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:

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

where c is the peptide concentration (mg/ml); A is the absorbance of the peptide measured at 280 nm; df the dilution factor; Mr the relative molecular mass of the peptide (g/moles) and ε 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

to allow for colour development. The reaction was terminated by the addition of 25% acetic acid and the absorbance was measured at 405 nm with a BioTek plate reader (Analytical and Diagnostic Products, Randburg, South Africa). Inhibition of LAL activation by the peptides was expressed as a percentage relative to the control containing LPS only (0% inhibition).

2.4   Bactericidal activity assay

The effect of LPS on the bactericidal activities of Os and Os-C was investigated as previously described [26]. *E. coli* was grown overnight, diluted 100 times in LB broth and allowed to proliferate at 37 ℃ until the OD_{600} of 0.5 was reached. The bacteria cells were centrifuged (14000 g for 90 seconds), washed twice, and re-suspended in 10 mM sodium phosphate (NaP) buffer (pH 7.4). The washed *E. coli* was diluted in NaP buffer to give approximately 1.125 x 10^6 CFU/ml. Os and Os-C were pre-incubated at their MBC of 1.88 µg/ml (0.76 µM) and 3.75 µg/ml (1.74 µM), respectively, with varying concentrations of LPS (0 – 50 µg/ml) in a volume of 20 µl for 2 hours at 37 °C. Diluted bacteria (80 µl) were then added to the mixtures and further incubated for 2 hours at 37 °C with shaking at 150 rpm. Growth controls containing bacteria only as well as bacteria and LPS alone were included. Thereafter, the incubated samples were diluted 500 times in NaP buffer and 100 µl of these dilutions plated on LB agar plates, incubated for 14-16 hours at 37 ℃ and the surviving colonies counted. 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).

2.5   Nitric oxide scavenging assay

To determine the direct NO scavenging ability of the peptides a modified quantitative chemical assay with Griess reagent was used [30]. A volume of 80 µl sodium nitroprusside (SNP) solution (5 mM) diluted in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4) was added to 20 µl of Os, Os-C, GSH and melittin, spanning a final concentration range of 0.78 - 25 µM, and incubated for 1 hour at room temperature in the dark. Thereafter, 50 µl sulfanilamide (1% SA in 2.5% phosphoric acid) was added and incubated for a further 10 minutes. A volume of 50 µl N-(1-Naphthyl)-ethylenediamine-dihydrochloride (0.1% NED in 2.5% phosphoric acid) was added. The absorbance was measured using a BioTek plate reader at 570 nm. The concentration (µM) of NO was determined using a standard sodium nitrite ($NaNO_2$) curve in PBS. 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).

2.6 Determination of NO and TNF-α production by RAW 264.7 cells

To determine the effect of the peptides on NO production, 70 µl of RAW 264.7 cells (final concentration of 1 x 10^6 cells/ml) were plated in sterile 96-well microtiter plates and stimulated for 24 hours at 37 °C with 20 µl LPS/IFN-γ (0.1 µg/ml; 25 U/ml in PBS) in the presence of 10 µl peptides (melittin and polymyxin B at 0.78 - 25 µM; Os and Os-C at 0.78 - 100 µM). To measure NO production the method of Hernandez-Ledesma *et al*. [31] was followed with slight modifications. Following stimulation, 50 µl aliquots of the supernatants were removed and combined with 100 µl Griess reagent (1:1 mixture (v/v) of 1% SA and 0.1% NED in 2.5% phosphoric acid) followed by incubation at room temperature for 10 minutes. The absorbance was measured with a BioTek plate reader at 570 nm. NO production was determined using a standard sodium nitrite curve in DMEM. Inhibition of NO production by the peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (0% inhibition).

