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Anti-inflammatory and anti-endotoxin properties of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*

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Abstract

Antimicrobial peptides (AMPs) are small, cationic peptides that possess a large spectrum of bioactivities, including antimicrobial, anti-inflammatory and antioxidant activities. Several AMPs are known to inhibit lipopolysaccharide (LPS)-induced inflammation *in vitro* and to protect animals from sepsis. In this study the cellular anti-inflammatory and anti-endotoxin activities of Os and Os-C, peptides derived from the carboxy-terminal of a tick defensin (OsDef2), were investigated. Both Os and Os-C were found to bind LPS *in vitro*, albeit to a lesser extent than polymyxin B and melittin, known endotoxin-binding peptides. Binding to LPS was found to reduce the bactericidal activity of Os and Os-C against *Escherichia coli* confirming the affinity of both peptides for LPS. At a concentration of 25 µM, the nitric oxide (NO) scavenging activity of Os was higher than glutathione (GSH), a known NO scavenger. In contrast, Os-C showed no scavenging activity. Os and Os-C inhibited LPS/IFN-γ induced NO and TNF-α production in RAW 264.7 cells in a concentration-dependent manner, with no cellular toxicity even at a concentration of 100 µM. Although inhibition of NO and TNF-α secretion was more pronounced for melittin and polymyxin B, significant cytotoxicity was observed at concentrations of 1.56 µM and 25 µM for melittin and polymyxin B, respectively. In addition, Os, Os-C and GSH protected RAW 264.7 cells from oxidative damage at concentrations as low as 25 µM. This study identified that besides previously reported antibacterial activity of Os and Os-C, both peptides have in addition anti-inflammatory and anti-endotoxin properties.

Keywords:

Sepsis; LPS; defensin; tick; anti-inflammatory; anti-endotoxin; antioxidant; NO scavenging

List of abbreviations:

AAPH, 2,2'-azobios(2-amidinopropane) dihydrocloride; AMPs, antimicrobial peptides; CD14, cluster of differentiation 14; DCFH-DA, dichloro-dihydro-fluorescein diacetate; DMEM, supplemented Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; LB, Luria-Bertani; ECACC, European Collection of Cell Culture; GSH, glutathione; MBC, minimum bactericidal concentration; IFN-γ, interferon- γ; IL-1, interleukin-1; LAL, *Limulus* amoebocyte lysate; LBP, LPS-binding protein; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NaP, sodium phosphate; NED, N-(1-Naphthyl)-ethylenediamine-dihydrochloride; NO, nitric oxide; ORAC, oxygen radical absorbance capacity; Os, synthetic peptide derived from the C-terminal of *O. savignyi* defensin isoform 2; Os-C, synthetic analogue of Os without cysteine residues; PBS, phosphate buffered saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; SA, sulphanilamide; SNP, sodium nitroprusside; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; TMB, 3,3',5,5'-tetramethylbenzidine; HRP, horse radish peroxidase

1. Introduction

Sepsis is a life-threatening condition with a high mortality rate that occurs when oxidative stress and inflammatory responses associated with the pathogenesis of infection spread from a local site throughout the body via the circulatory and/or lymphatic system [1, 2]. The inflammatory response that initially combats infection eventually leads to endothelial damage, multiple organ failure and septic shock if left untreated [3,4]. Inflammation is induced by LPS, an endotoxin present on the outer membrane of Gram-negative bacteria [5,6]. LPS is released from bacteria in several ways, for instance, when bacteria undergo cell division, cell death or when bacterial infections are treated with antibiotics [3,7]. Macrophages play a pivotal role in the onset of LPS-induced inflammation [8]. Released LPS binds to LPS-binding protein (LBP), forming a LPS-LBP complex that interacts with CD14 [3,9-11]. The LPS-LBP-CD14 complex binds to Toll-like receptor 4 (TLR4), present on macrophages, initiating a signalling cascade leading to the production and release of pro-inflammatory cytokines, such as TNF-α and interleukin-1 (IL-1), and other toxic mediator molecules, like NO. Due to overstimulation of macrophages by LPS, there is an unregulated expression and secretion of these pro-inflammatory cytokines and mediator molecules which play a critical role in the pathogenesis of septic shock [3,9]. Despite treatment of bacterial infections with antibiotics, antibiotic-induced release of LPS may contribute to sepsis itself [12,13]. A combination therapy is necessary for successful sepsis therapy. This therapy must include antibacterial activity, LPS-binding, inhibiting LPS-mediated LPS/IFN-γ induced pro-inflammatory cytokine and mediator formation, while creating an environment that promotes cellular and tissue repair.

