

Exploring the mode of action of an antimicrobial peptide, Os-C, in planktonic *Candida albicans* ATCC 90028

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Summary

Fungal pathogen resistance has increased rapidly in recent years, creating an urgent need for new antifungal drugs. Research has identified multifunctional antimicrobial peptides (AMPs) as promising candidates due to their broad-spectrum of cellular targets. The aim of this study was to further investigate the mode of action of a tick-derived AMP, Os-C, against planktonic *Candida albicans* (*C. albicans*).

Os-C has previously shown inhibitory activity against Gram-positive and -negative bacteria as well as planktonic and biofilm forms of *C. albicans* but activity was reduced in the presence of salts. Preliminary mode of action studies indicated that Os-C kills *C. albicans* cells within 30 minutes by interacting with cell wall polysaccharides, translocating to the cytosol in an ATP-dependent manner and inducing the production of reactive oxygen species (ROS).

For rapid screening and further mode of action studies of salt-sensitive AMPs a modified micro-broth dilution assay was developed. With this assay in 10% RPMI-1640 the minimum concentration of Amphotericin B (AmB; antifungal drug control) and melittin (peptide control) that inhibits 50% of planktonic *C. albicans* cells (MIC_{50}) was $0.5 \pm 0.06 \mu M$ and $0.98 \pm 0.14 \mu M$ respectively, and under these conditions the MIC_{50} of Os-C was $4.69 \pm 0.18 \mu M$.

The effect of AmB and Os-C at the MIC_{25} , MIC_{50} and MIC_{75} on the morphology and ultrastructure of *C. albicans* was determined with scanning (SEM) and transmission electron microscopy (TEM) respectively. AmB treated *C. albicans* presented with an irregular cell wall structure, budding scars, bud formation and eventual collapse of cellular structure at high concentrations. In contrast, Os-C caused cytoplasmic retraction, depression in cell surfaces, cellular extravasation, the formation of buds, small vacuoles adjacent to the cell membrane and intracellular granulation. The differences between the ultrastructural effects of AmB and Os-C indicate different modes of action.

The ability of Os-C to bind laminarin and mannan, components of the cell wall of *C. albicans*, was investigated with isothermal titration calorimetry (ITC). No binding occurred between Os-C and either of the polysaccharides at low, physiologically relevant concentrations. Using the modified micro-broth assay binding of Os-C ($20 \mu M$) to laminarin at 40 mM was observed. However, binding at such a high concentration of laminarin may not be of physiological value. The ability to induce membrane depolarization was evaluated using the probe, bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)]. Depolarization by Os-C was minimal compared with melittin, an AMP with well-described cell membrane depolarization and permeabilization activity.

Flow cytometry and confocal laser scanning microscopy (CLSM) with 5-FAM-labelled-Os-C and a vacuole specific dye, Cell tracker blue (CTr) was undertaken to evaluate uptake and effects on the vacuoles. Os-C was able to enter and successfully carry the fluorescent 5-FAM tag into the cells. Os-C accumulated intracellularly and caused moderate vacuolar break down independent of

concentration. This may be the result of ROS production, rather than the direct action of Os-C on the membrane or inhibition of associated biochemical processes.

In conclusion, this study showed that Os-C does not bind laminarin or mannan but accumulates on the cell membrane before translocating into the cytoplasm via ATP-mediated endocytosis. Os-C accumulates in the cytoplasm and induces ROS that cause moderate vacuole degradation. ROS production also leads to changes in cellular morphology, moderate membrane permeabilization and pore formation, small vacuole accumulation associated with the cell membrane, degradation of intracellular organelles and the formation of hyphal filaments. All these effects eventually lead to cell death probably due to apoptosis.

Keywords: Antifungal resistance, antimicrobial peptide, *Candida albicans*, mechanism of action, ROS production, ultrastructure, cell wall binding, membrane depolarization, intracellular targets.

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LIST OF ABBREVIATIONS

A

AFP	Antifungal peptide
AmB	Amphotericin B
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
ANOVA	One-way analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU	Absorbance unit

B

BIC	Biofilm inhibition concentration
-----	----------------------------------

C

CBM	Carbohydrate binding module
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
CMAC	7-amino-4-chloromethylcoumarin
CpMnBP1	Putative solute-binding peptide 1
CPP	Cell penetrating peptide
CTB	Cell titre blue

D

ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
DABCO	1,4-diazabicyclo[2.2.2]octane

E

<i>E. coli</i>	<i>Escherichia coli</i>
Em	Emission wavelength
EUCAST	European committee on antimicrobial susceptibility testing
Ex	Excitation wavelength
EV	Extracellular vesicles

F

FA	Formaldehyde
5-FAM	5-Carboxyfluorescein
FEG	Field emission gun

G

<i>g</i>	Gravity
GA	Glutaraldehyde

GABA	γ -aminobutyric acid
GFP	Green fluorescent protein
GlcNAc	β 1,4- <i>N</i> -acetylglucosamine
GNBP3	Gram-negative binding protein 3
GPI	Glycosylphosphatidylinositol
H	
<H>	Hydrophobicity
HAI	Hospital acquired infection
HBD	Human β -defensin
HGT	Horizontal gene transfer
HMDs	Hexamethyldisilazane
HNP	Human neutrophil peptide
I	
ITC	Isothermal titration calorimetry
K	
K _b	Binding affinity
M	
MFC	Minimum fungicidal concentration
MGE	Mobile gene element
MIC	Minimum inhibitory concentration
MW	Molecular weight
N	
NaP	Sodium phosphate buffer
nm	Nanometer
O	
OsDef	<i>Ornithodoros savignyi</i> defensin isoform
Os-C	Synthetic derivative of OsDef2 from C-terminus end by omitting 3 cysteine residues
OD	Optical density
P	
PBS	Phosphate buffered saline
PI	Propidium iodide
PMAP-23	Porcine myeloid antibacterial peptide
R	
RDA	Radial diffusion assay
ROS	Reactive oxygen species
rpm	Rotations per minute
RPMI	Roswell park memorial institute
RTD	Rhesus θ -defensin

S

SE	Standard error of the mean
SEE	Split-complementation endosomal escape
SEM	Scanning electron microscopy

T

TEM	Transmission electron microscopy
Trp	Tryptophan
Tyr	Tyrosine

V

VGSC	Voltage-gated sodium channel
v/v	Volume per volume

W

w/v	Weight per volume
-----	-------------------

Y

YPD	Yeast peptone dextrose
-----	------------------------

CHAPTER 1: LITERATURE REVIEW

1.1 Healthcare- associated infections

An ever-growing concern over the past few decades is the increase in nosocomial acquired fungal infections. Healthcare-associated infections (HAIs) do not only threaten the health of thousands of patients globally but also result in additional economic burdens to the patients and healthcare system including direct financial loss and prolonged hospitalization. The Centers for Disease Control and Prevention (CDC) have reported that nearly 1.7 million hospital patients suffer from HAI annually and that more than 98000 patients die in the United States of America (USA) alone (Haque *et al.*, 2018). The direct and indirect costs of HAIs in the USA are estimated to total \$97-147 billion annually. Dramowski *et al.* (2017) reported that in South Africa 22% of hospitalised children (1 in every 4.5) in the Northern Cape acquire HAIs which is similar to the 16.5% (1 in every 6) reported in Gauteng (Duse, 2005). The most common infections reported are pneumonia, septicaemia, urinary tract and skin infections (Lin-Hui *et al.*, 2003).

Reasons for the alarming increase in the incidence of HAIs may be an increase in the occurrence of many cancers and chronic illnesses, an increase in organ and bone marrow transplants as well as a growing need for medical implants especially in older patients (Perlroth *et al.*, 2007). Many of these patients are immune-compromised and therefore are easy targets for infection. In these patients nosocomial fungal infections can quickly turn into chronic infections which are difficult to treat because of their close association with biofilms (Zhao *et al.*, 2013). This leads to longer hospitalization periods, higher costs and increased mortality rates.

To reduce HAIs, all facilities are recommended to implement hygienic protocols such as sterilization of equipment, regular hand washing, monitoring air quality and ensuring overall sanitary conditions (Rabier *et al.*, 2008). However, the most challenging precautionary measure remains the controlled use of antibiotics and antifungals. The misuse of antimicrobial drugs leads to a substantial increase in antimicrobial resistance (AMR) and allows pathogens to proliferate uncontrollably.

1.2 Antimicrobial resistance

Due to their genetic flexibility microorganisms have a natural ability to withstand environmental challenges and the effects of harmful molecules, such as antimicrobial agents. Every time antimicrobial agents are used or misused, a selective pressure causes microorganisms to mutate or exchange DNA. These mutations collectively result in antimicrobial resistant microbes that can cause fatal infections which are difficult to prevent or treat (Levy, 2002).

According to the CDC (2013) every 10 minutes a patient in the USA or Europe dies because of ineffective antimicrobial drugs. These statistics are still undetermined for other countries but are

likely to be exceedingly higher in Africa and Asia. Consequently, the World Health Organization (WHO) recognised AMR as one of the major public health threats of the 21st century (WHO, 2014).

AMR allows microorganisms to thrive under unusual circumstances. Mutational resistance occurs when a susceptible microbial population contains a few cells with mutated genes that are resistant to the action of antimicrobial drugs. Subsequent exposure eradicates the susceptible cells leaving only the resistant mutants to proliferate (Woodford & Ellington, 2007). Mechanisms of resistance as summarized in Figure 1.1 include changes to the cellular targets (DNA, mRNA and protein), the development of efflux pumps to prevent uptake, inactivation of enzymes involved in drug metabolism and the use of alternative pathways (Ventola, 2015).

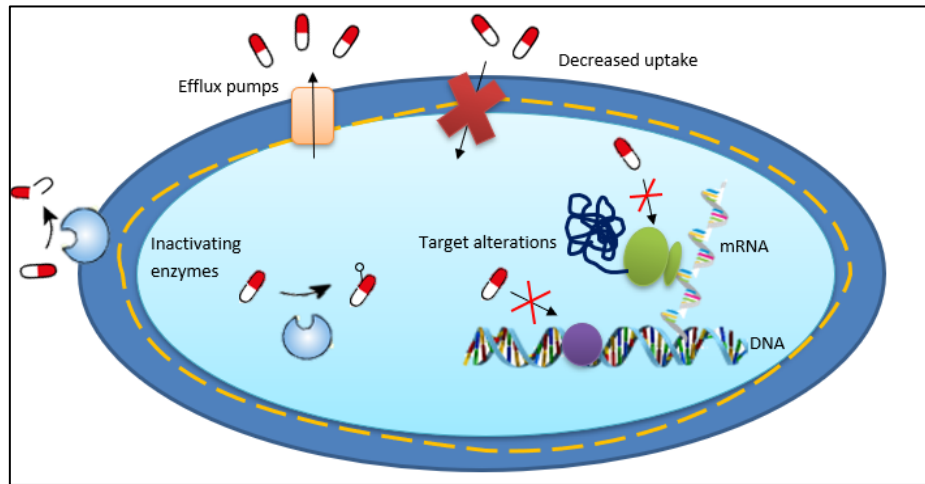


Figure 1.1: Mechanisms of antimicrobial resistance. Microorganisms can change intra- and extracellular targets, increase the expression of efflux pumps, decrease drug uptake, inactivate metabolic enzymes and alternate biochemical pathways, to resist the action of antimicrobial drugs. (Modified from: *Antibiotic resistance strategies in bacteria*, ReAct, 2015 used under CC-BY-NC 2.0 user license).

Resistance develops as a result of mutations in genes involved in the mechanism of action of antimicrobial drugs or through the acquisition of DNA that codes for resistance through horizontal gene transfer (HGT) (Munita & Arias, 2016). Resistance via HGT plays an important role in fungal evolution and is also responsible for antimicrobial resistance. The main strategy of HGT in hospital environments is conjugation, where genes are transferred by cell-to-cell contact and occurs at high rates when cells are faced with antimicrobial treatment. Mobile genetic elements (MGE) are the vehicles used to share genetic information between the cells during conjugation. The most common MGEs are plasmids and transposons which both play major roles in AMR development (Munita & Arias, 2016).

1.3 *Candida albicans* infections

One of the most common opportunistic yeasts that turn pathogenic in nosocomial settings is *Candida albicans* (*C. albicans*). This pathogen is a common component of the human gut microbiome and usually lives in a commensal relationship with the host (Coburn & Guttman, 2015). As an opportunistic yeast, *C. albicans* typically requires the host to be in an immunocompromised state before it can cause a systemic infection (Pfaller & Diekema, 2007). *C. albicans* infections

usually arise due to the ability of the yeast to attach to moist surfaces such as open wounds, the pelvic area and on artificial implants (Chandra *et al.*, 2001).

Candidiasis is a *Candida* infection that is caused by an overgrowth of *Candida spp.*, with *C. albicans* being the main causative agent (Table 1.1). *Candidiasis* causes minimal symptoms in healthy individuals such as itching, redness or discomfort, but can cause severe symptoms in immunocompromised individuals (Martins *et al.*, 2014).

Table 1.1: *Candida* species that cause *Candidiasis* (Adapted from Perltroth *et al.*, 2007)

Species	Percentage of cases
<i>C. albicans</i>	± 50%
<i>C. glabrata</i>	± 15-25%
<i>C. parapsilosis</i>	± 10-20%
<i>C. tropicalis</i>	± 15%
<i>C. krusei</i>	± < 3%
Others	± < 5%

Current treatments for *C. albicans* infections include topical antifungal agents such as nystatin, clotrimazole, amphotericin B (AmB), systemic oral azoles such as fluconazole, itraconazole or posaconazole or intravenous (IV) administration of caspofungin and micafungin (Bassetti *et al.*, 2018). Unfortunately, the efficacy of current treatments is limited due to the steady development of AMR that is associated with a 40% mortality rate (Singh & Chakrabarti, 2017). A consequence is that there is a dire need for new antifungal agents.

1.4 Antimicrobial peptides as potential alternatives for conventional therapies

The global antimicrobial resistance crisis led research to discover new potential antimicrobial agents that have several cellular targets making resistance development less probable. Antimicrobial peptides (AMPs) which are key components of the innate immune system of complex organisms are gaining increasing attention (Wang, 2010) due to their many advantages in treating resistant pathogens.

1.4.1 Properties and characteristics of AMPs

AMPs, also called host defence peptides, are short peptide molecules ranging from 5 to 100 amino acids in size. These peptides usually contain positively charged residues such as lysine and arginine with hydrophobic regions, making them amphipathic. The secondary structure of AMPs

varies greatly and can be α -helical, β -stranded, looped structures or combinations of these structures (Figure 1.2). AMPs are usually unstructured in solution and only fold into their final structure upon interacting with their target (Biljana & Havard, 2015).

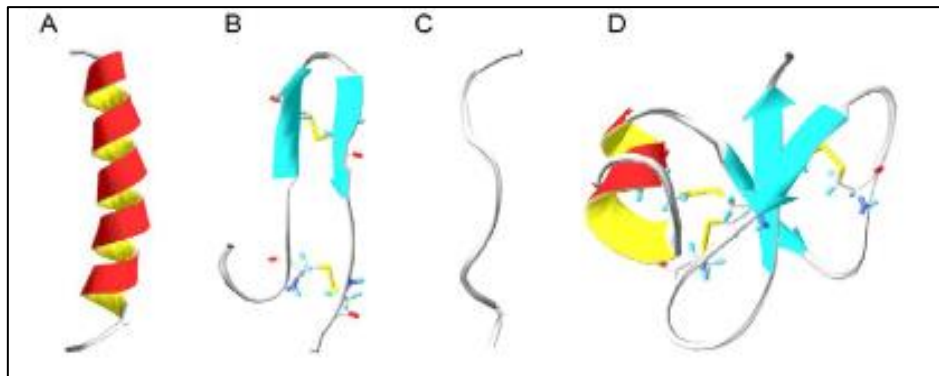


Figure 1.2: Four structural classes of AMPs. (A) α -helical structure, (B) β -pleated sheet, (C) extended structure and (D) mixed structure (By Biljana & Havard, 2015, used under CC-BY-NC 2.0 user license).

1.4.2 Mechanism of action of AMPs

Research into the mechanism of action of AMPs is essential for their development as therapeutic drugs. The mechanisms of action of AMPs can be divided into two categories: immune modulatory or direct killing (Kumar *et al.*, 2018). AMPs that can activate platelets, monocytes, lymphocytes and polymorphonuclear leukocytes to aid in pathogen clearance are said to be immunomodulating peptides (Jenssen & Hancock, 2010). Some of these immunomodulating peptides can be used as vaccine adjuvants due to the activation and attraction of immune cells. These AMPs have also been shown to reduce sepsis, stimulate angiogenesis and promote wound healing (Schuerholz, *et al.*, 2012; Biragyn, 2005; Mader and Hoskins, 2006 & Malan *et al.*, 2016).

The ongoing focus in this study was the direct killing mechanism of action and determining whether the peptide is membrane targeting, non-membrane targeting or both. Earlier studies suggested that direct killing by AMPs is due to membrane targeting and destabilization, but recently there is increasing evidence of additional intracellular targets (Raheem and Straus., 2019) such as the degradation of DNA, interrupting DNA or RNA synthesis, inhibition of RNA polymerase, degradation of vacuoles or causing mitochondrial dysfunction (Table 1.2).

1.4.2.1 Cell membrane targeting

The primary mechanism of action of most AMPs is the permeabilization of cell membranes through binding to negatively charged phospholipids on the outer membrane (Figure 1.3). Although anionic and cationic AMPs exist, most research focuses on cationic AMPs because of their ability to electrostatically interact with microbial membranes (Zasloff, 2002).

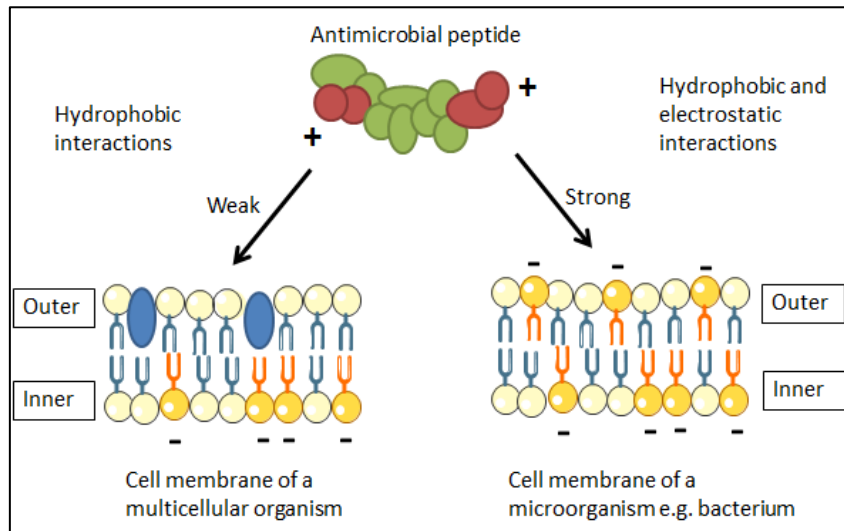


Figure 1.3: AMPs and their membrane binding specificity. This shows the cell membrane of a multicellular organism versus that of simpler organisms and the AMP specificity toward these membranes. The outer membrane of the bacterium is much more anionic than that of the multicellular organism, making it a target for AMP binding (Modified from: Molecular basis for membrane discrimination by antimicrobial peptides, Matsuzaki, 1999, used under CC-BY-NC 2.0 license).

The phospholipids in the bilayer membrane of microorganisms such as fungi are arranged with the polar phosphate groups on the outside, giving the cell an overall negative charge at physiological pH due to the presence of phospholipids such as phosphatidylserine and phosphatidylglycerol (Lodish *et al.*, 2008). In contrast, cell membranes of mammalian cells contain neutral phospholipids such as sphingomyelin, phosphatidylcholine and phosphatidylethanolamine. Thus, cationic peptides will selectively bind the cytoplasmic membrane of microorganisms at physiological pH. Whilst the cationic charges of AMPs allow them to interact with the polar membrane of these cells, the hydrophilic side chains allow them to insert into the membrane. This interaction destabilizes or depolarizes the membrane, making it more permeable (Zasloff, 2002).

Resistance development against membrane acting AMPs is less probable than against conventional antibiotics as microorganisms would have to rearrange their entire outer membrane in order to repel these lethal AMPs.

1.4.2.2 Intracellular targeting

Although most AMPs are membrane acting, many studies have demonstrated that some AMPs are able to exploit intracellular targets as a primary or supportive mechanism of killing. Examples of intracellular modes of action are shown in Table 1.2 and include the inhibition of nucleic acid synthesis, protein synthesis, enzyme activity and cell wall synthesis. Additional activities include immune modulation, ROS production, initiation of apoptosis, mitochondrial dysfunction and degradation of vacuoles (Lei *et al.*, 2019).

Table 1.2: Modes of action of intracellular targeting AMPs

AMP	Intracellular mechanism	Reference
Lactoferrin	Degrades DNA, increases ROS production	Andres <i>et al.</i> , 2008
Indolicidin	Inhibits DNA topoisomerase	Le <i>et al.</i> , 2017
Sordarin	Inhibitor of elongation factor – 2 during fungal protein synthesis	Dominguez <i>et al.</i> , 1998
Histatin 5	Induces oxidative stress and mitochondria malfunction	Puri & Edgerton, 2014
Microcin J25	Inhibits RNA polymerase	Le <i>et al.</i> , 2017
hGAPDH	Initiates apoptosis and inhibits proteases	Wagener <i>et al.</i> , 2013
Buforin II	Interrupts DNA and RNA synthesis	Park <i>et al.</i> , 1998

1.4.2.3 Additional functions and targets

Apart from their antimicrobial activity, AMPs have a broad range of additional functions. Some of these peptides can act as drug delivery vehicles, be used as anticancer agents, neutralize toxins and promote wound healing (Avila, 2016).

AMPs as drug delivery vehicles are a possible strategy that can improve antimicrobial treatment by enhancing cellular recognition and intracellular delivery of novel or conventional drugs (Gong *et al.*, 2017). These peptides are called cell penetrating peptides (CPPs) and have been extensively researched for the delivery of bioactive molecules into human cells. Several bioactive molecules such as nucleic acids, fluorescent labels, and antibodies have successfully been delivered intracellularly by CPPs (Stetsenko & Gait, 2000; Rajarao *et al.*, 2002 & Theisen *et al.*, 2006). CPPs can potentially increase the volume of drugs translocated into pathogen cells and new molecules that are membrane impermeable can now be explored. Table 1.3 shows CPPs that successfully transported bioactive molecules into fungal pathogen cells.

Table 1.3: CPPs that can translocate and carry molecules into *C. albicans* and *C. glabrata* (Gong *et al.*, 2017)

CPP	Net charge	Translocation mechanism	Vacuolar localization
Cecropin B	+9	Endocytosis/ Direct translocation	Low
Penetratin	+7	Endocytosis/ Direct translocation	Low
pVEC	+8	Macropinocytosis	Low
MAP	+5	Direct translocation/ Pore formation	Low
SynB	+6	Endocytosis	High

Gong *et al.* (2017) concluded that vacuoles play a notable part in CPP trafficking and that the translocation of CPPs into fungal cells may involve multiple mechanisms. Some of these translocation mechanisms may lead to the cytotoxicity observed for some peptides. Further research to investigate the translocation mechanisms of CPPs could improve their efficacy and toxicity features to make CPPs viable drug delivery vehicles.