The same conditions as described above were followed to determine the effect of Os and Os-C on TNF-α production, except that only polymyxin B at 6.25 µM was used as a positive control. To measure TNF-α production a mouse TNF-α ELISA assay was performed according to the manufacturer's protocol. Following stimulation, 100 µl aliquots of the supernatants were added to the antibody-coated ELISA 96 well plate and incubated for 2.5 hours at room temperature. The plate was washed four times with washing buffer. This was followed by the addition of 100 µl biotinylated detection antibody, incubation for another hour at room temperature and a wash cycle. Thereafter, 100 µl horseradish peroxidase (HRP)-Streptavidin was added to each well and incubated for 45 minutes at room temperature. The solution was discarded and the plate washed another four times. Thereafter, 100 µl ELISA colorimetric 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and incubated for 30 minutes at room temperature. To terminate the reaction, 50 µl of stop solution was added and the absorbance of the solution in each well was measured using a BioTek plate reader, at a wavelength of 450 nm. The concentrations (ng/ml) of TNF-α were determined using a standard TNF-α curve. Inhibition of TNF-α production by the peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (0% inhibition).

2.7 Cell viability assay

The cytotoxicity of peptides towards RAW 264.7 macrophage cells was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [32]. RAW 264.7 cells (70 µl; 1 x 10^6 cells/ml) were plated in sterile 96-well microtiter plates and stimulated for 24 hours at 37 °C with 20 µl LPS/IFN-γ (0.1 µg/ml; 25 U/ml in PBS) in the presence of 10 µl peptides (melittin and polymyxin B at 0.78 - 25 µM; Os and Os-C at 0.78 - 100 µM). Following incubation, 5 µl of MTT (91 µg/ml) solution was added to each well and incubated for 3 hours at 37°C and 5% CO_2. After incubation, the medium containing MTT was removed and the plate left at room temperature until dry. The purple formazan crystals were dissolved with 50 µl DMSO with shaking for approximately 10 minutes. The absorbance of the solution in each well was measured using a BioTek plate

reader using a wavelength of 570 nm. Cell viability in the presence of peptides was expressed as a percentage relative to the control containing LPS/ IFN-γ only (100% cell viability).

2.8 Cell-based antioxidant assay

To investigate whether the peptides are able to neutralize the oxidative damage induced by 2,2'-azobios(2-amidinopropane) dihydrocloride (AAPH) in RAW 264.7 cells, the method of Wang and Joseph [33] with some modifications was used. Cells (100 µl; final concentration of 1 x 10^6 cells/ml) were plated in sterile 96-well microtiter plates and incubated for 24 hours at 37°C. The cells were then incubated with 50 µl dichlorodihydrofluorescein diacetate (DCFH-DA) (25 µM) for 45 minutes, after which the cells were washed with sterile PBS. The cells were exposed to 50 µl of Os, Os-C and GSH (0.78 - 100 µM) and 50 µl of PBS or AAPH (2 mg/ml). Fluorescence was measured with a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), using an excitation wavelength of 485 nm and an emission wavelength of 520 nm, for 1 hour. Percentage protection by the peptides against AAPH oxidative damage was calculated using the equation below:

$$\% \, Protection = 100 - \frac{Sample - PBS}{Sample - AAPH} \, X \, 100$$

2.9 Data analysis

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

3. Results

3.1 Os and Os-C bind to free LPS

The ability of Os and Os-C to bind to free LPS was determined by measuring the capacity of the peptides to inhibit LPS-induced activation of the LAL enzyme. The physicochemical properties and minimum bactericidal concentration (MBC) of synthetic peptides are shown in **Table 1**. Polymyxin B and melittin both reported to have endotoxin-binding properties were used as positive controls [34, 35]. Os and Os-C showed inhibition of the LPS-induced activation of the LAL enzyme in a dose-dependent manner (**Figure 1**). The inhibition observed for Os-C at 25

µM was statistically higher (28%) than Os (19%), whereas melittin and polymyxin B at the same concentration inhibited activation by 88% and 84%, respectively.

3.2   LPS inhibits the bactericidal activity of Os and Os-C

An indirect way to confirm the observed affinity of the peptides for LPS is to determine whether pre-incubation of Os and Os-C with an excess of LPS reduces the antibacterial activity of Os and Os-C. Whereas both peptides killed *E. coli* in the absence of LPS, increasing concentrations of LPS abolished the activity of both peptides in a dose-dependent manner (**Figure 2**). This was shown by the increase in the % viability of *E.coli* as the LPS concentration was increased. The growth control consisting of *E. coli* and free LPS showed that LPS had no effect on bacterial growth.

