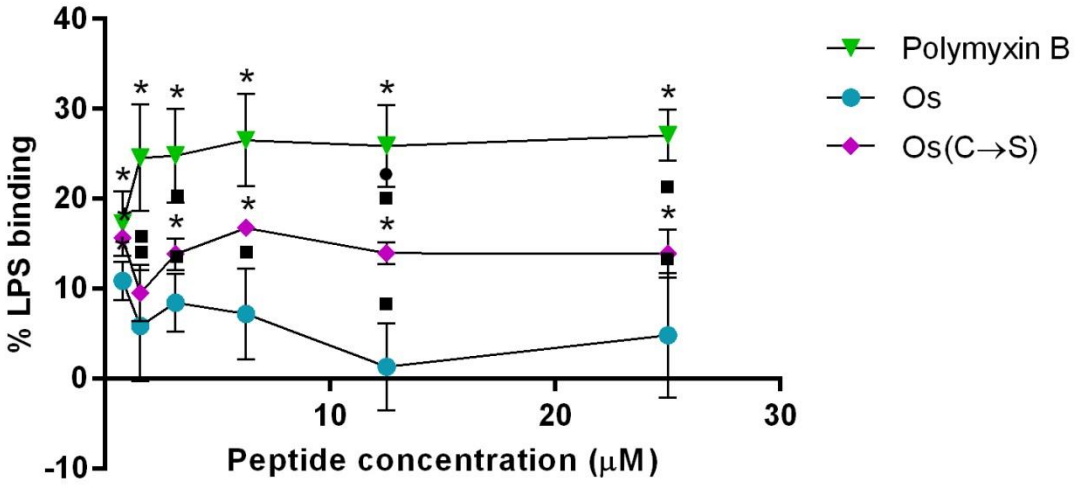


Figure 5.7: The LPS-binding capacity of polymyxin B, Os and Os(C→S). Data is expressed as a mean ± SE of two independent experiments performed in duplicate. An asterisks (*), solid square (■) and solid circle (●) indicates significant differences ($p \leq 0.05$) relative to the control containing $dddH_2O$ only (0% LPS-binding); polymyxin B and Os, respectively.

5.5 Discussion

NO is a gaseous signaling molecule (Korhonen *et al.,* 2005) crucial for the regulation of vascular tone, nuerotransmission, acute and chronic inflammation and host defence. In innate immunity, NO exerts considerable toxicity on pathogenic microbes, inducing or regulating the death and function of immune cells. NO is therefore capable of functioning as both a pro-inflammatory and anti-inflammatory or immunosuppressive agent depending on its inhibitory or apoptotic effects. The capacity of NO to assist in host defence is thought to be attributed to its reaction with iron or thiol groups of Cys residues on proteins, forming iron-nitrosyl or nitrosothiol complexes (Tracey *et al.*, 1988; Khansari *et al.*, 2009) that are capable of inactivating enzymes functioning in mitochondrial respiration or DNA replication. Additionally, NO is capable of exerting various effects on leukocyte function, including inducing macrophage apoptosis, stimulating macrophage cytoplasmic motility, modulating neutrophil adhesion and regulating cytokine production by leukocytes (Tripathi *et al.,* 2007).

NO is considered a free-radical due to its unpaired electrons capable of considerable reactivity with particular proteins and other free radicals, including superoxide. According to the spontaneous generation of NO from SNP and subsequent interaction with O_2 producing NO_2^-, the capacity of compounds to compete with O_2 and scavenge NO (Boora *et al.,* 2014), inhibiting a pro-inflammatory response, may be estimated *in chemico*.

The NO scavenging activity of Os was comparable to that observed for GSH (Figure 5.4 A and B). Polymyxin B and Os(C→S) similarly displayed comparable dose-dependent NO scavenging activity (Figure 5.4 C), although considerably less than that observed for Os and GSH. No significant difference between the molar ratios of polymyxin B and Os(C→S), or GSH and Os, at concentrations ranging from 1.56 - 50 µM, was observed. These results are consistent with data obtained by Malan *et al.* (2015) who evaluated the NO scavenging capacity of melittin, GSH, Os, and Os-C and found that both GSH and Os were capable of scavenging NO with comparable dose-dependency. The scavenging capacity of melittin and Os-C on the other hand, was largely negligible with no discernible dose-response.

Os was therefore, found to be capable of scavenging both ROS and NO, reducing RNS production. The absence of NO scavenging and *in vitro* increase in ROS production (Chapter 4, Section 4.4.2) by Os(C→S) emphasises the contribution of Cys to the protective effects of Os

against ROS and RNS. The susceptibility of Cys residues to ROS and RNS is largely determined by their reducing capacity. Cys residues may exist in various reversible oxidative states, i.e. inter- and intra-chain disulphide bonds; *S*-nitrosylation as well as sulphinic, sulphenic or sulphonic acids - modifying their reactivity and accessibility to various compounds and reaction (Coppo & Ghezzi, 2011).

Bacterial LPS plays a crucial role in the development of sepsis. Systemically released LPS complexes with LBP which subsequently binds to CD-14, a high-affinity LPS receptor present on the surface of numerous types of cells, inducing cellular responses through TLR-4. MD2, an extracellular adaptor protein, is essential for the recognition of LPS by TLR-4. Multiple adaptor proteins, including myeloid differentiation factor (MyD)-88, the MyD-88 adaptor-like protein (Mal), Toll/interleukin-1 receptor-like domain (TIR)-containing adaptor molecule (TRIF), and TRIF-related adapter molecule (TRAM) are additionally required for TLR-4-induced intracellular signalling. Although TLR-4 is the primary signal transducer for most LPS serotypes and TLR-2 is a major receptor for LTA from Gram-positive bacteria, TLR-2 also functions as a signal transducer for several types of Gram-negative bacteria. TLR-2 is expressed primarily in platelets and requires the MyD-88 pathway for signal transduction (Zhang *et al.,* 2009).

TLR-4 has also been found to be expressed in platelets, functioning critically in LPS-induced thrombocytopenia. LPS has been shown to enhance microvascular thrombosis and induce the accumulation of infused platelets in wild type mice, possessing active TLR-4 signalling. Despite this, numerous studies are in disagreement on the effects of LPS on platelet activation and function. A study performed by Zhang *et al.* (2009) however, suggests that LPS primarily stimulates platelet secretion of granular content, enhancing integrin-dependent platelet aggregation. Components of the TLR-4-MyD-88 receptor-signalling complex, required for LPS signalling, were additionally shown to be present in platelets with subsequent LPS-mediated platelet activation requiring TLR-4/MyD-88-dependent activation of the NO and cyclic guanosine monophosphate (cGMP)-dependent protein kinase pathway. Platelet activation is frequently accompanied by numerous morphological and biochemical changes, including discoid to spherical morphological modifications, remodelling of internal cytoskeletal components, and spread and release of storage granules (Zhang *et al.,* 2009).

The initial formation of platelet pseudopodia is thought to assist in the adherence of platelets to each other. The subsequent release of storage granules, stimulated by the fusion of α-granule

membranes to surface connected membranes of the OCS, induces additional platelet recruitment, promoting the formation of fibrin networks that form a stable, secondary haemostatic plug. OCS channels and pores, along with α-granules, additionally increases membrane surface area permitting surface expansion up to 4-fold in size and subsequent platelet spreading (Swanepoel & Pretorius, 2015). Alone, the OCS is thought to function primarily in the entry of exogenous elements into platelets, the release of granular content to the platelet exterior, platelet adhesion, the storage of plasma membrane GPs and as an internal membrane reservoir facilitating the formation of filopodia - thin cytoplasmic projections extending beyond the actin-containing lamellipodia (Thon & Italiano, 2012).

