Antifungal activity and mode of action of synthetic peptides derived from the tick OsDef2 defensin

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Abstract

Candida albicans is the principal opportunistic fungal pathogen in nosocomial settings and resistance to antifungal drugs is on the rise. Antimicrobial peptides from natural sources are promising novel therapeutics against *C. albicans*. OsDef2 defensin was previously found to be active against only Gram-positive bacteria, whereas derived fragments Os and its cysteine-free analogue, Os-C, are active against Gram-positive and Gram-negative bacteria at low micromolar concentrations. In this study, OsDef2-derived analogues and fragments were screened for anticandidal activity with the aim to identify peptides with antifungal activity and in so doing obtain a better understanding of the structural requirements for activity and modes of action. Os, Os-C and Os(11–22)NH_2, a Os-truncated carboxy-terminal-amidated fragment, had the most significant antifungal activities, with minimum fungicidal concentrations (MFCs) in the micromolar range (6–28 μM). *C. albicans* killing was rapid and occurred within 30–60 min. Further investigations showed all three peptides interacted with cell wall derived polysaccharides while both Os and Os(11–22)NH_2 permeabilized fungal liposomes. Confocal laser scanning microscopy confirmed that Os-C and Os(11–22)NH_2 could enter the cytosol of live cells and subsequent findings suggest that the uptake of Os and Os-C, in contrast to Os(11–22)NH_2, is energy dependent. Although Os, Os-C and Os(11–22)NH_2 induced the production of reactive oxygen species (ROS), co-incubation with ascorbic acid revealed that only ROS generated by Os-C and to a lesser extent Os(11–22)NH_2 resulted in cell death. Overall, Os, Os-C and Os(11–22)NH_2 are promising candidacidal agents.

Keywords: antimicrobial peptides; defensins; membrane permeabilization; reactive oxygen species; tick

Abbreviations

5-FAM, 5-carboxyfluorescein; AMP, antimicrobial peptide; CFU, colony-forming unit; DCFH-DA, 20 , 7-dichlorofluorescein diacetate; FU, fluorescence unit; MFC, minimum

fungicidal concentration; MIC, minimum inhibitory concentration; NaP, sodium phosphate; RD, radial diffusion; ROS, reactive oxygen species; YPD, yeast peptone dextrose

1 INTRODUCTION

Candida albicans is an opportunistic pathogen that can cause genital, oral and systemic infections[1] and in severely ill patients has a mortality rate of up to 50%. Antifungal therapy is currently limited to only four drug classes[2, 3] and resistance to all four classes has been reported.[3-8] Therefore, the need to develop effective antifungal agents with novel mechanisms of action is a priority.

Antimicrobial peptides (AMPs), key components of innate immunity of many organisms, are promising candidates to either replace or enhance the activity of conventional antimicrobial agents due to their numerous advantages. Mainly, AMPs have attracted attention due to their low susceptibility to resistance, conferred by their broad spectrum of activity and membrane-targeting abilities.[9] This is explained by the fact that it is metabolically costly for most microorganisms to mutate their membrane components to acquire AMP resistance.[10] So far, AMPs have been identified in humans, plants, bacteria and fungi[11] and these peptides and derivatives have a wide range of host–defense-related activities including antifungal properties. AMPs with antifungal activity are known to target cell wall polysaccharides,[12] plasma membrane components or act on intracellular targets.[13, 14]

Defensins, a broad group of Cys-rich peptides, are AMPs found in perhaps all eukaryotic organisms as part of the innate immune system.[15, 16] These AMPs are generally cationic, cyclic or open-ended, with a molecular weight of 3 to 5 kDa and contain 3 to 4 disulphide bridges[11, 15] with the conserved pairing Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6.[17] Among tick AMPs, defensins and their isoforms have been identified in various species including *Dermacentor variabilis*, *Ixodes ricinus* and *Haemaphysalis longicornis*.[18-20] Tick defensins display activity directed against both Gram-positive and Gram-negative bacteria, viruses, fungi and protozoan parasites.[20, 21] This broad spectrum of activity along with their low toxicity towards mammalian cells makes defensins suitable for therapeutic applications.[22-25] Additional valuable properties of defensins include anti-inflammatory properties,[23] involvement in cytokine release and regulation,[24] angiogenesis,[25] male fertility[26] and wound repair.[27] The plasma membrane remains the primary target of antifungal defensins.[14, 28] Following this primary interaction, antifungal defensins either remain at the cell periphery,[13] inducing death through a signalling cascade[13] or can gain entry into the fungal cytosol[29] and interact with intracellular targets. For some defensins, entry into the cytosol leads to the induction of reactive oxygen species (ROS) and/or apoptosis.[14]

AMPs, including defensins, have been criticized for their inability to achieve high antifungal activity under systemic conditions and their high production cost.[11] To confer proteolytic stability, AMPs may be modified through strategies such as cyclisation,[30] incorporation of D-amino acids,[31] amino acid end-tagging, substitutions[30] and carboxy-amidation,[32, 33] *N*-terminus acetylation or combinations of these strategies.[33] The carboxy-amidation modification has the additional benefit that it increases the net positive charge of peptides and has been associated with increased antimicrobial activity.[32] To address the issue of the high production cost of AMPs, shorter peptide derivatives with retained antimicrobial activity and selectivity represent a more immediate solution.[9, 34]

The parent peptide in this study OsDef2, a defensin from the soft tick *Ornithodoros savignyi*, was previously found to be active against only Gram-positive bacteria.[22] The C-terminal half (named Os, 22 amino acids) of this defensin and its cysteine-free analogue, Os-C (19 amino acids), were bactericidal to Gram-positive and Gram-negative bacteria in the low micromolar range, and nontoxic to mammalian cells at concentrations as high as 100 μM.[22] Investigations into their bactericidal modes of action revealed that both peptides could translocate into the cytosol of bacteria.[35] In addition, Os and Os-C were identified to possess antioxidant,[22] anti-endotoxin and anti-inflammatory properties.[36] Using overlapping peptide sequences of Os, two shorter peptide analogues, Os(3–12) and Os(11–22) of 10- and 12-amino acid residues, respectively, with Gram-positive and Gram-negative antibacterial activity were identified.[37] C-terminal amidation of Os(3–12) resulted in enhanced activity against Gram-positive and Gram-negative bacteria. Likewise, amidation of Os(11–22) also presented with improved activity although activity was reduced against *Staphylococcus aureus*.

In this study, 16 peptide analogues derived from the parent OsDef2 defensin were screened for antifungal activity using *C. albicans* as a model microorganism. To gain a better understanding of their modes of action, the most active peptides Os, Os-C and Os(11–22)NH_2 were investigated for their potential interaction with cell wall polysaccharides, effects on the cell membrane, translocation into cells and induction of intracellular ROS production. The research presented in this study showed that the peptides appear to interact with cell wall polysaccharides, Os and Os(11–22)NH_2 are membrane acting and the uptake of Os and Os-C and, to a lesser degree, Os(11–22)NH_2, into *C. albicans* cytosol, occurs via an energy-dependent pathway. Although the three OsDef2 analogues induce ROS production, only ROS generated by Os-C and to a lesser extent Os(11–22)NH_2, correlates with antifungal activity.

2 MATERIALS AND METHODS

2.1 *C. albicans* growth and culture conditions

C. albicans (ATCC 90028) was grown aerobically in yeast peptone dextrose (YPD) medium at 28°C. To obtain *C. albicans* cells in the mid-logarithmic phase, the cells were grown for 16 h, diluted 100 times in YPD broth and proliferated until an OD_{600} of 0.5 was reached.

2.2 Preparation of synthetic peptides

The peptides Os, Os-C, their overlapping 10-mer fragments as well as 5-carboxyfluorescein (5-FAM)-labelled Os, Os-C, Os(11–22)NH_2 and penetratin were obtained from GenScript (New Jersey, USA), while Os(3–12), Os(11–22) and their respective amidated forms, Os(3–12)NH_2, and Os(11–22)NH_2 were purchased from LifeTein (New Jersey, USA). The purity (GenScript >95%; LifeTein 95%) and molecular mass of the peptides were determined using reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry, respectively. Dithiothreitol (10 nmol) was added by the supplier to Cys containing peptides (Table 1) prior to lyophilization. Melittin (≥85% purity), a well-known AMP with lytic and ROS inducing properties, was purchased from Sigma Aldrich, South Africa (SA), and served as a positive control for membrane permeabilization and ROS studies. Stock peptide solutions were prepared in sterile deionized double distilled water (dddH_2O) and stored at −20°C. Peptide concentrations were determined as described previously.[36]

TABLE 1. Physicochemical properties and activities of selected peptide derivatives

PEPTIDE	SEQUENCE	CHARGE	MW (G/MOL)	MIC (MM)	MFC (MM)
OS	KGIRGYKGGYCKGAFKQTCKCY	+6	2460	1.9 ± 0.1	6
OS(3–12)	IRGYKGGYCK	+3	1144	22.8 ± 0.0***	—
OS(11–22)	CKGAFKQTCKCY	+3	1380	>200***	—
OS(3–12)NH_2	IRGYKGGYCK-NH_2	+4	1143	15.4 ± 0.6***	—
OS(11–22)NH_2	CKGAFKQTCKCY-NH_2	+4	1379	4.4 ± 1.7	22
OS-C	KGIRGYKGGY_KGAFKQT_K_Y	+6	2151	2.2 ± 0.9	28
MELITTIN	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	+5	2,845	3.0 ± 0.2	2.6

Note: Omitted Cys residues are replaced with underscores. Residues in Os comprising the γ-core region ($X_{1–3}GXCX_{3-9}C$/ $CX_{3–9}CXGX_{1–3}$/$CX_{3–9}GXCX_{1–3}$) are underlined. MIC determined with the RD assay and data are means ± SE of three independent experiments in triplicate.