1.4.3 Therapeutic applications of AMPs

Conventional antibiotics and antifungals normally have a specific mechanism of action that target intracellular structures or disrupt cellular processes without disturbing the cell membrane. Microorganisms have developed the ability to alter these specific targets or processes within the cell to avoid damage or death, thereby resisting common drug treatments (Figure 1.1).

Contrary to conventional antimicrobial drugs, AMPs have several targets including the cell membrane of microorganisms, making the attack non-specific and difficult to evade (Flamm *et al.*, 2015). They also originate from the innate immune system of organisms to protect against a broad spectrum of pathogens. Thus, this non-specific mechanism of killing of AMPs can be used to treat various bacterial, virus, fungal or parasitic infection and even cancer (Sarmasik *et al.*, 2002). Examples of AMPs and their various pathogen targets are listed in Table 1.4.

Table 1.4: Various AMPs and their targets

AMP	Target	Mechanism	Reference
Cecropins	Bacteria	Disrupts bacterial membrane integrity	Wang <i>et al.</i> , 2016
BMAP-12	Viruses	Destruction of viral RNA	Miao <i>et al.</i> , 2018
Rabbit defensin: NP-2	Fungi	Membrane permeabilization	Yeung <i>et al.</i> , 2011
SMAP-29	Parasites	Local membrane permeabilization	McGwire <i>et al.</i> , 2003
Temporin-1CEa	Cancer	Targets phosphatidylserine in cancer cell membranes	Wang <i>et al.</i> , 2016

Besides their antimicrobial activity, AMPs are multifunctional often with additional therapeutic benefits such as antioxidant activity (Park *et al.*, 1998). Examples of peptides with excellent antioxidant activity include several defensins derived from sweet potato (Huang *et al.*, 2012) and plantiricin A that protect cells against oxidative damage (Marzani *et al.*, 2012).

1.5 Antifungal peptides against fungal resistance

Current antifungal therapies are scant including only four chemical groups namely echinocandins, polyenes, triazoles and flucytosine. Antifungal peptides (AFPs) are AMPs active against fungal pathogens with several modes of action (Oshiro *et al.*, 2019). AFPs are thus promising candidates for the development of novel antifungal drugs.

1.5.1 Mode of AFP action

The majority of AFPs eliminate fungal pathogens through membrane-associated mechanisms of action. Fungal membranes differ from other eukaryotic membranes such as mammalian cells in terms of sphingolipid composition, polyimide content and ergosterol (Rautenbach *et al.*, 2016). Examples of membrane associated modes of action include increasing the permeabilization of

fungal membranes, decreasing membrane stability, impacting the membrane potential or direct pore formation (Van der Weerden *et al.*, 2010 & Moerman *et al.*, 2002).

In recent years, studies have also shown the capability of some AFPs to inhibit fungal biofilm formation or eradicate mature biofilms, where some AFPs have both inhibitory and eradication activities (Matejuk *et al.*, 2010). AFPs with biofilm inhibitory properties can potentially be used to coat medical devices, equipment and surfaces to prevent biofilm formation. AFPs with biofilm eradication properties can potentially be used to treat fungal biofilm infections or contaminations.

Several AFPs also have specific targets within fungal pathogens, including fungal proteins, DNA and RNA, mannosyldiinositol phosphorylceramide and glucosylceramide (Rautenbach *et al.*, 2016). These differences between fungal cells and eukaryotic cells provide insight into selecting AFPs for the treatment of human fungal infections, without risking cytotoxicity.

1.5.2 Different types of AFPs

AFPs have been identified in plants, invertebrates and mammals and can thus be grouped according to origin. AFPs can also be divided according to their functions (antimicrobial, antioxidant, immunomodulatory), amino acid sequences (linear, circular, looped), 3D structures (α -helix, β -pleated sheets, combinations) and modes of action (Wang, 2010).

1.5.2.1 Defensins

Defensins are a large family of evolutionary related AFPs that have been grouped together according to their structure. These peptides all contain six Cys residues linked by three disulphide bonds forming folded β -sheets (White *et al.*, 1995). Vertebrate defensins are further divided into subfamilies called α -defensins and β -defensins, depending on the length of peptide sequence between the Cys residues and how these Cys residues are linked. Both α - and β -defensins have a characteristic fold of triple-stranded β -sheets (Van Wetering *et al.*, 1999).

Later, a third subfamily of defensins called θ -defensins was identified with a distinct 3D structure. These defensins are generated by the splicing and cyclization of α -defensin precursors forming a circular structure. Although θ -defensins also contain six Cys residues, the way these residues are linked to each other differ greatly from both the α - and β -defensins (Ganz, 2003) (Figure 1.4).

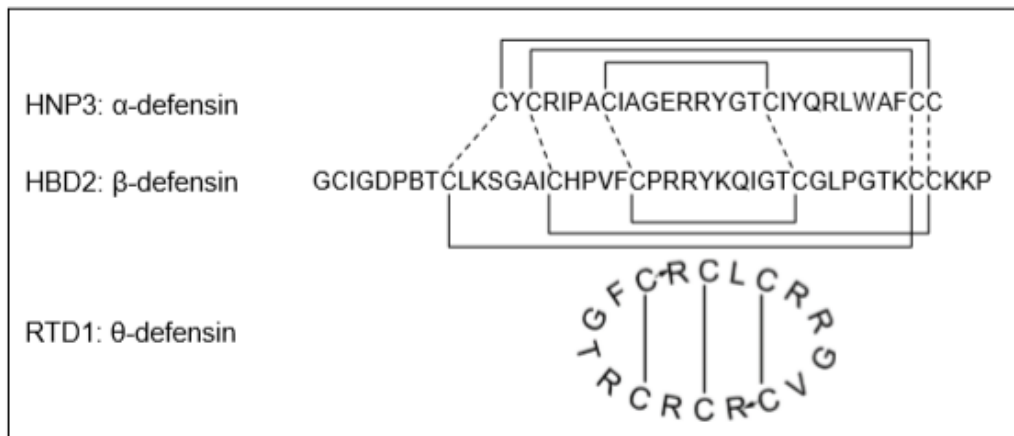


Figure 1.4: Sequences and the disulphide pairing of cysteines of α-, β- and θ-defensins (Ganz, 2003, used under CC-BY-NC 2.0 license). HNP3: human neutrophil peptide (α-defensin), HBD2: human β-defensin and RTD1: rhesus θ-defensin. Disulphide bonds between Cys residues are indicated by solid lines. Dash lines indicate matching Cys residues between the α- and β-defensin.

AFPs with defensin properties such as Cys rich sequences and disulphide bonds have also been identified in invertebrates and plants. These AFPs differ from each other and from vertebrate defensins in terms of structure. Insect defensins, for example, contain an α-helix linked to a β-sheet with disulphide bonds (Ganz, 2003).

1.5.5.2 Defensins active against *Candida*

Because of the similarity in overall 3D-structure of plant defensins, insect defensins and mammalian β-defensins, these peptides are believed to be ancient and conserved. It is possible that all defensins evolved from a single precursor molecule with the basic defensin structure (Thomma *et al.*, 2002). This suggests that defensins derived from a variety of organisms may all have similar targets and abilities, such as antifungal activity.

One example is the human oral cavity that is rich in AMPs, especially defensins, with potent anti-*Candida* activity. *C. albicans* is the main causative agent of oral *Candidiasis* in individuals with immunodeficiency. Human β-defensin 1 (HBD1) to HBD3 are expressed in salivary glands and excretions as well as other epithelial tissues. The most effective β-defensin identified to date is the HBD3 peptide, which is active against Gram-positive and -negative bacteria as well as fungi and certain viruses (Harder *et al.*, 2001).

Several θ-defensins found only in Old World monkeys, have also shown anti-*Candida* activity. The Rhesus θ-defensin 1 (RTD1) discovered in the neutrophils of rhesus macaque was screened against *C. albicans* and showed promising antifungal activity (Tongaonkar *et al.*, 2011).

Not only have the defensins derived from vertebrates been effective against *Candida*, but also some plant and insect-derived defensins. One specific plant defensin called HsAFP1, isolated from the seeds of coral bells, is known for its broad spectrum of activity against fungi including the human pathogen *C. albicans*. This defensin is non-toxic to plant and human cells which points to its potential for further drug development (Thevissen *et al.*, 2007).

In this study, however, the focus will be on the antifungal abilities of tick-derived defensins. As ticks are vectors for human arthropod-borne diseases, they rely greatly on AMPs to defend against pathogens. Numerous defensins that have been identified and isolated from hard and soft ticks proved to be bactericidal and fungicidal. An example is the novel defensin HIDS1 identified from the hard tick *Haemaphysalis longicornis*. This defensin showed lethal activity against microbes such as *Staphylococcus aureus* and the pathogen of interest *C. albicans* (Sun *et al.*, 2017).

1.6 Antifungal drug targets of *C. albicans*

Another challenge in treating resistant infections such as *Candidiasis* is the many similarities in the cell structure of eukaryotic fungal and mammalian cells. The distinct differences between fungal and mammalian cell structures play an important role in the development of novel antifungal drugs against resistant fungal infections. As the cell wall, membrane and fungal vacuoles are the potential targets investigated in this study the structure of these cellular components will be discussed in greater detail.

1.6.1 Cell wall

The *C. albicans* cell wall is a good antifungal drug target because of its importance in fungal pathogenicity by presenting virulence factors, antigenic cell wall proteins and polysaccharides, and serving as the connection between fungal-host interactions. A typical fungal cell (Figure 1.5) has a true nucleus, membrane-bound organelles and a cell wall consisting of mannoproteins, β -1,3/ β -1,6 glucans, chitin, and other polysaccharides (Free, 2013). The cell wall is a two-layered structure that makes up 40% of the fungal cell and is essential for fungal homeostasis. The inner layer consists of a conserved structural skeleton composed of chitin and glucans that can withstand the internal hydrostatic pressure of the cytoplasm and cell membrane (Gow *et al.*, 2017). The outer layer is more heterogeneous and consists of glycosylated mannoproteins with modified N- and O- linked oligosaccharides (Hobson *et al.*, 2004). Since the cell wall components are not present in mammalian cells, antifungal agents that target these components are more selective and less toxic and thus promising candidates for further development.

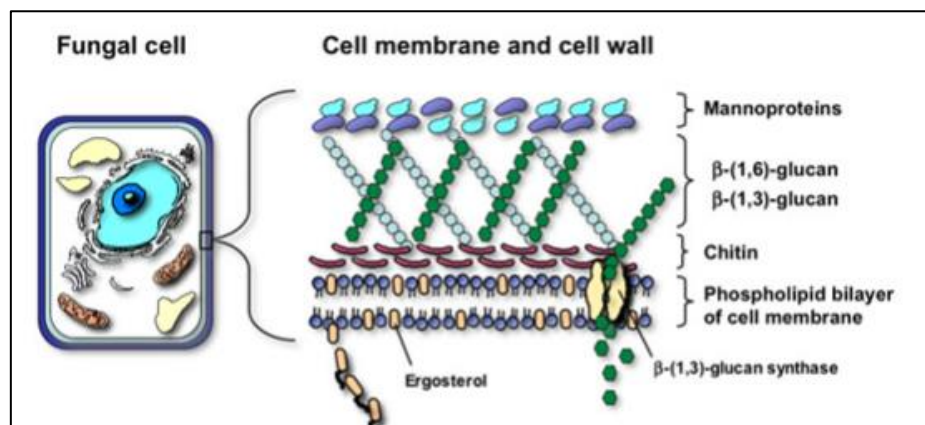


Figure 1.5: The general structure of a fungal cell (Thompson *et al.*, 2019, used under CC-BY-NC-ND 4.0 license). The fungal cell wall has two layers: The inner layer consists of chitin and β -1,3/1,6-glucans and the outer layer consists of mannoproteins.

1.6.1.1 Chitin

Within the inner layer of the cell wall a β -glucan-chitin skeleton is found (Figure 1.5), which is essential for the secure structure and shape of the cell (Gow & Hube, 2012). Chitin is a linear homopolymer of β -1,4-*N*-acetylglucosamine (GlcNAc) joined by β -1,4-linkages (Figure 1.6). It is an insoluble structure mainly composed of tight antiparallel hydrogen-bonded chains (Chaffin, 2008). The *C. albicans* cell wall contains 2-3% chitin under normal conditions, but the chitin content can increase in response to cell damage. It has been described that an increase in chitin content could be responsible for resistance against commercially available drugs (Lee *et al.*, 2012).

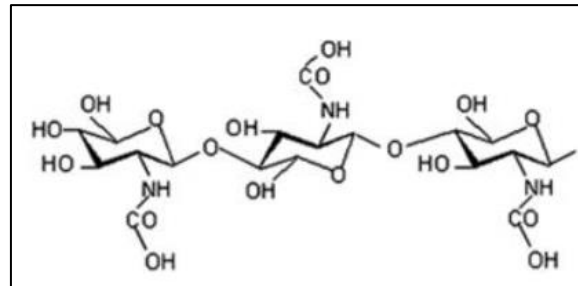


Figure 1.6: Chemical structure of the linear polysaccharide, chitin, found in the inner layer of *C. albicans* cell wall (Modified from: Chemical structures of chitin (a), β -1,3/1,6-glucan (b), a glycosyl phosphatidylinositol (GPI) protein (c) and CaPir1 (d), by Ruiz-Herrera *et al.*, 2006, used under CC-BY license).

1.6.1.2 Glucans

C. albicans cell walls contain only β -glucans, instead of α - and β -glucans, and they are the most abundant polysaccharides making up 58-60% of the inner cell wall. Glucans are glucose moieties linked with β -1,3 and/or β -1,6-glycosidic linkages (Figure 1.7). *C. albicans* cell walls contain both β -1,3- and β -1,6-glucans, but no mixed intrachain β -1,3/1,6 linkages. β -1,3-Glucan chains have a helical structure that could be single or triple helix stabilized by hydrogen bonds (Ruiz-Herrera *et al.*, 2006). The β -1,6-glucans connect the inner and the outer cell wall by binding β -1,3-glucans in the inner layer to various cell wall proteins (Klis *et al.*, 2001).

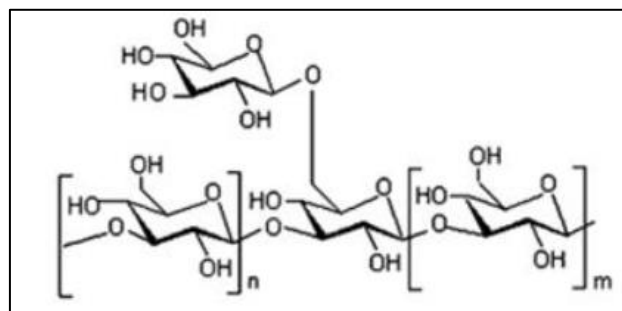


Figure 1.7: Chemical structure of β -1,3/1,6-glucan found in the inner layer of *C. albicans* cell wall. (Modified from: Chemical structures of chitin (a), β -1,3/1,6-glucan (b), a glycosyl phosphatidylinositol (GPI) protein (c) and CaPir1 (d), by Ruiz-Herrera *et al.*, 2006, used under CC-BY license).

1.6.1.3 Mannoproteins

The outer layer of *C. albicans* cell walls contains mostly mannoproteins that are glycosylphosphatidylinositol (GPI)-modified and linked to β -1,6- glucans (Shibata *et al.*, 2007). N-

linked mannans consist of α -1,6-mannose backbone and α -1,2-oligomannose sidechains capped with β -1,2-mono-, di-, tri- or tetra mannans. O-linked mannans are linked to cell wall glycoproteins (Shibata *et al.*, 2007). Mannans do not affect the overall cell shape as they are less rigid than β -glucans and chitin, but they do however increase the resistance of the cell to antifungal drugs due to their low permeability (Gow & Hube, 2012). It has also been reported that mannans play a role in immune evasion as it conceals β -glucans in the inner layers of the cell wall from host immune detection (Hernandez-Chavez *et al.*, 2017).

1.6.2 Cell membrane

Furthermore, eukaryotes such as fungi and mammalian cells both have a complex cell membrane that is important for structure, cell division, defence and metabolism. Sterols are the lipid polymers that make up 25% of eukaryotic cell membranes and are responsible for structure and stability of the cell. A key difference between mammalian and fungal cells is that mammalian cells contain cholesterol, whereas pathogenic fungal cells contain ergosterol (Figure 1.8). This difference in sterol type is a main target of antifungal agents such as azoles or polyenes that are currently in use (Gow *et al.*, 2017). An example is the antifungal drug, AmB, that selectively targets cell membranes containing ergosterol by binding with the sterol and forming pores in the cell membranes that lead to the rapid leakage of ions (Noor & Preuss, 2019).

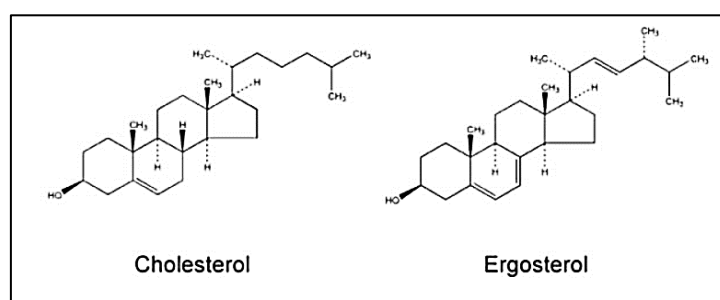


Figure 1.8: Chemical structure of cholesterol and ergosterol. These are two sterols found in eukaryotic cell membranes (Thompson *et al.*, 2019, used under CC-BY-NC-ND 4.0 license)

There are four different theories that describe how AMPs interact with microbial cell membranes: The aggregated model, the toroidal pore model, the barrel-stave model and the carpet model (Figure 1.9). The eventual outcome depends on the model of membrane interaction that a specific AMP uses (Jenssen *et al.*, 2006).

In the aggregate model (Figure 1.9A) AMPs insert perpendicularly into the membrane bilayer in a non-specific arrangement, resulting in the formation of pores that differ in size and lifetime. When these aggregates collapse, the peptides can translocate into the cell cytoplasm. The toroidal model (Figure 1.9B) is similar to the aggregate model in that the peptides insert perpendicularly into the membrane, but via a specific arrangement forming pores with a uniform size. These pores lead to cell leakage and eventual cell death. Peptides that use the barrel-stave model (Figure 1.9C) also arrange perpendicularly in the cell membrane but then form the staves of a barrel-shaped

pore. The lipid core will attract the hydrophobic regions of the peptide whilst the hydrophilic regions will face inwards, forming pores of uniform size.

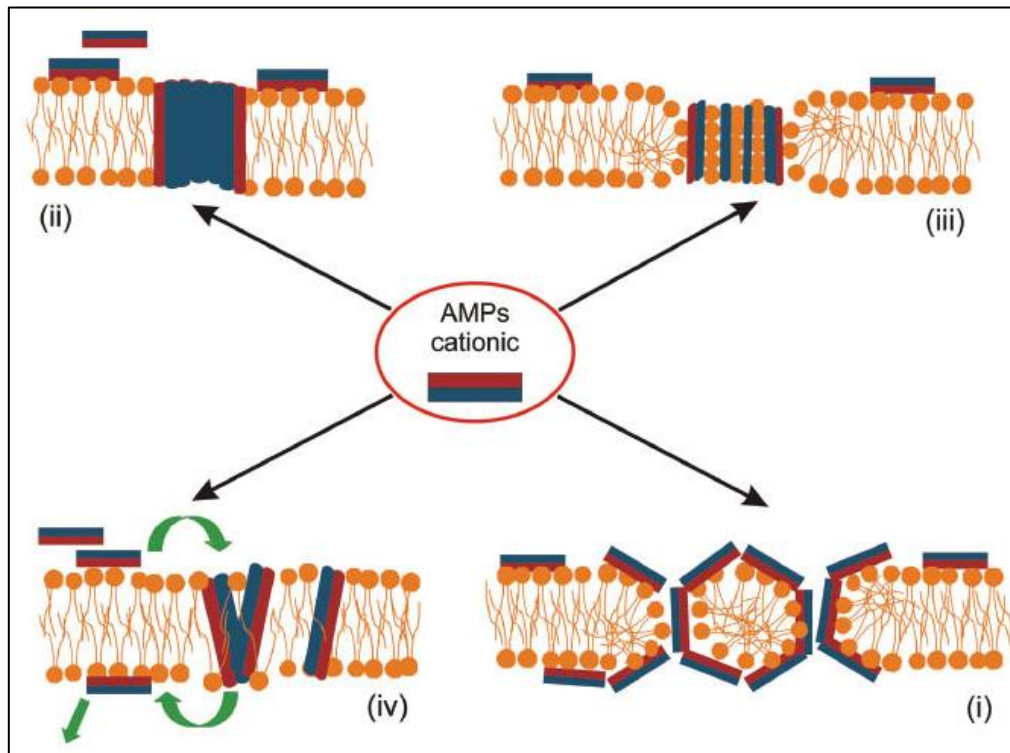


Figure 1.9: Models of AMP action on cell membranes. Micro-organism cell membrane is shown as a orange lipid bilayer with acting peptides in red (hydrophilic region) and blue (hydrophobic region). **(i)** Carpet model, **(ii)** Barrel-stave model, **(iii)** Toroidal pore model and **(iv)** Aggregate model (Modified from: Four basic models of cationic AMPs interaction with a PL bilayer (orange) by Cytryńska & Zdybicka-Barabas, 2015, used under CC-BY-SA license).

Lastly, peptides using the carpet model (Figure 1.9D) will aggregate on the cell surface until the concentration is high enough for detergent-like micelle formation. This model disrupts the membrane completely leaving big gaps in the bilayer resulting in irreversible cell leakage (Jenssen et al., 2006).

Another functional part of the *C. albicans* cell membrane is the existence of an electrostatic potential gradient (Figure 1.10). This potential gradient is created across the cell membrane due to an uneven distribution of ions on the inside and outside of the membrane (Arino et al., 2010). The transmembrane potential is regulated by protein transporters such as protein carriers or protein channels. Examples include α -helical protein channels, aquaporin, $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporters, $\text{Na}^+(\text{K}^+)\text{-ATPase}$ transporters, glucose transporters and many more (Arino et al., 2010). Antimicrobial agents that target these transporters, such as voltage-gated sodium channel (VGSC) blockers and γ -aminobutyric acid (GABA) (Kathwate et al., 2015) can disrupt the electrostatic potential gradient leading to increased concentrations of ions, a shortage of ions or disturbance of potential dependent processes such as glucose uptake, signal conduction or biofilm formation.

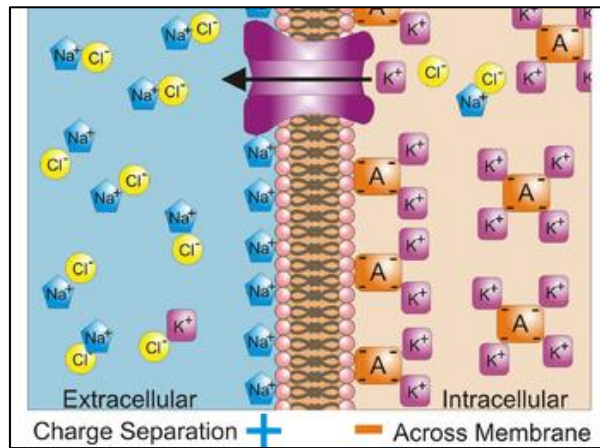


Figure 1.10: Electrostatic potential across the cell membrane due to membrane protein transporters. An AMP can disrupt transporters function and cause membrane depolarization (Bruce, 2014, used under CC-BY-NC).