Platelet activation characterised by the appearance of large pores associated with the OCS, the release of granules, the formation of pseudopodia, membrane spreading and platelet aggregation was observed, to varying degrees, for all peptides, both in the presence and absence of LPS (Figure 5.5). Co-stimulation of platelets with LPS and melittin (Figure 5.5 C and D) or polymyxin B (Figure 5.5 E and F) reduced the effects observed in platelets exposed to either peptide alone (Figure 5.5 M and N; O and P). The observed degree of platelet activation in the presence of LPS and polymyxin B may be attributed to the peptides capacity to bind endotoxins through direct covalent bonds, hydrophobic and ionic interaction and hydrodynamics, reducing blood concentrations of inflammatory cytokines (Mitaka & Tomita, 2011). Since lipid A components of endotoxins bind to platelet membranes, polymyxin B is not however capable of removing all endotoxin units (Mitaka & Tomita, 2011) resulting in the observed degree of LPS-induced platelet activation. Melittin, although highly toxic, similarly possesses strong surface amphipathicity that contributes to the peptides interaction with LPS. The binding of melittin to LPS predominantly involves the C-terminal region of peptide, resulting in a structural transformation towards helicity (Bhunia *et al.*, 2007). The unique morphological alterations observed in platelets exposed to melittin alone were consistent with the allergenic peptides capacity to promote lysis of plasma and intracellular membranes (Lee & Bae, 2016). Platelets exposed to Os (Figure 5.5 Q and R) and Os(C→S) (Figure 5.5 S and T) in the presence of LPS exhibited similar morphological characteristics as platelets exposed to Os (Figure 5.5 G and H) and Os(C→S) (Figure 5.5 I and J) alone, consistent with limited LPS-binding.

Traditionally, NO was considered a product of the endothelium that induced vasodilation. Freedman *et al.* (1997) however, showed that NO is capable of modulating platelet activation, in response to both strong and weak agonists, and inhibiting the recruitment of platelets to the

growing platelet thrombus. This was confirmed by Freedman *et al.* (1999) in an animal model of deficient platelet-derived NO. Endothelium- and platelet derived NO impairs platelet activation and recruitment to the growing thrombus (Freedman *et al.,* 2001) via the interaction between glutathione peroxidase (GPx)-3 and extracellular superoxide dismutase (SOD) on the surface of the endothelium, inactivating ROS intermediates that would otherwise directly or indirectly inactivate NO. The formation of $ONOO^-$ by the reaction of NO with SOD additionally limits the ROS-dependent thrombotic response. This inhibition of platelet adhesion in the presence of NO donors is thought to be a consequence of the inhibiting interactions between vWF and GP-IIb/IIIa (Loscalzo, 2001).

According to Freedman *et al.* (2001) the average concentration of platelet-derived NO in 1 x 10^8 platelets is 3.5 ± 1.2 pmol NO. Since WB contains approximately 3 x 10^8 platelets/ml (Invitrogen, 2008), the average concentration of platelets in 40 µl WB and 10 µl substrate, evaluated in the present study, was calculated as 9.6 x 10^6 platelets/50 µl. From this and results obtained by Freedman *et al.* (2001), the concentration of NO in activated platelets was determined to be 0.366 ± 0.1152 pmol NO/9.6 x 10^6 platelets. According to these estimates and the known NO scavenging capacity of polymyxin B, Os and Os(C→S) (Figure 5.4) NO was effectively scavenged, permitting the observed activation of platelets. Platelet activation observed in this study was therefore, considered twofold, i.e. direct NO scavenging and LPS-binding.

The effect of melittin, polymyxin B, Os and Os(C→S) on erythrocytes in WB was evaluated with SEM. Exposure of erythrocytes to peptides in the presence of LPS resulted in the appearance of several types of poikilocytes, including spherocytes, echinocytes and knizocytes. In the absence of LPS, however, no significant morphological modifications to erythrocyte membranes was observed, with the exception of melittin (Figure 5.6).

Knizocytes appeared to be the predominant poikilocyte associated with LPS (Figure 5.6 C and D). The observed modifications to erythrocyte morphology, associated with LPS, are consistent with the bacterial endotoxin's capacity to reduce erythrocyte deformability. Deformability, the capacity of erythrocytes to undergo structural modification in response to stress without eliciting any haemolytic response, is determined by mechanical properties associated with erythrocyte membranes, cytoplasmic viscosity, predominantly regulated by the mean cellular haemoglobin concentration (MCHC), as well as the surface area-volume ratio (S/V) (Cluitmans *et al.,* 2012). Both an increase and decrease in S/V with a subsequent increase in MCHC and decrease in

membrane elasticity results in the reduction of deformability and associated modifications to erythrocyte membrane structure (Cluitmans *et al.,* 2012).

Previous *in vivo* evaluations of the effects of LPS on erythrocyte deformability have produced conflicting results where no alterations to deformability as well as a significant reduction in deformability has been observed in isolated erythrocytes exposed to LPS. In a study performed by Pöschl *et al.* (2003) lipid A, the toxic core of LPS, and not intact LPS was found to impair erythrocyte deformability in the absence of plasma components. The observed impairment was attributed to the variations in LPS binding to erythrocytes, either binding directly to the surface or intercalating within the lipid bilayer of erythrocyte membranes.

NO has been thought to regulate erythrocyte deformability and aggregation, with NOS inhibitors demonstrating protective effects against erythrocyte deformability during septic shock and the acute phase of endotoxemia (Bor-Kucukatay *et al.,* 2003). Mesquita *et al.* (2002) characterised the effects fof NO on erythrocyte haemorheological properties, including aggregation and deformability; membrane dynamic and structure and O_2 Hb affinity and found that lower concentrations of NO (0.1 - 1 µM) did not alter membrane lipid fluidity and peroxidation but increased erythrocyte deformability - facilitating the passage of erythrocytes through narrow capillaries. At 10 µM, NO reduced membrane fluidity and increased lipid peroxidation - reducing O_2 dissociation from Hb and subsequent delivery to tissues. At the highest concentration of 10 mM, Mesquita *et al.* (2002) found that NO increased methaemoglobin concentrations and decreased O_2 dissociation from Hb and erythrocyte deformability - contributing to the impairment of tissue oxygenation.

The binding of AMPs to LPS is critical for antibacterial and anti-inflammatory activity. Both hydrophobicity and charge, determining the interaction between AMPs and bacterial phospholipid membranes, are essential parameters contributing to the bactericidal activity of AMPs. Since LPS is considered a constituent of the phospholipid membrane, the degree of hydrophobicity and positive polarity may modify the interaction between AMPs and LPS. Cationic AMPs are capable producing strong electrostatic interactions with negatively charged LPS, permitting neutralisation of the negative charges., while the hydrophobicity of AMPs enables their embedding within LPS micelles (Sun & Shang, 2015). Geometric compatibility between AMPs and LPS conformation has been shown to assist with the insertion of hydrophobic amino acid residues into the lipophilic core region of LPS (Sun & Shang, 2015). Rosenfeld *et al.* (2006) observed that AMPs possessing

greater ratios of hydrophobic amino acid residues and positively charged amino acid residues have improved affinity and a stronger binding capacity towards LPS. The binding affinity of AMPs to LPS was however dependent on the distance between positively charged amino acids. In known LPS-binding peptides, the distances between charged amino groups of Lys and Arg range from between 12 and 15 *ångström* (Å) or nm, consistent with the interphosphate distance of the lipid A moiety in LPS.