3.3   Os scavenges NO

To establish whether Os and Os-C can directly scavenge NO, a quantitative scavenging assay was performed. The activities were compared to GSH, a known NO scavenger [36, 37], and melittin. Os and GSH showed NO scavenging activity in a dose-dependent manner (**see supporting information Figure S1**). At 25 µM, the scavenging capabilities of Os, GSH and melittin were 49%, 38% and 13%, respectively, whereas Os-C showed no activity (**Figure 3**).

3.4   Os and Os-C inhibit NO and TNF-α production in LPS/ IFN-γ stimulated RAW 264.7 cells

NO and TNF-α are key mediators of inflammatory responses [38, 39]. To evaluate the effects of Os and Os-C on LPS/IFN-γ induced production of NO and TNF-α, RAW 264.7 cells were stimulated with LPS/IFN-γ in the presence of various concentrations of peptides and the amounts of NO and TNF-α released into the supernatants were measured. Os, Os-C, melittin and polymyxin B inhibited LPS/IFN-γ induced production of NO in a dose-dependent manner (**see supporting information Figure S2**). At 25 µM, Os, Os-C, melittin and polymyxin B inhibited NO production by 25%, 36%, 97% and 79%, respectively (**Figure 4**).   TNF-α secretion by RAW 264.7 cells was also significantly inhibited by both Os and Os-C in a dose-dependent manner (**Figure 6**). Compared to polymyxin B (6.25 µM) which caused 63% inhibition of TNF-α production, only Os-C showed slight activity (11%). However, at 100 µM, Os and Os-C inhibited TNF-α secretion by 31% and 38%, respectively.

3.5   Os and Os-C are non-toxic to RAW 264.7 cells

The effects of Os, Os-C, melittin and polymyxin B on the viability of RAW 264.7 cells were assessed with the MTT assay. Significant cytotoxicity was observed for melittin and polymyxin B at concentrations of 1.56 µM and 25 µM, respectively (**Figure 5**), whereas both Os and Os-C showed no cytotoxicity at these concentrations. To confirm lack of cytotoxicity, Os and Os-C were evaluated to higher concentrations. Even at a concentration of 100 µM, both peptides did not affect RAW 264.7 cell viability (**see supporting information Figure S3**).

3.6   Os and Os-C protect RAW 264.7 cells from free radicals

Activation of macrophages results in an increase in cellular oxidative stress associated with the excessive production of free radicals and depletion of antioxidants [37].   In addition, NO can combine with reactive oxygen species (ROS), to form reactive nitrogen species (RNS), which contribute to cellular damage [40].   We have previously found that Os and Os-C displayed antioxidant activity as assessed with the oxygen radical absorbance capacity (ORAC) assay [26]. The ability of both peptides to provide protection against free radicals in RAW 264.7 cells was determined and compared to GSH. Os, Os-C and GSH significantly protected RAW 264.7 cells against AAPH-induced oxidative damage in a concentration-dependent manner (**see supporting information Figure S4**).   At 25 µM, Os, Os-C and GSH protected against AAPH-induced oxidative damage by 21%, 27% and 25%, respectively (**Figure 7**). However at 100 µM, the activities of Os and GSH were similar, 71% and 79%, respectively, whereas Os-C protected cells by only 40%.   Melittin and polymyxin B had negligible antioxidant activity under these conditions (results not shown).

4   Discussion

LPS, LPS-binding sites and macrophage associated inflammatory pathways have been identified as therapeutic targets for the reduction of inflammation associated with sepsis [41].   Therefore, the search for antimicrobials with high affinity towards LPS thereby blocking LPS-induced activation of macrophages in early stages of inflammation is crucial [42].   Cationic peptides contain, in addition to their antimicrobial activity, the ability to suppress harmful inflammatory/septic responses and promote wound healing [43].