1.6.3 Vacuoles

Another key difference between fungal and mammalian cells is the large vacuoles in fungal cells that regulate a number of biological processes such as endocytosis, pH and salt balance, distribution of proteins throughout the cell, cell differentiation such as bud or hyphal formation, toxin degradation and pathogenicity (Figure 1.11) (Rane *et al.*, 2013). The vacuole is an acidic organelle and is similar in function to lysosomes in mammalian cells. Studies by Johnston *et al.* (2009) and Palmer (2011) have confirmed that vacuoles play an important part in the virulence and pathogenicity of *C. albicans*.

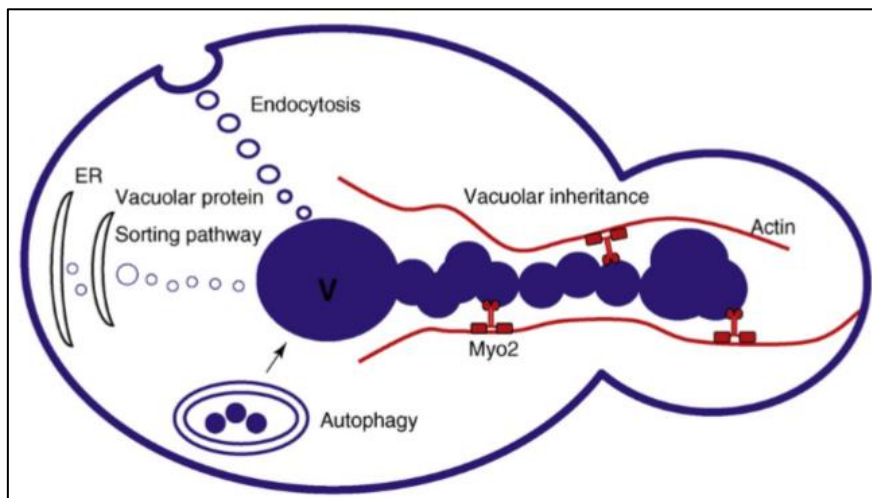


Figure 1.11: Illustration of some of the functions of a fungal vacuole. Shows the pathways for endocytosis, vacuole protein sorting, autophagy and vacuole inheritance in a budding yeast. Vacuole vesicles segregate from the mature vacuole and is mobilised by the Myo2-actin cytoskeleton into a forming bud (Veses *et al.*, 2008, used under CC-BY-SA license).

An example of an AMP that targets the fungal vacuoles at a concentration $\leq 1 \mu\text{M}$ is penetratin. *C. albicans* vacuoles are involved in the translocation and intracellular trafficking of penetratin within the cell (Gong *et al.*, 2017). This AMP permeabilizes cell membranes as well as organelle membranes such as vacuoles and then accumulates in the cytosol. Thus, antimicrobial agents that

can target and degrade intracellular organelles like the vacuoles of *C. albicans* can disrupt the virulence of the pathogen leading to eventual cell death. The differences between fungal cells and other eukaryotic cells provide insight into selecting AFPs for the treatment of human fungal infections, without risking cytotoxicity.

1.7 Tick derived peptides as a possible treatment for *C. albicans* infections

Previous studies showed that tick-derived defensins can be used to develop novel antimicrobial agents. Synthetic forms of the defensins OsDef1 and OsDef2 identified in the soft tick *Ornithodoros savignyi* (*O. savignyi*) were found to be active against Gram-positive bacteria (Prinsloo *et al.*, 2013). Several synthetic derivatives were developed from OsDef2 including Os-C (Os peptide with three cysteine residues removed). The sequences of these peptides are presented in Table 1.5. The hydrophobicity <H> was computed with HeliQuest. Hydrophobicity is an indication of the antimicrobial activity of the peptides as a higher <H> is associated with higher antimicrobial activity (Wieprecht *et al.*, 1997).

Table 1.5: Properties of OsDef2 and derived synthetic peptides

Peptide	Amino acid sequence	MW	<H>	No. amino acids	Net charge*
OsDef2	GYGCPFNQYQCHSHCKGIRGYKGGYCKGAFKQTCKCY	4185.80	0.371	37	+6
Os	KGIRGYKGGYCKGAFKQTCKCY	2459.90	0.249	22	+6
Os-C	KGIRGYKGGY_KGAFKQT_K_Y	2150.49	0.045	19	+6

*At a pH of 7.0

Compared to the original defensin identified from ticks, Os-C showed improved antibacterial activity against Gram-positive and -negative bacteria. This peptide was bactericidal but also nontoxic to mammalian cells making them potential alternatives to conventional antibiotics (Taute *et al.*, 2015). *In vitro* mode of action studies indicated that Os-C binds to lipopolysaccharides (LPS) (Malan *et al.*, 2016) and crosses bacterial membranes (Taute *et al.*, 2015).

Because of their promising antibacterial activity, Os and Os-C were also tested for antifungal activity. Os and Os-C were found to inhibit planktonic *C. albicans* cells (Mbuayama *et al.*, 2020). The latter study further found that Os-C potentially binds to cell wall polysaccharides and translocates into the cytoplasm of fungi in an energy-dependent process. In terms of mechanism of action, Os-C caused killing of *C. albicans* cells by inducing ROS-production. The known antimicrobial effects of Os-C are summarized in Table 1.6.

Table 1.6: Summary of known effects of Os-C

Activity	Effect	Reference
<i>Antimicrobial mode of action</i>		
Cell wall interaction	Interacts with excess laminarin	Mbuayama <i>et al.</i> , 2020
Cell membrane interaction	Accumulates on fungal cell membrane MIC ₅₀ causes 40% fungal membrane permeabilization and 25% liposome leakage Permeabilizes bacterial outer membranes	Mbuayama <i>et al.</i> , 2020 Taute <i>et al.</i> , 2015 Ramafoko, 2018
Translocation	ATP-dependent in fungal cells	Mbuayama <i>et al.</i> , 2020
Ultrastructure	Affects membrane integrity of Gram-positive and -negative bacteria Bacterial membrane blebbing Bacterial bud formation	Taute <i>et al.</i> , 2015 Ramafoko, 2018
Localization	Accumulates within the cytosol of <i>C. albicans</i>	Mbuayama <i>et al.</i> , 2020
ROS production	Induces ROS production that leads to <i>C. albicans</i> killing and autophagy in Gram-positive and -negative bacteria	Mbuayama <i>et al.</i> , 2020 Taute <i>et al.</i> , 2015
DNA binding	Binds Gram-positive and -negative plasmid DNA	Taute <i>et al.</i> (2015)
Salt sensitivity	Loses activity in physiological salt environments	Mbuayama <i>et al.</i> , 2020
<i>Cytotoxicity</i>		
Erythrocytes	No haemolysis or change to morphology Protects erythrocytes with antioxidant activity	Taute <i>et al.</i> (2015)
Leukocytes: Peripheral mononuclear cells Polymorphonuclear cells	Activates leukocytes Causes little cellular damage	Taute <i>et al.</i> (2015)
Mouse L929 cells	Non-toxic to mammalian cells	Ramafoko, 2018

1.8 Aim

The aim of this study was to further explore the mode of action of the antimicrobial peptide, Os-C, against planktonic *C. albicans* (ATCC 90028) cells.

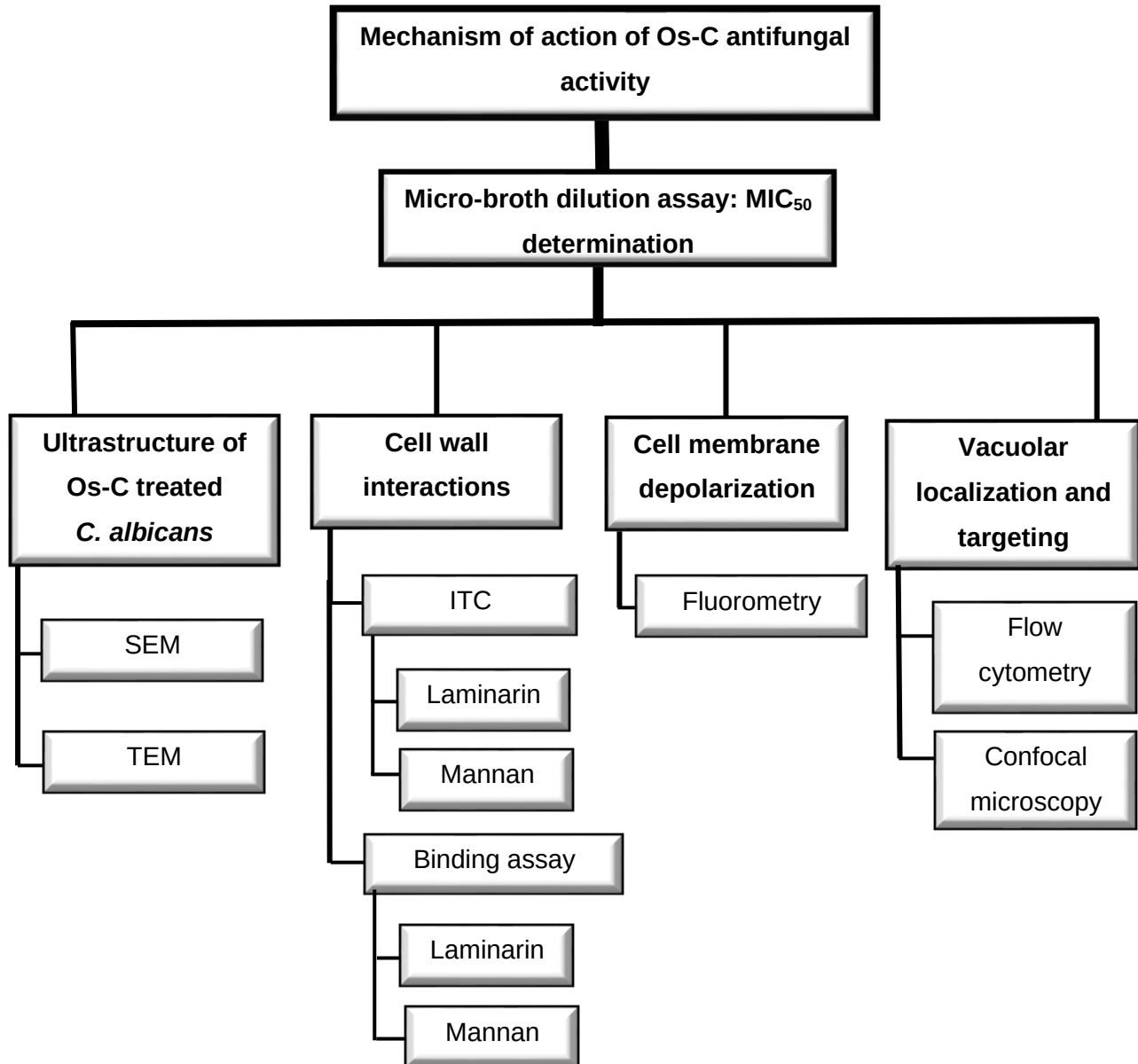
1.9 Objectives

1. To determine the MIC₅₀ of Os-C with a modified micro-broth dilution assay.

2. To study the effect of Os-C on the morphology and ultrastructure of planktonic *C. albicans* cells using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).
3. To determine the binding properties of Os-C with polysaccharides of the fungal cell wall by means of isothermal titration calorimetry (ITC) and the modified micro-broth dilution assay.
4. To evaluate the ability of Os-C to cause *C. albicans* cell membrane depolarization using the modified micro-broth dilution assay and the fluorescent DiBAC₄(3) dye.
5. To investigate the cellular uptake, distribution and vacuolar localization of 5-FAM labelled Os-C in planktonic *C. albicans* cells using flow cytometry and confocal laser scanning microscopy (CLSM).

CHAPTER 2: MATERIALS AND METHODS

2.1 Flow diagram



2.2 Experimental

2.2.1 Cultivation of *C. albicans*

C. albicans yeast strain ATCC 90028 was used as the target organism. The fungus was cultured aerobically for 16 hours at 30°C in yeast peptone dextrose [YPD; 0.5% (w/v)] yeast extract, 1% (w/v) peptone and 2% (w/v) dextrose with 149 rpm shaking. The cells were diluted 100 times in YPD and incubated for a further two and a half hours at 30°C to reach the exponential growth phase.

2.2.2 Peptide preparation

The synthetic Os-C peptide synthesized using FlexiPeptide technology was obtained from GenScript (New Jersey, USA). The purity and the molecular mass of Os-C was determined by the vendor using reverse phase HPLC and mass spectrometry, respectively (Appendix A). The lyophilized peptide was re-suspended in 300 µL double distilled water (ddH₂O) and the absorbance of the peptide suspension was measured at 280 nm (UV-1600 PC VWR, Separation scientific, USA). The concentration of the peptide was determined using the following equation:

$$c = \frac{MW \times df \times Abs}{no.Tyr (Ex) \times no.Trp (Ex)}$$

where c is the peptide concentration in mg/mL, MW is the molecular weight of the peptide (2150.49 g/mole), df is the dilution factor and Abs is the measured absorbance. The extinction coefficient of Tyr is 1200 AU/mmol/mL and of Trp is 5560 AU/mmol/mL (Lamichhane *et al.*, 2011). Stock solutions of the peptides were prepared in sterile, filtered ddH₂O and stored at – 20°C.

2.2.3 Micro-broth dilution assay

2.2.3.1 Optimization of the modified micro-broth dilution assay

Using the standard micro-broth dilution assay in RPMI-1640, Os-C showed no inhibition of planktonic *C. albicans* cells (Mbuayama *et al.*, 2020). RPMI-1640 consists of amino acids, vitamins, salts and minerals at pH 7.4 at concentrations that ensures the optimal *in vitro* growth of cells. The salt molarity of RPMI-1640 is 139 mM which represents the systemic physiological conditions in humans (Mater methods, 2013). Electrostatic interactions between AMPs and the negative surface of *C. albicans* are required for effective killing. High salt as in RPMI-1640 interferes with this interaction making Os-C ineffective. Despite the inhibitory effects of salts, Os-C can still be further investigated for topical use. Thus, to optimize the micro-broth dilution assay it was necessary to identify the optimal dilution of RPMI-1640 that allows significant growth of planktonic *C. albicans* cells while simultaneously creating an environment where Os-C can effectively kill *C. albicans* cells. *C. albicans* were cultured to the exponential growth phase as described in section 2.2.1, centrifuged at 2000 x g for 10 minutes and the supernatants discarded.

Thereafter the cell pellets were washed with 100, 75, 50, 25, 10, 5 and 1% RPMI-1640 solution diluted with 20 mM sodium phosphate buffer (NaP; 2 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 8 mM $\text{HNa}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; pH 7.4) by centrifugation at $14100 \times g$ for 2 minutes. Finally, the supernatants were removed, and the pellets re-suspended in the different percentages of RPMI-1640 in 20 mM NaP buffer solution with corresponding molarities of 139, 119.25, 79.50, 15.90, 7.95 and 1.59 mM.

These suspensions were further diluted 5 times and each respective solution added to a 96-well polypropylene plate. The cells were incubated for 2 hours at 37°C . After incubation, cell viability was determined with Cell Titre Blue (CTB) (Promega, USA) as described in Section 2.2.3.2 (Van den Driessche *et al.*, 2014).

2.2.3.2 Determination of the MIC_{50} of Os-C

To determine the MIC_{50} of Os-C a modified micro-broth dilution assay was used. The MIC_{50} is the minimum inhibition concentration of Os-C required to inhibit 50% of planktonic *C. albicans* cells. The modified micro-broth dilution assay was performed in polypropylene 96-well plates with the known antifungal drug, AmB, as a positive control (Figure 2.1).

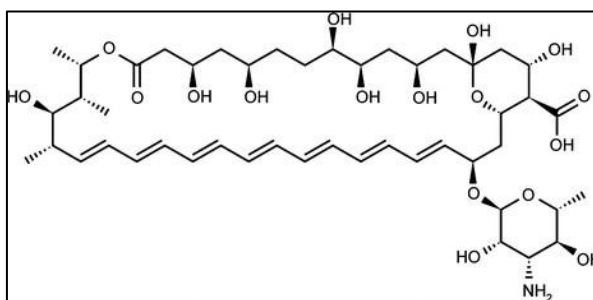


Figure 2.1: Chemical structure of Amphotericin B. This is a known polyene antifungal drug that will serve as the positive control in this study. (Finberg and Guharoy, 2012, used under CC-BY-NC license).

C. albicans cells in the exponential growth phase were centrifuged at $2000 \times g$ for 10 minutes and the supernatant was discarded. Thereafter the pellet was washed with a 20% RPMI-1640 in 20 mM NaP buffer (pH 7.4) (10% RPMI-1640/NaP) and centrifuged again at $14100 \times g$ for 2 minutes. Finally, the supernatant was removed, and the pellet was re-suspended in the 10% RPMI-1640/NaP buffer solution. This suspension was diluted 5 times with 10% RPMI-1640/NaP and the OD was measured at 620 nm. A cell density of 1×10^6 CFU/mL was obtained, and the cells were added to a 96-well polypropylene plate. The cells were treated with serial dilutions of Os-C (0.7 to 40 μM) or AmB (0.07 to 5 μM) and incubated for 2 hours at 37°C . After incubation viability was determined with the CTB assay.

The CTB viability assay uses fluorescence to quantify the number of viable cells that is present within multi-well plates. The assay relies on the metabolic conversion by viable cells of the indicator dye resazurin (dark blue) which shows little fluorescence to resorufin (pink) that is highly fluorescent (Figure 2.2). Nonviable cells will have no metabolic activity and will thus not be able to

reduce resazurin, resulting in no fluorescence. Advantages of this assay include that the reagent is generally nontoxic to cells, allowing extended incubation times if necessary and the easy three step format that reduces possible errors.

After incubation (section 2.2.3.1 and 2.2.3.2) 11 μ L CTB was added to each well to reach a final volume of 10% followed by incubation in the dark for an hour at 37°C. Fluorescence was measured using a Spectramax, multi-mode detection platform paradigm (Molecular devices, EU).

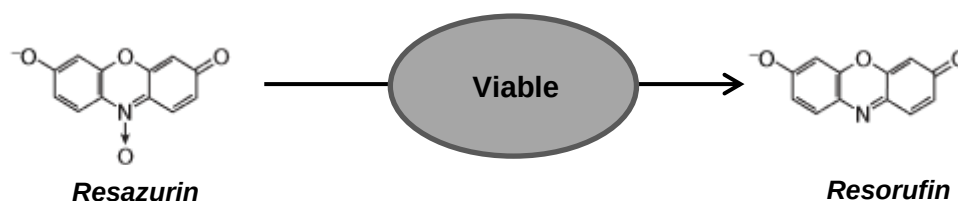


Figure 2.2: The metabolic conversion of resazurin to resorufin to generate a fluorescent product. Resorufin emits fluorescence at 590 nm. The number of viable cells is proportional to the fluorescence produced (Modified from: Chemical structures of resazurin and resorufin by Csepregi et al., 2018, used under CC-BY license).

2.2.4 Ultrastructural effects of Os-C

2.2.4.1 Coating of glass coverslips

SEM was performed to visualise the effect of Os-C treatment on the ultrastructure of planktonic *C. albicans* cells. For visualization with SEM the planktonic cells were attached to glass coverslips coated with positively charged poly-L-lysine (Figure 2.3) which improves the attachment of negatively charged cells through electrostatic interactions (Sitterly, 2008). Round coverslips with a 10 mm diameter (Lasec, Johannesburg, RSA) were washed with an alkaline solution of 10% NaOH (w/v) and 60% ethanol (v/v) by shaking for a minimum of 2 hours. The coverslips were rinsed several times with ddH₂O and sterilized with 99.9% ethanol (v/v) for 30 minutes. The dried coverslips were submerged in poly-L-lysine (7000 – 15000 kDa, Sigma-Aldrich, Johannesburg, RSA) for 2 hours. The poly-L-lysine solution was removed and the coverslips were rinsed several times with dddH₂O. The coated coverslips were allowed to dry in a sterile environment for at least 3 days prior to use.

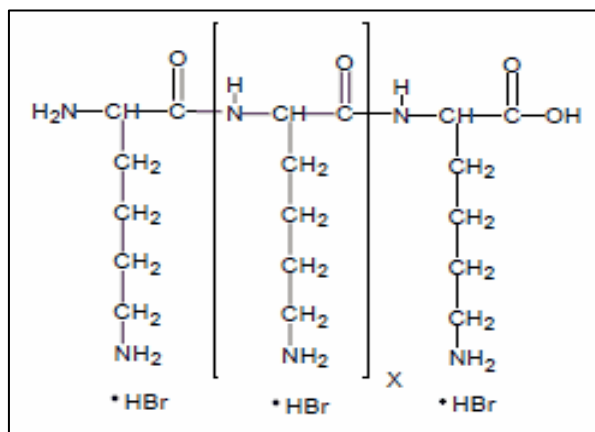


Figure 2.3: Chemical structure of poly-L-lysine. Poly-L-lysine is a charge enhancer used to coat many surfaces (Sitterly, 2008, used under CC-BY license).

2.2.4.2 Ultrastructure: Scanning electron microscopy

SEM was used to evaluate the ultrastructural morphology of planktonic *C. albicans* cells treated with Os-C. In SEM, a thin layer of metal is sputtered over a biological material, such as exposed *C. albicans* which increases conductance so that when a high-energy electron beam is focussed onto the sample a variety of signals are emitted from its surface (Figure 2.4 A). The signals include secondary electrons, backscattered electrons and x-rays. The secondary electrons are picked up by the detector of the microscope to generate a two-dimensional image of the sample. These images reveal information about the sample such as its topography, morphology, structure and orientation (Beane, 2004).