Since neither Os nor Os(C→S) were capable of inhibiting LPS-induced platelet activation, it was necessary to determine whether the absence of such anti-inflammatory activity was associated with the inability of each peptide to bind LPS. Both Os and Os(C→S) exhibited significantly less LPS-binding compared with polymyxin B. The cyclic lipopeptide, polymyxin B, possesses a high affinity for LPS and binds capable of binding to LPS on Gram-negative bacterial cell membranes via electrostatic interactions, where it is then internalised for subsequent binding to phosphatidylglycerol-rich membrane leaflets, inducing extracellular leakage of bacterial cytoplasmic contents at high concentrations (Domingues *et al.*, 2012). Compared with Os, Os(C→S) exhibited significantly greater LPS-binding at 12.5 µM (Figure 5.7), suggesting that the substitution of Cys residues with Ser residues enhanced the affinity of the peptide towards LPS. Although insignificant, the observed reduction in the capacity of both Os and Os(C→S) to bind LPS at 1.56 µM was likely as a result of either endotoxin impurity, instability of LAL enzyme or a saturation effect (Sandle, 2014). These results complement those obtained by Malan *et al.* (2015) who similarly observed a greater dosage effect in the inhibition of LAL enzyme activation by Os-C, suggesting that the omission of Cys residues from Os improved the binding of the peptide to LPS. In contrast, Ramamoorthy *et al,* (2006) who investigated the effect of the omission of four Cys residues from tachyplesin-I, a cyclic β-sheet AMP, and observed no significant difference in the affinity of the peptide towards LPS implying that Cys residues possess no functional contribution to the antimicrobial activity of AMPs.

This observed affinity of Os(C→S) towards LPS may in part be attributed to the physiochemical properties of each peptide. Bhunia *et al.* (2007) found that the C-terminal helical structure of melittin functions as an anchorage on the outer-membrane capable of disrupting outer membrane organisation and facilitating the intracellular translocation of the peptide, while the dynamic nature of the N-terminal region is ideal for the peptides interaction with the inner cytosolic membrane. Additionally, the interaction between melittin and lipid A, the active component of LPS, was predominantly attributed to the peptides hydrophobicity. This suggesting that both the helical

conformation and hydrophobicity of melittin is essential for the recognition and subsequent binding of LPS on the outer membrane of Gram-negative bacteria.

5.6 Conclusion

The *in chemico* NO scavenging capacity of Os(C→S) was significantly less than that of Os, despite the peptide producing moderate NO scavenging at high concentrations. The effect of Os(C→S) on LPS-induced platelet was similar to that observed for Os, consistent with NO scavenging and the inability of both peptides to bind LPS. Additionally, knizocyte formation in the presence of Os and Os(C→S) appeared to be a product of erythrocyte deformability.

Chapter 6: Concluding Discussion

Bacterial endotoxin, LPS, is a potent water soluble molecule capable of binding to macrophages and stimulating the synthesis and secretion of pro-inflammatory cytokines and associated mediators (Dinarello, 2000). The release of LPS, promoting endotoxinaemia and ultimately septic shock, is largely stimulated by direct killing of bacteria with antibiotics. The unnecessary and irresponsible use of antibiotics has introduced microbial adaptation by genetic mutation with subsequent transmission by horizontal gene transfer (Mølbak, 2004). This rapid emergence of MDR bacteria has stimulated the investigation of alternative antimicrobial agents.

Multifunctional AMPs have emerged as promising therapeutic candidates possessing a broad range of anitbacterial and immunomodulatory activity as well as rapid, nonspecific mechanisms that contribute to the prevention of developing MDR bacteria. The unique lipid composition of prokaryotic cell membranes serves as a major target of AMPs with the charge, size and hydrophobicity of the molecules ultimately determining their association with the cell membrane and subsequent exertion of their activity. Smaller AMPs for example, are capable of rapid diffusion and secretion, elucidating an almost immediate immune response (Hancock & Sahl, 2006).

AMPs derived from the carboxyl-terminal region of their parent peptide have been shown to either maintain or enhance their activity (Varkey *et al.,* 2006). In order to reduce the cost of synthesising these peptides, truncated peptide analogues are frequently designed. Truncated peptides have however, demonstrated significantly lower antimicrobial potential, stimulating investigations into the contribution of specific amino acids, by omisson, as well as the effect of minor structural or sequence-specific modifications. Examining the effect of such small incremental changes by single amino acid substitution in synthetic peptides enables rapid progression in the design of therapeutic peptides with improved selectivity and stability (Tan *et al.,* 2014).

Recent research has placed considerable emphasis on the antimicrobial and immunomodulatory functions of invertebrate defensins involved in innate immunity (Semple & Dorin, 2012). Tick defensins, in particular, function in maintaining the organisms' pathogenic and commensal microbes, commonly responsible for numerous human diseases including Lyme disease, tick-borne encephalitis and rickettsiosis; and zoonoses, without impairing their fitness or development. Such defence mechanisms in ticks have modified both their immune and inflammatory response to pathogens, facilitating both pathogen acquisition and transmission (Hajdušek *et al.*, 2013).

OsDef2, a defensin isoform previously identified in the midgut of the tick *O. savignyi*, was used as a template for the synthesis of several smaller analogues (Prinsloo *et al.,* 2013), including Os, Os-C and Os(C→S) derived from the carboxyl-terminal region. The omission of Cys residues from Os and subsequent substitution of Cys with Ser residues removed any uncertainty regarding the oxidative state of the peptide as well as the potential for inter-molecular disulphide bond formation. Previously, Prinsloo *et al.* (2013) investigated the cytotoxic, antibacterial and antioxidant activities of Os and Os-C and found that both peptides exhibited potent antibacterial activity against Gram-positive and Gram-negative bacteria, no significant cytotoxic effects on mammalian cells and significant antioxidant activity. Malan *et al.* (2015) further evaluated the anti-inflammatory potential of these peptides and found that Os-C did not exhibit any significant NO scavenging activity. The Cys residues, absent in Os-C, reducing the peptide's length and modifying its sequence were therefore thought to be attributed to direct NO scavenging by Os. To ascertain whether the absence of Cys residues or the resulting modification to the amino acid sequence was responsible for the difference in the NO scavenging capacity observed, a novel peptide containing Ser residues, instead of Cys, was synthesised – eliminating the contribution of reduced sequence length to the observed activity. The contribution of Cys residues to the various activities of Os, including the cytotoxic, antioxidant, and anti-inflammatory activity, was therefore evaluated using the Os(C→S) analogue and compared with Os. Selection of Ser was based on similarities in both the structure and chemistry of Cys residues.

