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Investigation into the mechanism of action of the antimicrobial peptides Os and Os-C derived from a tick defensin

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ABSTRACT

Os and Os-C are two novel antimicrobial peptides, derived from a tick defensin, which have been shown to have a larger range of antimicrobial activity than the parent peptide, OsDef2. The aim of this study was to determine whether the peptides Os and Os-C are mainly membrane acting, or if these peptides have possible additional intracellular targets in *Escherichia coli* and *Bacillus subtilis*. Transmission electron microscopy revealed that both peptides adversely affected intracellular structure of both bacteria causing different degrees of granulation of the intracellular contents. At the minimum bactericidal concentrations, permeabilization as determined with the SYTOX green assay seemed not to be the principle mode of killing when compared to melittin. However, fluorescent triple staining indicated that the peptides caused permeabilization of stationary phase bacteria and TEM indicated membrane effects. Studies using fluorescently labeled peptides revealed that the membrane penetrating activity of Os and Os-C was similar to buforin II. Os-C was found to associate with the septa of *B. subtilis*. Plasmid binding studies showed that Os and Os-C binds *E. coli* plasmid DNA at a similar charge ratio as melittin. These studies suggest membrane activity for Os and Os-C with possible intracellular targets such as DNA. The differences in permeabilization at lower concentrations and binding to DNA between Os and Os-C, suggest that the two peptides have dissimilar modes of action.

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1. Introduction

The increase in bacterial resistance to antibiotics is a major cause for concern all over the world [37]. The study of antimicrobial peptides (AMPs) strives to produce viable candidates for new antibiotics with novel mechanisms of action. AMPs are active against a wide range of microbes and the development of resistance against these agents is less frequent than against traditional antibiotics [18]. The mode of bacterial killing of an AMP needs to be fully understood if it is to be developed as an anti-infective agent.

The amphipathic structure of AMPs allows these molecules to be soluble in an aqueous environment, and also to penetrate lipid-rich membranes. In bacteria, the primary target of AMPs is the membrane. However, AMPs which do not kill by permeabilization may kill through a wide range of intracellular mechanisms [14]. Some intracellular targets include the stimulation of autolytic enzymes, the inhibition of DNA synthesis, protein synthesis, cell wall and membrane synthesis [8,15]. The use of microscopy may reveal important insights into the killing mechanism. Hartmann and colleagues used electron microscopy to observe that the peptides gramicidin S and PGLa caused damage of the bacterial envelope, disruption of osmoregulation and affected bacterial DNA [12]. Mangoni et al. described a fluorescence triple staining method with which permeability and viability of cells caused by peptides, could simultaneously be visually evaluated [20]. By fluorescently labeling buforin II analogs and observing the location with microscopy, Park and colleagues revealed that substitution or insertion of proline caused the peptide to become membrane-acting [24].

Disadvantages of using AMPs as antimicrobial treatments include susceptibility to proteases, limited knowledge on toxicity profiles and unknown systemic effects as well as the high cost of production [38]. Therefore, natural AMPs are often used

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMP, antimicrobial peptide; CFU, colony forming unit; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylene diaminete traacetic acid; 5-FAM, 5-carboxy fluorescein; FITC, fluorescein 5(6)-isothiocyanate; HPF, high pressure freeze; LB, Luria–Bertani; MBC, minimum bactericidal concentration; OD, optical density; TAE, tris-acetate-EDTA; TEM, transmission electron microscopy.

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as templates to develop structural analogs with decreased size and increased activity. New analogs of an AMP have to then be re-evaluated for activity and mode of action.

OsDef2, a defensin identified in the midgut of the soft tick *Ornithodoros savignyi*, has been shortened to the C-terminal peptide Os. Os was also modified to Os-C by removing the three Cys residues from the sequence (Table 1) [26]. Both Os and its analog, Os-C, were previously found to be strongly bactericidal to both Gram-positive and Gram-negative bacteria [26]. Os was found to be more active with a shorter killing time than Os-C, suggesting different modes of killing for the two peptides. Both peptides caused the collapse of bacterial cell structures and indentation of cell membranes [26]. Os and Os-C were found not to be toxic to mammalian cells at the concentrations tested, and were shown to possess antioxidant activity [26]. However, their bacterial killing mechanism is not yet fully understood.

The aim of this study was to determine whether the peptides Os and Os-C are mainly membrane acting, or if they affect additional intracellular targets.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli (ATCC 700,928) and *Bacillus subtilis* (ATCC 13,933) were used. Bacteria were grown aerobically in Luria–Bertani (LB) broth at 37 °C. To obtain bacteria in the mid-logarithmic phase, bacteria were cultured overnight, diluted 100 times in LB broth and proliferated until an OD_{600} of 0.5 was reached.