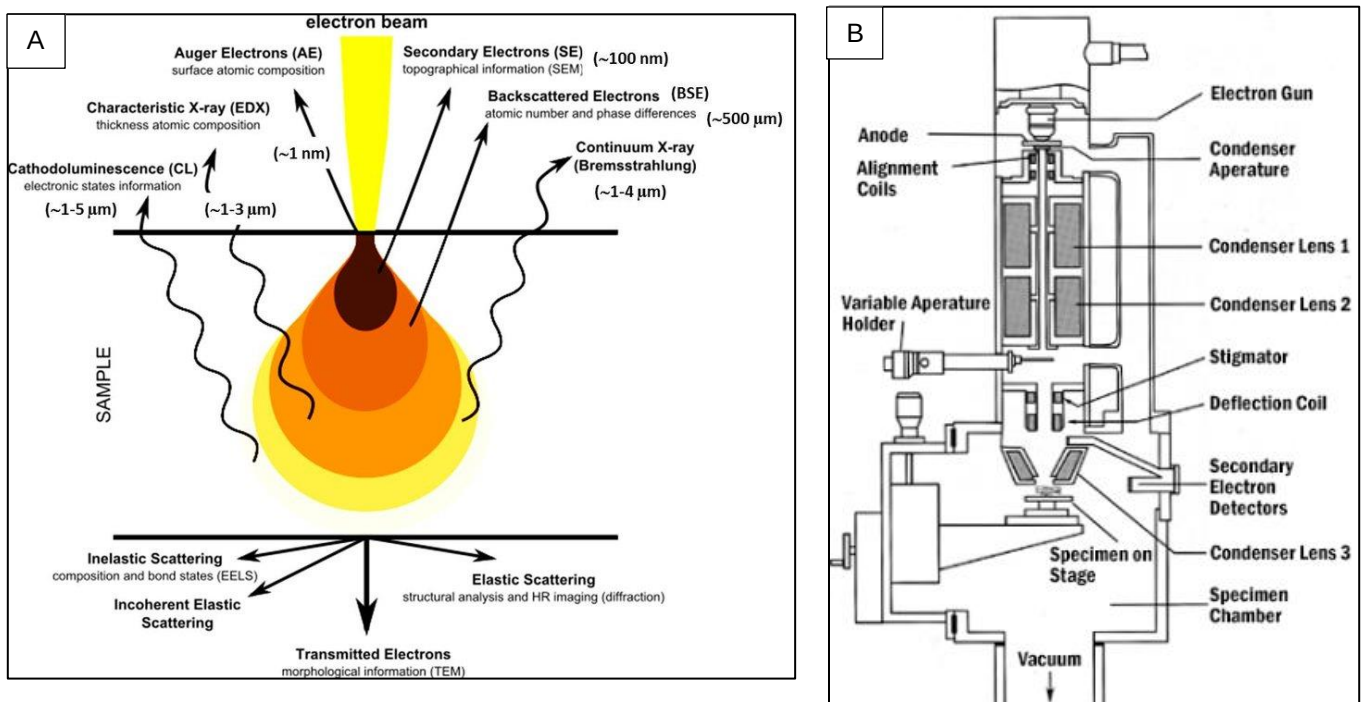


Figure 2.4: Scanning electron microscopy. (A) The scattering of high-energy electrons on the surface of a solid sample to collect the emitted secondary electrons and generate a three dimensional image for SEM (Modified from: Working principle of SEM by Zhang & Ulrey, 2018, used under CC-BY license). (B) A general design of the SEM showing the path of an electron beam towards the sample that is in a vacuum on the specimen stage (Public domain: <https://cmrf.research.uiowa.edu/scanning-electron-microscopy>)

After the planktonic cells were treated for 3 hours with Os-C at the MIC₂₅, MIC₅₀ and MIC₇₅, 50 μL of 2.5% glutaraldehyde/formaldehyde (GA/FA) fixative diluted with 75 mM NaP (pH 7.4) was added to the cells for 20 minutes. Thereafter 100 μL of the semi-fixed fungal suspension was transferred to the poly-L-lysine coated coverslips for 70 minutes to attach. Afterwards the coverslips were rinsed 3 times with 75 mM NaP buffer (pH 7.4) to remove unattached fungal cells and fixative solution. This was followed by performing a secondary fixation step with 1% osmium tetroxide for 45 minutes. The coverslips were rinsed again in 75 mM NaP buffer (pH 7.4) before being dehydrated with 30, 50, 70, 90 and 100% ethanol. The coverslips were dried overnight by adding 2 drops of hexamethyldisilazane (HMDS). With carbon tape the coverslips were mounted onto aluminium stubs, coated with carbon and viewed with an Ultra plus field emission gun (FEG) SEM (Zeiss, Oberkochen, Germany) (Figure 2.4 B).

2.2.4.3 Ultrastructure: Transmission electron microscopy

TEM was used to evaluate the internal ultrastructure of planktonic *C. albicans* cells treated with Os-C. This type of microscopy operates on the same principles as light transmission microscopy but instead uses a high-energy electron beam instead of light (Figure 2.5 A). Biological material is coated with electron dense metals such as osmium or lead and the interaction of electrons and a cation coated sample allows high resolution viewing of internal structures (Williams & Carter, 1996).

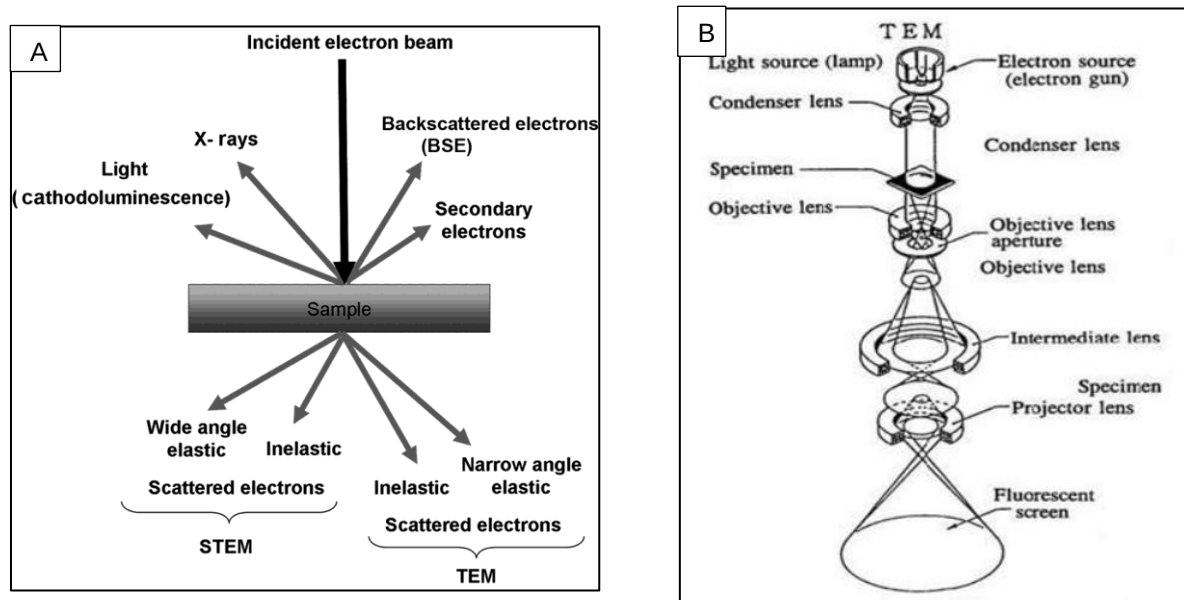


Figure 2.5: Transmission electron microscopy. (A) The transmission of high-energy electrons through a sample to collect all transmitted electrons and create a two-dimensional image. (Modified from: Signals generated from the interaction between a high energy electron beam and an electron transparent (very thin) sample by Sales et al., 2012, used under CC-BY license). (B) A general design of the TEM showing the path of an electron beam towards the sample and the subsequent projection on the fluorescent screen (From JEOL 2000FX Instruction manual).

C. albicans cells treated with Os-C at MIC₂₅, MIC₅₀ and MIC₇₅ for 3 hours, were fixed with a 2.5% GA/FA fixative for an hour. Thereafter the cells were centrifuged at 5000 x *g* for 2 min and the pellet was fixed with 2.5% GA/FA fixative for an hour. The cells were centrifuged again, and the pellet washed 3 times with 75 mM NaP buffer (pH 7.4). Osmium tetroxide (1%) was added to the cell pellets as a secondary fixative for 1 hour. After fixation the samples were centrifuged, the pellet washed 3 times with 75 mM NaP buffer (pH 7.4) and dehydrated with 30, 50, 70, 90 and 100% ethanol. The cells were gradually embedded with 50% Embed 812 resin (SPI supplies, Pennsylvania, USA) in ethanol and afterwards with 100% resin for about 4 hours. The resin was replenished and polymerized in moulds for up to 72 hours. Ultra-thin sections of 100 nm were made using the Leica Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) with a 45° diamond knife (Diatome, Pennsylvania, USA). These sections were placed on a copper grid and stained with 4% aqueous uranyl acetate and Reynolds' lead citrate for the best contrast. Sections were viewed with the JEM-2100 TEM (JEOL, Tokyo, Japan) (Figure 2.5 B).

2.2.5 Mode of action studies

2.2.5.1 Cell wall polysaccharide binding: Isothermal titration calorimetry

Glycoproteins, glycosphingolipids and polysaccharides located on the outer surface of fungal cells are involved in various types of interactions with the environment. These components are also important for cell structure, stability, growth, differentiation and signalling. The composition of the cell wall differs greatly between fungal species, but they all have very similar polysaccharide structures (Pinto *et al.*, 2008). This experiment focused on two of the most common polysaccharides in the cell wall of *C. albicans*, namely laminarin (β -1,3 glucan polymer) and mannan (mannose polymer) both which were obtained from Sigma-Aldrich (RSA) (Figure 2.6). Binding to these polysaccharides may be one of the first interactions that occurs between an AMP and fungi including *C. albicans*.

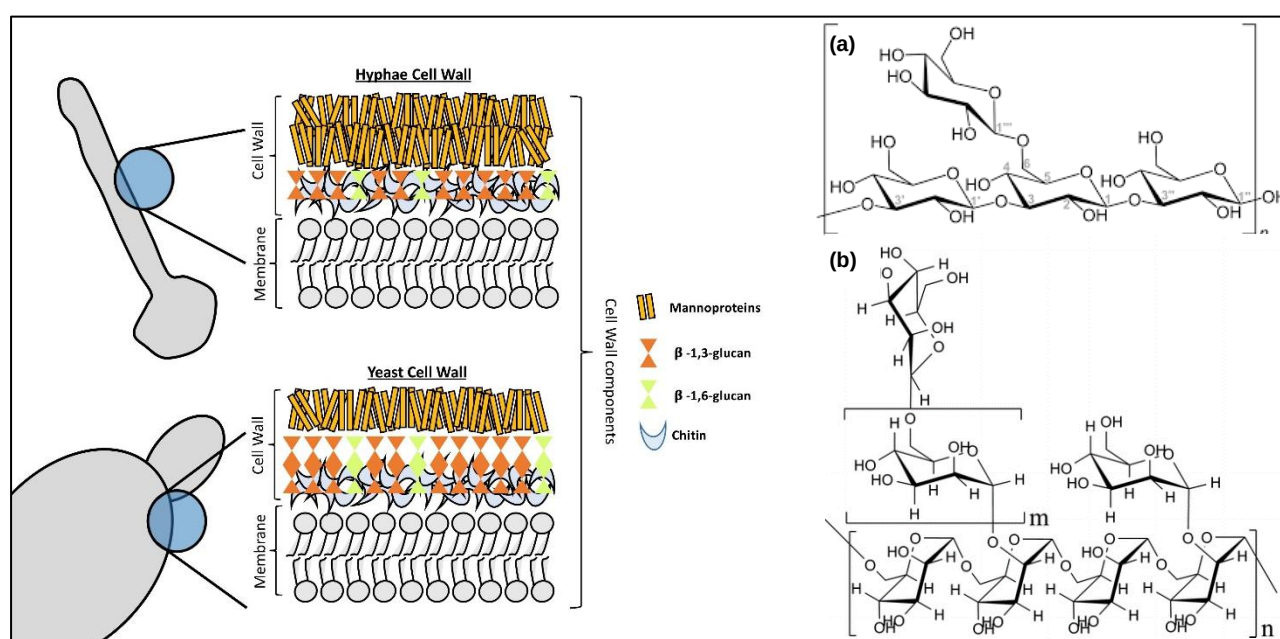


Figure 2.6: Structural organization and composition of *C. albicans* hyphae and yeast cell wall. Showing the chemical structures of the most common polysaccharides (A) laminarin (β -1,3 glucan polymer) and (B) mannan (Garcia-Rubio *et al.*, 2020, used under CC-BY-SA license).

Mbuayama *et al.*, (2020) showed that the antifungal activity of Os-C decreased, when Os-C was first mixed with either mannan or laminarin and then added to cultures of planktonic *C. albicans*. This indicated that Os-C may interact with these polysaccharides, thereby reducing the concentration of free Os-C that can inhibit the growth of *C. albicans*. The ability of the peptide to bind to polysaccharides of the fungal cell wall was determined using isothermal titration calorimetry (ITC).

ITC is an analytical technique that directly measures the heat energy that is absorbed or released during a molecular interaction. Heat changes are used to determine binding kinetics such as the enthalpy and entropy, as well as binding constants and stoichiometry (Saponaro, 2018) (Figure 2.7).

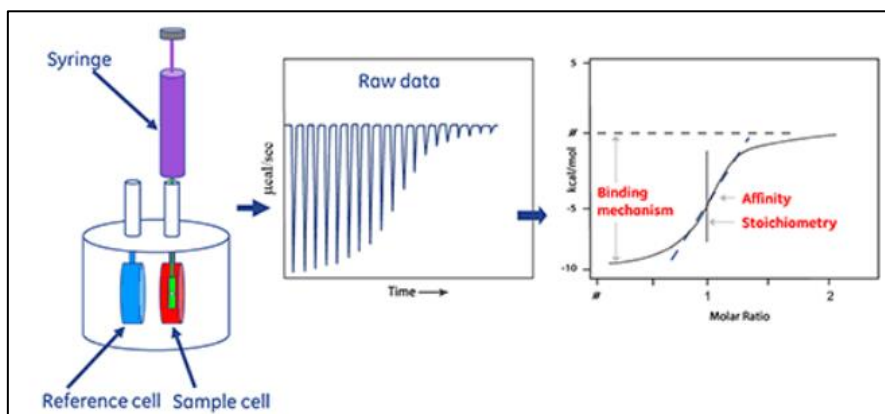


Figure 2.7: A simple illustration of an ITC system and the binding isotherms generated from heat changes (From Malvern panalytical, 2020, used under CC-BY license).

The ITC (MicroCal iTC200, Onderstepoort Veterinary Institute) sample chamber was filled with Os-C peptide solution at the MIC_{50} of $4.7 \mu M$ or $10 \times MIC_{50}$ of $47 \mu M$. An auto-syringe was used to titrate Os-C with a 1:20 or 1:40 ratio of representative cell wall constituents laminarin ($94 \mu M$ or 1.88 mM), mannan ($94 \mu M$ or 1.88 mM) (Table 2.1) or NaP buffer (10 mM , pH 7.4) at a set temperature of $30^\circ C$ and specific parameters (Table 2.2). To calculate the molarities of laminarin and mannan, we adopted 504.438 g/mol and 666.579 g/mol as the molecular mass respectively, as reported by the supplier Sigma-Aldrich (RSA).

Table 2.1: ITC titration ratios of Os-C with cell wall polysaccharides laminarin and mannan.

[Os-C] (μM)	[Polysaccharide] (μM)	Ratio
$4.7 \mu M$	$94 \mu M$ laminarin	1:20
$4.7 \mu M$	$94 \mu M$ mannan	1:20
$47 \mu M$	1.88 mM laminarin	1:40
$47 \mu M$	1.88 mM mannan	1:40

The control, NaP buffer (10 mM , pH 7.4) was titrated with NaP buffer alone, then with laminarin and mannan respectively to determine and eliminate background noise. Fluctuation in temperature was measured to determine binding properties such as the number of binding sites, the binding affinity and binding kinetics. All solutions used for ITC measurements were thoroughly degassed before use by stirring under vacuum for 10-15 min. The Origin 7.0. software (Originlab Corporation, Northampton, MA, USA) provided by MicroCal was used to analyse the experimental data.

Table 2.2: ITC experimental parameters.

Instrument setting	Parameter
Total injections	20
Injection volume (μL)	2
Duration (sec)	4
Spacing (sec)	120
Filter period	5
Cell temperature	30°C
Reference power (μcal/sec)	6
Initial delay (sec)	120
Syringe concentration (μM)	4.7 & 47
Sample cell concentration (μM)	94 & 1880
Stirring speed (rpm)	600

2.2.5.2 Cell wall polysaccharide binding: Micro-broth dilution assay

To confirm the results obtained from ITC, a cell wall polysaccharide binding was investigated with the modified micro-broth dilution assay. This assay measures the change in the inhibitory activity of Os-C against planktonic *C. albicans* cells in the presence of excess laminarin or mannan which may bind Os-C thereby reducing the activity of Os-C. *C. albicans* cells were treated with 10X the MIC of Os-C (20 μM) that, under normal conditions, ensures 100% inhibition. Before treatment Os-C was preincubated with a final concentration range of 0 and 20 mg/mL of laminarin and mannan, respectively for one hour. Afterwards 50 μL of the Os-C/laminarin or Os-C/mannan mixtures were added to 50 μL of planktonic *C. albicans* cells cultured to the exponential growth phase as described in section 2.2.1. After a 2 hours incubation at 37°C, cell viability was determined as described in section 2.2.3.2. From this data the change in the percentage inhibition was calculated.

2.2.5.3 Membrane depolarization

The effect of Os-C treatment on the membrane potential of planktonic *C. albicans* cells was investigated using a potential sensitive probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] (Figure 2.8). This dye can enter depolarized cell membranes where it binds to intracellular proteins and membranes. An increase in depolarization results in increased cellular influx of the red anionic dye associated with an increase in fluorescence (ThermoFisher, 2019).

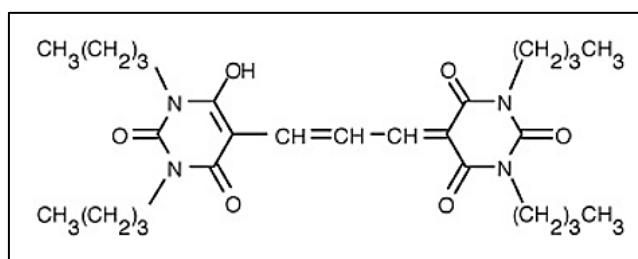


Figure 2.8: Chemical structure of DiBAC₄(3). Molecular formula: C₂₇H₄₀N₄O₆. Molecular weight: 516.64 g/mol (PubChem, 2019, used under CC-BY license).

Melittin, an AMP in bee venom, was used as a positive control (Figure 2.9). Melittin is a small cytolytic peptide of 26 amino acids that integrates into lipid bilayers causing cell membrane depolarization and eventually cell membrane lysis (Terwilliger & Eisenberg, 1981).

Planktonic *C. albicans* cells cultured to the exponential growth phase as described in section 2.2.1 were treated with a two-fold serial dilution of melittin (0.156 to 10 μ M) and Os-C (0.156 to 10 μ M) respectively. The treatment was performed in polypropylene Eppendorf tubes for an hour at 37°C. After treatment the DiBAC₄(3) dye (2 μ M) was added to each tube and incubated for 30 minutes in the dark at 37°C. The cells were centrifuged at 5000 x *g* for 10 minutes, the pellets washed with phosphate-buffered saline (PBS; 0.137 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄; pH 7.4) and then transferred to a 96-well polypropylene plate. The fluorescence was measured using a Spectramax, multi-mode detection platform paradigm (Molecular devices, EU) with an excitation wavelength (Ex) of 490 nm and an emission wavelength (Em) of 516 nm.

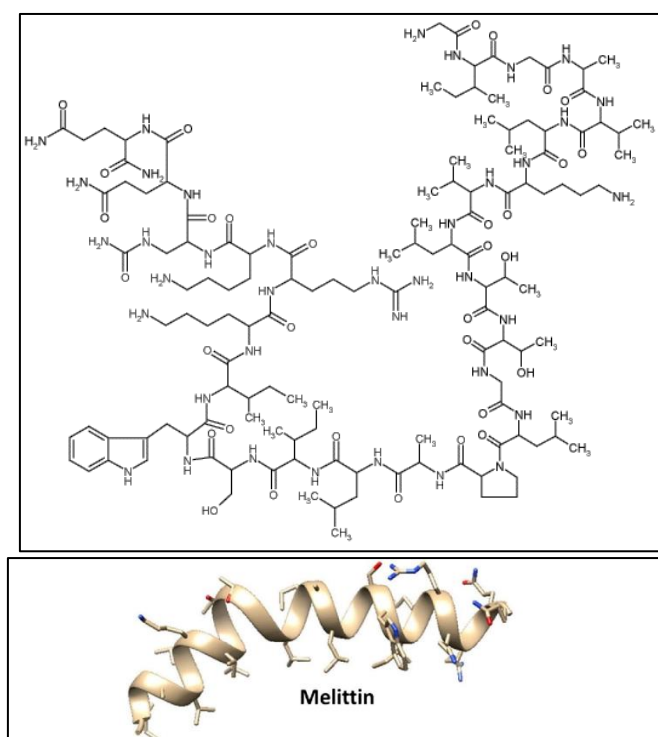


Figure 2.9: Chemical and 3D structure of melittin. Molecular formula: $C_{131}H_{229}N_{39}O_{31}$. Molecular weight: 2846.5 g/mol (PubChem,2019, used under CC-BY license).

2.2.5.4 Peptide trafficking and vacuole interaction

To understand Os-C trafficking and interaction with *C. albicans* vacuoles, staining was undertaken with CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin; CTr) (Figure 2.10). This fluorescent dye translocates across cell membranes and in an intracellular environment is transformed into a membrane-impermeant product (Gong & Karlson, 2017). In this experiment it was used to determine if Os-C accumulates in the vacuoles of *C. albicans*.

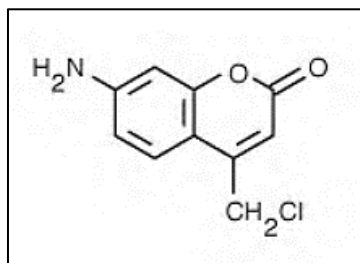


Figure 2.10: Chemical structure of CTr. Molecular formula: $C_{10}H_8NO_2Cl$. Molecular weight: 209.63 g/mol (ThermoFischer Scientific, 2019, used under CC-BY license).

Penetratin (RQIKIWFQNRRMKWKK-NH₂) labelled with 5-carboxyfluorescein (5-FAM) was used as a positive control in this experiment (Figure 2.11). This peptide has well described cell-penetrating properties and is used to transfer cargo (Dupont et al., 2011).

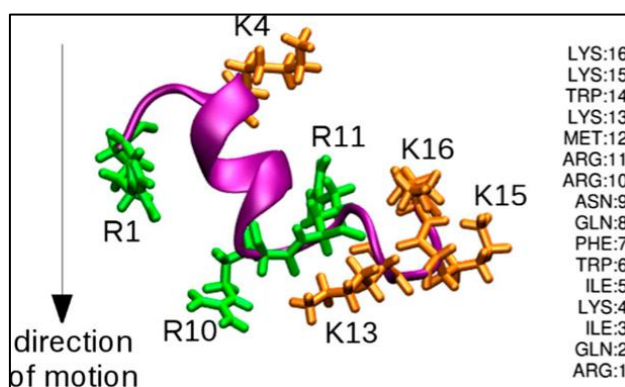


Figure 2.11: 3D structure of penetratin in bicellar solution. Residues R10 and K13 lead the way into the cell membrane. Molecular formula: $C_{104}H_{168}N_{34}O_{20}S$. Molecular weight: 2246.7 g/mol (Modified from: Secondary structure by General & Ascianto, 2017, used under CC-BY-NC license).

5-FAM is green fluorophore used to label peptides and proteins (Figure 2.12). 5-FAM has an Ex of 495 nm and an Em of 520 nm similar to FITC. It is a better peptide label than FITC due to the single isomer of carboxyfluorescein that contains a carboxylic acid used to react with the primary amine groups of the peptide forming a natural and stable carboxamide bond (Radu and Moldovan, 1991). For 5-FAM-Os-C the fluorophore is bound to the primary amine at the N-terminal. Detection of fluorescence stains was undertaken with fluorescence CLSM (LSM 880 Meta, Zeiss, Germany).

