The dual functionality of antimicrobial peptides Os and Os-C in human leukocytes

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Running title: Dual functionality of the peptides Os and Os-C

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Key words: Antimicrobial peptide, defensin, cell penetrating peptide, antioxidant, leukocyte activation, anti-inflammatory

Abbreviations

5FAM, 5-carboxyfluorescein

AB, Alamar Blue

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride

AMP, antimicrobial peptide

CPP, cell penetrating peptide

DCFH-DA, 2',7'-dichlorofluorescin diacetate

GM-CSF, granulocyte-macrophage colony-stimulating factor

HDP, host defence peptide

MBC, minimum bactericidal concentration

Mel, melittin

MN, mononuclear

NADPH, nicotinamide adenine dinucleotide phosphate

NET, neutrophil extracellular trap

NOX, NADPH oxidase

PMN, polymorphonuclear

SEM, scanning electron microscopy

Abstract

Antimicrobial peptides (AMPs), Os and Os-C have been identified as multifunctional peptides with antibacterial, anti-endotoxin and anti-inflammatory properties. For further development of Os and Os-C as therapeutic peptides it is essential to evaluate these effects in human mononuclear (MN) and polymorphonuclear (PMN) leukocytes. The cytotoxicity and the effects of both peptides on MN and PMN morphology were determined with the Alamar Blue assay and scanning electron microscopy, respectively. The ability of Os and Os-C to induce reactive oxygen species (ROS) and to protect against 2,2'-azobis(2-amidinopropane) dihydrochloride induced oxidative damage in both cell populations was evaluated using 2',7'-dichlorofluorescin diacetate (DCFH-DA). Using fluorescently labeled peptides, the ability of the peptides to cross the cell membranes of MN and PMN was also evaluated. At the minimum bactericidal concentrations of Os and Os-C neither peptide was cytotoxic. Os caused morphological features of toxicity at 100 µM, entered MN cells and also protected these cells against oxidative damage. Os-C caused MN and PMN leukocyte activation associated with ROS formation and was unable to penetrate cell membranes, indicating extracellular membrane interactions. This study confirms that both Os and Os-C at <100 µM are not cytotoxic. The MN-specific uptake of Os identifies it as a cell specific cargo carrier peptide, with additional anti-inflammatory properties. In contrast, the ability of Os-C to activate MN and PMN cells implies that this peptide should be further evaluated as an AMP which, in addition to its ability to eradicate infection, can further enhance host immunity. These novel characteristics of Os and Os-C indicate that these AMPs can be further developed for specific applications.

1. Introduction

Antimicrobial peptides (AMPs) have been identified as promising therapeutic agents that can be developed as new multifunctional antibiotic compounds, which may address antibiotic resistance. Some AMPs have been reported to have a wide range of bioactivities, including antimicrobial, antioxidant, anti-inflammatory and anticancer properties.[1-3] AMPs are thus potential agents for the treatment of topical and systemic acute or chronic infections.[4] In such a treatment strategy, AMPs must be highly specific for bacteria and not compromise cellular viability and proliferation.

Host defence peptides (HDP) have been identified as peptides with immunomodulatory properties that play an important role in these processes.[5] HDPs, released by epithelium and neutrophils, kill bacteria, promote the recruitment of immune cells, suppress the production of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α). Further cellular effects include the induction of macrophage and dendritic cell differentiation and activation. These events modulate the adaptive immune response and regulates processes such as autophagy and neutrophil extracellular trap (NET) formation.[5] AMPs derived from different sources are increasingly identified as multifunctional peptides, that can reduce infection and promote cell and tissue healing by creating an environment that promotes the clearance of endotoxins, reduces inflammation, promotes fibroblast chemotaxis for matrix synthesis and epithelial cell proliferation as well as angiogenesis and vasculogenesis.[6]

In *Streptococcus pyogenes* (group A, *Streptococcus* (GAS)) infections, GAS has developed several strategies to avoid the direct antimicrobial mechanisms of neutrophils[7] while in tumors, cancer cells have developed strategies to suppress the activation of cells of the innate and adaptive immune system by suppressing the activation and effector functions of these cells[8]. Therefore peptides that can enhance innate and/or adaptive immunity may have a beneficial effect in the treatment of infection or disease. In addition, cell penetrating peptides (CPP) are attractive candidates for the treatment of intracellular infections[9] or selective drug delivery.[10] Coupling of proteins, oligonucleotides or nanoparticles to CPPs increases the uptake of therapeutic agents into cells[10] which lowers toxic side effects of the coupled molecules.

Leukocytes are highly mobile cells and via the circulatory system can access most organs and tissues, including tumors. Neutrophils and monocytes can be recruited to leave the circulation and enter tissue at sites of inflammation.[11] Consequently, the inherent ability of leukocytes to access all tissue, except neurological tissue, makes leukocytes attractive cells for the modulation of inflammation or as cellular carriers of for example drugs, peptides and nano-particles to sites of infection, disease and injury.