Minimum fungicidal concentration (MFC) determined with the colony-forming unit (CFU) assay, four independent experiments were conducted in triplicate.

*** Minimum inhibitory concentration (MIC) of peptide is significantly higher ($p < 0.001$) than that of Os.

2.3 Radial diffusion assays

OsDef2 derivatives were initially screened for anticandidal activity by a radial diffusion (RD) assay as described by Lehrer et al.[38] with minor modifications. *C. albicans* was grown in 10-ml YPD for 16 h at 28°C. The culture was centrifuged for 10 min at 2000*g*, washed twice and resuspended in 10-mM sodium phosphate (NaP) buffer, pH 7.4. A volume containing 6.6×10^5 colony-forming units (CFUs) was added to 10 ml of a previously autoclaved underlay of agarose gel containing 1% (w/v) low electroendosmosis type I agarose gel (Lonza, Walkersville, USA) and 0.02% (v/v) Tween 20 (Sigma Aldrich, SA) prepared in NaP buffer and poured into 9-cm petri dishes. Wells of 4 mm, in diameter, were punched and subsequently filled with 10-µl peptide (25–200 µM) or $dddH_2O$. Following a 3-h incubation at 37°C, 7 ml of an overlay agarose gel containing 6% (w/v) tryptic soy broth (Sigma-Aldrich, SA) and 1% (w/v) Low-EEAOI prepared in $dddH_2O$, was poured over the bottom agarose layer. After 18 h of incubation at 28°C, the diameter of the clear zones around the wells were measured and the minimum inhibitory concentration (MIC) of the peptides was determined as described previously.[39]

2.4 CFU assays

To determine the minimum fungicidal concentration (MFC), the CFU assay method described by Nordin et al.[40] was followed with some modifications. Briefly, 90 µl of mid-logarithmic phase *C. albicans* (10^6 CFU/mL) in NaP buffer were incubated with 10 µl of $dddH_2O$ or serially diluted peptides (1.2–500 µM) at 28°C for 2 h. To detect a 3-log reduction (99.9% killing) of viable *C. albicans* cells, the untreated sample was first diluted 1000 times in NaP/YPD(10% [*v/v*]) buffer and plated out onto YDP agar while the treated cells were plated undiluted. After a 48 h incubation period, the minimum peptide concentration that allowed growth of less colonies than the untreated sample was retained as the minimum

fungicidal concentration (MFC), here defined as the lowest peptide concentration that resulted in 99.9% killing of *C. albicans*.

2.5 Time-killing assays

Mid-logarithmic phase *C. albicans* cells were incubated at the MFC of each peptide, as described in Section 2.4, for 0, 10, 30, 60 and 120 min. The cultures, both treated and untreated, were then diluted 500-fold in NaP and plated onto YDP agar. The plates were incubated at 28°C for 48 h, and the viable cells were counted. Effective killing time was defined as the time (min) required to kill 100% *C. albicans* cells.

2.6 Binding of peptides to cell wall component assays

The ability of Os, Os-C and Os(11–22)NH_2 to bind to cell wall polysaccharides with a subsequent loss of anticandidal activity was determined as previously described by Han et al.[12] In brief, peptides (100 μM) were pre-incubated with 40 mg/mL of the representative cell wall constituents laminarin (β-1,3 glucan polymer, Sigma-Aldrich, SA) and mannan (mannose polymer, Sigma-Aldrich, SA) for 1 h at 37°C. The effect of polysaccharide binding on peptide activity was then assessed by the RD assay as described in Section 2.3.

2.7 Liposome leakage assays

Liposomes were manufactured and permeabilization assayed similar to what has been described by Strömstedt et al.[41] with minor alterations. In brief, dry lipid films of *Saccharomyces cerevisiae* polar lipid extract with 30% ergosterol, were formed on round-bottom flask walls. This was achieved by dissolving lipids in chloroform and evaporating the solvent under agitation and nitrogen gas flow, followed by incubation in vacuum chamber. Lipid films were re-suspended by 30 min stirring at 55°C in an aqueous solution of 100-mM 5(6)-carboxyfluorescein in 10-mM Tris buffer (pH 7.4 at 37°C). Suspensions were subjected to repeated extrusion through a 100-nm mesh size polycarbonate membrane to reduce multilamellar structures and polydispersity. Un-trapped carboxyfluorescein was removed by gel filtration. Membrane permeability was measured by monitoring carboxyfluorescein efflux from the liposomes to the external low concentration environment, resulting in loss of self-quenching and an increased fluorescence signal at an excitation and emission wavelengths of 497 and 520 nm, respectively. Plates (96-well) were prepared with a two-fold serial dilution of the peptides in Tris buffer, as well as controls without peptides (background) and 0.16% Triton X-100 (maximum leakage). The plates were pre-heated to incubation temperature (37°C) and administered liposome solution, to a final lipid concentration of 10 μM in 200 μl. The effects of each peptide concentration on the liposomes were monitored for 45 min, at which point peptide induced leakage had largely plateaued.

2.8 Localization of 5-FAM-peptides in fungal cells

To determine whether Os, Os-C and Os(11–22)NH_2 can enter *C. albicans* cells, the peptides were labelled with 5-FAM and cellular localization was observed with confocal fluorescence microscopy as described previously.[42] Mid-logarithmic phase *C. albicans* (10^6 CFU/ml) were exposed to 2.5 μM of 5-FAM-Os, 5-FAM-Os-C, 5-FAM-Os(11–22)NH_2 and 5-FAM-penetratin for 50 min at 28°C in a shaking incubator. Penetratin, which can cross the membrane of *C. albicans*,[43] was used as a positive control. The cells were then immobilized for 70 min onto poly-l-lysine coated cover glass slides that were placed in wells of a 24-well

Cellstar polystyrene plate (Greiner Bio-One GmbH, Austria). The cells were rinsed with NaP buffer, counterstained with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, SA) for 30 min before the cover glass slides were mounted with polyvinyl alcohol mounting medium with DABCO® (Sigma Aldrich, SA). Confocal images were acquired with Zeiss LSM 880 Confocal laser scanning microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) at an excitation and emission wavelength of 490 and 520 nm, respectively.

2.9 Mechanism of translocation assays

For energy depletion experiments, cells were pre-incubated with 5-mM NaN_3 in 10-mM NaP buffer for 30 min at 37°C before the addition of peptides. The effects of azide on the antifungal activity of labelled peptides (2.5 μM) and unlabelled peptides (100 μM) were evaluated by confocal microscopy and by RD assays, respectively. To assess the effect of polysaccharide interaction on peptide translocation, the peptides were pre-incubated with laminarin (40 mg/ml) for 1 h at 37°C.

2.10 Measurement of cellular reactive oxygen species production

ROS formation in *C. albicans* was measured using the fluorogenic and cell-permeable probe, 2′, 7-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, SA). Mid-logarithmic phase *C. albicans* cells were resuspended in NaP buffer containing 20-μM DCFH-DA and incubated for 30 min at 37°C. Cells were collected by centrifugation and further resuspended in NaP buffer. Peptides (1.56–50 μM) alone or with 10-mM ascorbic acid were then added to cells (10^6 CFU/mL) and incubated for 1 h at 37°C. Fluorescence was read at excitation and emission wavelengths of 485 and 535 nm, respectively, while antifungal activity was determined by the CFU assay.

2.11 Data management and statistical analysis

Unless otherwise stated, the experiments were performed at least three times in triplicate. Data normality was tested using the Shapiro–Wilk normality test ($p < 0.05$). Further analysis was then conducted with either the Student *t* test (one-tailed) for MIC determination and ROS assays and the GraphPad two-way ANOVA with the Tukey multiple comparisons test for the effect of cell wall components and azide treatment on antifungal activity.

3 RESULTS

3.1 Antifungal activity of OsDef2 derived peptides

Initial screening of six shorter peptides derived from OsDef2 for antifungal activity with RD assays against *C. albicans* identified five active peptides (Table 1). Of these, peptides Os, Os-C and Os(11–22)NH_2 showed the highest activity with MIC close or equal to 4 μM. Interestingly, whereas Os(11–22) showed no activity even at 200 μM, amidation of the same peptide bestowed a MIC of 4.4 μM. In contrast, amidation of Os(3–12) only increased its antifungal activity by approximately 1.5-fold. Next, the candidacidal activity of the three most active peptides was determined using CFU assays. The candidacidal activity was in the order of Os > Os(11–22)NH_2 > Os-C with corresponding MFC values of 6, 22 and 28 μM, respectively. A kinetics study of the activity for Os, Os-C and Os(11–22)NH_2 at their respective MFC values was conducted (Figure 1). Both Os and Os-C eradicated 100% of *C. albicans* cells within 30 min while Os(11–22)NH_2 required 60 min.