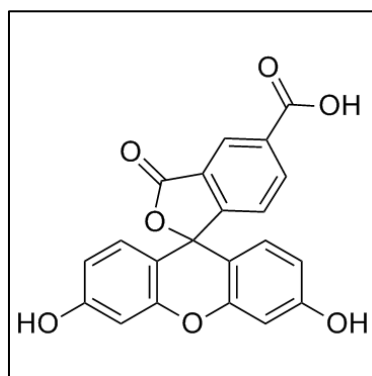


Figure 2.12: Chemical structure of 5-FAM. Molecular formula: $C_{21}H_{12}O_7$. Molecular weight: 376.3 g/mol (PubChem, 2019, used under CC-BY license).

2.2.5.4.1 Flow cytometry

Flow cytometry was used to quantify the translocation of the 5-FAM labelled Os-C into planktonic *C. albicans* cells. Flow cytometry is a technique that can measure or detect physical or chemical changes in cell populations. The cells are suspended in a fluid that is injected into the flow cytometer. These cells flow one by one through a laser beam and the scattered light is measured (Figure 2.13). Cells labelled with fluorescent markers will absorb the light from the laser beam and emit a new wavelength of light (Picot *et al.*, 2012). Using this technique single cells were identified and the percentage of cells positive for FAM fluorescence and/or CTr fluorescence was determined.

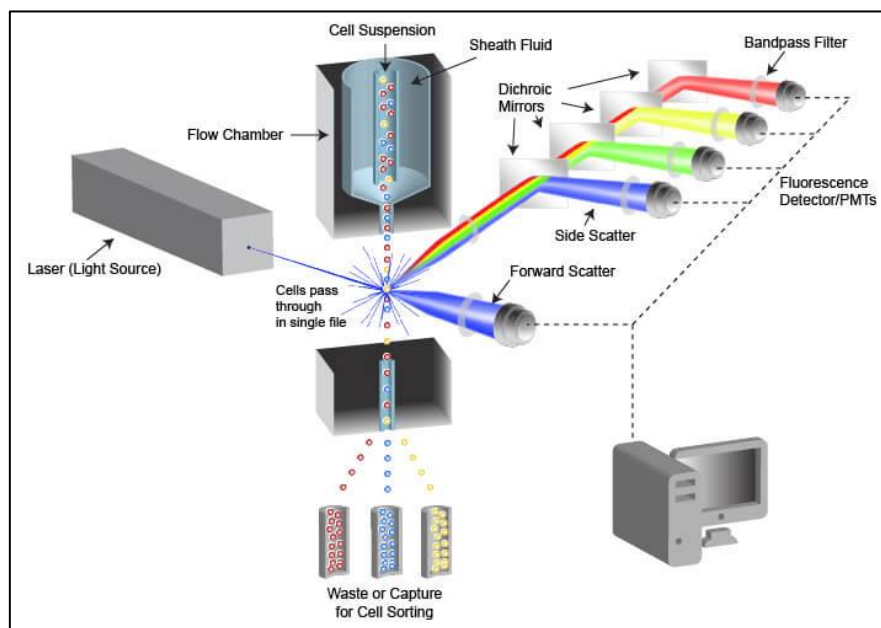


Figure 2.13: An overview of the flow cytometry system and its critical components (Adapted from Picot *et al.*, 2012, used under CC-BY-SA license). A single cell is passed through a laser beam and side scattered fluorescent light is detected. Fluorescently labelled cells will absorb the laser light and emit a new wavelength of light.

Exponentially cultured *C. albicans* cells (Section 2.2.1, without diluting the cells five times) were treated with 5-FAM labelled Os-C at the MIC₂₅ of 2.8 μ M and MIC₇₅ of 6.2 μ M for an hour in the dark at 37°C. The cells were centrifuged at 5000 x *g* for 10 min and the pellets were washed with NaP buffer (10 mM, pH 7.4). Thereafter 400 μ L of trypsin (0.025%, trypsin/EDTA 1X, Cascade Biologics, RSA) was added to the cell pellets for 10 min to degrade extracellular peptides. The cells were centrifuged and washed as before and 10 μ M CTr (Figure 2.10) was added to every pellet for 40 min. The cells were centrifuged and washed again, and the pellets were resuspended in 400 μ L NaP buffer. The cell suspensions were transferred to flow tubes and analysed for 5-FAM and CTr fluorescence using the BD FACS Aria Fusion cell counter (BD Biosciences, Johannesburg RSA) at the University of Pretoria, Prinshof. Data was processed and analysed using the BD FACSDiva Version 8.0.1 software.

2.2.5.4.2 Confocal microscopy

CLSM was used to determine whether Os-C can enter planktonic *C. albicans* cells and bind to intracellular membranes such as the vacuoles. CLSM is a type of laser scanning microscopy that increases the contrast and optical resolution of an image by blocking out-of-focus light (Figure 2.14). This type of microscopy focuses a smaller beam of light on one depth level at a time to construct a three-dimensional image (Minsky, 1988).

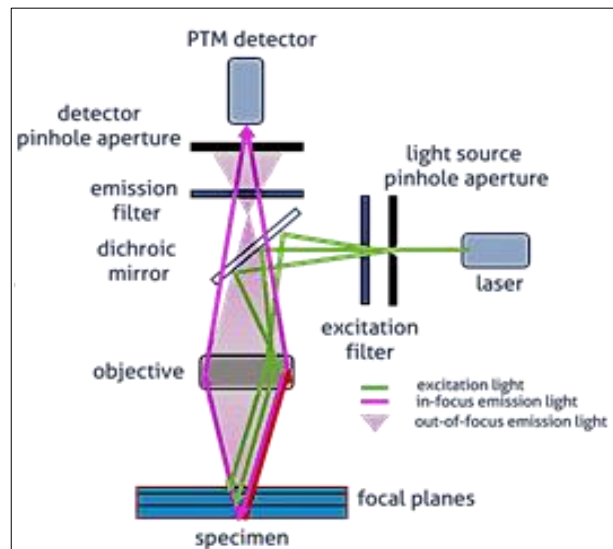


Figure 2.14: A general design of the confocal microscope. This shows the path of the fluorescent beam towards the sample (Modified from: Schematic principle of the confocal arrangement by Guthoff et al., 2009, used under CC-BY-SA license).

Procedures analogous to those for flow cytometry were followed. Exponentially cultured *C. albicans* cells (Section 2.2.1, without diluting the cells five times) were treated with 5-FAM (6.2 μM) and 5-FAM labelled Os-C at the MIC_{25} of 2.8 μM and MIC_{75} of 6.2 μM for an hour in the dark at 37°C. The cells were centrifuged at 5000 $\times g$ for 10 min and the pellets washed with NaP buffer (10 mM, pH 7.4). Thereafter 400 μL of trypsin (0.025%, trypsin/EDTA 1X, Cascade Biologics, RSA) was added to the cell pellets for 10 min to remove extracellular peptides. The cells were centrifuged and washed as before, then 10 μM of CTr was added to every suspended pellet for 40 min. The cells were centrifuged and washed again before the pellets were resuspended in 3 μL of NaP buffer. The resuspended cells were transferred to poly-L-lysine coverslips and mounted on a microscope slide using a polyvinyl alcohol mounting fluid (DABCO, pH 8.7, Sigma-Aldrich, Johannesburg, RSA). The edges of the coverslips were sealed with clear nail polish. The microscope slides were viewed with the Zeiss LSM 880 Meta Confocal microscope (Zeiss, Oberkochen, Germany). Images were analysed and processed using the AXIO VISION 4.8 software (Zeiss, Oberkochen, Germany).

2.2.6 Data analysis and statistics

The percentage inhibition was determined by using the following formula:

$$\text{Inhibition (\%)} = 100 - \frac{100 \times (\text{Avg. absorption} - \text{Avg. blank})}{\text{Avg. absorption of growth control} - \text{Avg. blank}}$$

GraphPad Prism 7 software (San Diego, USA) was used to obtain sigmoidal dose-response curves of log [inhibitor] versus response (inhibition %) using non-linear regression analysis and data normalization functions. This software was also used for the statistical analysis of all data. The analysis considered 95% confidence levels, sum of squares, standard error of the mean (SE) and one-way analysis of variance (ANOVA) using Dunnetts multiple comparison test. Data were obtained from two or three independent biological repeats each performed in triplicate and are shown as mean $\pm$ SE.

CHAPTER 3: RESULTS

3.1 Antifungal activity of Os-C

3.1.1 Optimization of the micro-broth dilution assay

The existing European Committee on Antimicrobial Susceptibility Testing (EUCAST) method (EUCAST Definitive Document EDef7.1, Rodriguez-Tudela *et al.*, 2008) for screening antimicrobial activity of specific compounds against planktonic *C. albicans* cells was optimized to be able to determine a MIC₅₀ for Os-C. As this peptide is inactive in high concentrations of salt present in RPMI-1640, lower RPMI-1640 concentrations are required to retain activity. The viability of planktonic *C. albicans* cells in serial dilutions of RPMI-1640, diluted with 20 mM NaP buffer was determined. Figure 3.1 shows no significant difference between the viability of cells incubated at 37°C for 3 hours in 100%, 75%, 50% and 10% diluted RPMI-1640 media. The 10% RPMI-1640/NaP was selected for further experiments as growth in 10% RPMI-1640/NaP was similar to growth in 100% RPMI (no statistical difference) after 3 hours (Figure 3.1). The final salt molarity of 10% RPMI-1640/NaP is 15.90 mM.

The effect of 10 µM AmB on *C. albicans* cultured in 100% RPMI 1640 was not statistically different from that cultured in 10% RPMI-1640/NaP, indicating that the sensitivity of *C. albicans* to AmB did not change in media with a lower salt content. It can be concluded that under the experimental conditions used in the present study the growth and properties of *C. albicans* in 10% RPMI-1640/NaP are the same as in 100% RPMI-1640.

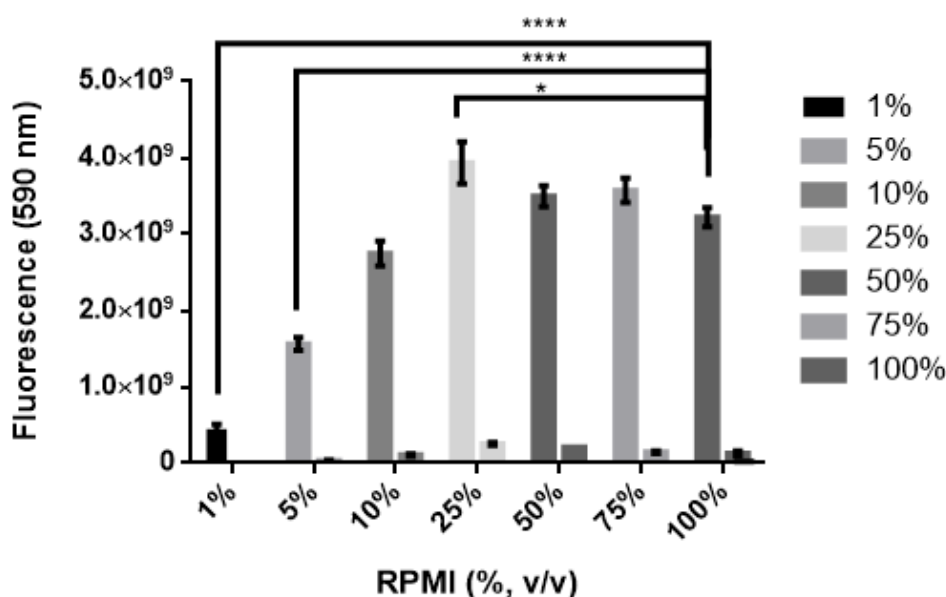


Figure 3.1: Evaluation of the % RPMI-1640 required for optimal growth of *C. albicans* and the evaluation of the antimicrobial effect of AmB on cell viability. Planktonic *C. albicans* were grown for 3 hours in different dilutions of RPMI-1640 diluted with NaP buffer. In addition, the effect of 10 µM AmB at each dilution was evaluated. Fluorescence values indicate the number of metabolically active *C. albicans* cells. Error bars show the mean ± SE. Significant difference indicated by *, **, *** and **** represents $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ values. Results are of four independent biological repeats each performed in triplicate.

3.1.2 MIC₅₀ of AmB, melittin and Os-C

The antifungal activity of AmB, melittin (used as the positive control in the membrane depolarization assays) and Os-C against planktonic *C. albicans* cells was determined using the modified micro-broth dilution assay. In 10% RPMI-1640/NaP medium the positive controls AmB and melittin showed dose-dependent antifungal activity against planktonic *C. albicans* cells (Figure 3.2 and 3.3).

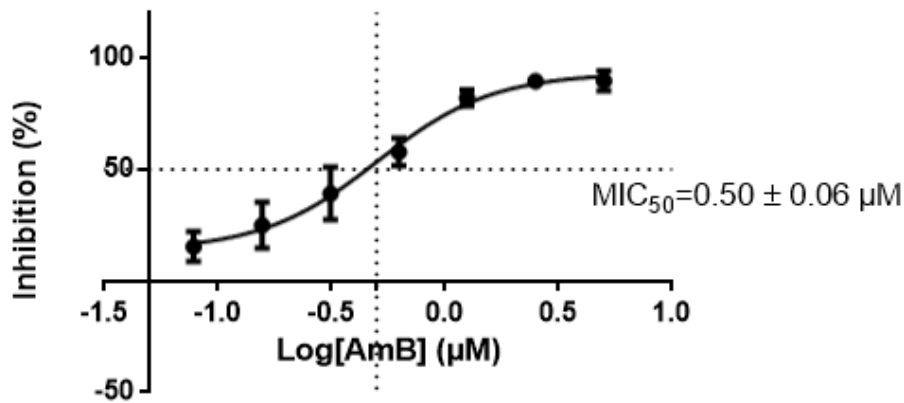


Figure 3.2: Antifungal activity of AmB against planktonic *C. albicans* cells. AmB was serially diluted to concentrations ranging from 0.08 μM to 5 μM . Error bars are the mean $\pm$ SE. Results are of four independent biological repeats each performed in triplicate.

At the two highest concentrations (2.5 and 5 μM) fungal inhibition of 100% was exhibited. The MIC₅₀ for AmB against planktonic *C. albicans* cells was $0.50 \pm 0.06 \mu\text{M}$ (Figure 3.2). For melittin, at the two highest concentrations of 5 and 10 μM , 100% fungal inhibition was achieved with an MIC₅₀ of $0.976 \pm 0.14 \mu\text{M}$ (Figure 3.3).

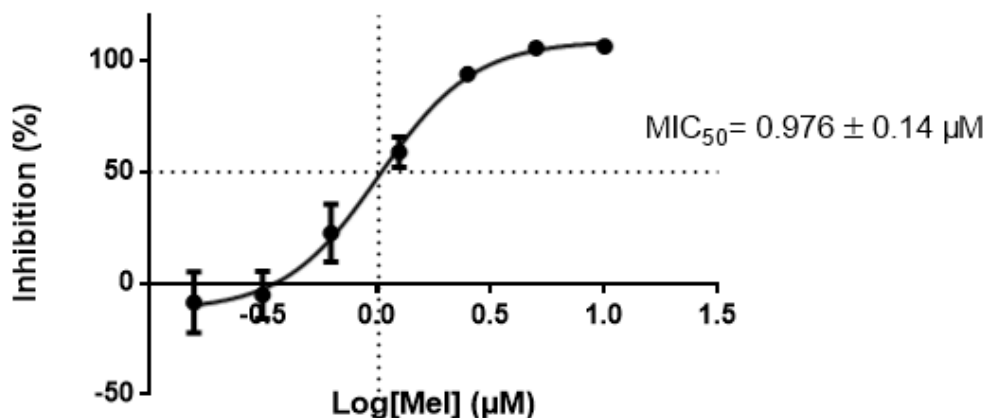


Figure 3.3: Antifungal activity of melittin against planktonic *C. albicans* cells. Melittin was serially diluted to concentrations ranging from 0.156 μM to 10 μM . Error bars are the mean $\pm$ SE. Results are of three independent biological repeats each performed in triplicate.

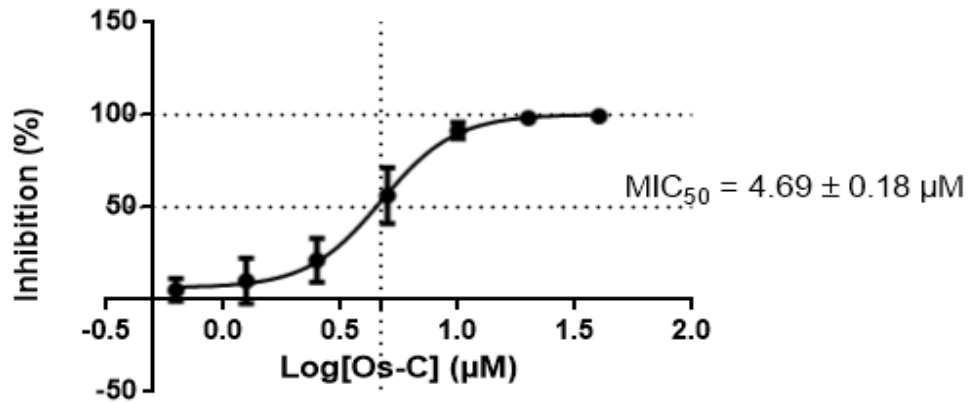


Figure 3.4: Antifungal activity of Os-C against planktonic *C. albicans* cells. Os-C was serially diluted to concentrations ranging from 1.25 μM to 80 μM . Error bars are the mean $\pm$ SE. Results are of four independent biological repeats each performed in triplicate.

For the peptide Os-C, a dose-dependent antifungal activity against the planktonic yeast cells was observed. A 100% inhibition was observed at 40 and 80 μM Os-C treatment and the MIC_{50} was determined as $4.69 \pm 0.18 \mu\text{M}$ (Figure 3.4).

From this data the MIC_{25} and MIC_{75} values for AmB and Os-C were calculated (Table 3.1) and these concentrations were used throughout this study.

Table 3.1: The MIC values of antifungal agents used in this study against *C. albicans* ATCC 90028

Antimicrobial agent	MIC_{25} (μM)	MIC_{50} (μM)	MIC_{75} (μM)
AmB	0.15	0.50 ± 0.06	1.00
melittin	N/A	0.98 ± 0.14	N/A
Os-C	2.80	4.69 ± 0.18	6.20
Penetratin	N/A	1.00 *	N/A

* Value obtained from Gong & Karlsson (2017). N/A = Not assigned

3.2 Cell morphology investigated by SEM

The effect of different concentrations of AmB and Os-C on the ultrastructural morphology of planktonic *C. albicans* cells was investigated using SEM. The untreated *C. albicans* cells displayed a typical cell shape with a smooth surface and an intact cell wall and cell membrane (Figure 3.5 A and B).

AmB, used as a positive control, is an antifungal drug that binds to ergosterol in the cell membrane inducing pore formation (Mesa-Arango *et al.*, 2012). Cells treated with 0.15 μM (MIC_{25}) of AmB showed no change in the overall shape, but distinct small extracellular vesicles (EVs) were observed on the surface as well as in the extracellular environment (white arrow heads) (Figure 3.5 C). Budding scars (asterisk) and cellular extravasation (white arrows) were also observed (Figure 3.5 D). Cells exposed to 0.5 μM (MIC_{50}) of AmB showed a collapse in the cell structure with cytoplasmic retraction (double-sided white arrows) and ruffling of the cell membrane. Active formation of buds and budding scars (asterisks) were also observed (Figure 3.5 E and F).

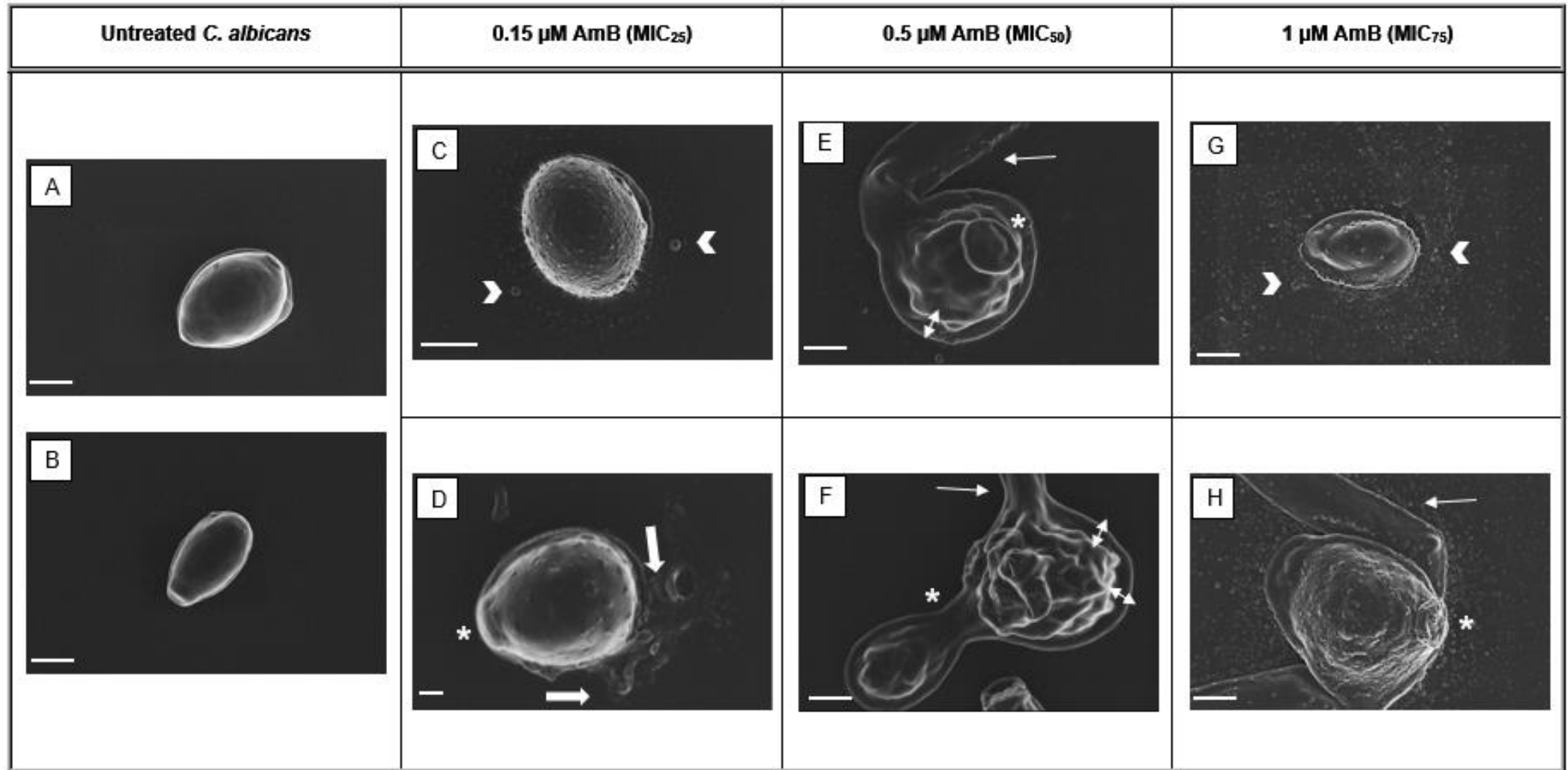


Figure 3.5: Effect of AmB on the morphology of planktonic *C. albicans* cells. Cells were exposed to concentrations equal to the MIC_{25} , MIC_{50} and MIC_{75} for 3 hours and viewed with SEM. Two images per concentration shows a comprehensive effect on the treated cells. This includes depression in cell surfaces, cytoplasmic retraction (double-sided white arrows), budding scars and formation (asterisk), hyphal formation (thin white arrows), extracellular vesicles (arrow heads) and cellular extravasation (thick white arrows). Scale bars = 1 μm .