Small cationic host-defence AMPs are key components of innate immunity and are present in all species of life such as bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals [14]. Cationic AMPs were initially identified as antimicrobial agents that display

antibacterial, antiviral, anti-parasitic and antifungal activities. These peptides have also been found to possess additional activities such as anti-cancer, anti-inflammatory and antioxidant activities, which make them promising candidates for the development of new therapeutic drugs [15-18]. Proposed anti-inflammatory roles of AMPs include the killing of Gram-negative bacteria as a result of AMP binding to LPS on the outer membrane, direct binding to free LPS and blocking of LPS-induced cytokine and NO production by macrophages [19]. Several peptides derived from AMPs that have anti-endotoxin activities are being tested in clinical trials [20].

Ticks are small, hematophagous arachnids that rely only on an innate immune defence for protection against microbes and therefore, produce a variety of AMPs, like defensins [21-24]. Tick defensins could potentially function as templates for the development of multifunctional peptides. Identification of defensin-like AMPs in the hemolymph of the tick *Ornithodoros savignyi* [25] resulted in the cloning and sequencing of midgut defensin isoforms (OsDef1 and OsDef2). Synthetic OsDef2 showed increased bactericidal activity compared to OsDef1 towards Gram-positive bacteria [26]. Consequently, OsDef2 was used as template for the synthesis of peptide Os which was derived from the carboxy-terminal and, its analogue Os-C in which the three cysteine residues were omitted. Previous research showed that Os and Os-C were bactericidal to both Gram-positive and Gram-negative bacteria, were non-toxic towards mammalian cells (human erythrocytes, Caco-2 and SC-1 cells) and exhibited antioxidant activity [26]. Further investigation into the mechanism of action showed that although both peptides are membrane acting, fluorescently labelled Os and Os-C were able to enter bacterial cells pointing towards intracellular targets [27].

Multifunctional peptides hold potential for the treatment of bacterial infections and may prevent the onset of unwanted inflammatory responses, as well as the oxidative cellular damage caused by such conditions [28]. In this study the anti-inflammatory properties of Os and Os-C were investigated using LPS/IFN-γ stimulated RAW 264.7 macrophages as an *in vitro* model of inflammation. The endotoxin-binding ability of Os and Os-C was determined and the effect of LPS on the bactericidal activity of these peptides examined. To ascertain whether binding of both peptides to LPS would result in inhibition of pro-inflammatory responses that usually occurs as a result macrophage stimulation, NO and TNF-α responses of RAW 264.7 cells after 24 hours of incubation with the peptides was investigated. In addition, the NO scavenging ability of Os and Os-C, and their capability to provide protection against oxidative damage as a result of radical formation was determined. To our knowledge, this is the first report of cellular anti-inflammatory and anti-endotoxin activities observed for tick defensin-derived peptides.

2. Materials and methods

2.1 Reagents

Os and Os-C were synthesized using FlexPeptideTM technology by GenScript (New Jersey, USA). The purity and molecular mass of these peptides were determined by the vendor by means of reverse-phase high-performance liquid chromatography and mass spectrometry, respectively. Melittin, polymyxin B and GSH which were used as positive controls as well as IFN-γ and LPS (*E. coli* 0111:B4), were supplied by Sigma-Aldrich (Johannesburg, South Africa). The kinetic, chromogenic *Limulus* amoebocyte lysate (LAL) kit was obtained from Pierce Biotechnology (Rockford, USA). RAW 264.7 (*Mus musculus*, doubling time 11 hours) macrophage cell line, obtained from the ECACC, was supplied by Sigma-Aldrich (Johannesburg, South Africa). The mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit was from Sigma-Aldrich (Johannesburg, South Africa). All peptides were re-suspended in sterile water and concentrations were determined using molar extinction coefficients for Tyr (1200 AU/mmole/ml) and Trp (5560 AU/mmole/ml), and the equation below:

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

where c is the peptide concentration (mg/ml); A is the absorbance of the peptide measured at 280 nm; df the dilution factor; Mr the relative molecular mass of the peptide (g/moles) and Ɛ the extinction coefficient (AU/mmole/ml). Peptide stock solutions were diluted in water to 1 mM, filter sterilized (0.45 µm membrane) and stored in 20 µl aliquots at -20 °C.