Two synthetic antimicrobial peptides, Os and Os-C (Table 1) derived from a tick defensin have been identified as multifunctional peptides, due to the ability of these peptides to effectively kill bacteria while showing absence of toxicity towards human peripheral erythrocytes and the SC-1, Caco-2 and RAW 264.7 cell lines.[12] Both Os and Os-C bound LPS, and inhibited nitric oxide (NO) and TNF-α production in RAW 264.7 macrophage cells.[13] Os but not Os-C in this cell line scavenged reactive oxygen species (ROS) and nitric oxide (NO). Erythrocytes are widely used to evaluate the cytotoxicity of AMPs against mammalian cells. However, this only provides information on their effect on mammalian membranes. Although erythrocytes are a physiologically relevant cell population, these cells are highly differentiated and lack nuclei and organelles. Although cell lines are valuable models that provide important information on possible intracellular targets, these are not physiologically relevant normal cells. To further develop AMPs as therapeutic agents, it is important to evaluate toxicity as well as the effects of AMPs in leukocytes, a cell type with a comprehensive transcriptional and translational system. Leukocytes are attractive therapeutic targets due to the involvement of these cells in infection and inflammation. In this study the effects of Os and Os-C on peripheral mononuclear (MN) and polymorphonuclear (PMN) leukocytes was further investigated to evaluate the anti-inflammatory effects of Os and Os-C in human leukocytes, and further identify based on mode of action, novel applications for future research.

Table 1

Properties of synthetic peptides [12]

Peptide	Sequence	Net charge
Os	KGIRGYKGGYCKGAFKQTCKCY	+6
Os-C	KGIRGYKGGYKGAFKQTKY	+6

2. Materials and methods

2.1. Reagents

Histopaque 1077 and Histopaque 1119 were obtained from Sigma-Aldrich (Johannesburg, South Africa (SA)). RPMI-1640 culture medium was obtained from Highveld Biological (Johannesburg, SA). The peptides Os and Os-C, as well as Os, Os-C and penetratin labelled with 5-carboxyfluorescein (5FAM) on the N-terminus were obtained from GenScript (Piscataway, New Jersey). The purity and molecular mass of the peptides were determined by the manufacturers by reverse-phase HPLC and mass spectrometry, respectively. Antibacterial activity of labelled peptides was confirmed with the colony forming assay (results not shown). The peptide melittin (Mel), obtained from Sigma-Aldrich (Johannesburg, SA) is a known lytic peptide and was used as a positive control for membrane damage. Stock peptide solutions of 1.2 mg/mL were prepared in sterile deionized double distilled water. Peptide concentrations were determined as previously described.[14]

2.2. Separation of blood cells

Ethical clearance was obtained from the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria to collect blood from healthy, consenting donors (reference nr 452/2014). Venous blood from healthy volunteers between the ages of 20 – 60 years was drawn into potassium EDTA collection tubes. Leukocytes were collected by layering onto a double-gradient of Histopaque 1119 and 1077. The vials were centrifuged for 30 minutes at 500 xg. The upper layer of MN and lower layer of PMN cells were collected into separate tubes and washed twice with RPMI-1640 containing 2% fetal bovine serum (FBS) at 250 xg for 10 minutes. The PMN pellet was treated with 3 mL of distilled H_2O for 30 seconds to lyse any remaining erythrocytes. To restore the physiological isotonic conditions, 3 ml of 1.8 % saline was immediately added. The leukocyte cell suspensions was again centrifuged and resuspended in RPMI-1640/FBS.

2.3. Leukocyte viability assay

To determine the toxic effect of the peptides, the Alamar Blue (AB) assay was modified from the methods of Catrina *et al.*[15] The cell concentration of both MN and PMN cells was adjusted to $1x10^6$ cells/ml, and a volume of 90 µl plated in a sterile tissue culture treated 96-

well plate. The cells were then exposed to a concentration range of Mel (positive control), Os and Os-C between 0.5 µM and 100 µM for 24 hours at 37°C and 5% CO_2. Sterile water was added to the untreated control wells. Sterile filtered AB was added to the leukocyte suspension to obtain a final concentration of 400 µM. The cell suspension was incubated for another 24 hours at 5% CO_2 and 37°C and the fluorescence measured with an excitation wavelength of 492 nm and emission wavelength of 590 nm (FLUOstar Omega, BMG Labtech, Ortenburg, Germany). Results were expressed as a percentage of the control.

2.4. Scanning electron microscopy

The effect of peptides on the ultrastructure of leukocytes was investigated using scanning electron microscopy (SEM). MN and PMN cells were attached to poly-L-lysine coated coverslips, exposed to 1 µM Mel (positive control), and 50 µM and 100 µM Os or Os-C for 20 hours at 37°C and 5% CO_2. Sterile water was added to the untreated control wells. Leukocytes were fixed with a mixture of 2.5% gluteraldehyde and 2.5% formaldehyde in 0.05 M phosphate-buffered saline (PBS; 40.5 mM $Na_2HPO_4.2H_2O$, 9.5 mM $NaH_2PO_4.H_2O$, 75 mM NaCl, pH 7.4). After one hour, the samples were rinsed three times with PBS for 10 minutes before being placed in a secondary fixative of 1% osmium tetroxide solution for 30 minutes. The samples were rinsed with PBS three times for 10 minutes and dehydrated with a series of ethanol dilutions (30%, 50%, 70%, 90% and three changes of 100%). The leukocytes were dried with hexamethyldisilazane after which the coverslips were air dried, coated with carbon and examined with an Ultra plus FEG SEM (Carl Zeiss, Oberkochen, Germany).