The main aim of this study was to investigate the potential anti-inflammatory and anti-endotoxin activities of Os and Os-C. Suppression of LAL activation by both peptides indicated that Os and Os-C can bind directly to LPS.   Electrostatic and hydrophobic interactions between peptides and LPS are necessary to ensure binding.   For successful binding, peptides must contain both hydrophilic and hydrophobic amino acids to facilitate these interactions [34, 44-47].   Melittin and polymyxin B bind LPS through electrostatic and hydrophobic interactions.

Melittin has been shown to bind LPS as a result of the presence of both cationic and hydrophobic amino acids present in the carboxy-terminal (A15-R24) of the peptide [47], whereas for polymyxin B, in addition to the positive charge and hydrophobic amino acids, the acyl chain plays a role in its interaction with LPS [42]. Binding of Os and Os-C may thus be similar to that observed for melittin, where both electrostatic and hydrophobic interactions are required for binding to LPS (**Table 1**). The hydrophobicity of these peptides might therefore be related to their ability to bind LPS. The higher mean hydrophobicity (<H>) of melittin (0.511) compared to that of Os (0.249) and Os-C (0.045), may explain its higher affinity for LPS (**Figure 1**).

The bactericidal activity assay was performed as an indirect method of confirming that Os and Os-C bind LPS (**Figure 2**). If Os or Os-C bind to LPS then addition of increasing amounts of LPS to a constant amount of peptide is expected to reduce the effective concentration of the peptide with concomitant reduction in bactericidal activity. Increasing concentrations of LPS markedly reduced the killing activity of both Os and Os-C towards *E. coli*, confirming the affinity of the peptides for LPS and the potential of both peptides to block LPS-mediated activation of inflammatory responses. Similarly, Lin, *et al.* [10], observed that the bactericidal activity of CM4, a peptide isolated from the Chinese silk worm, *Bombyx mori*, decreased with increasing concentrations of LPS. Furthermore, CM4 was found to block the release of inflammatory molecules from LPS-challenged RAW 264.7 cells. Reduction of the bactericidal activity of both Os and Os-C by increasing LPS concentrations also suggested that the initial interaction of both peptides with LPS on the surface of *E. coli* is important for its antibacterial role.

NO plays a pivotal role in the pathogenesis of inflammatory and infectious disorders. Besides being a mediator of inflammation, NO can combine with ROS to form RNS. Removal or scavenging of NO will reduce inflammation and RNS associated cellular and tissue damage [37]. NO is a highly reactive molecule which interacts with the thiol of GSH, a known NO scavenger, to form nitrosothiol [37, 39]. In this study, GSH caused a dose-related decrease in the amount NO generated *in vitro* from the decomposition of SNP (**see supporting information Figure S1**). Similarly, Os scavenged NO and followed the same trend as GSH, whereas the scavenging capability of Os-C was insignificant. The NO scavenging ability of Os, which contains three cysteine residues, is in agreement with the results obtained with GSH. Additionally, the non-existing NO scavenging activity of Os-C could be due to the fact that Os-C contains no cysteines.

Multifunctional cationic peptides that bind LPS, and inhibit LPS-induced inflammatory responses, are beneficial as these peptides target several key molecules involved in inflammation [43, 48]. Having established that these peptides bind to LPS, it was necessary to ascertain whether such binding would result in inhibition of pro-inflammatory responses such as NO and TNF-α production. Previous studies indicated that synergism between LPS and IFN-γ is more successful in supplying a favourable and sustainable source of NO due to the enhancement of iNOS transcription in RAW 264.7 macrophage cells [39]. In this study, an inflammatory response in RAW 264.7 cells was achieved with LPS/IFN-γ stimulation when compared to the

unstimulated cells. Os and Os-C were found to significantly reduce LPS/IFN-γ induced production of NO, and TNF-α by RAW 264.7 cells (**Figure 4 and Figure 6**). The mechanism of inhibition of NO production by Os is unknown. The observed inhibition could be due to its direct scavenging activity and requires further investigation.