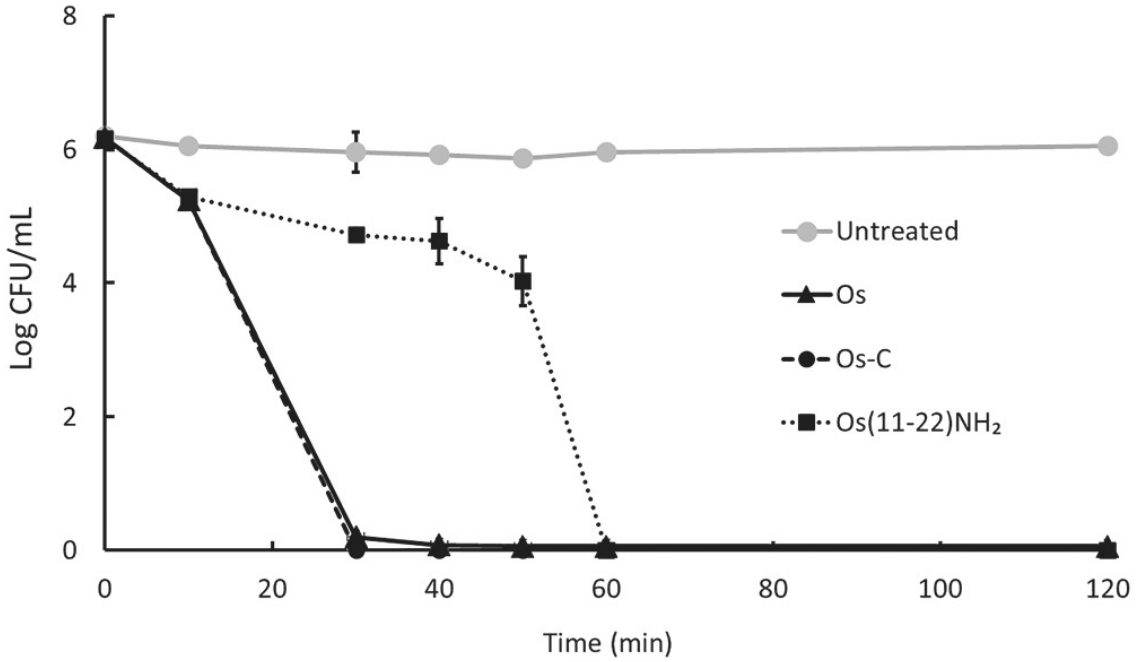

FIGURE 1. Killing kinetics of Os, Os-C and Os(11–22)NH_2. *Candida albicans* cells were incubated with Os, Os-C and Os(11–22)NH_2 at their respective MFC of 6, 28 and 22 μM. *C. albicans* viability was determined by the colony-forming unit (CFU) assay and compared with a growth control. Data are means ± SEM of two independent experiments, performed in triplicate

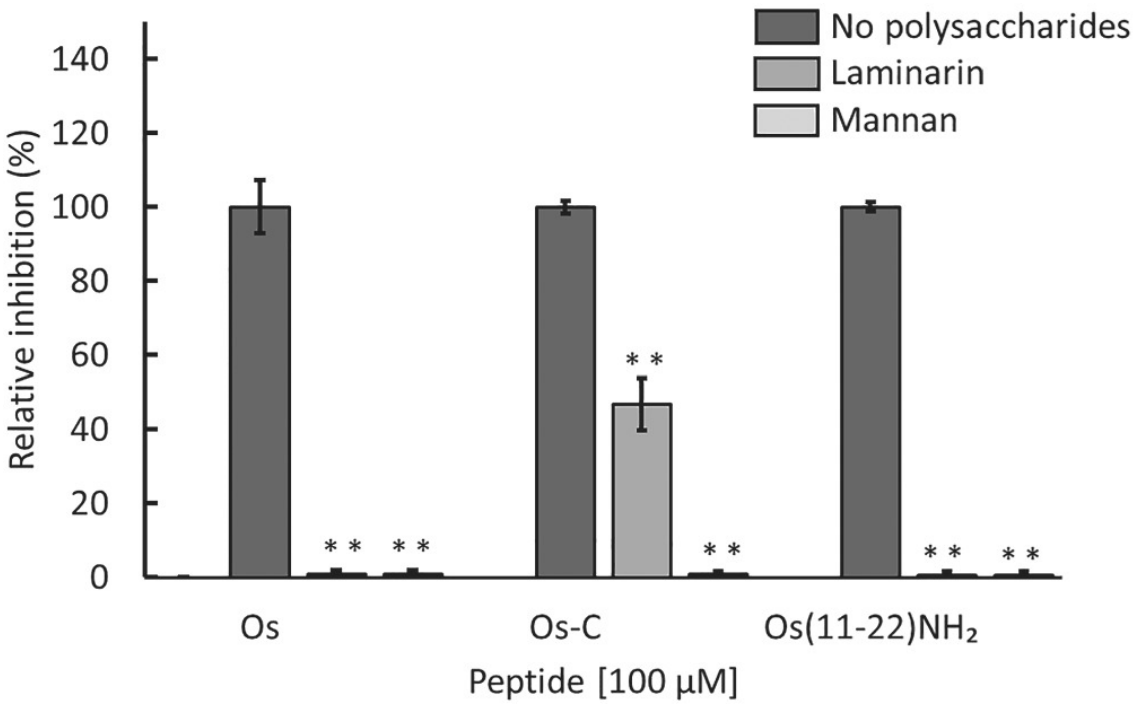

FIGURE 2. Effect of cell wall polysaccharides on the antifungal activity of peptides. Os, Os-C and Os(11–22)NH_2 at 100 μM were pre-incubated with 40 mg/ml laminarin or mannan for 1 h. Antifungal activity was determined by the radial diffusion (RD) assay. Data are means ± SEM of at least two independent experiments, performed in duplicate.** indicates statistically significant difference ($p < 0.01$) to peptide as a percentage of no polysaccharide added

3.2 Interaction of Os, Os-C and Os(11–22)NH_2 with cell wall polysaccharides

Initial binding of peptides to cell surface polysaccharides or glycosylated proteins is crucial for the antifungal activity of some AMPs.[43] We thus sought to investigate the possibility of such interaction with Os, Os-C and Os(11–22)NH_2 by assessing the inhibitory effect of 40 mg/ml laminarin (β-1,3 glucan polymer) and/or mannan (mannose polymer) on the antifungal activity evaluated by RD assays. The antifungal activity of Os and Os(11–22)NH_2 at 100 μM was completely suppressed following pre-treatment with both mannan and laminarin (Figure 2). Os-C at 100 μM also lost all activity after mannan pre-treatment, whereas its activity was only partially reduced following pre-treatment with laminarin. These results could indicate that the peptides interact with β 1,3-glucan and mannose residues to exert their antifungal activity before cell entry. It could also indicate that these cell wall components act as a peptide scavenging matrix that confers some level of protection to the cell from the peptides. None of the polysaccharides showed inherent antifungal activity.

3.3 Membrane permeabilization activity

Peptide-based antifungal agents may have specific targets in either the cell wall, cell membrane or within the cytosol. Biocidal peptides of ribosomal origin (i.e., AMPs) frequently act by disrupting the outer membranes of the cell types they have evolved to primarily interact and perforate, a mechanism that is typically not dependant on specific molecular affinity.[12, 44] Therefore, membrane permeabilization activity was evaluated using large unilamellar liposomes of a generic fungal lipid composition, *S. cerevisiae* polar lipid extract with 30% ergosterol. The peptide concentration that generated 50% liposomal leakage at 45 min (EC_{50}) for the benchmark membrane disrupting peptide melittin[45] was 0.5 μM (Figure 3). For melittin, being one of the most potent and broad-spectrum membrane disrupting peptides, this EC_{50} corresponds well with its EC_{50} on bacterial and mammalian liposome systems, which in turn correlates with its bioactivity on these cell types.[46] Both Os and Os(11–22)NH_2 exhibited membrane permeabilizing activity, in a sigmoidal dose-dependent manner, with EC_{50} values of 4 and 12 μM, respectively. This correlates with MFC values of 6 and 22 μM determined for Os and Os(11–22)NH_2, respectively, and the faster killing kinetics determined for Os compared with Os(11–22)NH_2. The Os-C peptide was the least potent of the three peptides, reaching approximately 30% leakage at 20 μM. Fungal membrane permeabilization by Os and Os(11–22)NH_2 can be considered as their main mechanism of action on candida cells. On the other hand, it is doubtful that the less pronounced lytic properties of Os-C alone can be responsible for the in vitro activity seen with this peptide. However, although the current liposome system attempts to mimic a fungal membrane, there are obvious limitations in direct comparisons with a living candida membrane.