Peptides evaluated in the present study were melittin, GSH, polymyxin B, penetratin, Os, and Os(C→S). Melittin, GSH, polymyxin B and penetratin were used as positive controls for the cytotoxic, antioxidant, anti-inflammatory, and intracellular localisation effects of Os and Os(C→S), respectively. Melittin, the primary toxic component of the venom of the honey bee, *Apis mellifera*, is a cationic (+5), linear, amphipathic, α-helical peptide composed of 26 amino acid residues, most of which are hydrophobic or uncharged with the exception of the peptides C-terminal region comprising only polar amino acid residues (Terwilliger & Eisenberg, 1982). The peptide is capable of integrating and disrupting both natural and synthetic phospholipid bilayers, and enhancing the expression of phospholipase A_2. The effect of melittin on membranes is associated with increased membrane permeability or, at high concentrations, lysis (Vogel & Jähnig, 1986). The strong lytic effect of melittin, attributed to both its hydrophobicity and amphipathicity, is associated with enhanced antimicrobial, cytotoxic and haemolytic activity. GSH is a tripeptide, comprising Cys, Glu and Gly. GSH functions as an antioxidant, free radical scavenger and detoxifying agent owing to the capacity of the thiol group of the Cys residue to donate an atom/electron to other molecules,

neutralising them. Polymyxin B, derived from *Bacillus polymyxa* strains, is a cationic (+5), cyclic lipopeptide composed of 10 amino acids, seven of which contribute to its polycationic ring and three making up a hydrophobic acyl side chain with a terminal fatty acid tail (Conrad & Galanos, 1989). Despite the association of polymyxin B with a considerably increased incidence of toxicity, particularly neurotoxicity, polymyxin B as polymyxin B sulphate has been available for topical, parenteral, intrathecal and inhalant applications (Falagas & Kasiakou, 2006). Penetratin is a 16 residue long PTD derived from the Antennapedia homeodomain of *Drosophila*. The mechanism of cellular uptake of penetratin and its conjugates has been shown to involve the endosomal pathway, facilitated by the binding of penetratin to GAGs present on the cell surface (Fischer *et al.,* 2004). Os and Os(C→S) exhibit several similar structural and chemical characteristics. Predictions of the physiochemical and structural properties of Os and Os(C→S) using the software programs, HeliQuest and Protein Sequence Analysis Workbench (PSIPRED), showed that both peptides are composed of 22 amino acids, of which 36% are hydrophobic residues, with a net positive charge of 6 at neutral pH. The hydrophobicity of Os was however, shown to be greater than that of Os(C→S), with 0.249 and 0.034, respectively. The predicted secondary structures of both Os and Os(C→S) contained random coils with some α-helical properties.

At high concentrations, numerous AMPs have been shown to exert some level of haemolysis or cytotoxicity towards mammalian cells (Bacalum & Radu, 2015; Edwards *et al.,* 2016), limiting their therapeutic development as antimicrobial agents. Cellular viability assays, both colorimetric and luminescent, are frequently employed during drug screening and tests of compound cytotoxicity. Colorimetric and luminescence based assays utilise specialised equipment for the direct quantification of viability according to specific parameters and products formed (Weyermann *et al.*, 2005). Parameters, including cell membrane permeability, enzymatic activity, cell adherence, ATP production and nucleotide uptake are common targets of such quantification. These assays are fundamental in determining harmful dose ranges, distinguishing the effects on biological function and developing therapeutic agents capable of targeting rapidly proliferating, carcinogenic cells (Graham-Evans *et al.*, 2003). Frequently, a combination of cellular viability assays, measuring various parameters of cellular function, is employed - enhancing the accuracy of the quantification of compound cytotoxicity.

To determine the effects of Os and Os(C→S) on mammalian cells, L929 mouse embryonic fibroblast cells were exposed to the increasing concentrations of Os and Os(C→S) (1.56 - 100 µM) and evaluated using both the CV and AB assay. The cytotoxic effects of GSH and polymyxin

B, at a concentration range of 1.56 - 100 µM, were additionally, evaluated to eliminate possible toxic involvement in subsequent *in vitro* antioxidant and anti-inflammatory investigations. Known lytic peptide, melittin, was used as a positive control. Light microscopic images of CV stained cells exposed to 100 µM melittin revealed a considerable reduction in cell density accompanied by the appearance of adherent, round, shrunken and sparsely distributed fibroblasts (Figure 3.3 C). No major difference in cell density or cell morphology was observed in cells exposed to Os (Figure 3.3 D and E) or Os(C→S) (Figure 3.3 F and G). Quantitative analyses of the results obtained for melittin, showed a significant reduction in cell density at concentrations ranging from 6.25 - 100 µM, while no significant toxic effects were observed in cells exposed to GSH, polymyxin B, Os and Os(C→S), even at the highest concentration of 100 µM (Figure 3.2). The effects of peptides on mitochondrial and cytoplasmic reductase activity, evaluated using the AB assay, were similar to that observed with CV staining (Figure 3.4).

These results were comparable to results obtained by Prinsloo *et al.* (2013), Malan *et al.* (2015) and preliminary data described by Minnaar (2015, unpublished). Using the CV assay, Prinsloo *et al.* (2013) found that neither Os nor Os-C induced any significant changes in cell density at any of the tested concentrations in either SC-1 (embryonic, *Mus musculus*, fibroblast) or Caco-2 cells (adult, *Homo sapiens*, epithelial, colorectal adenocarcinoma). At the highest concentration, melittin on the other hand, induced an approximate 2.5- and 1.25-fold reduction in the density of SC-1 and Caco-2 cells, respectively. Minnaar (2015, unpublished) similarly observed an almost 2.5-fold reduction in cell density of murine SC-1 cells exposed to the highest concentration of melittin and no significant cytotoxicity in cells exposed to Os or Os(C→S). Malan *et al.* (2015) found that both melittin and polymyxin B inhibited metabolic enzyme activity in RAW 264.7 murine macrophages, quantified using the MTT assay, at concentrations ranging from 1.56 and 25 µM, respectively, while no inhibitory effect was observed for Os and Os-C at any of the tested concentrations. Variation between the toxicity profiles of each peptide, described in the present study and by Malan *et al.* (2015), may be in part be due to differences in the physiological and biochemical properties and associated sensitivities of each cell line towards exogenous compounds.

Taute (2017) evaluated the viability of PBMCs and PMNs using the AB assay and observed a significant reduction in the viability of PBMCs for all tested concentrations of melittin (0.5 - 10 µM) as well as the highest concentration of Os (100 µM). At this concentration, Os reduced the viability of PBMCs approximately 4-fold. In PMNs, a reduction in the viability of cells exposed to melittin

was only observed at concentrations ranging from 2 - 10 µM. At 100 µM Os, no significant reduction in the viability of PMNs was observed. Os-C did not appear to influence the viability of either PBMCs or PMNs. Similarly, in the present study, no significant reduction in the viability of PBMCs and PMNs was observed in cells exposed to Os and Os(C→S) (Figure 3.8 A and B). Neither Os nor Os(C→S) therefore exhibited any cytotoxic effects at their MBCs against *E. coli* (ATCC 700928) - 1.52 and 3.11 µM, respectively.

Viscotoxins are cationic proteins belonging to thionins type II and isolated from the leaves and stems of *Viscum album* Loranthaceae (European mistletoe). Studies have shown that reducing the disulphide bonds of viscotoxins completely inhibits their biological activity - similar to that reported for Os, Os-C and Os(C→S). Additionally, the cytotoxicity of these compounds against human granulocytes and lymphocytes has previously been prevented by cleavage of their disulphide bonds, emphasising the significance of non-reduced conformations of viscotoxins for their biological activity. Viscotoxins are however, known to interact differently with different cell types and phospholipid membrane compositions, resulting in variations in their biological effects (Giudici *et al.,* 2003).