For therapeutic application, multifunctional AMPs must be non-toxic towards eukaryotic cells [43, 49]. In this study, the MTT assay showed that Os and Os-C (100 μM) had no effect on the cell viability of the RAW 267.4 cells when incubated with peptide only (data not shown), and simultaneously with peptide and LPS/IFN-γ (**see supporting information Figure S3**). This demonstrated that the inhibition of the NO response by Os and Os-C was not attributed to a direct cytotoxic effect of these peptides. In contrast, a significant reduction in cell viability was observed for melittin and polymyxin B at 1.56 μM and 25μM (**Figure 5**), respectively, confirming the non-selective toxicity of both peptides towards mammalian cells [36, 50].

There is also a great interest towards potential anti-inflammatory peptides that possess antioxidant properties. Inflammation is associated with ROS and RNS induced cellular and tissue damage [51, 52] but in addition ROS and RNS can also initiate and/or increase the inflammatory response [53, 54]. Using the ORAC assay it was previously found that Os and Os-C displayed equally potent antioxidant activities [26]. In this study, using a cell-based assay, Os, Os-C and GSH exhibited a dose- dependent increase in antioxidant activity, providing protection against free radicals (**see supporting information Figure S4**). At 100 μM, Os displayed equally potent activity when compared to GSH, whereas Os-C was found to possess less antioxidant activity (Figure 7). Several amino acids contribute to the antioxidant activity of peptides, such as Cys, Met, Phe, Tyr, His and Trp [55-57]. The sequences of Os and Os-C contain several of these antioxidant promoting amino acids (**Table 1**). Os contains more antioxidant amino acids (three Cys, one Phe and three Tyr) than Os-C (one Phe and three Tyr), and this may account for the higher cellular antioxidant activity found for Os. The antioxidant activity of these amino acids is attributed to the phenolic side chains, and the hydroxyl (Tyr) and thiol (Cys) groups of these amino acids that enables these amino acids to donate an electron to the radical species resulting in their stabilization. In a study by Madhyastha & Vatsala [57], the antioxidant activity of the Cys-rich cyanopeptide β2 isolated from *Spirulina fussiformis*, was due to the presence of the six Cys residues present in the peptide. This suggests that the presence of the three Cys residues in Os may be responsible for the higher antioxidant activity observed compared to Os-C. As previously explained, Os possesses NO scavenging activity due to the ability of Os to form nitrosothiols when exposed to NO. This confirms that Cys residues are also responsible for the observed activity. Additionally, BIOPEP analysis [http://uwn.edu.pl.biochemica/index.php/en/biopep] showed that the dipeptide sequence, IR, which is associated with antioxidant activity, is present in both Os and Os-C.

In conclusion, both Os and Os-C exhibited the ability to bind LPS as found by the LAL assay. Furthermore, the affinity of both peptides for LPS was confirmed by showing that the bactericidal activity of Os and Os-C against *E. coli* was inhibited following pre-incubation of the peptides with

LPS. Both Os and Os-C inhibited LPS-induced TNF-α and NO production by RAW 264.7 cells. Even at high concentrations, Os and Os-C, displayed no cytotoxic effects in RAW 264.7 cells. Moreover, in addition to the anti-endotoxin and anti-inflammatory properties, Os was able to scavenge NO directly and both peptides displayed antioxidant activity, which together may result in the reduction of oxidative stress associated with inflammation. This study identified Os and Os-C as multifunctional peptides that besides having antibacterial activities are capable of binding to LPS resulting in inhibition of inflammatory responses and may attenuate tissue injury by scavenging free radicals.