2.5 Leukocyte ROS production assay

To determine the effect of the peptides on leukocyte ROS production, the method of Honzel *et al.*[16] was followed. MN and PMN cells were exposed separately to a concentration range of Mel (positive control), Os and Os-C between 0.25 and 100 µM for 90 minutes at 37°C and 5% CO_2. An untreated control sample with the same volume of water was included. Thereafter, the cell suspension was rinsed twice with isotonic phosphate-buffered saline (isoPBS; 0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) to remove all extracellular peptide, and the cells incubated with 25 µM DCFH-DA in 0.1 M PBS (81 mM $Na_2HPO_4.2H_2O$, 19 mM $NaH_2PO_4.H_2O$, 0.15 M NaCl, pH 7.4). The amount of ROS

production by the leukocytes was detected by measuring the fluorescence intensity with the FLUOstar OPTIMA plate reader from BMG labtechnologies (Offenburg, Germany) for 60 min at 37°C. An excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. Decomposition of water-soluble azo compound 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), leads to a constant generation of peroxyl radicals[17] and all data is expressed relative AAPH (100% generation). The net area under the decay curve for each sample was calculated, and the final results were expressed as a percentage of the control (AAPH and water) with background fluorescence (water only) subtracted.

2.6 Leukocyte oxidative protection assay

The protective effect of the peptides against AAPH-induced oxidative damage of leukocytes was investigated according to the method of Honzel *et al.*[16] MN and PMN cells were exposed separately to a concentration range of Mel, Os, Os-C or glutathione (GSH) between 0.25 and 100 µM for 90 minutes at 37°C and 5% CO_2. GSH was used as a peptide antioxidant control. An untreated control sample with the same volume of water was included. The cells were incubated with 25 µM DCFH-DA in 0.1 M PBS for 60 minutes. The leukocytes were then exposed to 1.25 mM AAPH for 45 minutes. The amount of ROS production by the leukocytes was detected by measuring the fluorescence intensity with the FLUOstar OPTIMA plate reader (BMG labtechnologies, Offenburg, Germany) for 60 min at 37°C. An excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. The net area under the decay curve for each sample was calculated, and the final results were expressed as a percentage of the control in the same manner as for the ROS production assay.

2.7 Localization of peptides

To establish whether the peptides are able to enter MN and PMN leukocytes, the cells were exposed to fluorescently labeled peptide based on the modified methods of Letoha *et al.*[18] and Maiolo *et al.*[19] The cell concentration was adjusted to 1 x 10^6 cells/ml and exposed to a final concentration of 10 µM 5FAM-penetratin (positive control), 20 µM 5FAM-Os and 5FAM-Os-C for 60 minutes at 37°C. The cells were pelleted by centrifugation at 500 xg for 2 minutes and resuspended in 10 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 30 min at 37°C. Excess DAPI was rinsed off with isoPBS twice by centrifugation at 500 xg for 2 minutes. A volume of 5 µl of the pelleted cells was placed onto a glass slide, covered with a

coverslip and viewed immediately with a LSM 510 Meta Confocal Microscope (Carl Zeiss, Oberkochen, Germany). The excitation and emission wavelengths used were 488 nm and 520 nm for 5FAM and 405 nm and 460 nm for DAPI. To observe the cell borders, differential interference contrast images was also taken using a 488 nm laser.

2.8 Statistical analysis

For all quantitative methods each experiment consisted of three repeats of each concentration, and the experiment was repeated three times, resulting in nine data points. The data is presented as means ± standard error of the mean. Multiple comparisons were tested by one-way ANOVA followed by the Tukey post hoc test to test for significant difference between the different peptides. Linear regression fit and comparison of the slopes for statistical significance was done on a scatterplot (GraphPad Prism v6.01, San Diego, California).

3 Results

3.1 Leukocyte viability in the presence of peptides

The possible toxicity of Os and Os-C was tested on both MN and PMN leukocytes with the AB assay. The MN group contains mostly the agranulocytes that consist of the lymphocytes, and a few monocytes. The PMN group of cells contains mostly the granulocytes, the neutrophils, eosinophils and basophils.[20,21] The AB assay measures the reduction potential of the cell, as the non-fluorescent molecule is reduced within an active cell to fluoresce by cellular enzymes. Mel was previously found to be cytotoxic to human peripheral lymphocytes and was used in the present study as a positive control for damage.[22] Mel caused a decrease in the viability of both MN and PMN leukocytes (Fig. 1). MN cells were more sensitive, showing complete toxicity at 2 µM and significant differences compared to the untreated control were observed at all concentrations (Fig. 1A). In PMN cells, Mel at 2 µM caused a significant decrease in viability of 61.56% compared to the untreated control (Fig. 1B) and the observed effects for 2, 5 and 10µM were significantly lower compared to the untreated control.