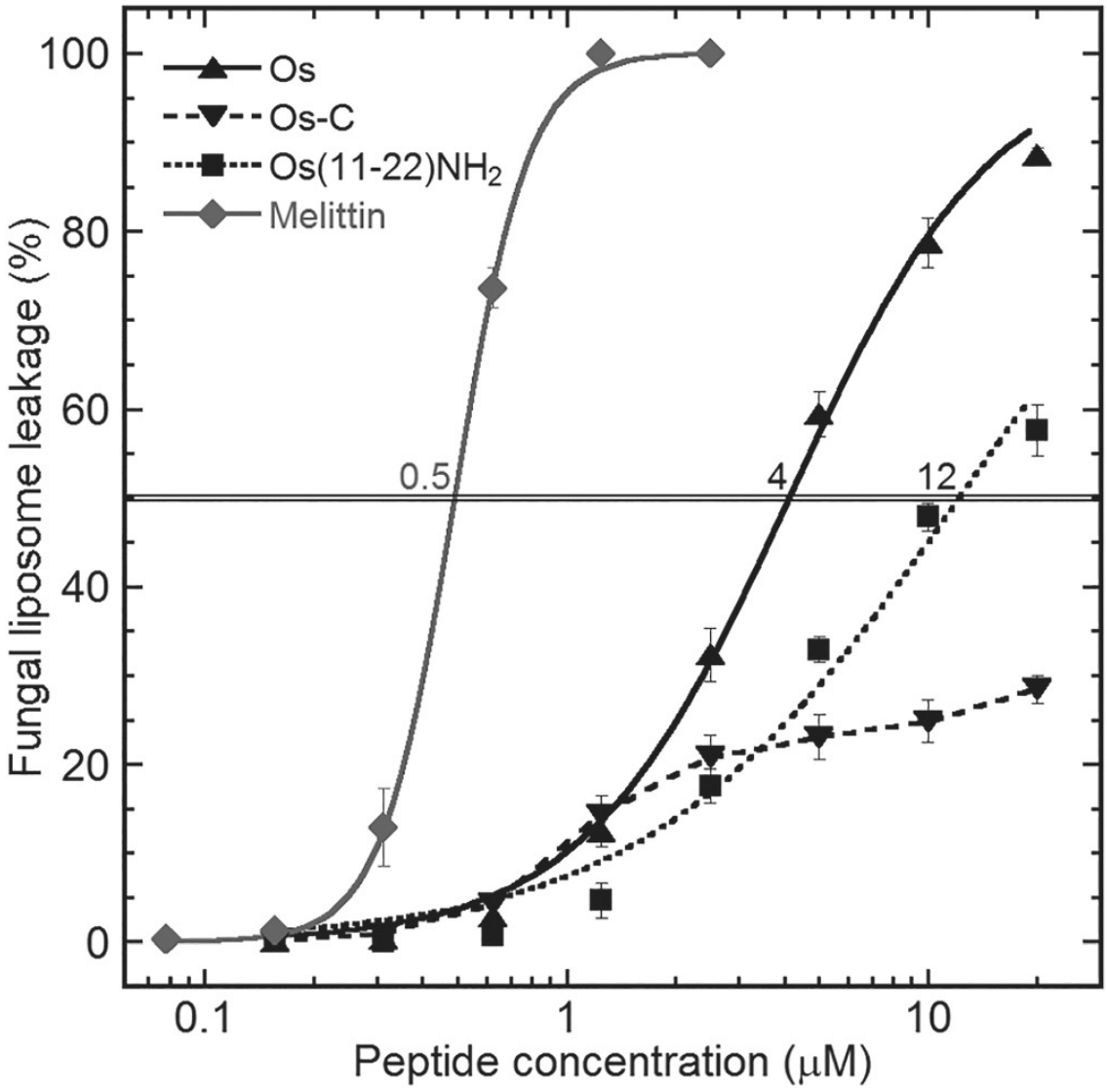

FIGURE 3. Fungal membrane permeabilization quantified by the release of carboxyfluorescein at 45 min of peptide incubation. The fungal plasma membrane model is liposomes composed of *Saccharomyces cerevisiae* polar lipid extract with 30% ergosterol. Each marker represents the mean leakage at 37°C in 10-mM Tris buffer (pH 7.4) with standard deviations from three experiments done at individual peptide concentrations, that is, no cumulative additions. Sigmoidal dose–response curves are fitted when $R > 0.95$, that is, not for Os-C. The EC50 level is highlighted with a double line and their respective values indicated when applicable

3.4 Translocation of peptides into fungal cells

To determine whether the peptides translocate across membranes, the peptides were labelled with fluorescent 5-FAM and cellular localization was observed with confocal fluorescence microscopy. The cells were counterstained with DAPI, a double stranded DNA-binding dye that stains both viable and non-viable cells.[47] After 50 min of incubation, the positive control penetratin, Os, Os-C and Os(11–22)NH_2 accumulated within the cytosol (Figure 4, also Figure S1). 5-FAM-labelled Os and Os(11–22)NH_2, like penetratin, stained both the membrane and the cytosol uniformly. For Os-C, a few cells showed strong staining throughout the cell while most cells showed strong staining only at specific regions within the cytoplasm. Interestingly, Os-C and Os(11–22)NH_2 treated cells appeared flocculated. DAPI

staining of cells treated with all three Os Def2 peptides revealed some evidence of nuclear fragmentation encountered during apoptosis.[48] This included the appearance of irregular and fragmented DNA (Figure 4, short white arrow, also Figure S2) for all three peptides as well as some tubular-shaped DNA (Figure 4, long white arrow) for Os and Os-C treated cells.

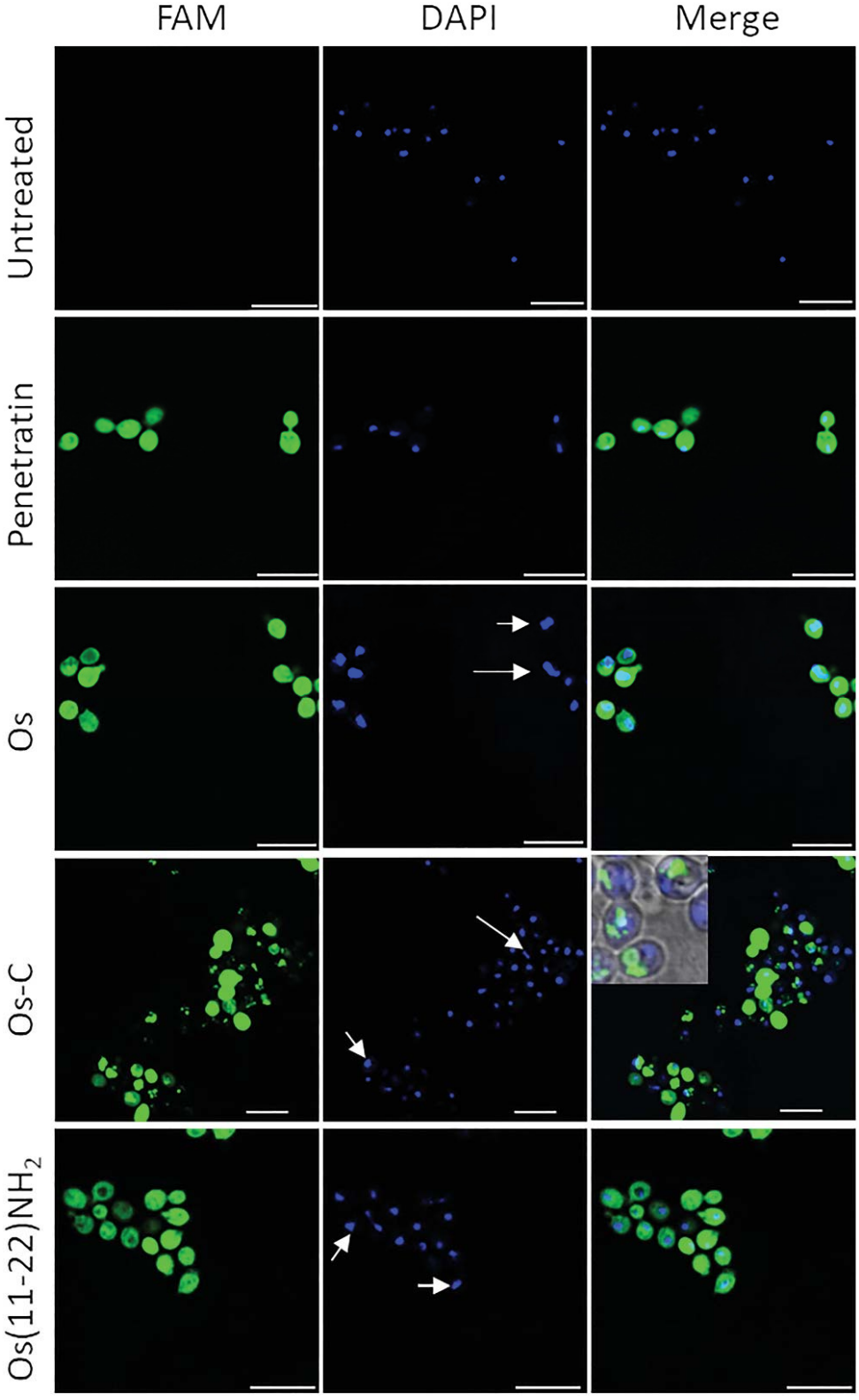

FIGURE 4. Localization of 5-FAM-peptides in *Candida albicans*. Cells were exposed for 50 min to 2.5 μM of 5-FAM-peptides (green), counterstained with DAPI (blue) and visualized by confocal microscopy. Cells treated with the peptides exhibit both irregular, fragmented nuclear DNA (short white arrow) and tubular-shaped DNA (long white arrow) in contrast to single and round nucleus observed in untreated cells. The box in the merged image section of 5-FAM-Os-C shows the transmitted light image of 5-FAM Os-C bound separately to the nucleus. Images are representatives of three independent experiments, in duplicate. Scale bars are 10 μm

3.5 Mechanism of peptide translocation into fungal cells

To investigate whether peptide translocation involves endocytosis, the translocation process was performed using conditions that inhibit ATP synthesis.[49] Concurrently, we sought to determine the link between reduced ATP availability and the antifungal activity of these peptides. *C. albicans* cells were pre-incubated for 30 min with 5-mM NaN_3 and then exposed to 100 μM of each peptide for antifungal activity determination with RD assays (Figure 5A) or incubated for 50 min with 2.5 μM of each 5-FAM-peptide for localization studies (Figure 5B). The RD assay results revealed that the antifungal activity of Os was significantly decreased ($p < 0.05$) by ATP depletion (Figure 5A) and the peptide was located on the membrane periphery of most cells (Figure 5B). In contrast to Os, the antifungal activity of Os(11–22)NH_2 was unaffected by ATP depletion (Figure 5A), and although at lower intensity, the peptide was still present in the cytoplasm (Figure 5B). For Os-C, its antifungal activity was completely suppressed ($p < 0.01$) (Figure 5A) and no staining of cells with labelled Os-C was observed (Figure 5B), which is in line with its lower membrane activity on liposomes (Figure 3). These results suggest that the uptake of Os and Os-C and, not, Os(11–22)NH_2 is energy dependent.