2.2 Cell cultures

E. coli (ATCC 700928) cells were grown aerobically overnight in Luria-Bertani (LB) broth (1% NaCl, 1% Tryptone, 0.5% yeast, pH 7.4) at 37 °C. RAW 264.7 cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum and 1% antibiotics (streptomycin/penicillin/fungizone) at 37 °C in an atmosphere of 5% CO_2.

2.3 Binding of peptides to free LPS

The ability of the peptides to bind LPS was assessed using a quantitative chromogenic LAL kit and was carried out following the protocols recommended by the manufacturer [29]. Stock solutions of Os, Os-C, polymyxin B and melittin were prepared in pyrogenic free water provided with the kit. Various concentrations of peptides (0.098 - 25 µM) were incubated with a constant concentration of LPS (100 ng/ml *E. coli* O111:B4) at 37 °C for 5 minutes using a non-pyrogenic sterile 96-well microtiter plate. A total of 25 µl of this mixture was added to 10 µl of the LAL reagent, and the mixture was incubated for 5 minutes at 37 °C. This was followed by the addition of 20 µl of the LAL chromogenic substrate solution that was incubated for 10 minutes

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Table 1: Physicochemical properties and MBC of synthetic peptides

Peptide	Sequence	Number of residues	Net charge	MBC for *E. coli*[a] (μM)	Number of residues with antioxidant activity[b]	Mean hydrophobicity <H>[c]
Os	KGIRG**Y**KGG**YC**KGA**F**KQT**C**K**CY**	22	+6	0.76	7	0.249
Os-C	KGIRG**Y**KGG**Y**- KGA**F**KQT- K- **Y**	19	+6	1.74	4	0.045
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	+5	nd	na	0.511

nd- not determined; na- not applicable; Os-C sequence- Os with cysteine residues omitted

[a] Prinsloo *et al*. [26]

[b] Amino acid residues that may contribute to antioxidant activity are indicated in bold in the primary sequences [55-57]

[c] Data obtained from HeliQuest [heliquest.ipmc.cnrs.fr]

Legend for figures

Figure 1: *In vitro* inhibition of LPS-induced activation of LAL enzyme by Os and Os-C. Various concentrations of Os, Os-C, melittin and polymyxin B (0.098 - 25 µM) were incubated with a constant concentration of LPS (100 ng/ml) at 37 °C for 5 minutes. Each sample was added to the LAL reagent and further incubated for 5 minutes. LAL chromogenic substrate was added, and the absorbance measured at 405 nm. Inhibition of LAL activation by the peptides was expressed as a percentage relative to the control containing LPS only (0% inhibition). Data was expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant inhibition of LAL activity for melittin and polymyxin B (0.39-25 µM), and for Os and Os-C (0.78-25 µM) compared to the control containing LPS only ($p \leq 0.05$).

Figure 2: The effect of LPS on the bactericidal activity of Os and Os-C against *E. coli*. Os (at MBC of 0.76 µM) and Os-C (at MBC of 1.74 µM) were incubated with varying concentrations of LPS (0 – 50 µg/ml) for 2 hours at 37 °C. Diluted bacteria were added to the mixtures and further incubated for 2 hours at 37 °C. Samples were plated and surviving colonies counted after 14-16 hours at 37 ºC. The bacterial viability in the presence of peptide and LPS was expressed as a percentage relative to the control containing peptide only (0% bacterial viability). Data was expressed as the means ± SEM of three independent experiments in triplicate.