Since some peptides have been shown to induce haemolysis in erythrocytes (Pacor *et al.,* 2002), the cytotoxicity of Os and Os(C→S), compared to positive control melittin, was additionally evaluated in human erythrocytes. Interestingly, the concentration of peptides within the blood has been shown to be largely reduced by the binding of peptides to cellular and plasma components (Pacor et al., 2002). Evaluating erythrocytes suspended in buffer does however, eliminate the interaction between plasma components and peptide. Melittin produced a dosage-related increase in haemolytic activity with complete erythrocyte lysis observed at concentrations ranging from 6.25 - 100 µM (Figure 3.6). Os and Os(C→S) did not display any significant cytotoxicity, compared to the untreated control, even at high concentrations (Figure 3.6). The observed haemolytic effect of melittin was confirmed using SEM (Chapter 5, Section 5.4.2, Figure 5.6 E and F). The haemolytic effects of Os and Os(C→S) were consistent with results obtained by Prinsloo *et al*. (2013) which showed no significant induction of erythrocyte haemolysis at any of the measured concentrations of Os and Os-C while total haemolysis was observed at concentrations rangin from 12.5 µM. Disruptions in the Leu zipper motif of melittin, resulting in a conformational change of the peptide's α-helical structure, subsequently compromising its membrane permeability, is thought to be responsible for the observed membranolytic effects (Asthana, et al., 2004; Zhang et al., 1999).

Peptide hydrophobicity is correlated with haemolytic activity, owing to a mechanism of membrane discrimination. Peptides with higher hydrophobicity tend to penetrate deeper into the hydrophobic core of cells, inducing stronger cytotoxic effect by the formation of pores or channels. Reducing the hydrophobicity of peptides to combat their cytotoxic effects does, however, reduce their antimicrobial activity. Conversely, increasing peptide hydrophobicity will improve antimicrobial activity to a certain extent, after which increased dimerisation will prevent peptide access to prokaryotic membranes and subsequent exertion of their antimicrobial activity (Chen *et al.,* 2007).

AMPs possessing additional cell-penetrating properties, such as lactoferricin (Splith & Neundorf, 2011), may be capable of targeting intracellular biochemical pathways, eradicating residual pathogens and acting as carrier molecules with no additional effects (Bahnsen *et al.,* 2015). The ability of such peptides to induce membrane permeabilisation in target cells has been correlated to the toxicity of thiol-containing AMPs. Some studies suggest that these AMPs create ion channels in the plasma membrane while others suggest peptide-induced membrane destabilisation (Coulon *et al.,* 2002). Studies of thionin toxicity have suggested that electrostatic interactions are required for the interaction between positively charged proteins with negatively charged membrane phospholipids, modifying the structure and integrity of the membrane. Differences in amino acid composition and charge, resulting in variations in structural conformation between peptides, are thought to be associated with unique biological behaviour (Giudici *et al.,* 2003).

Confocal laser scanning microscopy is capable of qualitatively assessing the cellular interaction and distribution of peptides, using multiple fluorophores, for the identification of CPPs. Colocalisation of peptides with DAPI, a cell permeable fluorescent dye that binds to adenine-thymine (A-T) rich regions of the DNA within the cell nucleus, showed considerable heterogeniety in both PBMCs and PMNs. In PBMCs, positive control penetratin exhibited extensive nuclear interaction and limited intracellular clustering suggesting that the peptide was capable of escaping the endosomal vesicles and becoming available for nucleic acid binding and subsequent nuclear localisation (Figure 3.7 C and D). Both Os (Figure 3.7 E and F) and Os(C→S) (Figure 3.7 G and H) on the other hand, exhibited membrane interaction with occasional internalisation. The degree of membrane interaction and subsequent internalisation of Os(C→S) in PBMCs was however, considerably less than that observed for Os. This interaction of peptides with the cell membrane is thought to initiate internalisation, following the binding of positively charged Arg and Lys residues to the polyanionic surface of the cell membrane (Bahnsen *et al.,* 2015). In PMNs,

penetratin appeared confined to endocytic vesicles, with limited nuclear and organellar interaction (Figure 3.7 K and L). Os exhibited slight cellular uptake and localisation (Figure 3.7 M and N), although considerably more than observed for Os(C→S) (Figure 3.7 O and P). The difference in membrane permeability between peptides may be attributed to the tendency for helical structuring of each peptide, their charge, hydrophobicity and amphipathicity as well as the stereochemistry of each peptide (Pacor *et al.,* 2002). These results were comparable to that obtained by Rodrigues *et al.* (2013) who showed that a minimal change in peptide structure resulting from Ser-substitution reduces both the rate of uptake and subsequent internalisation of the peptide in PBMCs. The reduction in the uptake and cellular internalisation of Os(C→S), in the present study, may therefore be attributed to the absence of Cys, further influencing both the *in vitro* antioxidant and anti-inflammatory activity of the peptide.

Increasing emphasis has recently been placed on the ability of therapeutic agents to scavenge ROS due to their involvement in processes such as senescence, pathogenesis and hypertension (Ou *et al.*, 2001). To be considered an antioxidant, particular amino acids must oxidise preferentially to polyunsaturated fatty acids. The antioxidant activity of proteins and peptides is therefore, dependent on the capacity of amino acid residues to interact with lipid-derived free radicals as well as $HOO^{\bullet}$. Amino acid residues vary considerably in their individual oxidative stability (Elias *et al.,* 2005). The relative reactivity rate of several amino acids is estimated to be Cys > Trp, Tyr > Met > Phe > His > Ile > Leu > Pro (Elias *et al.,* 2005). Oxidatively sensitive amino acid residues, including Cys, Tyr, Trp and Met, have the greatest potential for the inhibition of lipid oxidation reactions (Nakao *et al.,* 2003; Elias *et al.,* 2005).

Evaluation of the antioxidant activity of GSH, Os and Os(C→S), using the ET-based, TEAC assay, revealed comparable dosage effects for both Os and Os(C→S) (Figure 4.4 B). Additionally, the antioxidant activity of Os and Os(C→S) was significantly higher than that of GSH, which did not exhibit any significant free radical scavenging, compared to the untreated control (Figure 4.4 A). Further evaluation of the radical chain breaking capacity of Os and Os(C→S), compared to known antioxidant, GSH, using the ORAC assay similarly showed no significant difference in antioxidant activity between Os and Os(C→S) across all measured concentrations, with the exception of 50 and 100 µM (Figure 4.5 A and B). A high correlation between data obtained from both the ORAC and TEAC assay was observed for all peptides (Figure 4.6).

Following AAPH-induced oxidative damage in L929 cells, both GSH and Os exhibited considerable protective effects against radical production (Figure 4.7 B). The protective effects of GSH against AAPH-induced oxidative damage was however significantly greater than that of Os at concentrations ranging from 25 - 100 µM . At the highest concentration, the protective effects of GSH was approximately 8.59-fold greater than that of Os. Polymyxin B and Os(C→S) exhibited a significant increase in radical production at high concentrations (Figure 4.7 A), implying no significant protective effects against AAPH-induced oxidative damage. A high correlation between the ORAC and DCFH-DA assay as well as the TEAC and DCFH-DA assay was observed for Os (Figure 4.8), while for both Os(C→S) and GSH the correlation between the ORAC and DCFH-DA assay as well as the TEAC and DCFH-DA assay was absent.