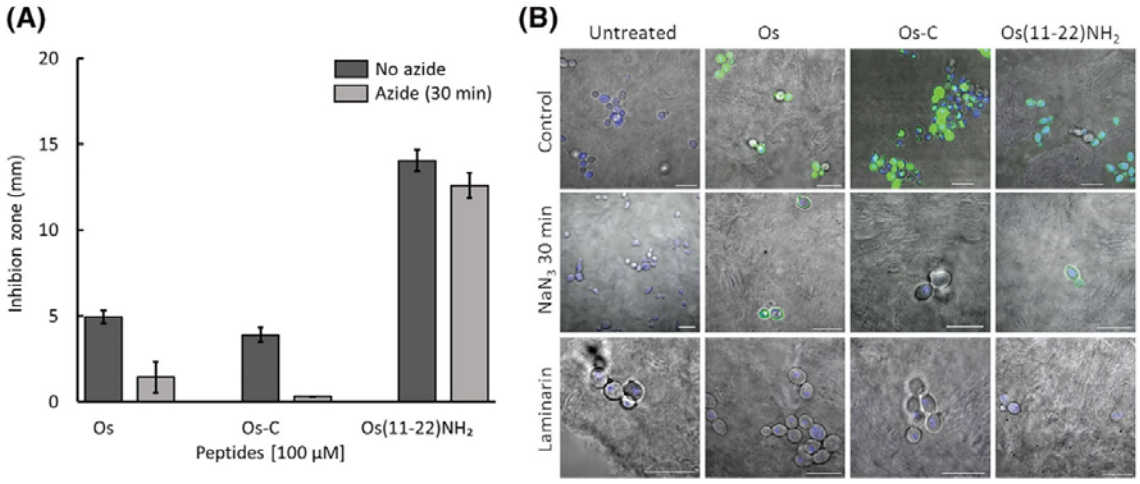

FIGURE 5. (A) Effect of sodium azide on antifungal activity. Radial diffusion (RD) assay was performed by loading 5 μl of 100-μM peptides into the wells of agar plates containing *Candida albicans* cells that were pre-treated for 30 min with 5-mM NaN_3. Data are means ± SEM of at least two independent experiments, performed in duplicate. Asterisks indicate statistically significant difference (**$p < 0.01$) to no NaN_3 added. (B) Mechanism of peptide translocation. *C. albicans* cells were pre-incubated with 5-mM NaN_3 for 30 min and treated with the labelled peptides for 50 min (second panel) or cells were exposed for 50 min to a pre-incubation mixture of 2.5-μM 5-FAM-peptides (green) and 40 mg/ml laminarin (third panel). The cells were counterstained with DAPI (blue) and visualized by confocal microscopy. Images are representatives of three independent experiments, in duplicate. Scale bars are 10 μm

To assess the effect of polysaccharide interaction on peptide translocation, the labelled peptides were pre-incubated with laminarin before incubation with cells. Confocal microscopy revealed that the interaction between the peptides and free laminarin resulted in

no intracellular accumulation of the labelled peptides (Figure 5B). These results imply that the peptides interact with the β-1,3-glucans. These branched polysaccharides could play a role in peptide cell entry or in direct antifungal activity, likewise it could indicate that such polysaccharides convey a scavenging effect on AMPs so that the level of free peptide is depleted before reaching the cytoplasmic membrane.

3.6 Reactive oxygen species induction in fungal cells

ROS formation is a potential mode of action for AMPs on *C. albicans*.[50] For example, ROS production and subsequent apoptosis has been observed following translocation of defensins into the cell.[14, 49] Therefore, ROS was quantified for 1 h following exposure to 25 μM of each peptide using the DCFH-DA assay. A time-dependent increase in ROS was observed for all peptides (Figure 6A). Lower levels of ROS were produced in Os-C and Os(11–22)NH_2 treated cells compared with the control melittin and Os (Figure 6B). To determine whether ROS production is a key player in antifungal activity, the ability of the antioxidant, ascorbic acid, to reduce ROS was evaluated at 25 μM peptide. There was a statistically significant decrease ($p < 0.05$) in peptide-induced ROS formation in the presence of ascorbic acid (Figure 6B). However, colony count assays revealed that there was only a significant ($p < 0.05$) loss in antifungal activity for Os(11–22)NH_2 and Os-C but not for melittin nor Os (Figure 5C). Interestingly, the reduction in activity of Os-C was approximating 90% loss, suggesting that ROS production might possibly be a main mechanism of action for Os-C but only a secondary effect of Os activity.

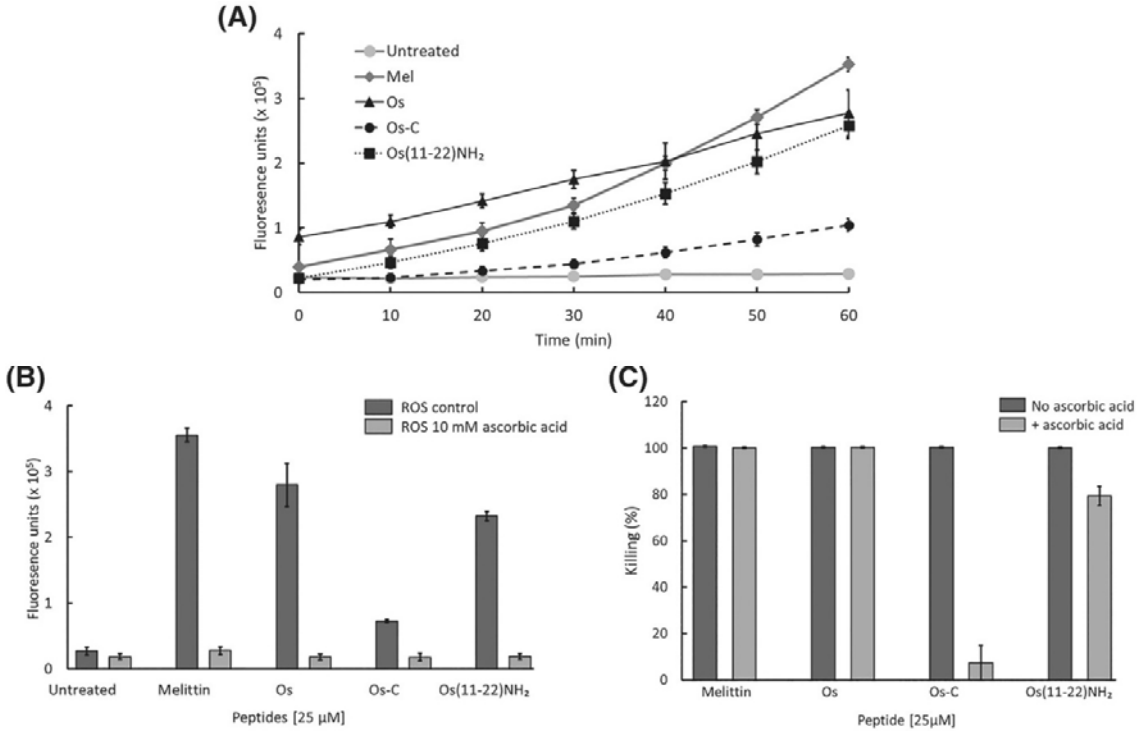

FIGURE 6. ROS levels in *Candida albicans* following exposure to peptides. (A) Cells were exposed to DCFH-DA (20 μM) and monitored over 1 h after 25-μM peptide administration. (B) ROS levels reached at 1 h with 25-μM peptide with or without 10-mM ascorbic acid, before recording the increase in fluorescence emission. (C) Cells were incubated with the combination in (B), and the colony-forming unit (CFU) assay was performed. Data are means ± SEM of three independent experiments, performed in triplicate. Asterisks indicate statistically significant decrease (*$p < 0.05$; **$p < 0.01$) relative to treatment in the absence of ascorbic acid

4 DISCUSSION

Many of the current antifungal drugs targeting *C. albicans* have shown limitations, such as undesirable side effects and notable inefficiency towards drug-resistant emerging strains.[3] AMPs are suitable alternatives in the treatment of *Candida*-related infections due to their broad-spectrum of activity and specific membrane targets, which render fungal pathogens less likely to develop resistance. However, the high cost of AMP synthesis hinders their therapeutic application, thus the identification of shorter peptides from lead compounds, that retain antifungal activity is the focus of much research.[34, 51] Our previous study showed that tick defensins are promising templates for shorter AMPs.[22] Whereas the defensin isoform, OsDef2, was bactericidal against only Gram-positive bacteria, Os derived from the carboxy-terminus of OsDef2 and its analogue, Os-C, were bactericidal against both Gram-positive and Gram-negative bacteria. Therefore, we first undertook to screen OsDef2 derivatives Os and Os-C, along with 10-mer shorter peptides spanning their respective sequences for antifungal activity. Starting with the 10-mer *C*-terminus sequence of Os termed Os(13–22), two *N*-terminus and *C*-terminus amino acids were respectively added and deleted at a time, to generate further derivatives. A 12-mer sequence termed Os(11–22) was also included to contain all three C-terminal Cys residues. For Os-C, the *C*-terminus sequence Os-C(9–18) was the starting point (Table S1).