Figure 3: NO scavenging capacity of Os and Os-C. Sodium nitroprusside solution was added to Os, Os-C, melittin and GSH (1.56 and 25 µM) and incubated for 1 hour. Thereafter, sulphanilamide was added and incubated for a further 10 minutes. This was followed by the addition of NED, and the absorbance measured at 570 nm. The concentration NO was measured using a standard sodium nitrite curve. The amount NO scavenged in the presence of peptide was expressed as a percentage relative to the control containing all reagents except peptide (0% NO scavenged). Data was expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant difference between the scavenging ability of the peptides at their respective concentrations compared to the control containing all reagents except peptide ($*p \leq 0.05$).

Figure 4: The effect of Os and Os-C on NO production of RAW 264.7 cells. Cells were treated with LPS/IFN-γ for 24 hours at 37°C in the presence of Os, Os-C, melittin and polymyxin B at 1.56 and 25 µM. The culture media were collected and the nitrite concentration measured. Inhibition of NO production by the peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (0% inhibition). Data was expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant differences between the inhibitory effects of the peptides compared to the control containing LPS/ IFN-γ only ($*p \leq 0.05$).

Figure 5: The cytotoxic effect of Os and Os-C on RAW 264.7 cells. Cells were treated as in **Figure 4**, followed by the addition of MTT to each well and incubated for 3 hours at 37ºC and 5% CO_2. Formazan crystals were dissolved, and the absorbance measured at 570 nm. Cell viability in the presence of peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (100% viability). Statistical analysis was performed and showed a significant decrease in cell viability in the presence of peptides compared to the control containing LPS/ IFN-γ only ($*p \leq 0.05$).

Figure 6: Inhibition of TNF-α production by Os and Os-C. RAW 264.7 cells were treated with LPS/IFN-γ for 24 hours at 37°C in the presence of Os and Os-C (1.56 - 100 µM) and, polymyxin B (6.25 µM). The culture media were collected and TNF-α measured with an ELISA kit. Inhibition of TNF-α production by the peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (0% inhibition). Data was expressed as means ± SEM of two independent experiments in duplicate. Statistical analysis was performed and showed

significant difference between the inhibitory effects of the peptides at their respective concentrations compared to the control containing LPS/ IFN-γ only (*p≤0.05).

Figure 7: Protective effects of Os and Os-C against oxidative damage of RAW 264.7 cells. Cells were incubated with DCFH-DA for 45 minutes, washed and exposed to Os, Os-C and GSH (25 and 100 µM) and AAPH. Fluorescence was measured at a wavelength of 485 nm and an emission wavelength of 520 nm. Protection by the peptides against AAPH oxidative damage was expressed as a percentage relative to the control containing AAPH only (0% protection) and the control without AAPH (100% protection). Data was expressed as means ± SEM of four independent experiments in duplicate. Statistical analysis was performed to compare each peptide concentration to the control containing AAPH only (*p≤0.05).

Figure S1: NO scavenging capacity of Os and Os-C. Sodium nitroprusside solution was added to Os, Os-C, melittin and GSH (0.78 – 25 µM) and incubated for 1 hour. Thereafter, sulphanilamide was added and incubated for a further 10 minutes. This was followed by the addition of NED, and the absorbance measured at 570 nm. The concentration NO was measured using a standard sodium nitrite curve. The amount NO scavenged in the presence of peptide was expressed as a percentage relative to the control containing all reagents except peptide (0% NO scavenged). Data was expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant difference between the scavenging ability of the peptides at their respective concentrations compared to the control containing all reagents except peptide (*p ≤ 0.05).

Figure S2: The effect of Os and Os-C on NO production of RAW 264.7 cells. Cells were treated with LPS/IFN-γ for 24 hours at 37°C in the presence of Os and Os-C (0.098 – 100 µM), melittin and polymyxin B (0.098 – 25 µM). The culture media were collected, and the nitrite concentration determined. Inhibition of NO production by the peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (0% inhibition). Data was expressed as the means ± SEM of three independent experiments in triplicate. Statistical analysis was performed and showed significant differences between the inhibitory effects of the peptides compared to the control containing LPS/ IFN-γ only (*p≤0.05).