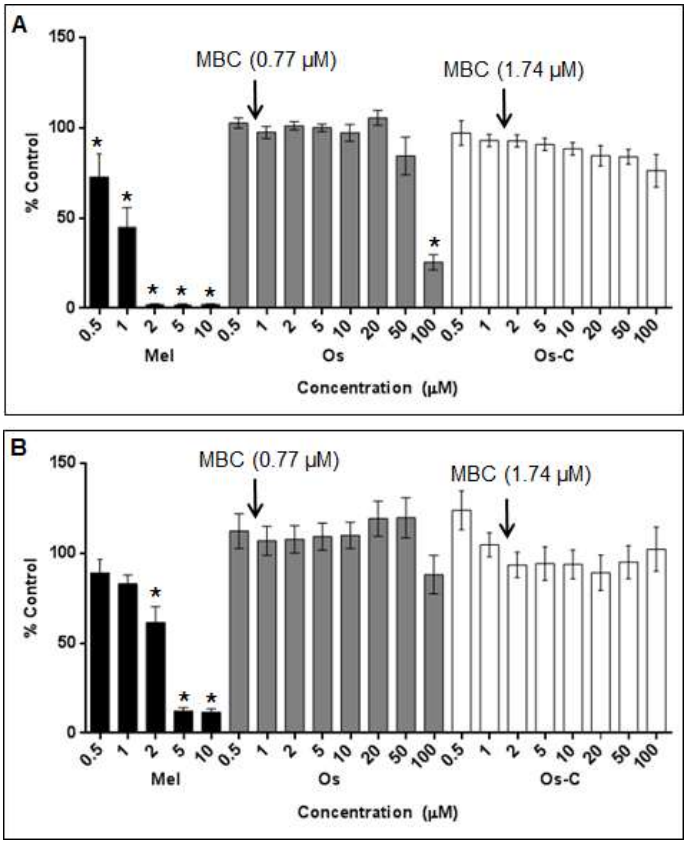

Fig. 1. Viability of A) mononuclear and B) polymorphonuclear leukocytes incubated with melittin (0 – 10 µM), Os and Os-C (0 – 100 µM) for 24 hours, measured with the Alamar Blue assay. Asterisks indicate significant difference to the untreated control (100% viability), MBC, minimum bactericidal concentrations against *Bacillus subtilis* and *Escherichia coli*, ($p<0.05$).

No toxicity to MN cells was observed from 0.5 – 50 µM Os (Fig. 1A). At all concentrations evaluated, Os did not alter the viability of PMN cells (Fig. 1B). However, Os at 100 µM, which is approximately 130 times the minimum bactericidal concentration (MBC) of Os[12], resulted in a significant loss of MN viability. In contrast, compared to the untreated control, Os-C did not alter the viability of MN and PMN cells even at 100 µM (Fig. 1A and 1B).

3.2 Ultrastructural effects of peptides on leukocytes

The effect of the peptides Mel, Os and Os-C on the ultrastructure of leukocytes was evaluated with SEM (Fig. 2 and 3). In the MN controls not exposed to peptides, two main types of morphology were observed. These were smaller round cells with microvilli covering the surface of the cell (Fig. 2A) and cells which had a more flattened appearance, often with pseudopodia (Fig. 2B). The former morphology is consistent with normal lymphocytes.[23] Mel caused severe membrane damage or complete loss of MN cell membranes when treated for

20 hours at a concentration of 1 µM (Fig. 2C and D). Os caused membrane damage at both 50 µM and 100 µM (Fig. 2F), after 20 hour exposure time. However, at 50 µM, fewer cells were affected (Fig. 2E). A concentration of 50 µM Os-C had no observable effect on MN cells (Fig. 2G). MN cells exposed to 100 µM Os-C appeared to be in a more activated state, with more cells with protrusions or pseudopodia extending from the cell body than the corresponding control leukocytes (Fig. 2H).

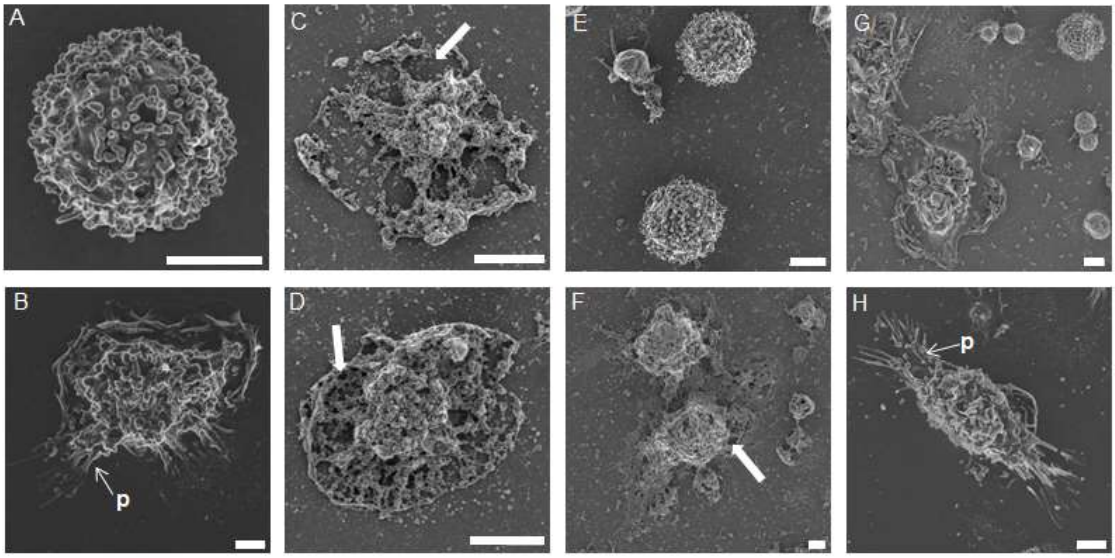

Fig. 2. Membrane effects of mononuclear leukocytes exposed to peptides for 20 hours and imaged with scanning electron microscopy. A, B) Controls, C, D) 1 µM melittin, E) 50 µM Os, F) 100 µM Os, G) 50 µM Os-C, H) 100 µM Os-C. Arrows indicate membrane damage, p) pseudopodia. Scale bars = 2 µm.