Acknowledgements

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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**Y**CKGA**F**KQT**C**K**C****Y**	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 °C. 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 37°C 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).
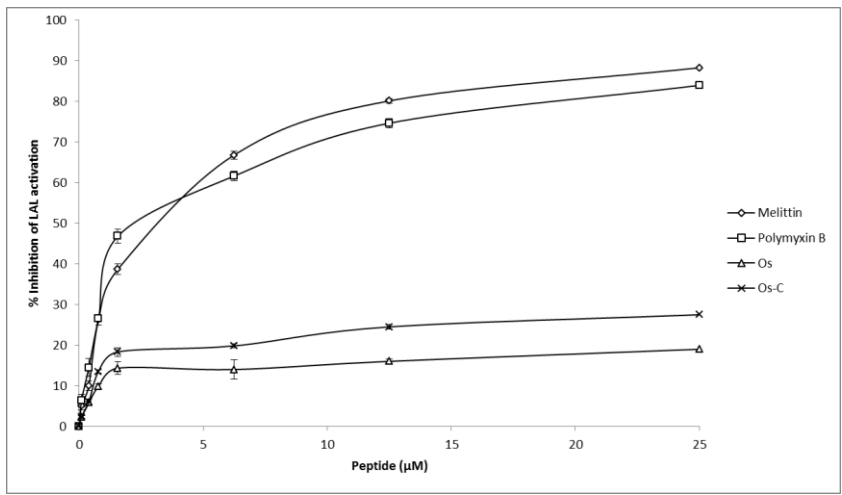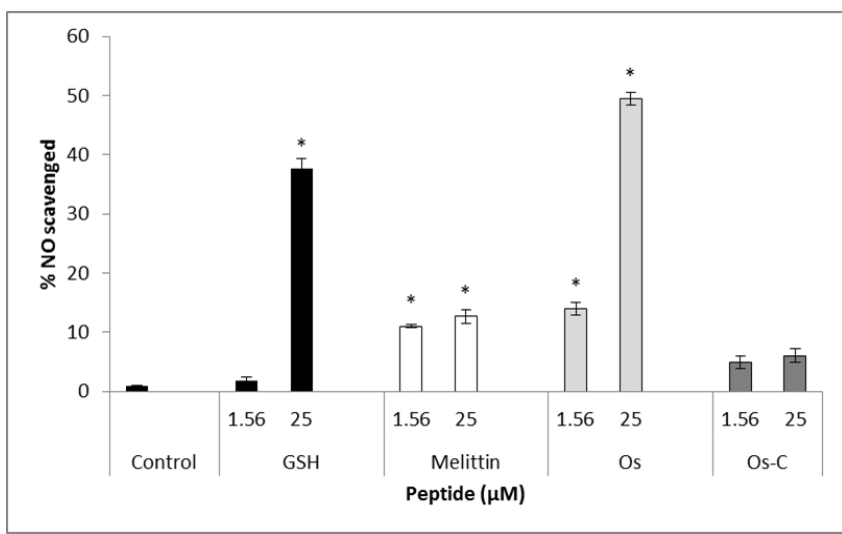