Figure S3: The cytotoxic effect of Os and Os-C on RAW 264.7 cells. Cells were treated as in **Figure S2**, followed by the addition of MTT to each well and incubated for 3 hours at 37ºC and 5% CO_2. Formazan crystals were dissolved, and the absorbance measured at 570 nm. Cell viability in the presence of peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (100% viability). Statistical analysis was performed and showed a significant decrease in cell viability in the presence of peptides compared to the control containing LPS/ IFN-γ only (*p≤0.05).

Figure S4: Protective effects of Os and Os-C against oxidative damage of RAW 264.7 cells. Cells were incubated with DCFH-DA for 45 minutes, washed and exposed to Os, Os-C and GSH (0.39 – 100 µM) and AAPH. Fluorescence was measured at a wavelength of 485 nm and an emission wavelength of 520 nm. Protection by the peptides against AAPH oxidative damage was expressed as a percentage relative to the control containing AAPH only (0% protection) and the control without AAPH (100% protection). Data was expressed as means ± SEM of four independent experiments in duplicate. Statistical analysis was performed to compare each peptide concentration to the control containing AAPH only (*p≤0.05).

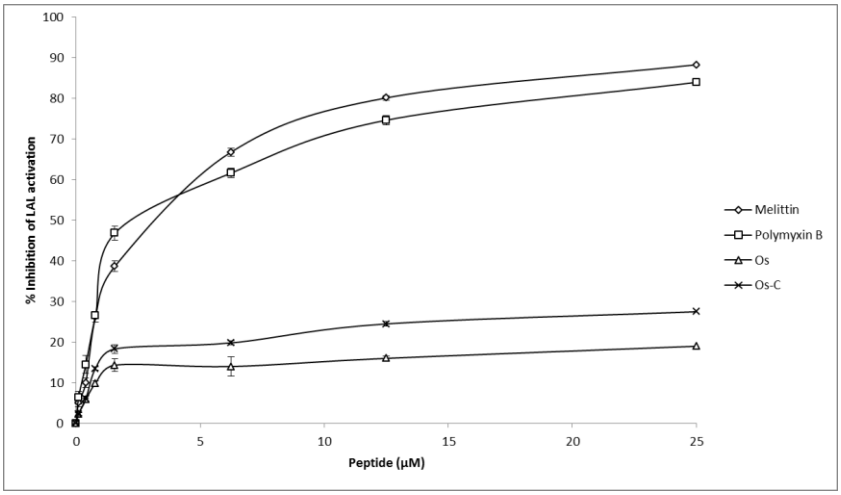
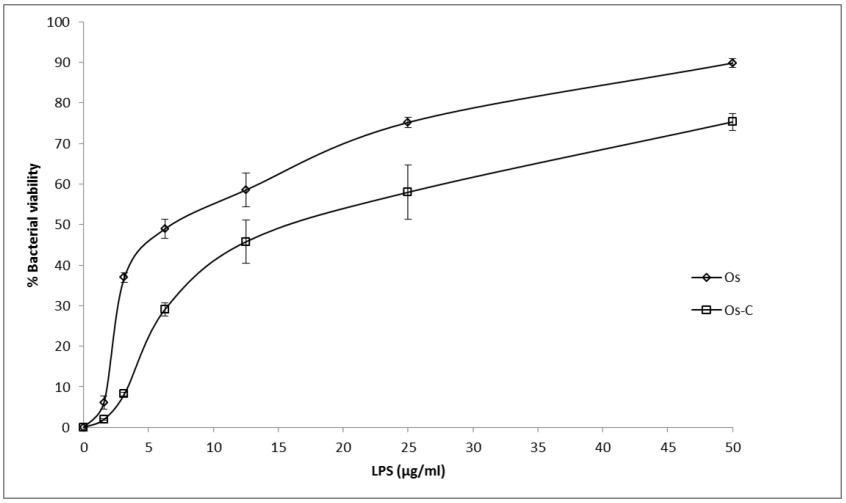