At a concentration of 1 μM (MIC_{75}) the few remaining cells exhibited acute blebbing on the surface, indentations in the cell membrane, cell budding (asterisk) and hyphal formation (thin white arrows) (Figure 3.5 G and H). Large amounts of extracellular debris were present including the production of EVs (arrow heads). Most of these cells collapsed inward due to the leakage of the cytosol and disintegration of the cell membrane. (Figure 3.5 G).

C. albicans cells treated with 2.8 μM (MIC_{25}), 4.7 μM (MIC_{50}) and 6.2 μM (MIC_{75}) Os-C showed loss in cell wall and membrane integrity (Figure 3.6). Cells exposed to 2.8 μM Os-C displayed small irregularities in their shape and cytoplasmic retraction (white arrows). Bud formation was also observed (asterisk) (Figure 3.6 C and D). After treatment with 4.7 μM Os-C depressions in the cell surfaces were visible and cell membrane retraction from the cell wall was observed (Figure 3.6 E and F, white arrows). These characteristics were more pronounced after treatment at the MIC_{75} . The cells appeared shrivelled and completely deformed (Figure H), with cellular extravasation (white arrows), and buds and blebs on the cell surface (Figure 3.6 G and H). Hyphal filaments were also formed (thin white arrows), which is a process indicative of the stress response.

Both AmB and Os-C affected the overall structure and morphology of the *C. albicans* cells in a concentration-dependent manner but in different ways, indicating a difference in mode of action.

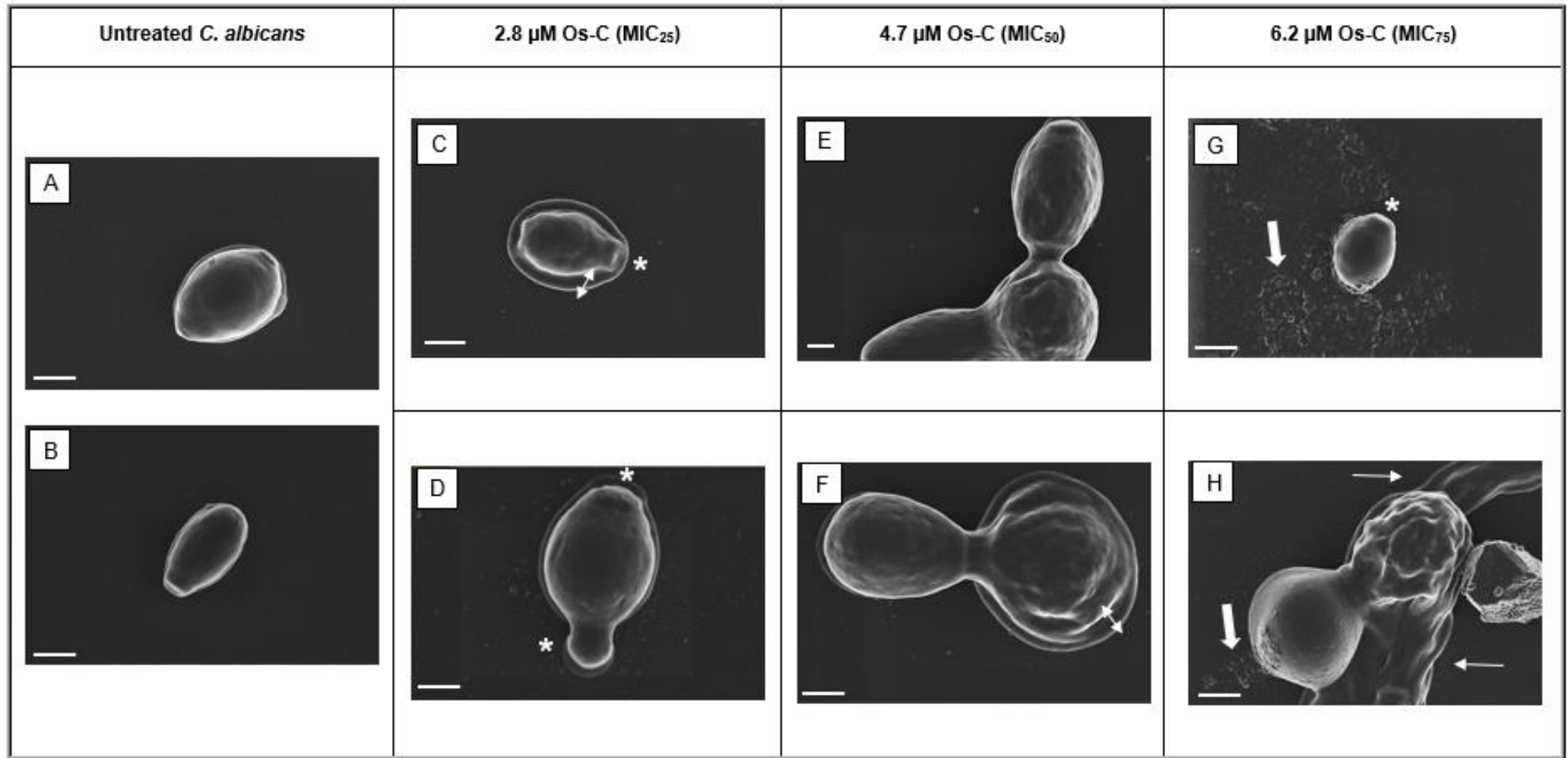


Figure 3.6: Effect of Os-C on the morphology of planktonic *C. albicans* cells. Cells were exposed to Os-C at concentrations equal to the MIC₂₅, MIC₅₀ and MIC₇₅ for 3 hours and viewed with SEM. Two images per concentration shows a comprehensive effect on the treated cells. The effects on the treated cells include depression in cell surfaces, cytoplasmic retraction (double-sided white arrows), bud formation/scars (asterisk), hyphal formation (thin white arrows) and cellular extravasation (thick white arrows). Scale bars = 1 μ m.

3.3 Cell ultrastructure investigated by TEM

TEM was used to determine the ultrastructural and internal effects of different concentrations of AmB and Os-C on planktonic *C. albicans* cells. The cells were treated with 0.15 μM (MIC_{25}), 0.5 μM (MIC_{50}), and 1 μM (MIC_{75}) AmB and 2.8 μM (MIC_{25}), 4.7 μM (MIC_{50}) and 6.2 μM (MIC_{75}) Os-C respectively for three hours. The subsequent damage to the cell wall, membrane and internal cell components is shown in Figures 3.7 and 3.8.

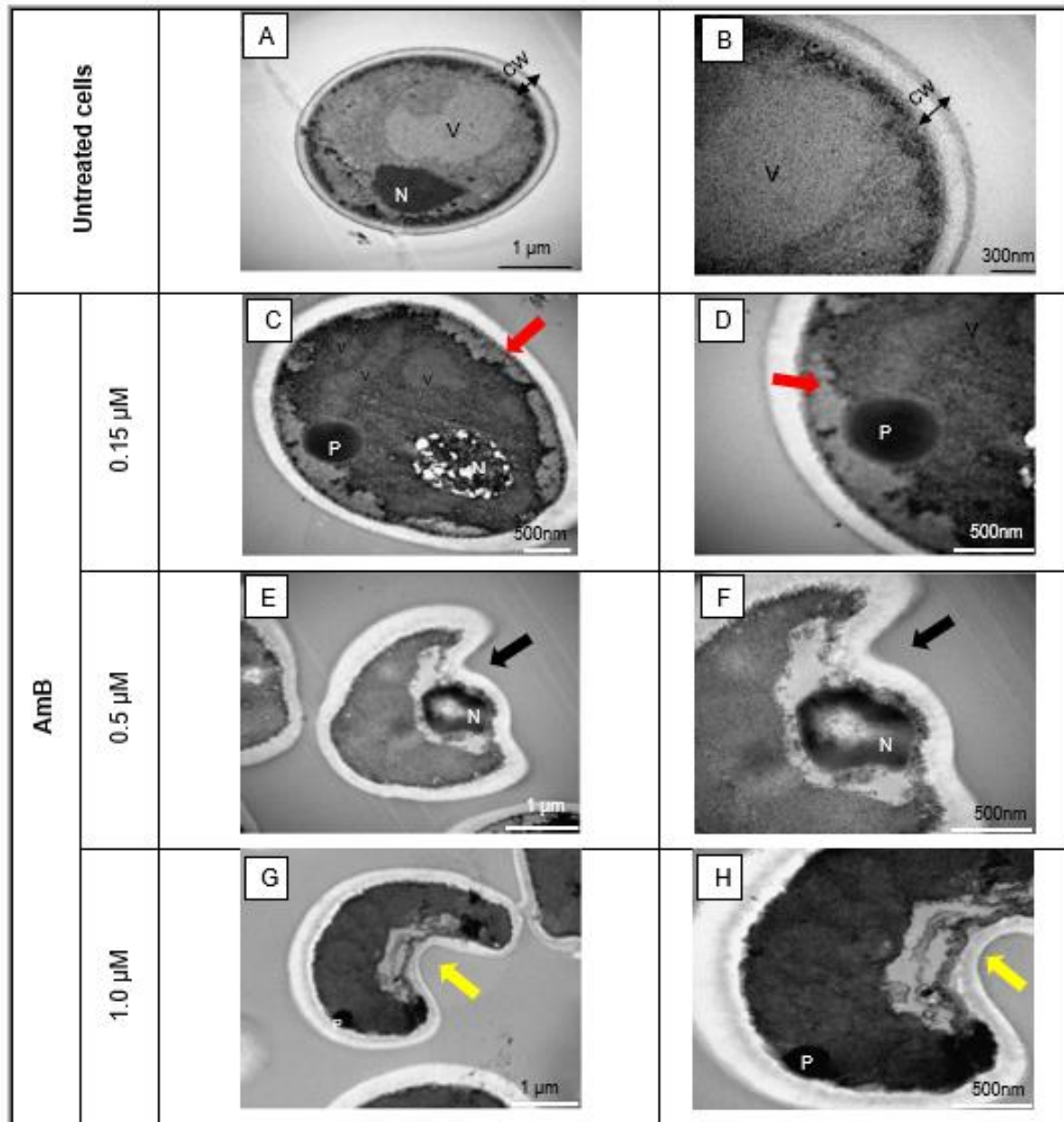


Figure 3.7: Effect of AmB on the ultrastructure of *C. albicans* cells. Cells were exposed to AmB at concentrations equal to the MIC_{25} , MIC_{50} and MIC_{75} for 3 hours and viewed with TEM. CW: cell wall, N: nucleus, V: vacuole, P: peroxisome. Effects on the treated cells include cell membrane detachment from cell wall (red arrows), depression in cell surfaces (black arrows) and final inward collapse (yellow arrows).

The morphology of the untreated *C. albicans* cells was normal with a uniform distribution of the cell content, an electron dense nucleus, a large vacuole and a cytoplasm with other membrane bound organelles. The cell is enveloped by an intact cell wall and a regular double layer cell membrane (Figure 3.7 A and B).

After three hours of treatment with AmB at a concentration equal to the MIC₂₅ the cell membrane has started to detach from the cell wall (red arrows) and the cell content appeared more electron dense. Enlarged and distinct peroxisomes were also formed (Figure 3.7 C and D). Cells exposed to AmB at its MIC₅₀ showed a collapse in the cell structure with definite indentations and irregularities in the cell membrane and cell wall (Figure 3.7 E and F, black arrows). Cells treated with AmB at its MIC₇₅ appeared completely altered due to cellular extravasation and final inward collapsing of the cell membrane and wall (Figure 3.7 G and H, yellow arrows). Widening of the space between the cell membrane and wall corresponds to the retraction observed with SEM.

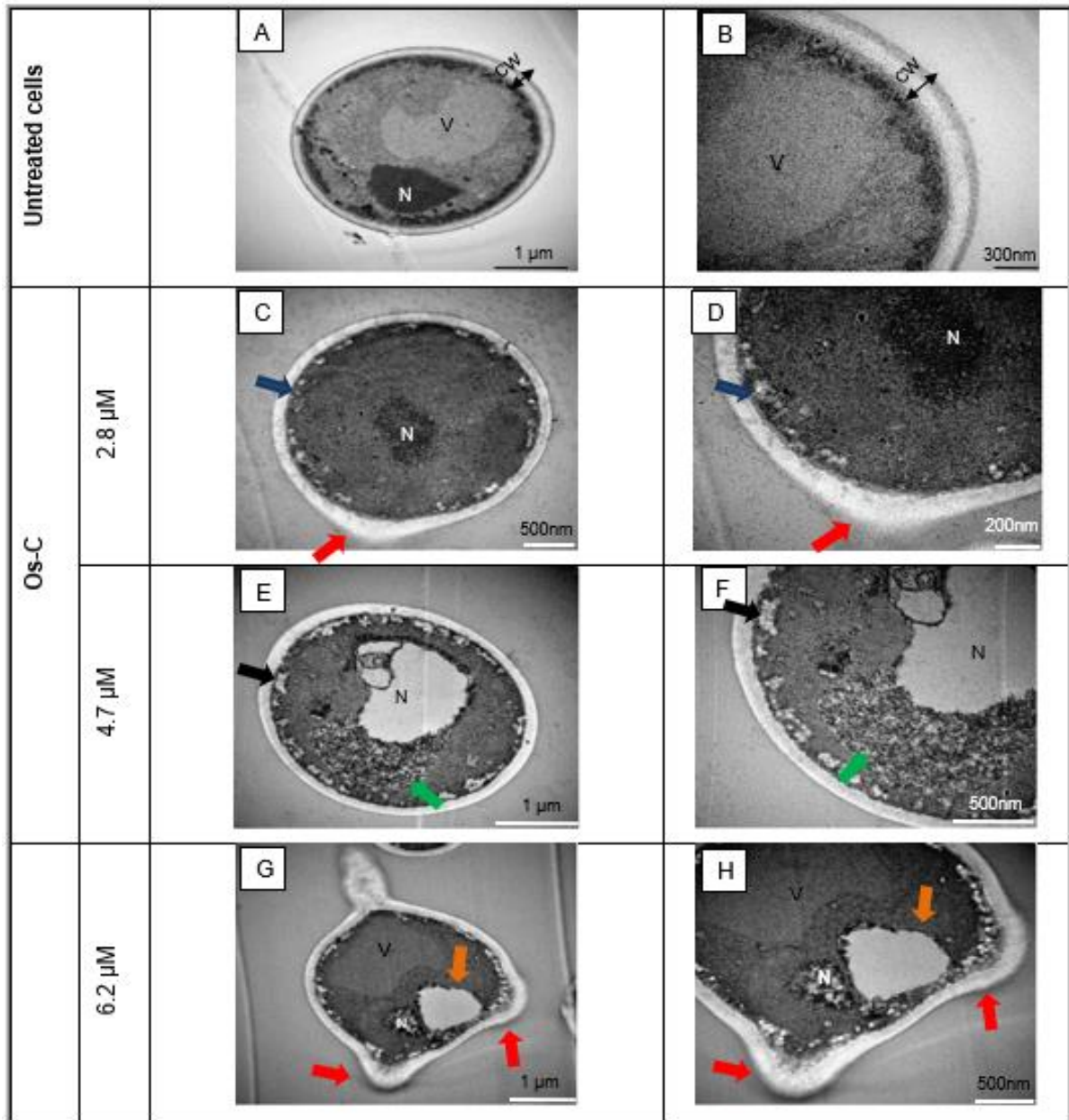


Figure 3.8: The effect of Os-C on the ultrastructure of planktonic *C. albicans* cells. Cells were exposed to Os-C at concentrations equal to the MIC₂₅, MIC₅₀ and MIC₇₅ for 3 hours and viewed with TEM. CW: cell wall, N: nucleus, V: vacuole. Effects on the treated cells include bud formation (red arrows), small vacuoles (blue arrows), fused small vacuoles (black arrows), intracellular granulation (green arrows), damaged organelles (orange arrows).

C. albicans cells exposed to 2.8 µM (MIC₂₅) Os-C showed small irregularities in their shape and cell wall as well as the formation of buds (red arrows). Small spherical vacuoles appeared close to

the cell membrane (Figure 3.8 C and D, blue arrows). After treatment with 4.7 μM (MIC_{50}) Os-C the small vacuolar bodies close to the cell membrane increased in size and number (black arrows). Intracellular organelles such as the nucleus are degraded, and intracellular granulation was detected (green arrows) (Figure 3.8 E and F). At 6.2 μM (MIC_{75}), membranes had retracted further, tearing from the cell wall, granulating bodies appeared, organelles were damaged (orange arrows) and buds or blebs started to form (red arrows) possibly due to the destabilization of the cell wall (Figure 3.8 G and H).

3.4 Cell wall polysaccharide binding: Isothermal titration calorimetry

The ability of Os-C to bind to polysaccharides, laminarin and mannan, of the fungal cell wall was investigated using ITC measurements at 30°C. Each peak in the binding isotherm represents a single 2 μL injection of the polysaccharide (94 μM or 1.88 mM) into the 350 μL Os-C solution (4.7 μM or 47 μM).

Figure 3.9 shows the ITC curve of the buffer runs as well as the interaction between EDTA and Ca^{2+} to confirm that the MicroCal iTC200 was accurate and conditions were optimized. Both laminarin and mannan were inert in the presence of 10 mM NaP buffer (pH 7.4) and only background noise was detected. The titration of EDTA with CaCO_3 showed a definite binding profile and the expected binding isotherm. Ca^{2+} bound to EDTA with a high binding affinity (K_b) of $6.49 \pm 1.74 \times 10^7 \text{ M}^{-1}$, 1.47 binding sites and the enthalpy for this interaction was determined as $-7.96 \pm 0.022 \text{ kcal.mol}^{-1}$.

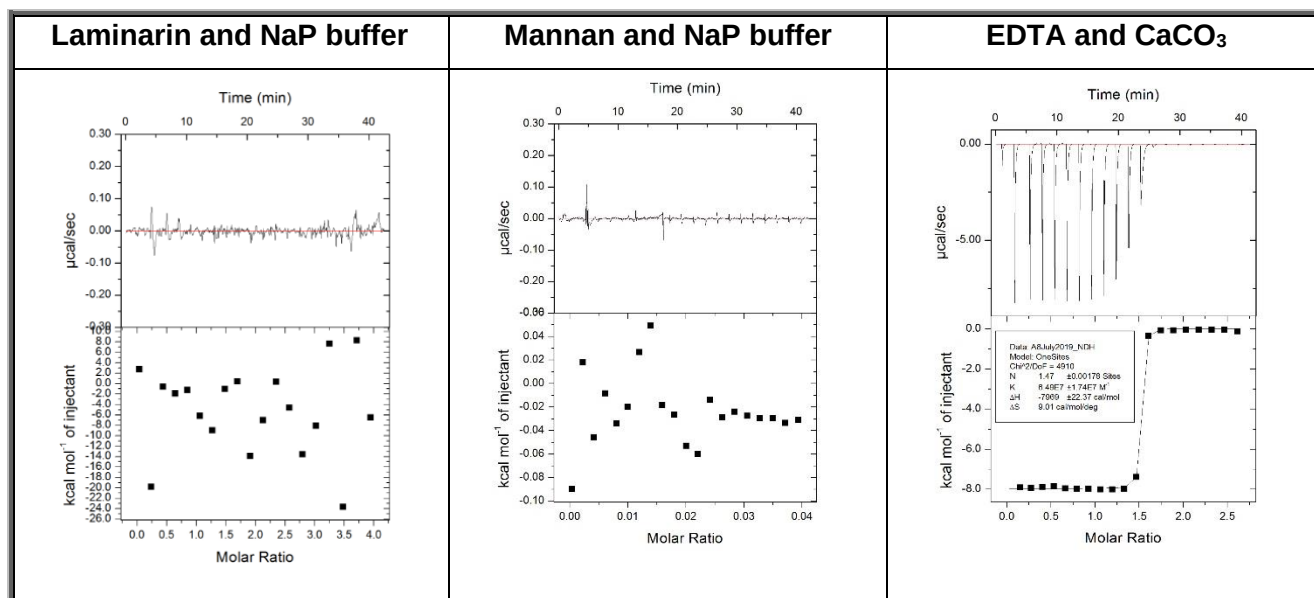


Figure 3.9: ITC binding isotherms of the positive and negative controls. The heat change is indicated after mixing laminarin and mannan with 10 mM NaP buffer alone and the metal chelator EDTA with CaCO_3 . The top half of every image shows the heat change data obtained after every 2 μL automatic injection whilst the bottom half of every image shows an integrated binding curve.

Os-C at 4.7 μM (MIC_{50}) was titrated with 94 μM laminarin and mannan, respectively, at a 1:20 ratio. These titrations resulted in negligible heat release or uptake indicating no interaction between Os-C and the cell wall polysaccharides at the tested concentrations (Figure 3.10 A and C).

Increased concentrations of both Os-C (47 μM) and the polysaccharides (1.88 mM) at a ratio of 1:40, did not change the outcome and no binding was detected (Figure 3.10 B and D).