PMN control cells also showed two types of morphology, a smaller round cell with a mostly smooth membrane (Fig. 3A) and a more flattened larger cell type with pseudopodia (Fig. 3B). The effect of 1 µM Mel on PMN leukocytes was similar to that observed for MN leukocytes (Fig. 3C and D). A concentration of 50 µM Os had no effect on PMN cells (Fig 3E), however, extensive cellular damage was observed with 100 µM Os (Fig. 3F). After exposure to 50 µM Os-C, the cellular morphology of PMN cells was similar to the control although there was an increase in the amount of long thin filopodia extending from the cell membrane (Fig. 3G). PMN cells exposed to 100 µM Os-C underwent a change in cell shape, with the formation of pseudopodia similar to cells responding to a chemotactic molecule (Fig. 3H).

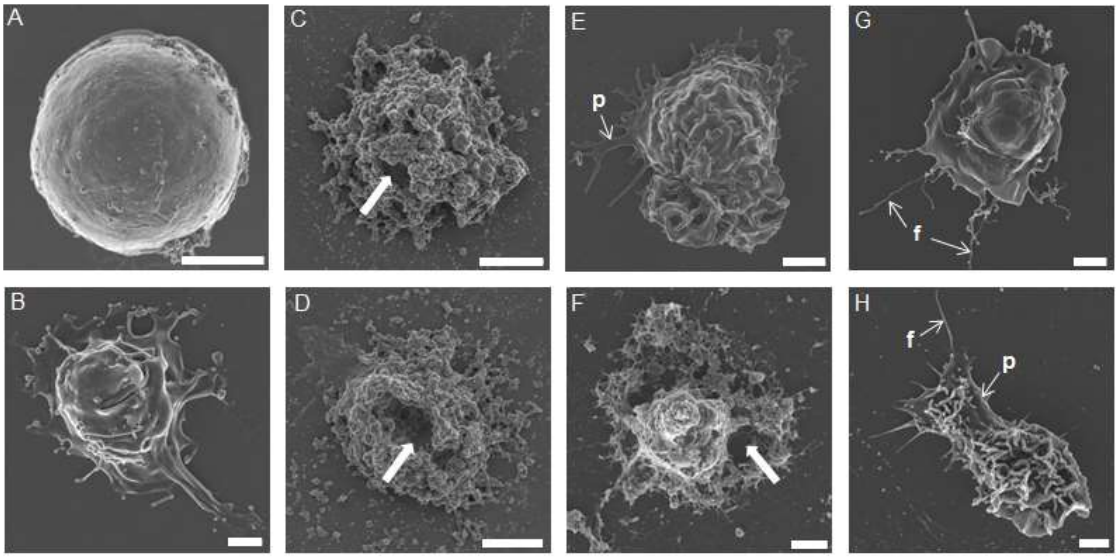

Fig. 3. Membrane effects of polymorphonuclear leukocytes exposed to peptides for 20 hours and imaged with scanning electron microscopy. A, B) Controls, C, D) 1 µM melittin, E) 50 µM Os, F) 100 µM Os, G) 50 µM Os-C, H) 100 µM Os-C. Arrows indicate membrane damage, f) filopodia, and p) pseudopodia. Scale bars = 2 µm.

3.3 Leukocyte ROS production by peptides

Hydrogen peroxide (H_2O_2) is a secondary messenger in leukocyte activation and this process is an important component of innate immunity although excessive ROS formation can cause inflammation.[24] Intracellular H_2O_2 is converted to ROS and these levels can be quantified using DCFH-DA.[16] All peptides tested caused a slight dosage-dependent increase in ROS production by MN cells (Fig. 4A). Compared to the untreated control a statistically significant increase in ROS production was observed for Mel from 2 µM, Os only at 100 µM and Os-C from 50 µM. In PMN cells, Mel caused a statistically significant increase in ROS production only at 100 µM (Fig. 4B) and Os caused no increase in ROS. In contrast, Os-C led to a marked increase in ROS production by PMN cells at 100 µM of 31%.

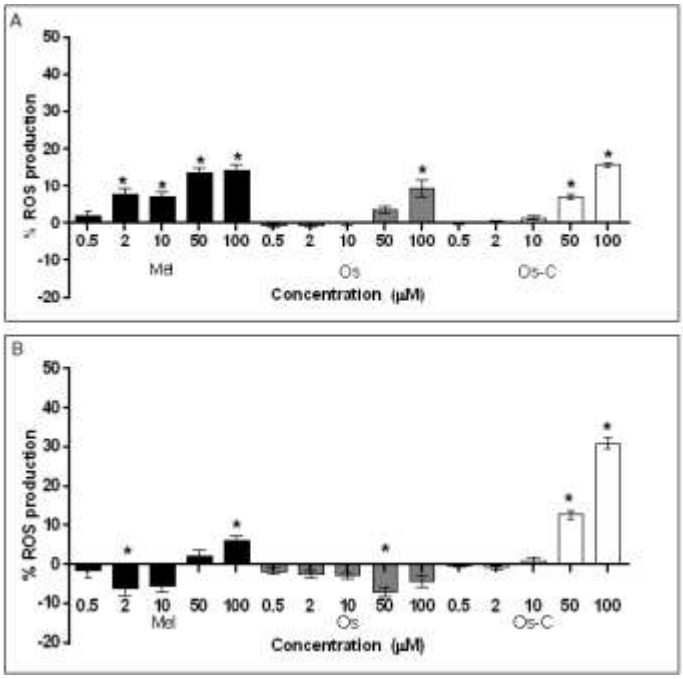

Fig. 4. ROS production in A) mononuclear, and B) polymorphonuclear leukocytes stimulated by melittin, Os and Os-C. Asterisks indicate significant difference to the untreated control (0% ROS production) ($p<0.05$).