Figure 1



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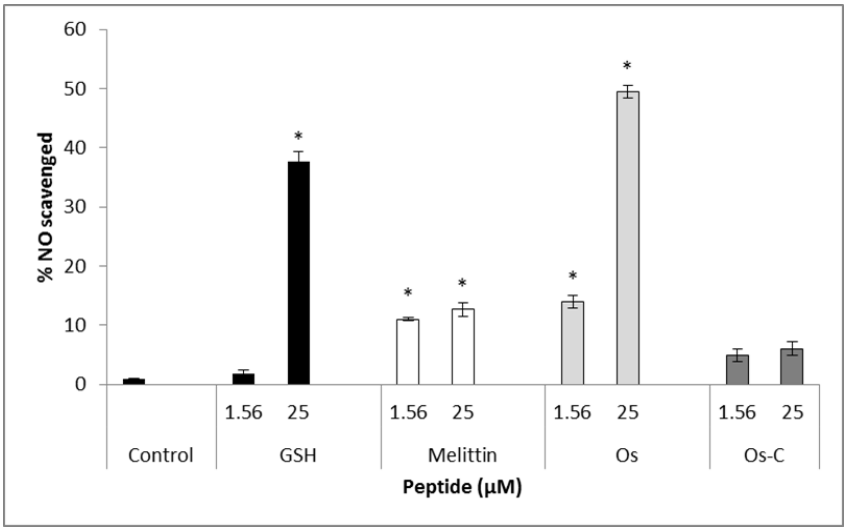

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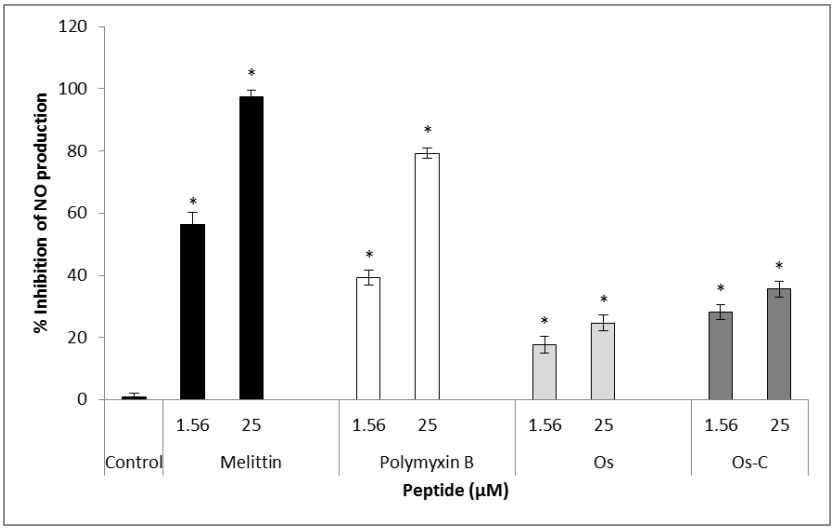