Charge and hydrophobicity that govern the initial interaction of AMPs with cell membranes are two of the main parameters that contribute to peptide antifungal activity.[52] As expected, both peptides Os and Os-C with the highest positive charge (+6) were the most potent, exhibiting the lowest MIC values (Table 1). Among the seven overlapping 10-mer peptides derived from Os, the MIC could only be determined for Os(3–12) using a threshold concentration of 200 μM. This finding identified that the sequence 3 to 12 of Os, is essential for antifungal activity. Because additional Os fragments such as Os(1–10), Os(5–14), Os(9–18) and Os(13–22), all with an equal charge to Os(3–12) of +3 were not active against *C. albicans* (Table S1), additional structural features might be required for the antifungal activity of OsDef2 derivatives. One such feature could be the presence of the functional region or γ-core motifs of Cys-stabilized proteins, $X_{1–3}GXCX_{3-9}C/CX_{3-9}CXGX_{1–3}/CX_{3–9}GXCX_{1–3}$ as observed for the defensins brazzein and charybdotoxin.[53] Os, which contains the full γ-core motif, and a + 6 charge, was more potent than all its derivatives. However, Os(3–12) containing an equal charge of +3 but fewer Os residues comprising the γ-core region than the peptides Os(7–16) and Os(11–20) was more potent than the latter peptides. This implies that components of the γ-core motif alone cannot be used to predict the antifungal activity of these OsDef2 derivatives. In line with this assumption, Os-C, which lacked all three Cys residues present in the γ-core motif, was more potent than any of the Os-derived 10-mer fragments. Noteworthy, the fact that none of the shorter 10-mer peptides derived from Os-C was active against *C. albicans* (Table S1) might suggest chain length as an important factor, in the absence of Cys residues, for the activity of OsDef2 derivatives.

Many naturally occurring bioactive molecules, including AMPs are carboxy-amidated, an important modification necessary for functionality and in addition stability against exopeptidase activity is increased.[54] Functional effects are increased peptide charge[32] and the promotion of helix formation.[55] The contribution of carboxy-amidation on the interaction of CM15, a α-helical, 15 amino acid peptide on a DPPG model bilayer was investigated by Ma et al.[56] Carboxy-amidation resulted in the rapid adsorption of CM15 onto the surface of a negatively charged DPPG model bilayer, resulted in the disruption of the outer layer and less ordered orientation of insertion. Carboxy-amidation can have variable effects on the

antimicrobial activity of peptides. For example, the activity of PGLa-NH_2 Gram-negative bacteria was 8- to 16-fold greater than PGLa-COOH[35] and the activity of Hb33-61a and Hb40-61a against *C. albicans* was four-fold greater when compared with Hb33-61 and Hb40-61 analogues.[57] In contrast, in other instances, carboxy-amidation has no effect as was reported for MSI-103-NH_2 against Gram-negative bacteria compared with MSI-103-COOH[35] and the antifungal activity of ANP-NH_2 compared with ANP-COOH.[57]

Carboxy-amidation increased the net charge of Os(3–12) from +3 to +4, with a slight effect on the MIC with a slight decrease from 22.8 ± 0.0 to 15.4 ± 0.6 μM. In contrast, the MIC of Os(11–22) increased with carboxy-amidation from >200 to 4.4 ± 1.7 μM to levels comparable to both melittin and Os. For many peptides, carboxy-amidation promotes helix formation, but this effect was not observed for either shorter analogue. Circular dichroism revealed that Os forms a α-helix and Os-C a β-sheet in a membrane mimicking environment, predicted to disrupt membrane structure by different models or modes of action reviewed by Nguyen et al.[58] The shorter analogues and the amidated versions of Os(3–12) and Os(11–22) are unstructured and random coiled, respectively.[37] The lack of propensity to form an α-helical structure may be related to the length of the peptides where the critical length for α-helical formation is 9–17 amino acids.[59] PMAP-36, is a naturally carboxy-amidated peptide of 36 amino acids. Shorter derivatives of 21, 18, 15 and 12 were α-helical in a membrane mimicking environment although when the chain length decreased to below 15 residues, activity against *C. albicans* was diminished and was not related to α-helical structure but rather a reduction of charge. For Os(3–12), shorter peptide length rather than increased charge may limit the membrane disruptive effect of carboxy-amidated Os(3–12). For Os(11–22)NH_2, secondary structure and length may also not contribute to the observed increase in activity. Liposome membranes indicate that this peptide is membrane active and that carboxy-amidation may favour the initial interaction between the peptide and the outer membrane prior to the disruption of membrane integrity through a mechanism that will be elucidated in future studies.

Nonetheless, these findings strongly validate carboxy-amidation as a simple and valuable method to confer or increase the antifungal activity of tick defensin derivatives. Thus, the antifungal activity of the parent peptides Os and Os-C as well as the amidated Os(11–22) was further investigated in terms of their MFCs (Table 1). Interestingly, the peptide Os (MFC of 6 μM) with only 22 residues exhibited antifungal activity similar to the 37 residues long LL-37 with an MFC of 10 μM under equivalent experimental conditions.[49] Similarly, Os(11–22)NH_2 and Os-C with only 12 and 19 residues, respectively, were only two- and three-fold less potent than LL-37.[49] These results point to the fact that Os, Os-C and Os(11–22)NH_2 are promising candidates as novel antifungals. Furthermore, the anticandidal activity of the peptides was fast-acting (Figure 1). The time to reach complete killing was 30 min for Os and Os-C and 60 min for Os(11–22)NH_2 and was determined at the MFC values of each peptide rather than the MIC values. Determination of killing time at the MFC provides information on therapeutically relevant killing rather than inhibition and provides insight into the mode of action where differences in killing time for Os and Os(11–22)NH_2 indicates a difference in the killing mechanism. In addition, with the haemolysis assay, a previous study showed that both Os(3–12)NH_2 and Os(11–22)NH_2 did not induce haemolysis compared with melittin.[37]

To reach the fungal plasma membrane, AMPs must cross a thick cell wall composed mainly of polysaccharides. In *C. albicans*, glucans account for 60%–65% while mannans make up for 20%–25% of the total polysaccharides.[60] Han et al.[12] found that the antifungal activity of Hst 5 and several of its derived hybrid fragments was highly dependent on its interactions

with β-1,3 glucans polymers but not with mannans. Therefore, the ability of Os, Os-C and Os(11–22)NH_2 to interact with laminarin (β-1,3 glucan polymer) and mannan (mannose polymer) was investigated. This was achieved by assessing the antifungal activity of the peptides in the presence of exogenous polysaccharides. The results suggest that, in addition to mannose residues, Os and Os(11–22)NH_2 strongly interact with *β*-glucans residues, whereas much less interaction occurs with Os-C, which may have a stronger affinity towards mannose residues. These findings are particularly noteworthy, considering that *β*-glucans become readily exposed during the infection process.[61] Therefore, the initial interaction of these peptides with cell wall polysaccharides may be necessary for their antifungal activity. Further experiments are currently underway in our laboratories to study these peptide-polysaccharide interactions and their requirements.

The fungal plasma membrane is the primary interaction site of antifungal defensins.[14, 28, 62] Most AMPs that permeabilize living membranes likewise disrupt both artificial bilayers and liposomes at low concentrations, thus providing a tool for investigating peptide–membrane interaction.[63] Therefore, the membrane permeabilizing activity of Os, Os-C and Os(11–22)NH_2 was investigated with a *S. cerevisiae* polar lipid extract. We found that the positive control, melittin, effectively disrupted the fungal liposomes as reported elsewhere.[46] Both Os and its shorter amidated analogue, Os(11–22)NH_2, exhibited distinct membrane disruptive activity on fungal liposomes, whereas Os-C had a less pronounced permeabilizing property (Figure 3). This finding identified membrane permeabilization as a mode of antifungal activity for Os and Os(11–22)NH_2, while for Os-C it appears this is a less relevant mode of action. This is corroborated by the EC_{50} of Os being three-fold lower than that of Os(11–22)NH_2, a ratio that corresponds well with the three-fold difference in the MFC of the same peptides. For melittin, a MIC of 3 μM and an EC_{50} of 0.5 μM identifies membrane disruption as predominant mode of action confirming the membrane effects of melittin.[46] However, for Os and Os(11–22)NH_2, these two values are reversed in that the EC_{50} are higher than their corresponding MIC. Although this could in theory result from the simplified nature of the liposome model compared with living cells affecting the Os-peptides disproportionally, the activity levels reached on the fungal liposomes in comparison to their MIC and MFC does not substantiate that membrane permeabilization is the sole mode of action. This is further corroborated by the longer time required by Os(11–22)NH_2 to completely eradicate *C. albicans* cells, indicating that additional cellular targets may also be involved in the antifungal activity of Os and Os(11–22)NH_2.

AMPs can also induce death through signalling cascades via the cell surface[13] or enter the cytosol[29] to interact with intracellular targets. This was observed with the plant defensin NaD1 (10 μM) which localized on the cell surface after 5 min, then translocated the *C. albicans* membrane gaining entry into the cytosol after 20 min where it induced ROS production from the mitochondrion inner membrane.[14] In this study, 2.5-μM 5-FAM-Os, like the control 5-FAM penetratin, caused uniform staining of the membrane and the cytoplasm of intact cells, indicating that Os can cross the cell membrane (Figure 4). Also, labelled Os(11–22)NH_2, at 2.5 μM, was present inside the cell while the cytoplasm of most cells treated with 5-FAM-Os-C was only partially stained. The presence of the peptides Os, Os(11–22)NH_2 and Os-C inside most cells should not be considered only as a result of cell lysis, as the peptide concentration used in this assay (2.5 μM) was equivalent to half, nine and eleven times less the MFC of Os, Os(11–22)NH_2 and Os-C, respectively, and would probably include both dead and living cells.