3.4 Leukocyte protection against oxidative damage by peptides

The peptides were investigated for their ability to protect leukocytes from AAPH-induced oxidative damage (Fig. 5). The tripeptide, GSH, was only able to effectively scavenge ROS in a dose-dependent manner in PMN cells (Fig. 5B). Mel and Os-C showed no significant protection in either cell groups but an increase in ROS production. No antioxidant activity was observed with Os for MN cells (Fig 5A), however for PMN leukocytes Os scavenged ROS by 23.4% and 25.5% at 50 µM and 100 µM, respectively (Fig. 5B).

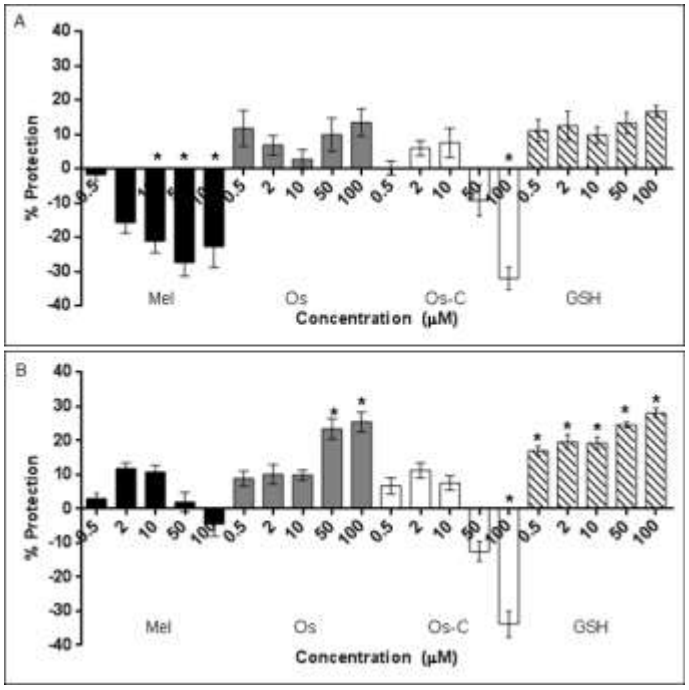

Fig. 5. Protection against AAPH-induced oxidative damage in leukocytes by melittin, Os, Os-C and glutathione. A) mononuclear leukocytes, B) polymorphonuclear leukocytes. Asterisks indicate significant difference to AAPH (0% ROS protection) ($p<0.05$).

3.5 Localization of peptides

Using fluorescently labelled peptides the ability of these peptides to cross leukocyte membranes was evaluated at a non-cytotoxic concentration of 20 µM. Penetratin, a peptide known to be able to cross the plasma membrane of eukaryotic cells[18], was used as a positive control. 5FAM-penetratin was found to cross both PMN and MN cell membranes and accumulate intracellularly in the cytoplasm (Fig. 6A and D).

Labelled Os was able to enter some MN leukocytes and was found to accumulate in the nucleus of activated MN leukocytes (Fig. 6B) but did not cross the plasma membrane of PMN leukocytes (Fig. 6E). Labelled Os-C was unable to cross the membranes of PMN or MN leukocytes (Fig. 6C and F).