Hydrophobic amino acid residues, such as Pro and Leu, are known to enhance the capacity of peptides to interact with hydrophobic cellular targets, increasing their cellular antioxidant activity (Chen *et al.,* 1998). Predictions of the physiochemical properties and secondary structures of Os and Os(C→S) using the software programs, HeliQuest and PSIPRED, respectively, showed that the net charge (+6), amino acid chain length (22 amino acids) and percentage hydrophobic residues (36%) were the same in both Os and Os(C→S) while the overall hydrophobicity of Os(C→S) was considerably less than that of Os, differing by 0.215. This difference in hydrophobicity between Os and Os(C→S), likely owing to the presence of Cys residues in Os, may be associated with the observed differences between the *in vitro* radical scavenging capacity of each peptide. Several other amino acids, including Tyr, Met, Phe, His and Trp are capable of acting as antioxidant agents (Elias *et al.*, 2008). The three Tyr residues as well as the Phe residue present in Os, may therefore, additionally be responsible for the observed antioxidant activity. The antioxidant activity of Tyr has been attributed to its phenolic OH^- group functioning as a good hydrogen donor (Saito *et al.*, 2003). The reduction in the protective effects of Os against AAPH-induced oxidative damage and further increase in radical production at high concentrations of GSH and Os, may be attributed to the capacity of free radicals to selectively oxidise Cys sulfhydryl groups forming disulphide bonds (Wu *et al.,* 2017).

Malan *et al.* (2015), who similarly evaluated the capacity of GSH, Os and Os-C to reduce free radical formation following AAPH-induced oxidative damage in the murine RAW 264.7 cell line, found that all peptides elicited considerable protective effects against AAPH-induced oxidative damage. At 100 µM, the protective effects of Os was greater than Os-C, although significantly less than that produced by GSH at the same concentration. Differences between current results

and data obtained by Malan *et al.* (2015) may predominantly be as a result of the physiological and biochemical differences between L929 and RAW 264.7 cells as well as the difference between the hydrophobicity of Os, Os-C and Os(C→S) (0.249, 0.045 and 0.034, respectively). Despite this, both studies observed the contribution of Cys residues to the protective effects of Os.

Inflammation is frequently associated with the accumulation of free radicals and overproduction of ROS resulting in localised tissue damage. NO, a gaseous signaling molecule, and TNF-α are essential mediators of such pro-inflammatory responses and are involved in the initiation of septic shock resulting from unregulated macrophage stimulation (Korhonen *et al.,* 2005; Choi *et al.*, 2013). Counteracting the overproduction of NO and resulting pro-inflammatory cytokines with peptides is therefore essential for the prevention of such consequences (Park *et al.*, 2004; Rosenfeld *et al.*, 2006). NO is spontaneously generated from SNP and subsequently interacts with O_2 producing NO_2^- (Boora *et al.,* 2014). The capacity of GSH, polymyxin B, Os and Os(C→S) to compete with O_2, generating S-nitrosothiols (Vriesman *et al.*, 1997) and inhibiting a subsequent pro-inflammatory response, was determined. At concentrations ranging from 1.56 – 50 µM, both Os and GSH exhibited comparable, dose-dependent, NO scavenging activity (Figure 5.4 B). At the highest tested concentration (100 µM) a significant reduction in NO scavenging was observed for Os and GSH consistent with the contribution of thiols to the production of NO radicals from SNP (Guzik *et al.*, 2003) (Figure 5.4 A). Os(C→S) reduced NO scavenging activity similar to that observed for polymyxin B (Figure 5.4 C) with significant NO scavenging occurring at concentrations greater than 12.5 µM of Os(C→S) (Figure 5.4 A).

Malan *et al.* (2015) evaluated the NO scavenging activity of Os and Os-C and observed moderate, dose-dependent, NO scavenging for Os, with 49% activity at the highest tested concentration i.e. 25 µM. At the same concentration, the NO scavenging activity of Os reported in this study was 1.2-fold higher. The difference may be attributed to modifications of the SNP assay performed in each investigation. Malan *et al.* (2015) added SNP to the peptides immediately following its preparation and the resulting reactants were incubated at room temperature, in the dark, for 1 hour. During the present study, SNP was incubated for 1.5 hours in natural light prior to being added to the peptide samples (Figure 5.3). This is thought to have generated significantly more NO radicals for more effective scavenging. Os(C→S) on the other hand displayed considerably less NO scavenging activity, comparable to the NO scavenging activity produced by Os-C at the same tested concentration. The absence of significant NO scavenging activity in both Os-C and

Os(C→S) therefore confirms the functional contribution of Cys to the NO scavenging activity of Os.

Bacterial LPS, typically released during cell division, cell death or antibiotic treatment of infection (Rosenfeld *et al.*, 2006); binds to LPB forming a complex that interacts with a primary receptor, CD-14 (Li *et al.*, 2012). CD-14 may either be soluble, present in tissue fluid, or bound to the surface of macrophages (Cohen, 2002). The complex initiates TLR signalling, by binding to transmembrane protein, TLR-4 (Cohen, 2002), ultimately activating the NF-κB pathway. The NF-κB pathway activates the transcription of genes encoding inflammatory cytokines, adhesion molecules and other innate immune response effectors, further activating systems of haemostasis (Kindt *et al.*, 2007; Brauckmann *et al.,* 2016). Generalised inflammation and activation of coagulation subsequently promotes disturbances in the microcirculation, ischemia and organ failure. TLR-4 antagonists are capable of reducing serum cytokine concentrations implicated in endotoxinaemia. In sepsis however, the effects of TLR-4 may be both beneficial and detrimental, owing to the toxicity of Hb and its degradation products resulting from LPS-induced haemolysis.

The effects of peptides, melittin, polymyxin B, Os and Os(C→S) on LPS-induced platelet activation and erythrocyte membranolysis was assessed using SEM. LPS-mediated platelet activation involves the TLR4/MyD-88-dependent activation of NO and cGMP-dependent protein kinase pathway (Zhang *et al.,* 2009). Platelet activation, characterised by the appearance of pores associated with the OCS; pseudopodia, membrane spreading and platelet aggregation was observed for all peptides, both in the presence and absence of LPS (Figure 5.5).

Co-stimulation of platelets with LPS and melittin (Figure 5.5 C and D) or polymyxin B (Figure 5.5 E and F) reduced the effects observed in platelets exposed to either peptide alone (Figure 5.5 M and N; O and P) resulting in platelet morphology similar to that observed in platelets exposed to LPS alone. Both melittin and polymyxin B have known LPS binding affinities (Bhunia *et al.*, 2007; Mitaka & Tomita, 2011) and therefore, likely became saturated with LPS permitting only the interaction between free/unbound LPS and platelets. Platelets exposed to Os (Figure 5.5 Q and R) and Os(C→S) (Figure 5.5 S and T) in the presence of LPS exhibited similar morphological characteristics as platelets exposed to Os (Figure 5.5 G and H) and Os(C→S) (Figure 5.5 I and J) alone, consistent with limited LPS-binding and peptide availability.