The liposome assay was primarily used to identify membrane disruptive activity. Besides the inadequacies of comparing a simplified model system with living cells, there are also peptide-to-lipid ratio and media constituents that can affect perceived AMP potency. Directly comparing concentration performance in liposome leakage with more complex cell culture systems is not straightforward. In our experience, liposome systems tend to appear more sensitive than their live cell counterparts.

AMPs can cross the membrane bilayers at sub-permeabilizing concentrations[64] and at 2.5 μM well below their EC50 on liposomes Os, Os(11–22)NH_2 and Os-C were found present within most *C. albicans* cells. AMP translocation can occur either through a direct mechanism or via endocytosis, an energy-dependent mechanism of cell penetration. Sodium azide impairs ATP synthesis in mitochondria by inhibiting cytochrome c-oxidase.[65] In this study, *C. albicans* cells were pre-treated for 30 min with 5-mM NaN_3 before peptide addition. Variable effects were observed for Os, Os-C and Os(11–22)NH_2 after 30 min exposure to NaN_3. The translocating ability of Os and Os-C like penetratin (Figure S1) was suppressed for most cells, while that of Os(11–22)NH_2 was unaffected. Similarly, the antifungal activity of Os was decreased, that of Os-C was suppressed completely while the activity of Os(11–22)NH_2 was unaffected. These findings indicate that Os-C entry into the cells most probably occurs via an energy-dependent mechanism while Os(11–22)NH_2 translocation follows membrane permeabilization. Os, on the other hand, seems to penetrate cells via both an energy-dependent pathway and following membrane permeabilization as evidenced by the presence of most cells with peripheral staining and few with cytoplasmic staining. Gong and Karlsson[42] found that the cell-penetrating peptides MAP and cecropin B showed contrasting results that were consistent with both direct as well as energy-dependent cell penetration. Future studies need to confirm, specifically if Os penetrates the cytoplasm by both membrane permeabilization and an energy-dependent mechanism. Furthermore, the staining pattern of the peptides after NaN_3 treatment, that is, peripheral staining for Os, no staining for Os-C and some staining for Os(11–22)NH_2, concurs with the finding that Os and Os(11–22)NH_2 binding to β-glucan residues was a possible requirement prior to membrane permeabilization, translocation and cell death.

The translocation of Os, its amidated analogue Os(11–22)NH_2, and Os-C into *C. albicans* cells strongly suggested that these peptides might also possess intracellular targets. Hence, the induction of ROS production was investigated. ROS are required for normal cellular function, however in excess can damage lipids, proteins and DNA as well as alter intrinsic membrane properties such as ion transport, fluidity and protein cross-linking, resulting in cell death.[66] ROS formation is increasingly reported as a mode of action of antifungal peptides,[14, 50] including the well-known membranolytic peptide, melittin.[67] The peptides melittin, Os and to a lesser degree Os(11–22)NH_2 and Os-C caused an increase in intracellular ROS levels, which for all peptides was significantly reduced in the presence of the antioxidant ascorbic acid (Figure 6). ROS production in *C. albicans* might occur following morphogenesis[68] or if intracellular potassium levels are low.[69] Therefore, it was necessary to determine if the scavenging of ROS with ascorbic acid would reduce the ability of the peptides to kill *C. albicans*. The results showed that Os-C and to a lesser degree Os(11–22)NH_2 but not Os, mediated a ROS-dependent killing. Many other plant defensins induce ROS in *C. albicans*, resulting in cell death such as the peptides NaD1 and RsAFP2.[14, 50] Moreover, ROS formation is a key event in the induction and regulation of apoptosis in yeasts.[70, 71] In the late stages of this process, DNA damage and chromatin condensation occur,[48, 72] and DAPI staining confirmed the occurrence of these events and thus apoptosis in some of Os, Os-C and Os(11–22)NH_2 treated cells. Melittin has also been found to trigger apoptotic events in *C. albicans*

through a ROS-mediated mitochondria/metacaspase-dependent pathway.[67] Likewise, for Os, Os-C and Os(11–22)NH_2, the apoptotic pathways need to be further elucidated in future studies.

In conclusion, this study has identified three promising antifungal peptides, Os, Os-C and amidated Os(11–22) from the *C*-terminus of a tick defensin isoform and has further substantiated the role of amidation on increasing the activity of shorter derivatives. Os and Os(11–22)NH_2 are predominantly membrane acting with possible targets in the fungal cell wall, while the killing effect of Os-C was mainly ROS-mediated.

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CONFLICT OF INTEREST

None.

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REFERENCES

1 Berman J, Sudbery PE. *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet*. 2002; 3(12): 918- 930.

2 Srinivasan A, Lopez-Ribot JL, Ramasubramanian AK. Overcoming antifungal resistance. *Drug Discov Today Technol*. 2014; 11: 65- 71. https://doi.org/10.1016/j.ddtec.2014.02.005

3 Denning DW, Bromley MJ. How to bolster the antifungal pipeline. *Science*. 2015; 347(6229): 1414- 1416.

4 Costa-de-Oliveira S, Rodrigues AG. *Candida albicans* antifungal resistance and tolerance in bloodstream infections: the triad yeast–host–antifungal. *Microorganisms*. 2020; 8(2): 154-172.

5 Vandeputte P, Ferrari S, Coste AT. Antifungal resistance and new strategies to control fungal infections. *Int J Microbiol*. 2012; 2012: 1- 26. https://doi.org/10.1155/2012/713687

6 Park S, Kelly R, Kahn JN, et al. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. *Antimicrob Agents Chemother*. 2005; 49(8): 3264- 3273.

7 Hope WW, Tabernero L, Denning DW, Anderson MJ. Molecular mechanisms of primary resistance to flucytosine in *Candida albicans*. *Antimicrob Agents Chemother*. 2004; 48(11): 4377- 4386.

8 Sangeorzan JA, Bradley SF, He X, et al. Epidemiology of oral candidiasis in HIV-infected patients: colonization, infection, treatment, and emergence of fluconazole resistance. *Am J Med*. 1994; 97(4): 339- 346.

9 Seo M-D, Won H-S, Kim J-H, Mishig-Ochir T, Lee B-J. Antimicrobial peptides for therapeutic applications: a review. *Molecules*. 2012; 17(10): 12276- 12286.

10 Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature*. 2002; 415(6870): 389- 395.

11 Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. *Clin Microbiol Rev*. 2006; 19(3): 491- 511.

12 Han J, Jyoti MA, Song H-Y, Jang WS. Antifungal activity and action mechanism of histatin 5-halocidin hybrid peptides against *Candida* ssp. *PLoS ONE*. 2016; 11(2): e0150196-e0150213.

13 Thevissen K, de Mello Tavares P, Xu D, et al. The plant defensin RsAFP2 induces cell wall stress, septin mislocalization and accumulation of ceramides in *Candida albicans*. *Mol Microbiol*. 2012; 84(1): 166- 180.

14 Hayes BM, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL. Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against *Candida albicans*. *Antimicrob Agents Chemother*. 2013; 3667-75(8): 3667- 3675.

15 Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*. 2003; 3(9): 710- 720. https://doi.org/10.1038/nri1180

16 Ganz T. Defensins and host defense. *Science*. 1999; 286(5439): 420- 421.

17 Chrudimská T, Chrudimský T, Golovchenko M, Rudenko N, Grubhoffer L. New defensins from hard and soft ticks: similarities, differences, and phylogenetic analyses. *Vet Parasitol*. 2010; 167(2–4): 298- 303.

18 Chrudimská T, Slaninová J, Rudenko N, Růžek D, Grubhoffer L. Functional characterization of two defensin isoforms of the hard tick *Ixodes ricinus*. *Parasit Vectors*. 2011; 4(1): 63- 71.

19 Johns R, Sonenshine DE, Hynes WL. Identification of a defensin from the hemolymph of the American dog tick, *Dermacentor variabilis*. *Insect Biochem Mol Biol*. 2001; 31(9): 857-865.

20 Lu X, Che Q, Lv Y, et al. A novel defensin-like peptide from salivary glands of the hard tick, *Haemaphysalis longicornis*. *Protein Sci*. 2010; 19(3): 392- 397.

21 Tsuji N, Battsetseg B, Boldbaatar D, et al. Babesial vector tick defensin against *Babesia* sp. parasites. *Infect Immun*. 2007; 75(7): 3633- 3640.

22 Prinsloo L, Naidoo A, Serem J, et al. Structural and functional characterization of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. *J Pept Sci*. 2013; 19(5): 325- 332. https://doi.org/10.1002/psc.2505

23 Wilmes M, Sahl H-G. Defensin-based anti-infective strategies. *Int J Med Microbiol*. 2014; 304(1): 93- 99.

24 Boniotto M, Jordan WJ, Eskdale J, et al. Human β-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob Agents Chemother*. 2006; 50(4): 1433- 1441.

25Jin G, Kawsar HI, Hirsch SA, et al. An antimicrobial peptide regulates tumor-associated macrophage trafficking via the chemokine receptor CCR2, a model for tumorigenesis. *PLoS ONE*. 2010; 5(6): e10993– e11006.

26 Tollner TL, Venners SA, Hollox EJ, et al. A common mutation in the defensin DEFB126 causes impaired sperm function and subfertility. *Sci Transl Med*. 2011; 3(92): 92ra65- 92ra87.

27 Roupé KM, Nybo M, Sjöbring U, Alberius P, Schmidtchen A, Sørensen OE. Injury is a major inducer of epidermal innate immune responses during wound healing. *J Invest Dermatol*. 2010; 130(4): 1167- 1177.