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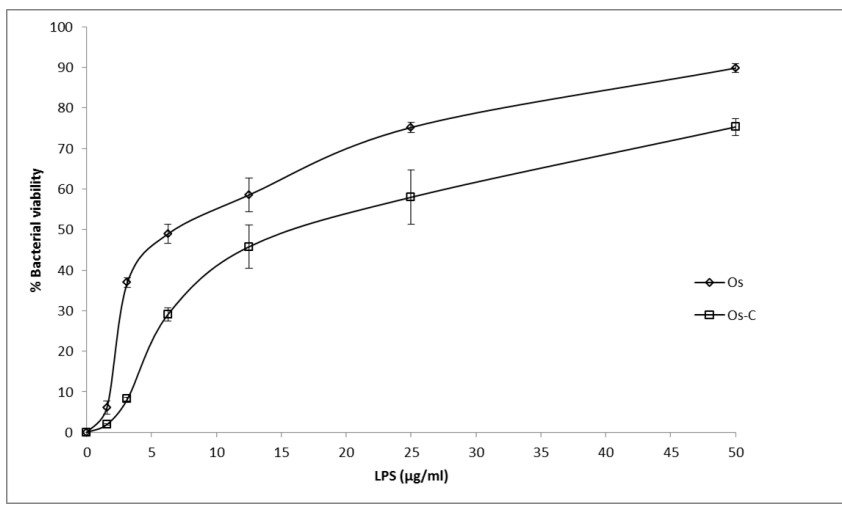
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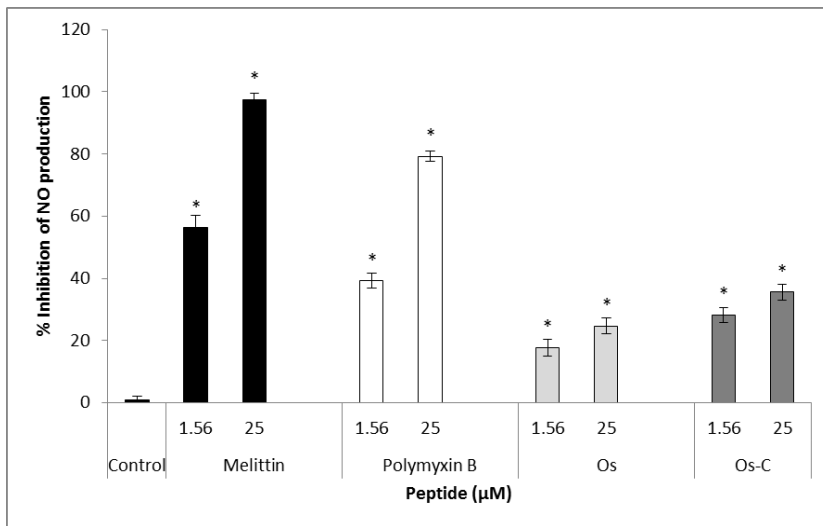
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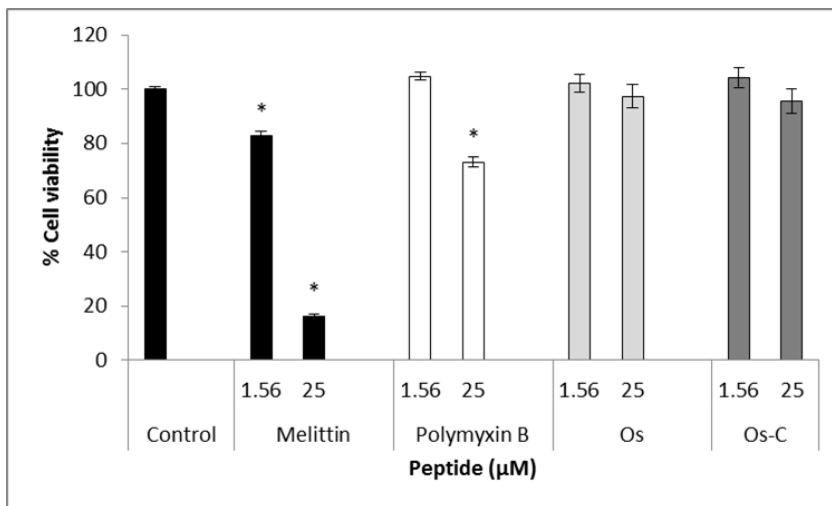
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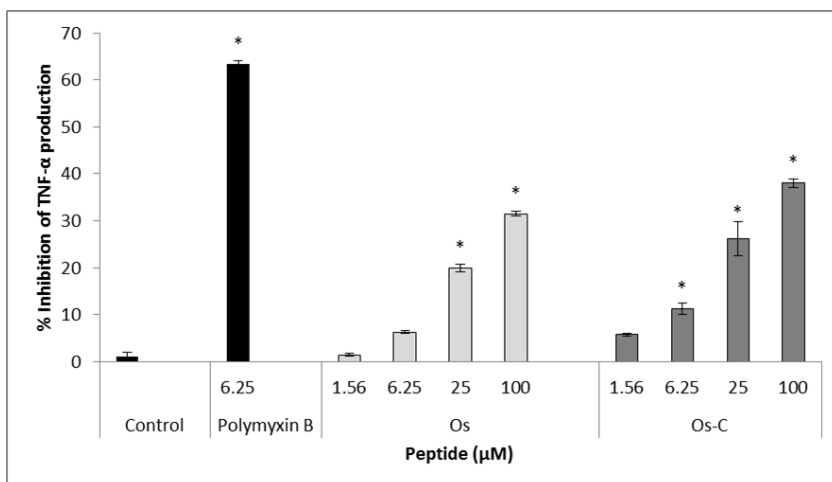