Traditionally, NO was considered a product of the endothelium that induced vasodilation however, Freedman *et al.* (1997) showed that NO is capable of modulating platelet activation and inhibiting the recruitment of platelets to the growing platelet thrombus. According estimates determined from Freedman *et al.* (2001), the concentration of NO in activated platelets in the present study was 0.366 ± 0.1152 pmol NO/9.6 x 10^6 platelets - permitting sufficient NO scavenging by polymyxin B, Os and Os(C→S) (Figure 5.4) and the observed activation of platelets.

Exposure of erythrocytes to peptides in the presence of LPS (Figure 5.6), resulted in the appearance of several types of poikilocytes (Figure 5.6 E and F). Knizocytes appeared to be the predominant poikilocyte associated with LPS (Figure 5.6 C and D) while extensive echinocyte formation was observed in erythrocytes exposed to melittin alone (Figure 5.6 G and H) and melittin in combination with LPS (Figure 5.6 M and N). No significant morphological modifications was observed in erythrocytes exposed to polymyxin B, Os and Os(C→S) in the absence of LPS.

Haemolysis has been thought to enhance thrombin formation and induce a severe procoagulant effect. Peng *et al.* (2012) evaluated the haemostatic effect of melittin using thromboelastography (TEG) and found that the peptide produced an increased procoagulant effect with increasing concentrations, although with a reduction in the onset and maximum strength of clot formation, early fibrinolysis and reduction in platelet function. As a result, melittin was thought to act on blood coagulation through erythrocyte haemolysis and interaction with plasma proteins, without significant influence from platelets. This haemostatic effect of melittin was considered to be strongly associated with the peptide's helicity and amphipathic properties.

AMPs possessing potent bactericidal activity and a high affinity for LPS are particularly attractive candidates for therapeutic development. Numerous such AMPs are cationic, exhibiting an overall positive charge, with well-defined secondary structures, including α-helical or β-strands. Both the amphipathic and hydrophobic properties of cationic AMPs enhances their interference with bacterial cell membrane stability, inducing extracellular leakage of bacteria contents. Several AMPs are additionally capable of neutralising LPS directly, inhibiting the production of pro-inflammatory cytokines such TNF-α, IL-6 and IL-8; regulating immune response and reducing inflammatory injury through modified immune regulation (Sun & Shang, 2015).

Since neither Os nor Os(C→S) was capable of inhibiting LPS-induced platelet activation, it was necessary to determine whether the absence of such activity was associated with the inability of

each peptide to bind LPS. At concentrations ranging from 1.56 - 25 µM, known LPS-binding peptide, polymyxin B, exhibited significantly greater LPS-binding than Os and Os(C→S), with the exception of Os(C→S) at 6.25 µM, while the binding of Os(C→S) to LPS at high concentrations was significantly greater than that observed for Os (Figure 5.7) suggesting that substitution of Cys residues with Ser residues enhanced the affinity of the peptide towards LPS. These results complement those obtained by Malan *et al.* (2015) who similarly observed a greater dosage effect in the inhibition of LAL enzyme activation by Os-C, implicating the omission of Cys residues in improved binding of the Os-C to LPS. These results do however, conflict with that obtained by Ramamoorthy *et al,* (2006) who investigated the effect of the omission of four Cys residues from tachyplesin-I, a cyclic β-sheet AMP, and observed no significant difference in the affinity of the peptide towards LPS implying that Cys residues possess no functional contribution to the antimicrobial activity of AMPs. This observed differences in the affinity of peptides towards LPS may in part be attributed to the physiochemical properties of each peptide, predominantly the hydrophobicity and propensity for helical structuring (Bhunia *et al.,* 2007).

In conclusion, neither Os nor Os(C→S) elicited any significant toxic effect against mammalian L929 cells or human erythrocytes. The enhanced cell-penetrating capacity of Os, compared with Os(C→S), although limited appeared to be associated with the presence of Cys residues, shown to be implicated in the overall hydrophobicity of the peptide. *In chemico,* Os and Os(C→S) were capable of inhibiting free radical production using both ET- and HAT-based reactions. *In vitro,* however, the protective effects of Os(C→S) against AAPH-induced oxidative damage was largely negligible implying some functional contribution of Cys residues to the antioxidant activity of Os. The *in chemico* NO scavenging potential determined for Os(C→S) was significantly less than that of Os compatible with observations by Malan *et al.* (2015). The effect of Os on LPS-induced platelet activation, on the other hand, appeared similar to that of Os(C→S), indicating a reduced capacity to bind LPS. The absence of significant antioxidant and anti-inflammatory activity by Os(C→S) correlates with the peptide's low hydrophobicity and associated inability to translocate across the cell membrane reducing its intracellular availability and subsequent exertion of its effects. The thiol groups of sulphur-containing Cys residues present in Os therefore, play a significant role in the cell-penetrating and antioxidant activity of the peptide. The mechanism of bacterial killing and subsequent immunomodulatory effects of Os is however, not likely to be associated with the presence of Cys residues or direct LPS binding (Table 6.1).

Table 6.1: Summary of the effect of peptides determined in this study.

	Melittin	Polymyxin B	GSH	Penetratin	Os	Os(C→S)	Os vs Os(C→S) ($p \leq 0.05$)	Activity
Antibacterial activity								
MBC of *E. coli* (ATCC 700928; µM)	0.17	n.d.	n.d.	n.d.	1.52	3.11	√	Os and Os(C→S) are capable of killing *E. coli* at low concentrations*
Cellular effects								
L929 cells (density)	√	x	X	n.d.	X	x	x	Neither Os no Os(C→S) affects the density of L929 cells
L929 cells (viability)	√	x	X	n.d.	X	x	x	Neither Os nor Os(C→S) affects the viability of L929 cells
Erytrocytes (membranolysis)	√	n.d.	n.d.	n.d.	X	x	x	Neither Os nor Os(C→S) induces any membranolytic effects
Leukocytes (viability)	√	n.d.	n.d.	n.d.	X	x	x	Neither Os nor Os(C→S) affects the viability of leukocytes
Leukocytes (penetration)	n.d.	n.d.	n.d.	√	Min.	Min.	x	Neither Os nor Os(C→S) penetrates leukocytes effectively
Antioxidant activity								
ET-based (*in chemico*)	n.d.	n.d.	√	n.d.	√	√	x	Os and Os(C→S) scavenges $ABTS^{\bullet}$
HAT-based (*in chemico*)	n.d.	n.d.	√	n.d.	√	√	x	Os and Os(C→S) scavenges $ROO^{\bullet}$
HAT-based (*in vitro*)	n.d.	n.d.	√	n.d.	√	x	√	Os scavenges $ROO^{\bullet}$; Os(C→S) produces a prooxidant effect at high concentrations
Anti-inflammatory capacity								
NO scavenging (*in chemico*)	n.d.	Min.	√	n.d.	√	Min.	√	Os scavenges NO; Os(C→S) is inactive
Ex vivo platelet activation	√	√	n.d.	n.d.	√	√	x	Neither Os nor Os(C→S) prevents LPS-induced platelet activation
LPS-binding	n.d.	√	n.d.	n.d.	x	x	√	Neither Os nor Os(C→S) binds LPS effectively; binding of Os(C→S) is greater than Os

*Minnaar (2015, unpublished), Min: minimal; n.d.: not determined.