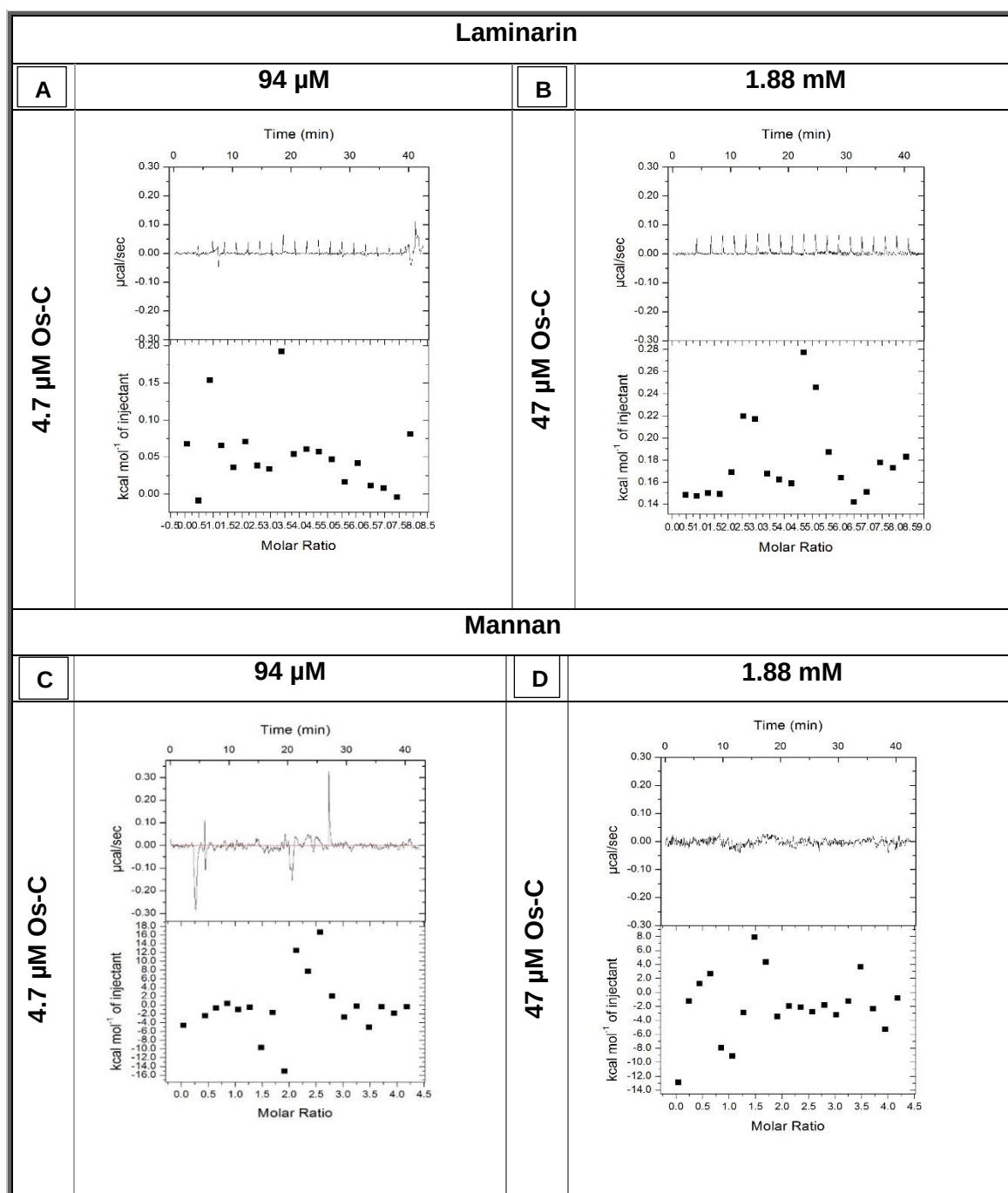


Figure 3.10: Cell wall polysaccharide binding determined with ITC. Analysis of binding of Os-C at 4.7 μM (A & C) and 47 μM (B & D) with laminarin at 94 μM (A) and 1.88 mM (B) or mannan at 94 μM (C) and 1.88 mM (D) at 30°C. The top half of every image shows the heat change data obtained after every 2 μL automatic injection whilst the bottom half of every image shows an integrated binding curve.

3.5 Cell wall polysaccharide binding: Micro-broth dilution assay

To confirm the results obtained from ITC, a cell wall polysaccharide binding assay was performed. Under normal conditions the MIC of Os-C (20 μM) causes 100% planktonic *C. albicans* cell inhibition. The antifungal activity of 20 μM Os-C remained at 100% inhibition even after the addition of 800 μM excess laminarin or excess mannan (Figure 3.11). This supports the findings of ITC

showing no binding between Os-C and laminarin or mannan at low and physiologically relevant concentrations.

However, at a higher concentration of laminarin (39.6 mM) the inhibition of Os-C on cell viability was reduced from 100% to 35.7% (Figure 3.11) whilst for the higher concentration of mannan (30.0 mM) the slight decrease was insignificant. This indicates that excess laminarin had a greater effect on the Os-C inhibition activity than excess mannan.

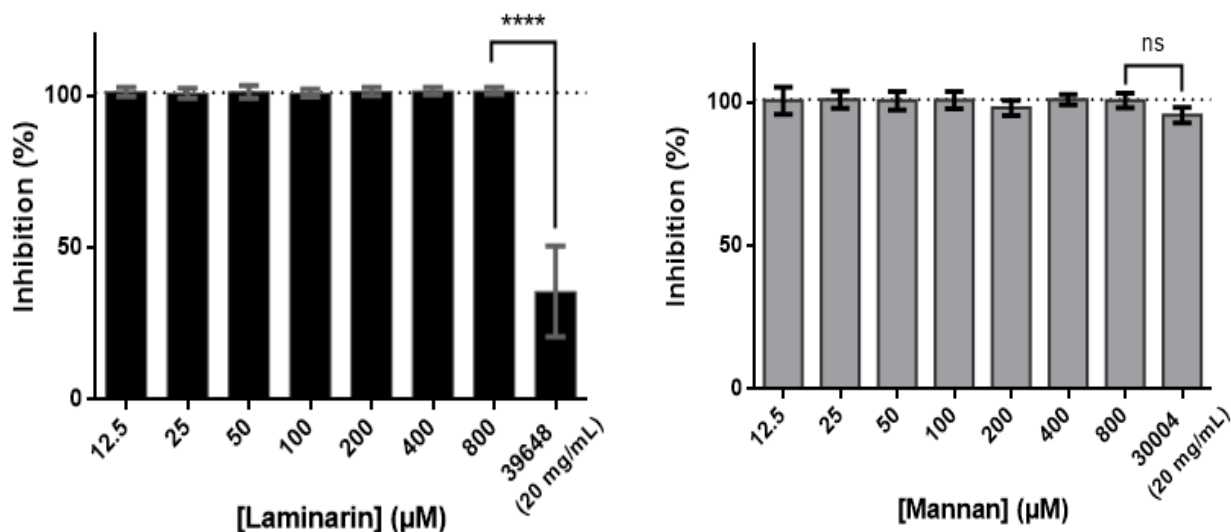


Figure 3.11: Cell wall polysaccharide binding assay. The inhibition activity of Os-C (20 μM) against planktonic *C. albicans* cells in the presence of increasing concentrations of cell wall polysaccharides laminarin (12.5 μM to 40 mM) and mannan (12.5 μM to 30 mM). Error bars are the mean ± SE. Significant difference is indicated by ns, *, **, *** and **** which represents no significant difference, $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ values, respectively. Results are of three independent biological repeats each performed in triplicate.

3.6 Membrane depolarization

Most AMPs are known to target the membranes of pathogenic microorganisms (Shai, 2002). Many studies have identified that membrane damage is the primary mode of action based on membrane permeabilization assays. The membrane depolarization assay evaluates the effect of AMPs on the polarity of cell membranes. The effect of Os-C treatment on the membrane potential of planktonic *C. albicans* was investigated using the potential sensitive probe DiBAC₄(3) and melittin as the positive control (Figure 3.12).

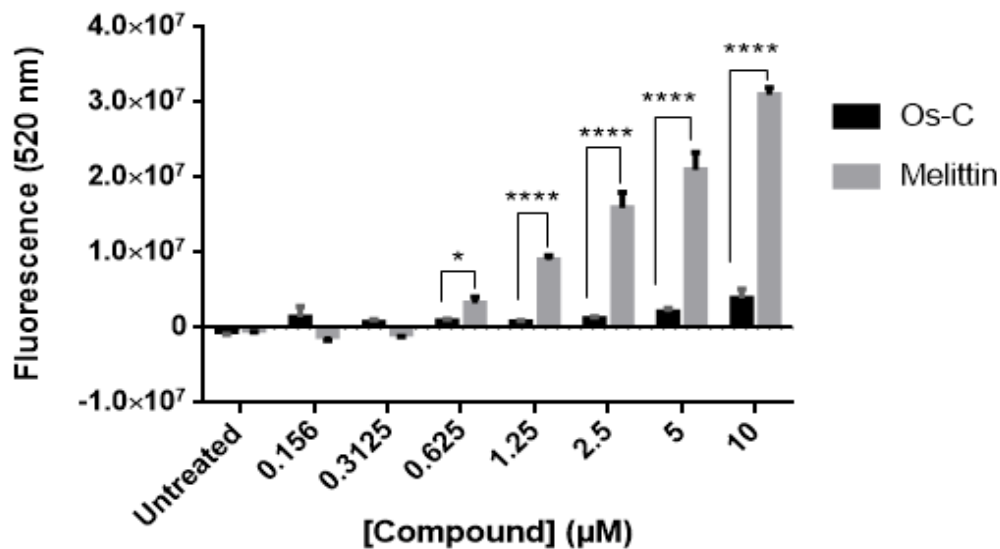


Figure 3.12: Depolarization of *C. albicans* cell membranes by Os-C and melittin (control). Error bars are the mean $\pm$ SE. Significant difference is indicated by *, **, *** and **** which represents $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ values, respectively. Results are of four independent biological repeats each performed in triplicate.

Figure 3.12 shows that melittin was able to depolarize the planktonic cell membranes at a concentration as low as 0.625 μ M, which was comparable to the extent of depolarization observed at 10 μ M for Os-C. Compared to melittin, Os-C did not have a significant effect on the potential of *C. albicans* membranes.

3.7 Os-C interaction with vacuoles

Fungal vacuoles play a key role in transmembrane transport, the immunity of cells and maintaining homeostasis (Klionsky *et al.*, 1990). To identify the vacuoles of planktonic *C. albicans* cells as potential intracellular targets of Os-C, flow cytometry and CLSM experiments were undertaken. Cells were treated with Os-C at 2.8 and 6.2 μ M respectively, concentrations equal to the MIC₂₅ and MIC₇₅ of the unlabelled peptide. Free 5-FAM and penetratin served as controls.

Flow cytometry was employed to quantify subcellular localization of Os-C within planktonic *C. albicans* cells. The data in Figure 3.13 shows that the untreated cells were negative for 5-FAM fluorescence and positive for CTr vacuolar fluorescence. Cells treated with 5-FAM were also negative for 5-FAM fluorescence and positive for CTr vacuolar fluorescence indicating that free 5-FAM is unable to enter cells on its own but requires a carrier. For both the untreated and 5-FAM treated cells, 85.11% and 85.06% of the cell population stained positively for the vacuole stain CTr. Figure 3.13 shows that 5-FAM labelled penetratin entered planktonic *C. albicans* and permeabilized the vacuoles, thereby preventing CTr staining. Consequently, of the 99.97% of the cells staining positively for 5-FAM-penetratin, only 0.08% of the vacuoles remained.

Planktonic *C. albicans* cells treated with 5-FAM labelled Os-C was positive for 5-FAM and CTr fluorescence at both concentrations. At 2.8 μ M, 96.96% of the cell population stained positive for

5-FAM-Os-C and 53.85% of that population had intact vacuoles and CTr staining. Thus, at 2.8 μ M Os-C 31.26% (85.11% - 53.85%) of the vacuoles are degraded compared to the untreated population. Staining after exposure to 6.2 μ M Os-C showed 96.89% of the cells were positive for 5-FAM-Os-C and of those only 50.79% had intact vacuoles. Thus, the higher concentration of Os-C degrades 34.32% (85.11% - 50.79%) of the vacuoles compared to the untreated population.

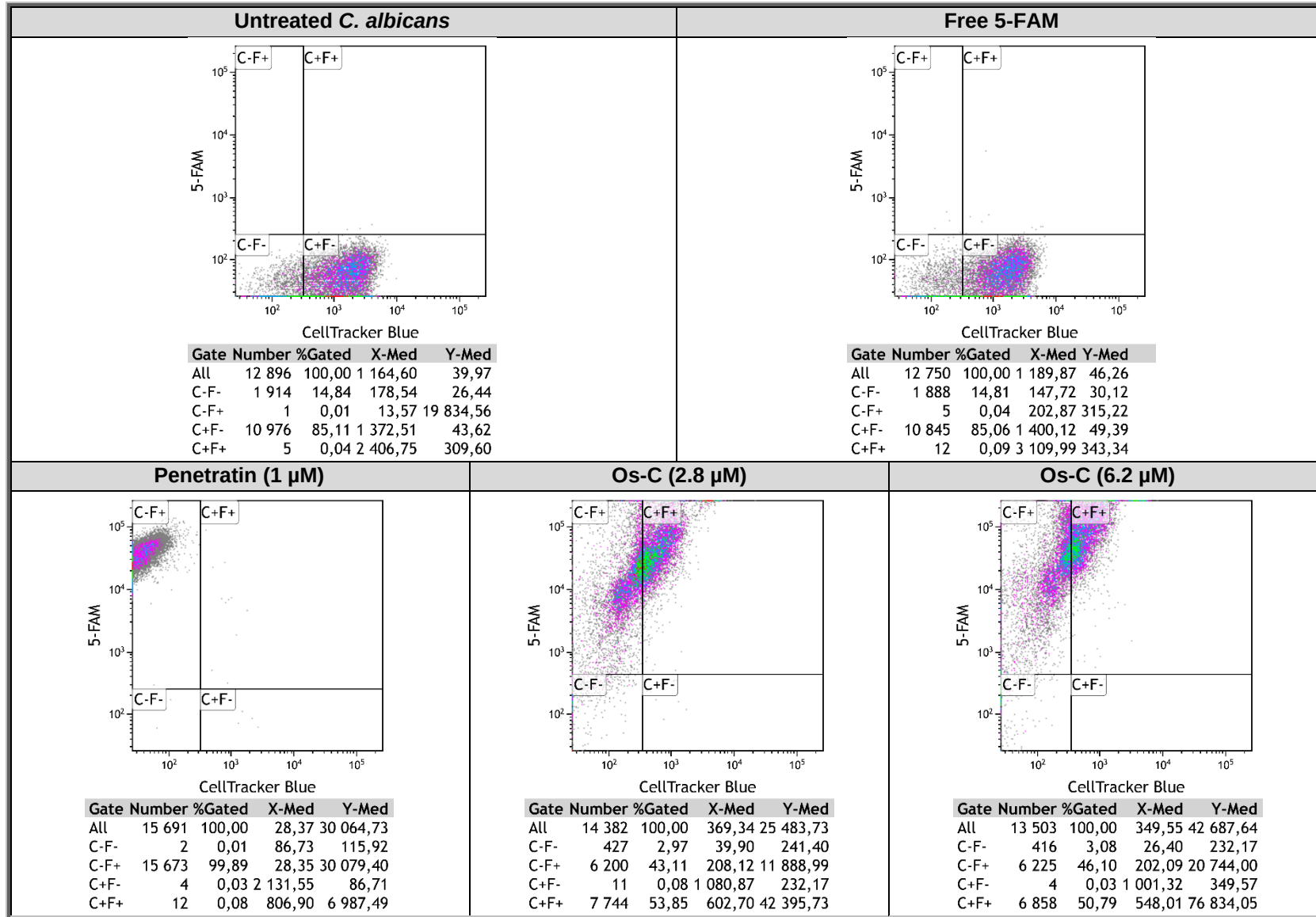


Figure 3.13: Flow cytometry data showing 5-FAM and vacuolar fluorescence. Cells treated with H₂O or free 5-FAM at 6.2 μ M showed 0% 5-FAM fluorescence and 85% vacuole stain, whilst cells treated with penetratin at 1 μ M and Os-C at 2.8 and 6.2 μ M (MIC₂₅ and MIC₇₅ of unlabelled peptide) showed 99%, 43% and 46% 5-FAM and 0%, 54% and 51% 5-FAM/vacuole stain.

The numerical data obtained from flow cytometry is also graphically presented in Figure 3.14. Intracellular localization was quantified by gating two populations of cells: one with strong 5-FAM fluorescence intensity and the other with strong CTr vacuole stain. Of all the cells positive for 5-FAM labelled Os-C, 53.85% and 50.79% of the cells had an intact vacuole. Figure 3.14 shows no significant differences in the percentage of cells positive for vacuole stain between the lower and higher Os-C treatments. Therefore, Os-C enters *C. albicans* cells and degrades the vacuoles but not as rapidly or to the same extent as penetratin.

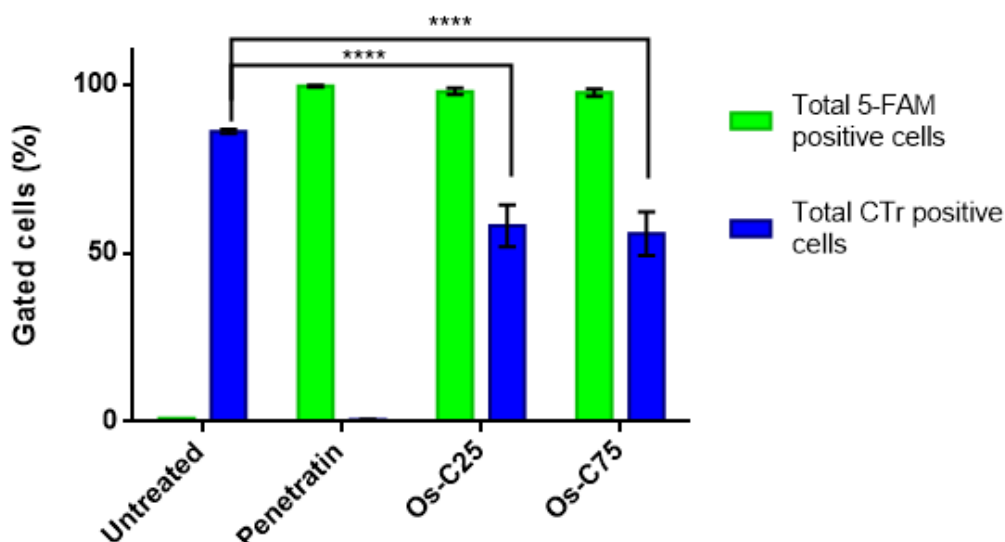


Figure 3.14: The quantification of cellular location of Os-C and CTr stain in *C. albicans* cells. Cells were treated with 5-FAM-penetratin at 1 μ M and 5-FAM-Os-C at 2.8 and 6.2 μ M (MIC_{25} and MIC_{75} of unlabelled peptide). Of all the cells positive for 5-FAM-Os-C, 53.85% (MIC_{25}) and 50.79% (MIC_{75}) of the cells contained Os-C in their vacuoles. Error bars are the mean $\pm$ SE. Significant difference is indicated by *, **, *** and **** which represents $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ values, respectively. Results are of three independent biological repeats each performed in triplicate.

The intracellular trafficking and localization of these peptides were also evaluated with CLSM (Figure 3.15). CTr alone entered the untreated cells and localized to the vacuoles (Figure 3.15, Untreated). Figure 3.15 also confirms that no 5-FAM fluorescence was detected for untreated cells but only blue CTr fluorescence.

C. albicans cells treated with free 5-FAM showed no intracellular green fluorescence indicating that 5-FAM is unable to enter *C. albicans* cells on its own and requires Os-C to cross the cell membrane (results not shown). This suggests that Os-C acts as a carrier peptide that can transport cargo such as 5-FAM across fungal cell membranes.

Both penetratin, and Os-C were able to enter and localize within the *C. albicans* cells. Green 5-FAM fluorescence was detected within the few remaining cells showing that the labelled penetratin was able to cross the membrane (Figure 3.15, penetratin). Green 5-FAM-penetratin was present throughout the cytoplasm. Figure 3.15 (overlay) shows the green fluorescence overlapping with the spots of blue fluorescence (white arrows), confirming that penetratin is able to enter the vacuoles. Subsequent vacuole degradation is indicated by cells containing only 5-FAM-penetratin and no CTr fluorescence (red arrows).

Cells treated with 5-FAM labelled Os-C at 2.8 μM displayed green fluorescence along the cell membrane of the cells and/or within the intracellular space (Figure 3.15). The white arrows indicate that Os-C targets the cell membrane at lower concentrations and then accumulates in the cytoplasm with CTr fluorescence in the vacuoles.

Cells treated with 5-FAM labelled Os-C at 6.2 μM also displayed both green and blue fluorescence similar to *C. albicans* cells exposed to 2.8 μM 5-FAM-Os-C. The only difference between the concentrations is that at the higher concentration no cell membrane localization was observed (Figure 3.15). The fluorescence intensity, indicating the amount of 5-FAM-Os-C located in the intracellular environment, is concentration-dependent.

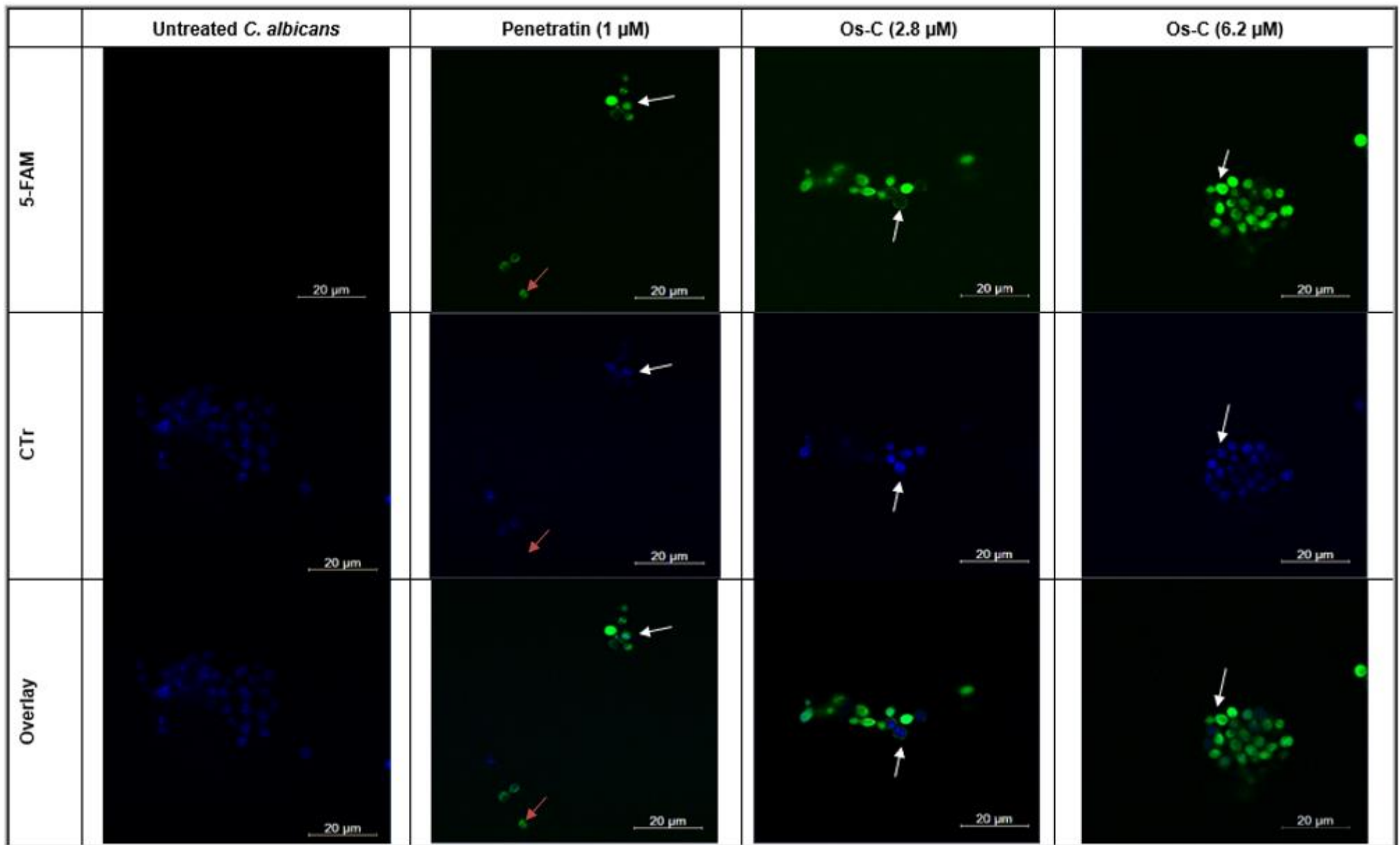


Figure 3.15: Localization of 5-FAM labelled Os-C and CTr stained vacuoles in cells. White arrows indicate the localization of 5-FAM labelled Os-C versus the CTr stain in the same cells.