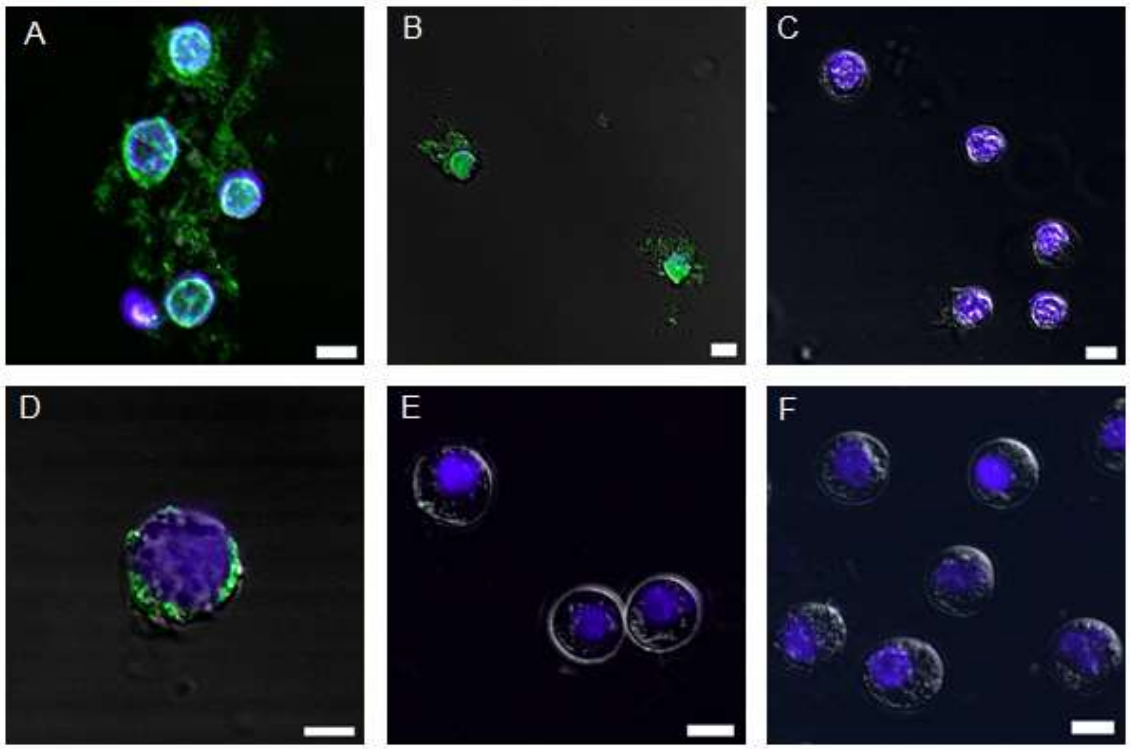

Fig. 6. Mononuclear leukocytes (A - C) and polymorphonuclear leukocytes (D - F) exposed to fluorescently labelled peptides (green) for 60 min. A, D) 10 µM 5FAM-penetratin, B, E) 20 µM 5FAM Os, C, F) 20 µM 5FAM Os-C. Nuclei stained blue by DAPI. Scale bars = 5 µm.

4 Discussion

Previous studies indicated that neither synthetic peptide, Os nor Os-C, were toxic to erythrocytes, fibroblast, epithelial or macrophage cell lines, and possess antioxidant and anti-inflammatory activity[12,13]. Phagocytic leukocytes, neutrophils, eosinophils and basophils play important roles in the innate immune response to pathogens. Therefore the effects of Os and Os-C was evaluated on MN and PMN leukocytes. In this study the MN fraction consisted of the agranulocyte leukocyte population of lymphocytes and monocytes[25] which are 25-35% and 2-8% respectively of the leukocyte cell population.[26] The monocytes, when stimulated leave the circulation and differentiate into macrophages. The PMN fraction represented the granulocyte leukocyte population of neutrophils, eosinophils and basophils[25] and are 50-70%, 1-3% and 0.4-1% of the leukocyte cell population, respectively.[26] Therefore, the predominant cell types in the MN and PMN cell fractions represent cells involved in the adaptive and innate immune systems respectively.

The cytotoxicity of Os and Os-C in both cell populations was determined and Mel was used as a control. Dosage dependent loss of cell viability was observed at 1 µM Mel, large gaps or

pores in the cell membranes of human MN and PMN leukocytes was also observed. Pratt *et al.*[27] reported that 1.56 µM Mel caused the disruption of the plasma and nuclear membranes of sheep lymphocytes due to a membrane detergent-like effect. Many AMPs lyse cells via the formation of transmembrane pores which allow water to enter the cell leading to swelling and rupture in a process known as colloid-osmotic cell lysis or oncosis.[27-29] The ultrastructural characteristics of MN and PMN leukocytes exposed to Mel are typical of leukocytes that have undergone oncosis.

Exposure of MN and PMN cells to 0.5 - 50 µM Os caused no changes to leukocyte viability as measured with the AB assay. The loss of viability at 100 µM Os in MN leukocytes was significant and was confirmed with SEM. At 100 µM Os PMN viability was reduced but the effect was not statistically significant, however, evaluation of the ultrastructure of these cells showed extensive cellular damage. Malan *et al.*[13] found no toxicity of Os to mouse macrophages (RAW 264.7), however lower concentrations were used in that study. There may also be differences in toxicity between mouse and human cells as well as cell lines and ex vivo cell populations. Shenoy *et al.*[30] found that drugs with antioxidant activity interfere with cell viability assays based on the reduction of resazurin to resorufin such as the Cell Titer and AB assays. In previous studies we have shown that Os and to a lesser degree Os-C has antioxidant activity measured with the TEAC and ORAC antioxidant assays.[12,13] Likewise, Os can reduce AB resulting in false negative results. Therefore, it is important especially if peptides have antioxidant activity to confirm cell toxicity using techniques such as electron microscopy that evaluates effects on cell morphology. In contrast, Os-C did not cause damage to MN or PMN cells at any concentration tested.

Several membrane models have been proposed for the killing of bacteria by AMPs.[31] However, AMPs may also indirectly kill microbes via the induction of macrophages and neutrophils. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) mediates the release of ROS. The ability of leukocytes to generate ROS via membrane-associated NOX is an important factor in the innate immune response. Electrons derived from intracellular NADPH are used by NOX to generate superoxide anion, which dismutates to H_2O_2 and other ROS molecules in macrophages and neutrophils that protects the host against bacterial and fungal pathogens.[32] During the inflammatory stage of wound healing, there is also an oxidative burst where PMN cells and macrophages release superoxide radicals upon migration into the wound. Os-C but not Os caused leukocyte activation observed with SEM

and this was associated with an increase in ROS formation by MN and PMN leukocytes following exposure to 50 and 100 µM Os-C. A longer exposure to low concentrations may generate increased ROS and consequently increased leukocyte activation. Therefore Os-C has dual activity by killing bacteria and activating intracellular pathways of immune cells leading to increased ROS formation which either directly kills bacteria or activates phagocytic cells.[32] Mel and Os also caused a slight increase in ROS production by MN cells. However, at these concentrations, Mel and Os was found to be cytotoxic, and the increased ROS may be due to cell lysis.