Os and Os(C→S) therefore show promising therapeutic potential due to their rapid bactericidal activity at low concentrations, as preliminarily determined by Minnaar (2015, unpublished), and the absence of toxicity in mammalian cells even at a concentration as high as 100 µM. The therapeutic potential of Os in particular is further enhanced by the peptides' high antioxidant capacity, providing possible applications against the adverse effects of oxidative stress. Both peptides are however, limited by their inability to translocate efficiently across the cell membrane of primary immune cells for intracellular targeting. Additionally, therapeutic development of Os and Os(C→S) should be confined to superficial or topical application due to systemic NO scavenging-induced platelet activation stimulating thrombosis typically associated with thromboembolic complications of atherosclerosis, such as heart attacks, strokes, and peripheral vascular disease (Packham, 1994).

Platelets mediate both the intrinsic (tissue factor) and extrinsic (contact activation) pathways of thrombosis responsible for activating blood coagulation. Phosphatidylserine (PS) exposed to the membranes of activated platelets stimulates the production of thrombin by inducing a negatively charged surface capable of binding coagulation factors (Hou *et al.,* 2015). Thrombin is a potent inducer of platelet aggregation, associated with the conversion of GP-IIb/IIIa to a receptor for fibrinogen, as well as platelet secretion (Packham, 1994). During blood coagulation, thrombin converts fibrinogen to fibrin (Hou *et al.,* 2015), stabilising platelet aggregates that rapidly form haemostatic plugs (Packham, 1994) and initiates signalling through protease activated receptor (PAR)-1 and -4 and GP-Ibα, resulting in further platelet activation and PS exposure – creating a positive feedback loop for the production of thrombin and blood coagulation (Hou *et al.,* 2015).These thrombi are.

Fibroblasts are considered gold standards for the evaluation of material biocompatibility (Schmidt *et al.*, 1993; Theerakittayakorn & Bunprasert, 2011). To determine the possible implications of Os(C→S) in topical application, the ability of the peptide to penetrate the membranes of mammalian L929 fibroblasts should be investigated. AMPs in topical application have the advantage of being directly delivered to the site of infection, escaping enzymatic digestion; achieving high local tissue levels following aggressive dosing and may be combined with conventional antibiotic strategies (Gordon & Romanowski, 2005). Systemically, AMPs are limited by their capacity to induce apoptosis, mast cell degranulation and extracellular DNA transfer (Giuliani *et al.,* 2007). The development of systemic AMP-based haemostatic vasoactive drugs may however, provide several advantages in the treatment of haemorrhage associated with

anticoagulation therapies and chronic, non compressible wounds. Such compounds frequently exhibit a biphasic or hormetic response - enhancing blood coagulation through increased fibrin polymerisation and reduction of proteolysis at low concentrations while inhibiting platelet receptors necessary for the interaction with fibrinogen, resulting in anticoagulation, at high concentrations (Peng *et al.,* 2012).

To optimise the efficacy and intracellular behaviour of Os as a potential drug carrier, the mechanism of uptake and intracellular trafficking should be further investigated. The importance of endocytosis in the translocation of Os and Os(C→S) across the cell membrane of primary immune cells should be evaluated using flow cytometry at 4°C and 37°C. Since endocytosis is an active process, low temperatures reduce the rate of uptake (Fernando *et al.,* 2010). Additionally, flow cytometry should be used to monitor the rate of uptake of peptides over 2 hours at 10 minute intervals (Rodrigues *et al.,* 2013).

Numerous α-helical AMPs have been reported to act on bacterial membranes via pore formation, ultimately inducing the destruction of bacterial membrane integrity (Kim *et al.,* 2014). Since preliminary data obtained by Minnaar (2015, unpublished) showed that Os(C→S) has potent antibacterial activity, the capacity of the peptide to permeabilise bacterial membranes should be evaluated using a model microorganism, e.g. *E. coli*. The β-galactosidase assay, making use of ortho-nitrophenyl- β-D-galactopyranoside (ONPG), should be performed (Schaefer *et al.,* 2016) to determine the mechanism by which Os(C→S) exerts its antibacterial activity. Additionally, bacterial membrane damaging effects of Os(C→S) should be confirmed using SEM.

The effect of Os(C→S) on LPS-induced platelet activation should be quantitatively confirmed using an *in vitro* model of inflammation i.e. RAW 264.7 macrophages (Aldridge *et al.,* 2009) or THP-1 promonocytes, differentiated using 1.25-dihydroxylcholecalciferol (Azofeifa *et al.,* 2013), measuring NO production following LPS/IFN-γ stimulation. The LPS binding capacity of Os(C→S) should be re-evaluated using the LAL assay, controlling for reagent purity and stability. Additionally, the effect of Os(C→S) on iNOS and TNF-α production and gene expression should be investigated using the enzyme-linked immunosorbent assay (ELISA) (Lee *et al.,* 2014; Malan *et al.,* 2015), Western blotting and real time-polymerase chain reaction (RT-PCR) (Lee *et al.,* 2014), respectively.

The production of NO from activated platelets should be determined, as described by Freedman *et al.* (2001) who measure platelet NO production, following platelet activation, using an NO-selective microelectrode. Additionally, the mechanism of peptide-induced platelet activation should be investigated. A dosage-effect study should be performed to determine the correlation between NO scavenging and platelet activation. Platelet activation related to NO scavenging would be indicative of adverse systemic effects, although necessary for the superficial release of inflammatory mediators and growth factors, including TNF-α, NFκB and TGF-1β, during acute inflammation. Using Western blotting and immunostaining, potential biochemical targets of Os and Os(C→S) in platelets, including P-selectin, PSGL-1, GP-Ib and GP-IIb/-IIIa (Stokes & Granger, 2012); should be identified.

The haemostatic effects of Os and Os(C→S) should be evaluated using TEG. TEG measures global haemostasis, from the initiation of coagulation to clot formation and finally fibrinolysis, using reaction time (time to first clot formation) associated with plasma clotting factors and circulating inhibitory activity; coagulation time (rate of clot strengthening) related to the activity of intrinsic clotting factors, fibrinogen and platelets; maximum amplitude (maximum strength of the clot) as a direct function of the maximum dynamic properties of fibrin and platelet concentration; rate of clot polymerisation; and α-angles which is considered a primary function of platelets, fibrinogen and plasma components on the surface of platelets, as parameters (Peng *et al.,* 2012).

Since a major limitation of the clinical use of AMPs is their inactivation by endogenous and exogenous proteases the resistance of peptides to various proteases, including trypsin, chymotrypsin and aureolysin, should be evaluated by analysing digestive fragments using tricine-SDS-polyacrylamide gel electrophoresis (PAGE) (Kim *et al.,* 2014). Additionally, the conformational behaviour of Os and Os(C→S) in an aqueous- and membranous-mimicking environment, using PBS and SDS respectively, should be investigated using circular dichroism secondary structure determination (Kim *et al.,* 2014).

Chapter 7: References

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Chapter 8: Appendix

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

28/09/2017

Approval Certificate
New Application

Ethics Reference No: 417/2017

Title: The role of cysteine residues on the bioactivity of the antimicrobial peptide os derived from the tick Ornithodoros savignyi

Dear Rene Stanley

The **New Application** as supported by documents specified in your cover letter dated 25/08/2017 for your research received on the 25/08/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 27/09/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**417/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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