2.2. Preparation of synthetic peptides

The peptides Os, Os-C as well as 5-carboxyfluorescein (5-FAM)-labeled Os, Os-C and buforin II were obtained from GenScript (New Jersey, USA). The purity and molecular mass of the peptides were determined by reverse-phase HPLC and mass spectrometry, respectively. Dithiothreitol (DTT, 10 nmol) was added to Os prior to lyophilization. The peptide melittin (Mel) (SigmaAldrich, South Africa) 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 by measuring the absorbance (Abs) of tyrosine or tryptophan residues at 280 nm and using the equation below:

$$c = \frac{\mathrm{MW} \times \mathrm{df} \times \mathrm{Abs}}{\text{no. of Try/Trp} \times \text{Extinction coefficient}}$$

where c is the peptide concentration in mg/mL, MW is the molecular weight of the peptide, and df is the dilution factor. The extinction coefficients of tyrosine and tryptophan are 1200 and 5560 AU/mmole/mL, respectively [17]. The concentration of the fluorescently labeled peptides was determined with the same equation, by measuring the absorbance of 5-FAM labeled peptide at the excitation wavelength (492 nm) and using its extinction coefficient (78 000 AU/mmole/mL) [5].

2.3. Transmission electron microscopy

The methods for high pressure freezing (HPF) and freeze substitution were based on the methods described by Venter et al. [35]. *E. coli* and *B. subtilis* cells in the mid-logarithmic phase were adjusted to a cell density of 64×10^6 CFU/mL and exposed to a final concentration of 2 μM Mel, Os and Os-C for 10 min at 37 °C. After exposure of selected bacteria to the peptides, the suspension was centrifuged to prepare a dense pellet and 1 μl of the bacteria pellet was used to fill the cavity of gold plated flat specimen carriers from Leica Microsystems. This was followed by high pressure freezing using the Leica EM Pact (Leica Microsystems GmbH, Wetzlar, Germany). To remove all water from the samples, freeze substitution (FS) was carried out with the Leica EM AFS2 (Leica Microsystems GmbH, Wetzlar, Germany) in 2% osmium tetroxide, 0.1% uranyl acetate and 99.88% acetone. The samples were kept at −90 °C for 42 h, heated to −60 °C over a 15 h period, left at −60 °C for 8 h, heated to −30 °C over a 15 h period, left at −30 °C for a further 8 h after which the samples were allowed to warm up to room temperature.

Samples were infiltrated with, and embedded in Embed 812 (SPI Supplies, Pennsylvania, USA). Ultra-thin sections (100 nm) were prepared with the Leica Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). These sections were picked up on copper grids and contrasted with 4% aqueous uranyl acetate and Reynolds' lead citrate and rinsed with water. The contrasted sections were viewed and images taken on the JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan).

2.4. SYTOX Green assay

To quantify membrane permeabilization of *E. coli* and *B. subtilis* caused by the peptides the SYTOX green assay, adapted from Roth et al. was used [27]. Bacterial suspensions in the mid-logarithmic phase of growth were diluted to a cell density of 1×10^6 CFU/mL and incubated with SYTOX green (Life Technologies, South Africa) at a final concentration of 0.1 μM in 10 mM sodium phosphate (NaP) buffer pH 7.4 for 15 min at 37 °C in a shaking incubator. The bacteria and SYTOX green mixture was then exposed to a concentration range of Mel, Os and Os-C (0.1–10 μM) for 1 h at 37 °C in a shaking incubator in a black, flat-bottom, polystyrene costar 96-well plate (Corning, New York, USA). The plate was then transferred to the SpectraMax Paradigm microplate reader (Molecular Devices, California, USA) and the fluorescence of each well measured using an excitation of 488 nm and emission of 530 nm. The data is presented as mean ± standard error of mean. Multiple comparisons were tested by one-way ANOVA followed by the Tukey's post hoc test (http://statistica.mooo.com/).

2.5. Localization of peptides

To determine whether Os and Os-C are able to enter intact *E. coli* and *B. subtilis*, cells exposed to fluorescently labeled peptide were observed with confocal fluorescence microscopy. Mid-logarithmic phase *E. coli* and *B. subtilis* were adjusted to a cell density of 64×10^6 CFU/mL and exposed to a final concentration of 7.6 μM of 5FAM-Os, 5FAM-Os-C and 5FAM-buforin II for 2 h at 37 °C in a shaking incubator. Buforin II is a non-membrane acting AMP that is known to cross the cell membrane and was used as a positive control [23]. The bacterial cells were immobilized onto poly-L-lysine coated cover glass slides which were placed in a 24-well Cellstar polystyrene plate (Greiner Bio-One GmbH, Kremsmünster, Austria). The cover glass slides were rinsed with NaP buffer pH 7.4, mounted on glass slides with antifade mounting medium and viewed with the Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). For visualization, an excitation wavelength of 490 nm and emission wavelength of 520 nm was used.

2.6. Gel retardation assay

To investigate the effect of peptides on plasmid DNA (pDNA), 2.5 μl of 10 μg/mL pBR322 vector from *E. coli* (SigmaAldrich, South Africa) was exposed to 2.5 μl of different concentrations of Mel, Os

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