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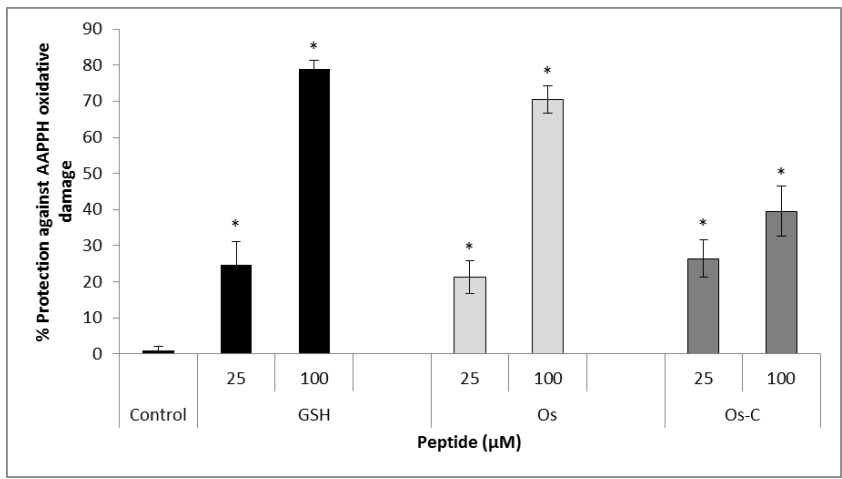
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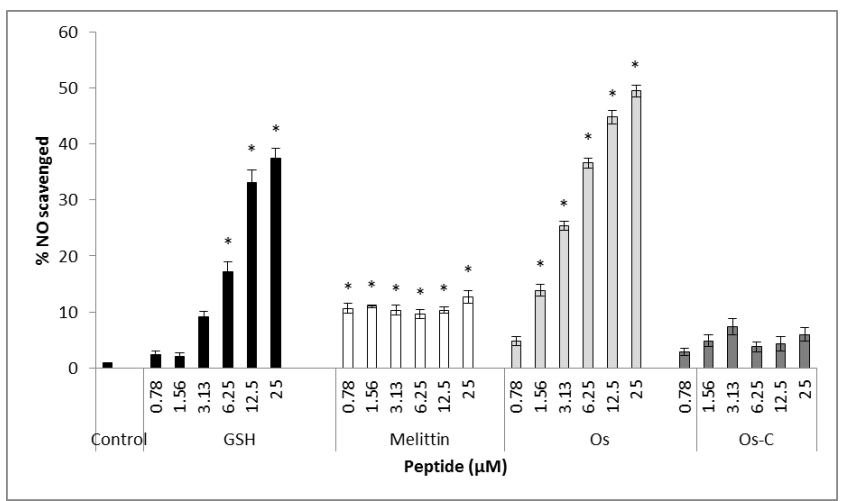
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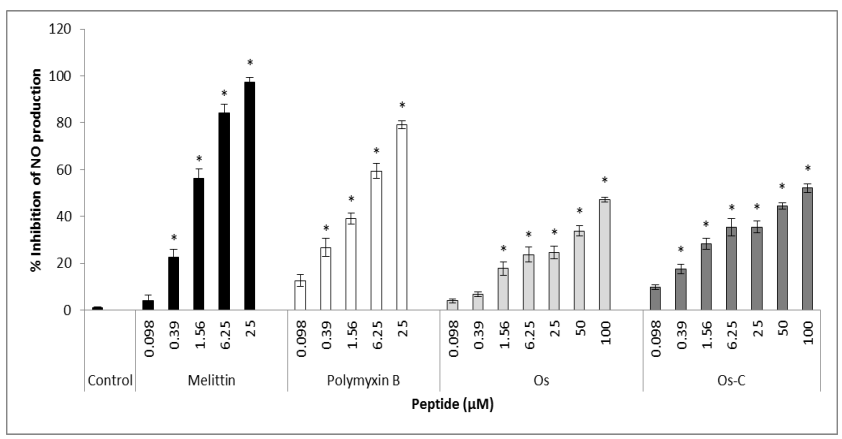
Figure S2

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Figure S4