Figure 4

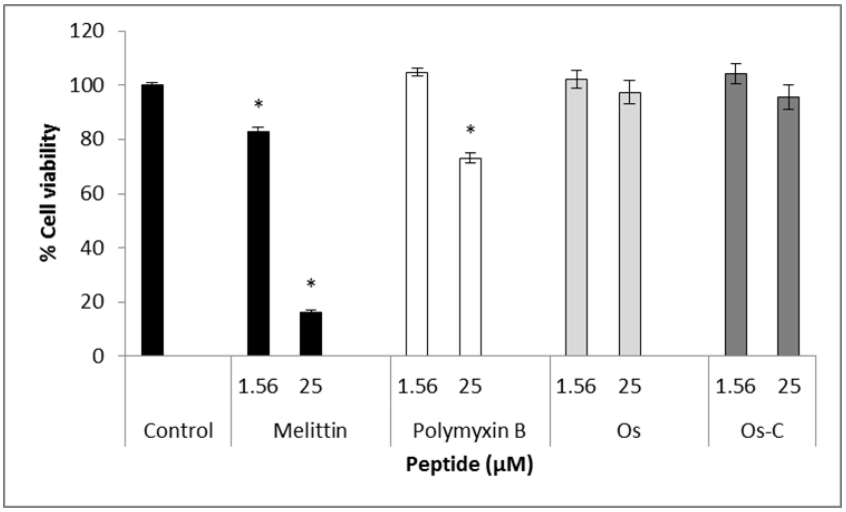


Figure 5

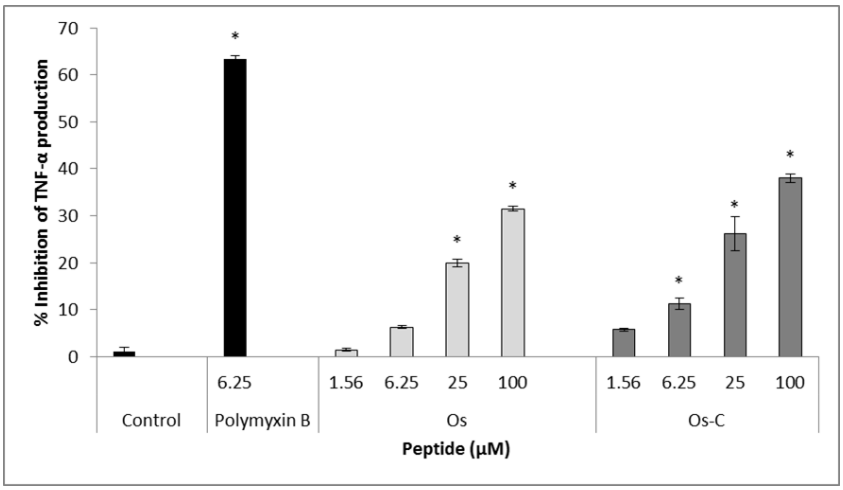


Figure 6

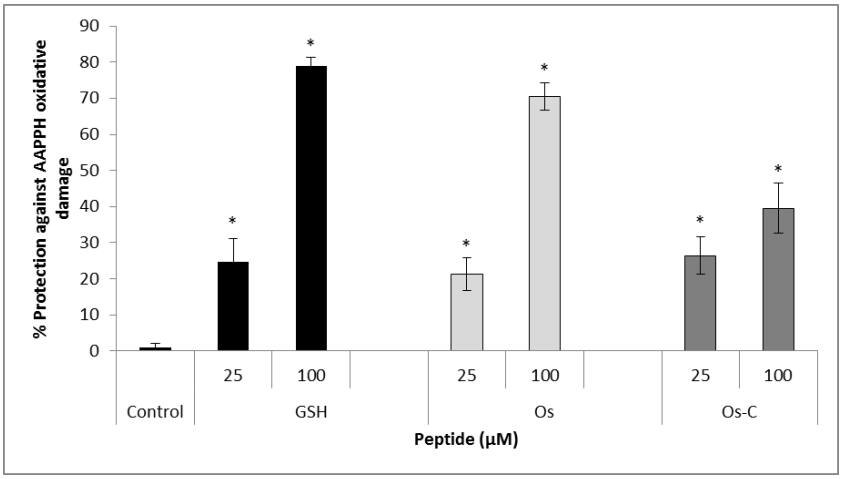


Figure 7

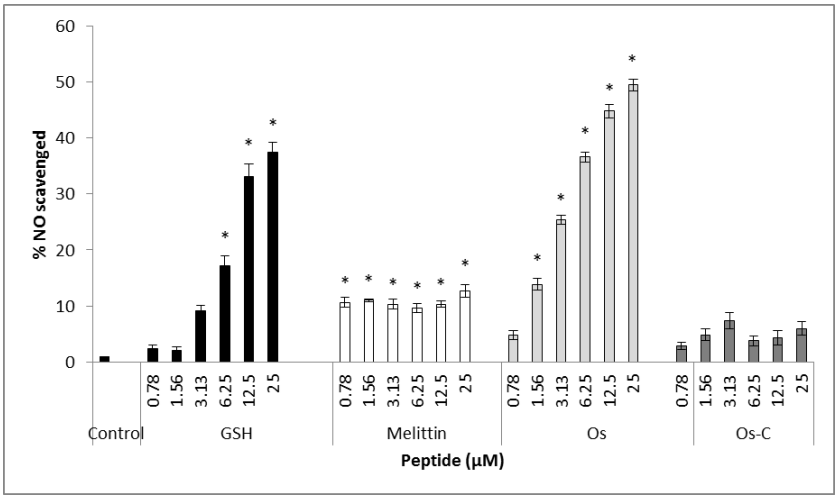
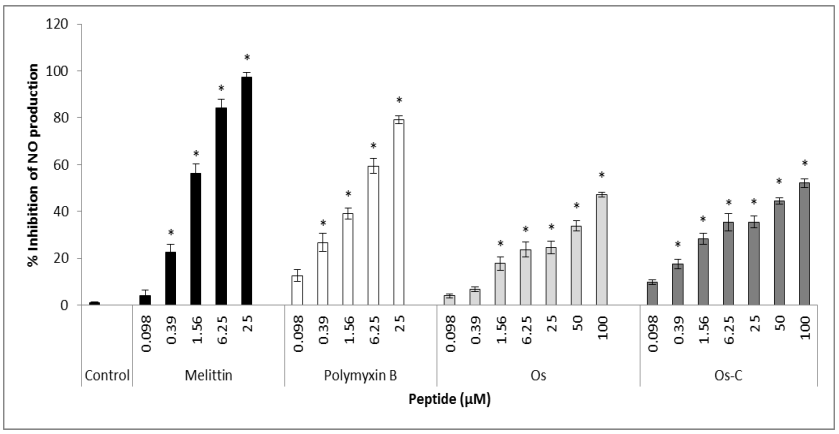