Excessive ROS formation and the failure of cellular antioxidant systems to clear ROS leads to oxidative stress, which can prolong inflammation and inhibit wound healing.[33] Malan *et al.*[13] reported that at 100 µM Os had better antioxidant activity than Os-C at the same concentration in RAW 264.7 cells, a macrophage cell line. The presence of cysteine residues in Os may be responsible for this difference in antioxidant activity. Os protects PMN cells against oxidative damage but not MN leukocytes. Macrophages as well as the PMN cellular fraction which consists of basophils, eosinophils and neutrophils are phagocytic cells and therefore the antioxidant activity of Os seems to be cell type specific, favoring cells with a phagocytic function. The antioxidant effect of GSH was greater for PMN than MN cells. These results indicate that size and possibly the presence of cysteine residues are responsible for cellular antioxidant activity. Differences in cellular damage effects of Os compared to the antioxidant effects are related to exposure time where the toxicity studies were evaluated after 20 hours and the antioxidant studies' exposure was for 90 min. Mel as well as Os-C at the highest concentration had a pro-oxidant effect. With Mel this is most probably due to cell lysis. However, Os-C caused no cellular damage, and similar results were observed in both the ROS production and leukocyte oxidative protection assays.

The leukocyte penetrating effects of Os and Os-C was determined using 5FAM- labelled peptides. 5FAM-penetratin entered both MN and PMN leukocytes. Penetratin lacks cysteine residues, but contains arginine that favours cell penetration.[34] HeLa as well as undifferentiated and differentiated SH-SY5Y cells were previously exposed to 22 – 178 µM penetratin for 24 hrs and toxicity was observed only at 178 µM in the SH-SY5Y, neuroblastoma cell lines.[35] Based on these results leukocytes were exposed to 10 µM 5FAM-penetratin and 20 µM 5FAM-Os and 5FAM-Os-C that shows no cytotoxicity. 5FAM-penetratin was found localized in the cytoplasm of both MN and PMN cells. Thorén *et al.*[36]

observed that when PC-12 (rat adrenal pheochromocytoma) cells were exposed to 5 µM 5FAM-penetratin for 1hr, peptide uptake was via endocytosis and the internalized peptide was mainly accumulated at the centrosomal region of the cell. Typical of endocytotic uptake, fluorescent staining associated with the plasma membrane was punctate. A similar pattern of staining was observed for PMN cells exposed to 10 µM FAM-penetratin for 1hr. Staining was also observed for MN although these leukocytes appeared activated. 5FAM-Os selectively entered MN and not PMN leukocytes, which indicates selective uptake of this peptide. In contrast Os-C lacked the ability to enter MN or PMN leukocytes. However, Os-C interaction with extracellular or transmembrane proteins such as NOX[32] can occur resulting in leukocyte activation and increased ROS formation.

Although both peptides are derived from the same tick species and effectively kill both Gram- negative and Gram-positive bacteria, this study clearly shows that the effect of these AMPs on leukocyte functioning is very different. Os has been found to have very selective cell specific penetrating properties, which indicates that it could be further developed as a cell-penetrating peptide (CPP). As a CPP, Os can be used for the delivery of therapeutic proteins such as peptides, oligonucleotides and nanoparticles to specific sites or as a CPP for the treatment of leukocyte specific infections or disorders.[37] Coupling of proteins, oligonucleotides or nanoparticles to CPPs using several different design approaches increases the uptake therapeutic agents into cells[10] or in the case of Os a specific cell population. Inflammation is a key feature of many diseases such as cardiovascular, autoimmune diseases and cancer. Leukocytes inherently migrate to the site of inflammation and therefore can serve as cellular carriers of therapeutic agents. In addition, inherent NO and ROS scavenging and TNF inhibitory activity of Os can further reduce inflammation associated with autoimmune disease and tumor growth.[38]

In contrast, Os-C activates leukocytes and this activation is associated with increased ROS formation. Some of the antimicrobial strategies used by neutrophils is phagolysomal degradation of bacteria via ROS, and degranulation with the release of antimicrobial molecules.[7,39] It was found that Os-C caused peripheral leukocyte activation and associated increase in ROS production. Leukocyte NOX2 is a transmembrane protein that under normal conditions is inactive, stimulation of formylated peptide receptors activates the oxidase leading to O_2^- formation.[40] Recently, Holdfeldt *et al.*[41] reported that a novel FPR2-interacting peptidomimetic, F2M2, activates transmembrane human neutrophil NOX. Although

morphological features and increased ROS production indicates that Os-C induces leukocyte activation via ROS formation it would be of value in future studies to determine the effect of Os-C on NOX activity.

The antimicrobial defensin, alarmin human neutrophil peptide 1 (HNP-1) found in neutrophils has antimicrobial activity as well as multiple effects on leukocytes.[42] The MBC for HNP-1 was 15 µM against *E. coli* and *S. aureus*, and 20 µM against *P. aeruginosa*.[43] This peptide at a concentration of 2 µM increased INFα RNA and protein levels of peripheral dendritic cells which are part of the mononuclear phagocytic system.[42] A concentration of 120 µM caused 25% cytotoxicity in rat MN leukocytes.[43] Compared to HNP-1, a highly active immunomodulatory defensin, Os-C has similar low cytotoxicity, better antibacterial activity, inhibits INFα/LPS induced NO and TNF-α in RAW264.7 cells at 1.56 µM as found previously[13], induces ROS formation by peripheral leukocytes at 50 and 100 µM while structural changes indicating activation was observed at 100 µM. This implies that Os-C is an AMP with both antibacterial and possible immunomodulatory effects.

This study confirms that the cytotoxicity of Os and Os-C is low compared to Mel and is not toxic to blood cells at concentrations that kill bacteria. Os crosses the plasma membrane of MN cells and consequently has been identified as a peptide that can be used as a CPP. In contrast, Os-C activates both MN and PMN cells resulting in an associated increase in ROS. Therefore, Os-C should be further evaluated as activators of immune response infections based on the antimicrobial and immune activating properties.

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