28 Thevissen K, Ferket KK, François IE, Cammue BP. Interactions of antifungal plant defensins with fungal membrane components. *Peptides*. 2003; 24(11): 1705- 1712.

29 Hayes BM, Bleackley MR, Anderson MA, van der Weerden NL. The plant defensin NaD1 enters the cytoplasm of *Candida albicans* via endocytosis. *J Fungi*. 2018; 4(1): 20- 34.

30 Nguyen LT, Chau JK, Perry NA, De Boer L, Zaat SA, Vogel HJ. Serum stabilities of short tryptophan-and arginine-rich antimicrobial peptide analogs. *PLoS ONE*. 2010; 5(9): e12684- e12691.

31 Zhao Y, Zhang M, Qiu S, et al. Antimicrobial activity and stability of the D-amino acid substituted derivatives of antimicrobial peptide polybia-MPI. *AMB Express*. 2016; 6(1): 122- 132.

32 Strandberg E, Tiltak D, Ieronimo M, Kanithasen N, Wadhwani P, Ulrich AS. Influence of C-terminal amidation on the antimicrobial and hemolytic activities of cationic α-helical peptides. *Pure Appl Chem*. 2007; 79(4): 717- 728.

33 Strömstedt AA, Pasupuleti M, Schmidtchen A, Malmsten M. Evaluation of strategies for improving proteolytic resistance of antimicrobial peptides by using variants of EFK17, an internal segment of LL-37. *Antimicrob Agents Chemother*. 2009; 53(2): 593- 602.

34 Lyu Y, Yang Y, Lyu X, Dong N, Shan A. Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and *Candida*. *Sci Rep*. 2016; 6(1): 27258- 27269.

35 Taute H, Bester MJ, Neitz AW, Gaspar AR. Investigation into the mechanism of action of the antimicrobial peptides Os and Os-C derived from a tick defensin. *Peptides*. 2015; 71: 179- 187.

36 Malan M, Serem JC, Bester MJ, Neitz AW, Gaspar AR. Anti-inflammatory and anti-endotoxin properties of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. *J Pept Sci*. 2016; 22(1): 43- 51.

37 Ismail NO, Odendaal C, Serem JC, et al. Antimicrobial function of short amidated peptide fragments from the tick-derived OsDef2 defensin. *J Pept Sci*. 2019; 25(12): e3223- e3231.

38 Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J Immunol Methods*. 1991; 137(2): 167- 173.

39 Ciornei CD, Sigurdardóttir T, Schmidtchen A, Bodelsson M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. *Antimicrob Agents Chemother*. 2005; 49(7): 2845- 2850.

40 Nordin SL, Sonesson A, Malmsten M, Mörgelin M, Egesten A. The epithelium-produced growth factor midkine has fungicidal properties. *J Antimicrob Chemother*. 2012; 67(8): 1927-1936.

41 Strömstedt AA, Kristiansen PE, Gunasekera S, Grob N, Skjeldal L, Goransson U. Selective membrane disruption by the cyclotide kalata B7: complex ions and essential functional groups in the phosphatidylethanolamine binding pocket. *Biochim Biophys Acta Biomembr*. 2016; 1858(6): 1317- 1327. https://doi.org/10.1016/j.bbamem.2016.02.013

42 Gong Z, Karlsson AJ. Translocation of cell-penetrating peptides into *Candida* fungal pathogens. *Protein Sci*. 2017; 26(9): 1714- 1725.

43 Wang K, Dang W, Xie J, et al. Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. *Biochim Biophys Acta Biomembr*. 2015; 1848(10): 2365- 2373.

44 Jang WS, Kim HK, Lee KY, Kim SA, Han YS, Lee IH. Antifungal activity of synthetic peptide derived from halocidin, antimicrobial peptide from the tunicate *Halocynthia aurantium*. *FEBS Lett*. 2006; 580(5): 1490- 1496.

45 Lee W, Lee DG. Fungicidal mechanisms of the antimicrobial peptide Bac8c. *Biochim Biophys Acta Biomembr*. 2015; 1848(2): 673- 679.

46 Strömstedt AA, Park S, Burman R, Göransson U. Bactericidal activity of cyclotides where phosphatidylethanolamine-lipid selectivity determines antimicrobial spectra. *Biochim Biophys Acta Biomembr*. 2017; 1859(10): 1986- 2000.

47 Hao B, Cheng S, Clancy CJ, Nguyen MH. Caspofungin kills *Candida albicans* by causing both cellular apoptosis and necrosis. *Antimicrob Agents Chemother*. 2013; 57(1): 326- 332.

48 Ordonez SR, Amarullah IH, Wubbolts RW, Veldhuizen EJ, Haagsman HP. Fungicidal mechanisms of cathelicidins LL-37 and CATH-2 revealed by live-cell imaging. *Antimicrob Agents Chemother*. 2014; 58(4): 2240- 2248.

49 Aerts AM, François IE, Meert EM, Li Q-T, Cammue BP, Thevissen K. The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. *J Mol Microbiol*. 2007; 13(4): 243- 247. https://doi.org/10.1159/000104753

50 Lv Y, Wang J, Gao H, et al. Antimicrobial properties and membrane-active mechanism of a potential α-helical antimicrobial derived from cathelicidin PMAP-36. *PLoS ONE*. 2014; 9(1): e86364- e86375.

51 Rahman MM, Tsuji N, Boldbaatar D, et al. Structural characterization and cytolytic activity of a potent antimicrobial motif in longicin, a defensin-like peptide in the tick *Haemaphysalis longicornis*. *J Vet Med Sci*. 2010; 72(2): 149- 156.

52 Yount NY, Yeaman MR. Multidimensional signatures in antimicrobial peptides. *Proc Natl Acad Sci U S A*. 2004; 101(19): 7363- 7368.

53 Nuijens T, Piva E, Kruijtzer JA, Rijkers DTS, Liskamp RMJ. Enzymatic C-terminal amidation of amino acids and peptides. *Tetrahedron Lett*. 2012; 53(29): 3777- 3779.

54 Mura M, Wang J, Zhou Y, et al. The effect of amidation on the behaviour of antimicrobial peptides. *Eur Biophys J*. 2016; 45(3): 195- 207.

55 Ma L, Luo Y, Ma L-H, Lu X. Interaction between antimicrobial peptide CM15 and a model cell membrane affected by CM15 terminal amidation and the membrane phase state. *Langmuir*. 2021; 37(4): 1613- 1621.

56 Machado A, Sforça ML, Mriand A, et al. Truncation of amidated fragment 33-61 of bovine α-haemoglobulin: effects on the structure and anticandidal activity. *Biopolymers (Peptide Science)*. 2007; 88(3): 413- 426. https://doi.org/10.1002/bip.20688

57 Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol*. 2011; 29(9): 464- 472.

58 Qin Z, Fabre A, Buehler MJ. Structure and mechanism of maximum stability of isolated alpha-helical protein domains at a critical length. *Eur Phys J E*. 2013; 36(5): 53. https://doi.org/10.1140/epje/i2013-13053-8

59 Sullivan PA, Yin CY, Molloy C, Templeton MD, Shepherd MG. An analysis of the metabolism and cell wall composition of *Candida albicans* during germ-tube formation. *Can J Microbiol*. 1983; 29(11): 1514- 1525.

60 Wheeler RT, Kombe D, Agarwala SD, Fink GR. Dynamic, morphotype-specific *Candida albicans* β-glucan exposure during infection and drug treatment. *PLoS Pathog*. 2008; 4(12): e1000227– e1000238.

61 Kim J-Y, Park S-C, Yoon M-Y, Hahm K-S, Park Y. C-terminal amidation of PMAP-23: translocation to the inner membrane of Gram-negative bacteria. *Amino Acids*. 2011; 40(1): 183- 195.

62 van der Weerden NL, Hancock RE, Anderson MA. Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process. *J Biol Chem*. 2010; 285(48): 37513- 37520.

63 Wimley WC. Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem Biol*. 2010; 5(10): 905- 917.

64 Noumi T, Maeda M, Futai M. Mode of inhibition of sodium azide on H+-ATPase of *Escherichia coli*. *FEBS Lett*. 1987; 213(2): 381- 384.

65 Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Aust J Bot*. 2012; 2012(217037): 1- 26.

66 Lee J, Lee DG. Melittin triggers apoptosis in *Candida albicans* through the reactive oxygen species-mediated mitochondria/caspase-dependent pathway. *FEMS Microbiol Lett*. 2014; 355(1): 36- 42.

67 Schröter C, Hipler U-C, Wilmer A, Künkel W, Wollina U. Generation of reactive oxygen species by *Candida albicans* in relation to morphogenesis. *Arch Dermatol Res*. 2000; 292(5): 260- 264.

68 Zhou X, Yin W, Doi SQ, Robinson SW, Takeyasu K, Fan X. Stimulation of Na, K-ATPase by low potassium requires reactive oxygen species. *Am J Physiol Cell Physiol*. 2003; 285(2): C319- C326.

69 Madeo F, Fröhlich E, Ligr M, et al. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biochem*. 1999; 145(4): 757- 767.

70 Munoz AJ, Wanichthanarak K, Meza E, Petranovic D. Systems biology of yeast cell death. *FEMS Yeast Res*. 2012; 12(2): 249- 265.

71 Dobrucki J, Darzynkiewicz Z. Chromatin condensation and sensitivity of DNA in situ to denaturation during cell cycle and apoptosis—a confocal microscopy study. *Micron*. 2001; 32(7): 645- 652.

72 Mangoni ML, Niv P, Barra D, et al. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of Escherichia coli. *Biochem J*. 2004; 380(3): 859- 865.