Figure S1



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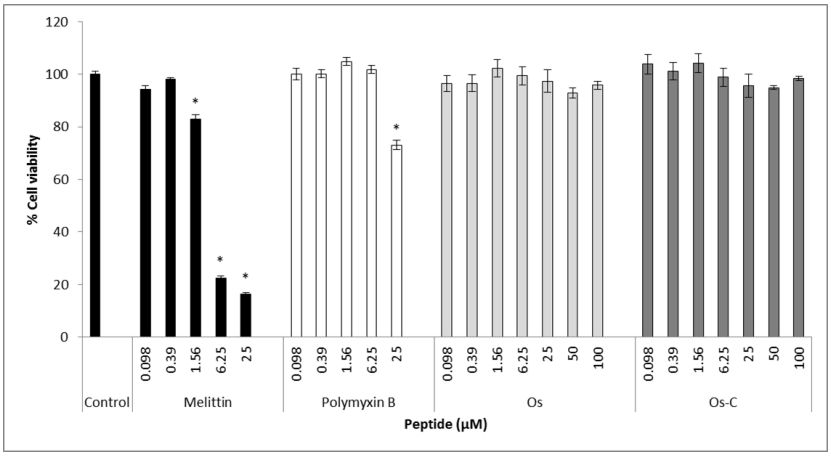

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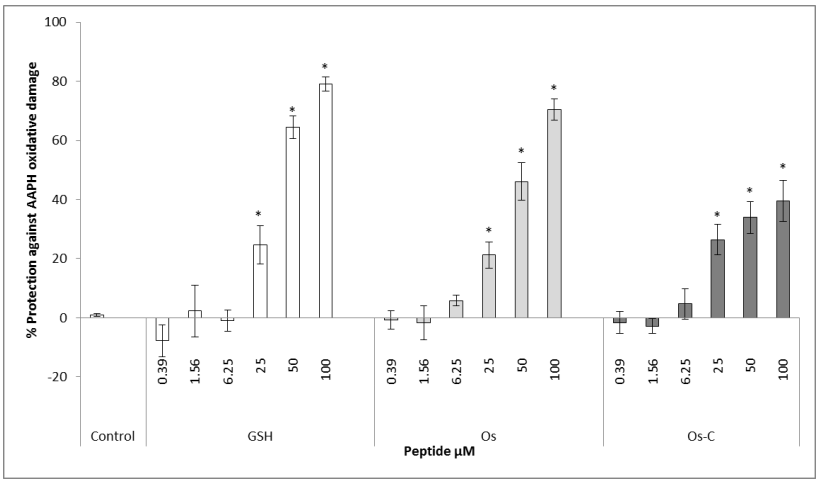

Figure S4