CHAPTER 4: DISCUSSION

There is an urgent need for new antimicrobial therapeutic agents due to the global AMR crisis. Current antimicrobial drugs are failing to keep severe infections such as *Candidiasis* at bay due to the development of resistance to existing antifungal drugs (Levy, 2002). The misuse of antimicrobial drugs induces genetic variation of microorganisms causing only resistant strains to survive (Holmes *et al.*, 2016). Fungal species such as *C. albicans* grow into highly resistant biofilms and can cause fatal infections especially in immunocompromised individuals (Park *et al.*, 1998). AMPs are a possible solution to the AMR crisis as these peptides are amphipathic, short and cationic in character enabling them to target microbial cell walls and membranes (Brogden, 2005). Furthermore, the development of resistance against AMPs is less probable than for conventional antimicrobial drugs (Zasloff, 2002).

Os-C, the AMP investigated in the present study, consists of 19 amino acids and has a net charge of +6 due to the presence of five lysine and one arginine residues. These positive residues help to maintain the cationic properties and enhance peptide solubility. Os-C is rich in glycine residues and its <H> value of 0.045 is much lower than the parent peptide OsDef2 (<H> value of 0.371) and the other derivative Os (<H> value of 0.249) (Table 1.5). Although a higher <H> value is associated with higher antimicrobial activity, recent studies have shown that AMPs with higher <H> values are more likely to form dimers and aggregate on membrane surfaces whereas AMPs with lower <H> values retain their α -helical conformation (Yin *et al.*, 2012). Higher <H> values are also associated with increased cytotoxicity and haemolysis of human erythrocytes (Chen *et al.*, 2007). Thus, peptides with lower <H> values such as Os-C are not inclined to self-associate and are able to cross cell membranes without causing mammalian cytotoxicity. This has been confirmed in laboratory-based studies where Os-C was found to have antibacterial (Prinsloo *et al.*, 2013 & Taute *et al.*, 2015) and antifungal activity (Mbuayama *et al.*, 2020) but did not cause erythrocyte haemolysis or cytotoxic effects in several cell lines (Prinsloo *et al.*, 2013).

In a previous study, Mbuayama *et al.* (2020) determined the MIC ($2.2 \pm 0.9 \mu\text{M}$) and minimum fungicidal concentration (MFC; $28.0 \mu\text{M}$) of Os-C for *C. albicans* using the radial diffusion assay (RDA) and the colony forming unit assay, respectively. The micro-broth dilution assay is preferred over the RDA, as it allows many samples to be tested, with simple, direct and rapid analysis of data (Steinberg & Lehrer, 1997). Further advantages are that this assay allows the quick and cost-effective screening of antimicrobial agents. In this assay the minimum concentration of a drug that inhibits the growth of planktonic cells by 50% within a specific time period compared to the growth control, is defined as the MIC₅₀ (Rodriguez-Tudela *et al.*, 2008). Mbuayama *et al.* (2020) first conducted the micro-broth dilution assay according to the method in the EUCAST definitive document (Rodriguez-Tudela *et al.*, 2008), with minor modifications. In the protocol RPMI-1640 containing 1% glucose is used for the cultivation of *C. albicans*. In this environment, even the

highest concentration of Os-C (100 μM) was inactive. RPMI-1640 is rich in salts and other cations that can bind to the negatively charged cell wall and membranes (Yacoub *et al.*, 2015 & Bals, 1998) preventing the binding of cationic molecules such as Os-C. Other studies also concluded that the presence of salts and other cations may affect the activity of defensin-derived peptides (Yacoub *et al.*, 2015 & Bals, 1998). Consequently, Mbuayama *et al.* (2020) used the RDA and colony forming unit assays for all further studies. In this study, a modified version of the micro-broth dilution assay was developed for the rapid screening and mode of action studies of Os-C in a low salt environment.

For the development of the modified micro-broth dilution assay it was necessary to determine the minimum concentration of RPMI-1640 required to ensure *C. albicans* growth comparable to cells grown in 100% RPMI-1640. It was also necessary to determine the sensitivity of the *C. albicans* cells to an antifungal agent, known to target ergosterol in the cell wall, in the reduced RPMI-1640 media. Increased sensitivity would indicate that cell wall synthesis is compromised in a low salt environment. To achieve this, the effect of serial dilutions of RPMI-1640 diluted in NaP on the growth of *C. albicans* was determined (Figure 3.1). The lowest concentration of RPMI-1640/NaP that supported the growth of planktonic *C. albicans* at 37°C for 3 hours similar to 100% RPMI-1640 was the 10% RPMI-1640/NaP medium. To evaluate *C. albicans* sensitivity, the antifungal activity of 10 μM AmB at all RPMI-1640/NaP dilutions was determined. No significant differences were observed and in 10% RPMI-1640/NaP the antifungal activity of AmB was the same as in 100% RPMI-1640 (Figure 3.1).

Using the modified micro-broth dilution assay, the MIC_{50} of the control AmB and of Os-C was determined against planktonic *C. albicans* cells. Both AmB and Os-C caused a dose-dependent increase in antifungal activity in the low salinity environments. Under these conditions the MIC_{50} of AmB, $0.50 \pm 0.06 \mu\text{M}$, was similar to the MIC_{50} value of $0.386 \pm 0.004 \mu\text{M}$ determined using the standard micro-broth dilution assay. Moreover, the MIC_{50} value was similar to the value of $0.54 \mu\text{M}$ reported by Chandra *et al.* (2001) determined with the XTT reduction assay, $0.52 \mu\text{M}$ reported by Zarif *et al.* (2000) using the micro-broth dilution assay and $0.52 \mu\text{M}$ reported by Tang *et al.* (2014) using the paper-plate technique. This confirmed that in the low salt environment in the modified micro-broth assay, the MIC_{50} value obtained for AmB is valid.

The MIC_{50} of the positive control, melittin, was determined to be $0.976 \pm 0.14 \mu\text{M}$ (Table 3.1) while the MIC_{50} of Os-C was determined as $4.69 \pm 0.18 \mu\text{M}$ (Figure 3.3). Although melittin has a lower MIC_{50} , haemolytic effects have limited the development of this AMP as a therapeutic agent. The MIC_{50} value for Os-C is in the same range as cecropin B ($4 \mu\text{M}$) against *C. albicans* strain SC5314 (Gong *et al.*, 2017). Cecropin B is a naturally occurring AMP isolated from the Cecropia moth (*Hyalophora cecropia*) and consists of 35 amino acids, lacking cysteine residues. The structure (KWKIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL) is mainly α -helical with an amphipathic basic N-terminal α -helical segment linked by a hinge region to a hydrophobic C-terminal. This AMP has

a broad spectrum of activity against Gram-positive and -negative bacteria as well as fungi and is already in clinical use in the porcine industry (Hu *et al.*, 2013).

The MIC₅₀ value for Os-C is also similar to that of the synthetic multiple antigenic peptide (MAP; MIC₅₀ of 4 µM) with a net charge of +5 (Gong *et al.*, 2017). MAPs (KLALKLALKALKALKLA) are actively used for immunodiagnosis of viral diseases such as Acquired Immunodeficiency Syndrome (AIDS) and Hepatitis C (Brattegaard *et al.*, 1995 & Saravanan *et al.*, 2004). From these results it can be deduced that the potency of Os-C is comparable to AMPs that have already been developed and used clinically in the biomedical industry.

In this study, the mode of action of the tick-derived Os-C peptide was further explored in planktonic *C. albicans*. The mode of action of AMPs can be divided into two categories: direct killing or immune modulation (Kumar *et al.*, 2018). The effects and targeting of antimicrobial agents are concentration-dependent as has been described by Munoz *et al.* (2006) for an antifungal hexapeptide and Grela *et al.* (2019) for AmB. Therefore, the effect of Os-C on the morphology of planktonic *C. albicans* cells was determined at concentrations equal to the MIC₂₅, MIC₅₀ and MIC₇₅ using both SEM and TEM providing information on the extra- and intracellular morphology of the cells, respectively.

AmB which was used as the positive control is a known antifungal drug that binds to ergosterol in the cell membrane inducing pore formation (Mesa-Arango *et al.*, 2012). At the MIC₂₅ of AmB, cell morphology investigated by SEM (Figure 3.5) was generally normal but small EVs were observed on the surface as well as in the extracellular environment. At the MIC₅₀ cell structure had collapsed, cytoplasmic retraction was present, and the cell membrane was ruffled. The few remaining cells exposed to AmB at the MIC₇₅ exhibited acute blebbing on the surface and indentations in the cell membrane. Large amounts of extracellular debris were present including the presence of EVs. Most cells had collapsed inwards due to the leakage of the cytosol and disintegration of the cell membrane. These results seem to correlate with the SEM images of *C. albicans* treated with AmB found by Grela *et al.* (2019) who observed collapsed mother cells with indentations in the overall shape, deformed phenotypes, irregular shapes, budding scars and bud formation.

The typical morphology of *C. albicans* cells evaluated with TEM included a uniform distribution of the cell content, an electron dense nucleus, a large vacuole and a cytoplasm with other membrane bound organelles (Figure 3.7). At the MIC₂₅ of AmB the cell wall had started to expand, and the cell content appeared more electron dense. Enlarged and distinct peroxisomes had also formed. At the higher MIC₅₀ concentration, AmB caused the collapse in cell structure with the formation of indentations and irregularities in the cell membrane and wall. At a concentration equal to the MIC₇₅, AmB caused cellular extravasation and final inward collapsing of the cell membrane and wall. The space between the cell membrane and wall corresponds to the retraction observed with SEM.

Grela *et al.* (2019) also reported irregularities and expansion of the cell wall, a denser cytoplasm and anamorphous cell shape.

Cells treated with Os-C at a concentration equal to its MIC₂₅ displayed small irregularities in their shape, uneven cell surfaces and cytoplasmic retraction from the cell wall (Figure 3.6). At the MIC₅₀, Os-C caused visible depression in the cell surfaces, further cytoplasmic retraction and bud formation. The few cells left after treatment at the MIC₇₅ appeared shrivelled and completely deformed with cellular extravasation, the formation of buds and blebs on the cell surface and the formation of hyphal filaments. The changes in the surface morphology are most probably due to a change in cell permeability. This agrees with previous permeabilization studies by Mbuayama *et al.* (2020) that reported a 54% increase in cell permeability of *C. albicans* (ATCC 90028) in the presence of Os-C at 3 μ M. The results confirm that the first changes are localized at the cell wall and cell membrane before any alterations can be detected in the cell interior.

TEM evaluation revealed that *C. albicans* treated with Os-C (Figure 3.8) appeared completely different than those treated with AmB suggesting that these antifungal treatments have different modes of action. At the MIC₂₅, Os-C caused small irregularities in the shape and cell wall of *C. albicans* as well as the formation of buds. Small vacuoles appeared adjacent to the cell membrane, transferring Os-C from the extracellular environment to the intracellular space. After treatment with the MIC₅₀ of Os-C an increase in size and number of small vacuoles were observed close to the cell membrane and intracellular granulation was detected. At the MIC₇₅ the cell membrane had retracted further, tearing from the cell wall, granulating bodies appeared, organelles such as the nucleus were damaged, and buds started to form. These findings suggest that Os-C causes stress in *C. albicans* planktonic cells and as a stress response bud and hyphal cell formation occur. Maurya *et al.* (2011) reported similar effects on the overall shape and cell wall caused by a ROS inducing peptide VS2 (KFWFKFVKFVK, net charge of +4) at 62.5 μ M against the same *C. albicans* strain. Effects included wrinkling and indentation of the cell surface, breakage in the cell wall and membrane and cellular extravasation.

To be an effective antifungal agent, AMPs such as Os-C must interact and transverse the cell wall to reach the cell membrane. Once the peptide has reached the cell membrane subsequent depolarization and permeabilization can alter membrane structure and stability leading to cell lysis. Two of these events, namely the ability of Os-C to bind cell wall polysaccharides and cause cell membrane depolarization, was further investigated.

Mbuayama *et al.* (2020) showed that the antifungal activity of Os-C decreased in the presence of high concentrations of fungal cell wall polysaccharides laminarin and mannan using the RDA, suggesting that Os-C may interact with the cell wall. To better understand this binding effect the ability of the Os-C peptide to bind to the most common cell wall polysaccharides, laminarin (β -1,3 glucan polymer) and mannan (mannose polymer), was investigated using ITC. Results showed a negligible release and uptake of heat (Figure 3.10) when the MIC₅₀ and 10X the MIC₅₀ of Os-C was

titrated with 20X and 40X higher concentrations of the polysaccharides. This indicated that there was no binding interaction between Os-C and free cell wall polysaccharides at concentrations where Os-C is normally active against *C. albicans*. However, when Os-C was incubated with an excess of laminarin (40 mM/ 20 mg/mL) in the modified micro-broth dilution assay, a 64% inhibition of Os-C activity occurred (Figure 3.11). No effect was observed for mannan. The concentrations used in the ITC procedure are similar to the 50 μ M putative solute-binding peptide (CpMnBP1) and 0.5 mM mannan that Chekan *et al.* (2014) used and the 53 μ M Gram-negative binding protein 3 (GNBP3) and 2 mM laminarin that Mishima *et al.*, (2009) used in their respective ITC studies. Both these articles reported that at these concentration ranges strong 1:1 binding interactions were obtained.

Certain structural features are required for binding of cell wall polysaccharides. Guillen *et al.* (2009) reported that non-catalytic carbohydrate binding modules (CBMs) are found in proteins that recognize and bind polysaccharides such as cellulose, β -glucans, chitin etc. CBMs are auxiliary domains that can be found at the N- or C-terminal end of these proteins and may differ in substrate specificity (Abe *et al.*, 2004). The most important conformational element of most CBMs that is related to the type of ligand they recognize, is the β -sheet and the fold thereof (Hashimoto, 2006). Studies by Gopalakrishnapai *et al.* (2006) and Miki *et al.* (2017) showed that tryptophan and asparagine residues play crucial roles in protein-carbohydrate recognition. The asparagine residues mainly contribute to laminarin binding, while tryptophan increases binding preference for β -glucans. Os-C has some α -helical properties and contains no tryptophan or asparagine residues and this could be the explanation of the poor binding affinity of Os-C for these cell wall polysaccharides.

Very few researchers use ITC to investigate the interaction between AMPs and laminarin or mannan. Binding is observed when high concentrations of the polysaccharides are used in the RDA (Mbuayama *et al.*, 2020, Han *et al.*, 2016). Only at a laminarin concentration of 20 mg/mL was the antifungal activity of Os-C reduced by 60% while no effect was observed for mannan. Jang *et al.* (2006) investigated the ability of the diK19Hc peptide (a homodimeric version of K19Hc) at 1.25 μ g/mL (0.303 μ M) (MIC 1 - 2 μ g/mL against *C. albicans* KCTC7121) to bind increasing concentrations of mannan, laminarin and pustulan (β -1,6-glucan) with the RDA. At a concentration of 1 - 8 mg/mL laminarin a significant loss in antifungal activity was observed indicating that binding to laminarin is required for activity. No binding of mannan or pustulan was observed. Likewise, Han *et al.* (2016) showed that the antifungal activity of peptides Hst5, P113, and di-18Hc at 180 μ g/mL (59.3 – 115 μ M) co-incubated with 0.5 – 20 mg/mL laminarin and mannan against *C. albicans* CCARM 14020 using the RDA, were reduced due to laminarin binding but was totally unaffected by mannan. These results indicate that most peptides bind laminarin, although only at high concentrations, while no binding of mannan occurs.

Lack of binding with ITC analysis and cell wall binding assay could signify that Os-C only binds to cell wall polysaccharides when they are part of a complete cell in a biological environment or that very high concentrations of polysaccharides are required for binding. This could explain that the polysaccharides might not be a primary target for binding, but high concentrations of laminarin could have an effect on the fungicidal activity of the Os-C peptide by obstructing the peptide from the cell membrane. Another explanation could be that in the presence of high concentrations of laminarin, carbohydrate-protein complexes form as described by Takahashi *et al.* (2014). The question is raised whether these high concentrations of laminarin and mannan are the same as that found in the cell wall of *C. albicans*.

Membrane depolarization is associated with the loss of charge across the cell membrane. AMP mediated membrane permeabilization results in increased cell membrane depolarization. In a study by Mbuayama *et al.* (2020), the control melittin caused a linear increase in membrane permeabilization of *C. albicans* cells reaching 60% at 6 μ M. In contrast, for Os-C a sigmoidal increase was observed reaching a plateau of 40% at 6 μ M. Using fungal liposomes, permeabilization of fungal liposomes by melittin was rapid and reached 100% at 1 μ M, whereas permeabilization by Os-C was minimal with 10% at 1 μ M and 20% at 10 μ M. When membrane depolarization was evaluated a linear increase was observed for melittin, but no effect was observed for Os-C (Figure 3.12). The MIC₅₀ of melittin is 0.98 μ M (Table 3.1) and at this concentration, the permeabilization of fungal liposomes was 100% and notable depolarization had occurred. For Os-C the observed effect was different. At the MIC₅₀ permeabilization of the fungal liposomes was 25%, the permeabilization of *C. albicans* membranes 40% and only minimal depolarization had occurred. The significant difference between the depolarization effects of melittin and Os-C suggests that membrane depolarization is not a primary mode of action for Os-C (Figure 3.12).

The membrane effects of Os-C are similar to the MCh-AMP1 peptide investigated by Sadat *et al.* (2020). At the MIC₅₀ (13.32 μ M) against *C. albicans* (ATCC 10231) MCh-AMP1 caused 56.2% membrane permeabilization and negligible depolarization. It was concluded that the increase in membrane permeabilization by MCh-AMP1 is too slow to be direct permeabilization or membrane damage such as pore formation, but rather as an effect of ROS. Mbuayama *et al.* (2020) proposed that *C. albicans* killing by Os-C could be due to increased ROS production. An accumulation of ROS causes oxidative stress and can lead to a loss in signalling ability, unspecific damage of cellular macromolecules and eventual cell death (Milkovic *et al.*, 2019).

Another peptide that causes moderate membrane damage to planktonic *C. albicans* cells and accumulates in the cytoplasm is the amphipathic penetrating peptide (APP) (GLARALTRLLRQLTRQLTRA) (Li *et al.*, 2016). This is a 20 amino acid AMP that was derived from an amphipathic α -helical cell-penetrating peptide ppTG20 (GLFRALLRLLRSLWRLLLRA) by sequence modification method. *C. albicans* ATCC 10231 cells treated with APP (MIC 8 μ M)

showed some membrane depolarization ($12.32 \pm 0.87\%$) and permeabilization ($21.52 \pm 0.48\%$), strong genomic DNA binding affinity and S-phase cell cycle arrest.

Not only do AMPs target the cell membrane of fungal cells but also intracellular organelles compromising their function. AMPs can bind to DNA affecting transcription and consequently translation. Taute *et al.* (2015) showed that Os-C can bind bacterial plasmid DNA. In the present study the ability of Os-C to accumulate in the vacuoles of *C. albicans* was investigated. A key difference between mammalian and fungal cells is the presence of large vacuoles in fungal cells. Vacuoles are responsible for numerous processes within fungal cells, including translocation and intracellular trafficking of various molecules. Some peptides can escape the vacuoles and localize in the cytoplasm which causes toxicity (Gong & Karlsson 2017).

Gong and Karlsson (2017) evaluated the translocation of eleven cell penetrating peptides (CCPs) including the control in the present study, penetratin. Findings were that generally at low concentrations the vacuoles stained positive with CTr and colocalization of CTr and 5-FAM labelled peptide was observed. At higher concentrations vacuole staining was lost as the vacuole was degraded by the peptides tested and an increase in the intensity of 5-FAM peptide staining occurred.

In the current study, flow cytometry showed that 5-FAM-penetratin entered planktonic *C. albicans* and has limited vacuolar localization but permeabilized vacuole membranes. Of the 99.97% cells positive for 5-FAM-penetratin, only 0.08% of the cells still contained intact vacuoles. At 1 μM penetratin, 85.03% of vacuoles were degraded compared to the untreated population. This is confirmed by the findings of Gong and Karlsson (2017), where less than 20% of the 80% 5-FAM-penetratin positive *C. albicans* cells (strain SC5314) still had intact vacuoles. With CLSM, 5-FAM-penetratin was located throughout the cytoplasm with no specific vacuole localization but some vacuole degradation.

Planktonic *C. albicans* cells were positive for both 5-FAM-Os-C and CTr fluorescence at the low and high concentrations. At 2.8 μM (the MIC_{25} of unlabelled Os-C), 96.96% of the cell population was positive for 5-FAM-Os-C and 53.85% of those cells still had intact vacuoles. Similar results were obtained at 6.2 μM (the MIC_{75} of unlabelled Os-C) where 96.89% of the cells were positive for 5-FAM-Os-C while only 50.79% of those cells still had intact vacuoles. Thus, at each concentration there was a 31.26% and 34.32% degradation of vacuoles compared to the untreated control. Therefore, Os-C enters *C. albicans* cells and causes moderate vacuole degradation that does not increase with concentration. The vacuole degradation caused by Os-C is not as rapid or as direct as the vacuole degradation caused by penetratin indicating, that an indirect effect due to ROS production may occur.

CLSM showed that Os-C targets the cell membrane at lower concentrations and gradually enters the cells (Figure 3.15). The only difference between cells treated with the 2.8 μM and 6.2 μM was

that at the lower concentration some peptide is located to the cell membrane while at a higher concentration Os-C accumulated in the intracellular space (Figure 3.15).

This above finding confirms that the effect of Os-C is different from that of penetratin and based on the findings of Gong and Karlsson (2017) is similar to the effects of MPG (N-methylpurine-DNA glycosylase) a derivative of two viruses (Morris *et al.*, 2001). The latter is a short peptide vector, consisting of 27 amino acids and a charge of +5, and contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen. MPG exhibits a relatively high affinity for both single- and double-stranded DNA in the nanomolar range (Morris *et al.*, 1997) similar to Os-C (Taute *et al.*, 2015). The MIC₅₀ of MPG against *C. albicans* (strain SC5314) is 10 µM. The mode of action of this peptide as summarized by Gong and Karlsson (2017) is associated with a moderate degree of vacuolar localization and degradation ($\pm$ 50%), with low propidium iodide (PI) permeability and identified mechanism of uptake is via endocytosis. In this study combined flow cytometry and CLSM experiments confirmed that Os-C causes moderate degradation of the vacuoles of planktonic *C. albicans* cells which may be an indirect effect of ROS production.

CHAPTER 5: CONCLUSION AND FUTURE PERSPECTIVES

Drug resistance is a fast-approaching global threat to the successful treatment of fungal, viral and bacterial infections, and thus new therapeutic strategies are imperatively required. AMPs are potential candidates that fulfil the requirements for overcoming the threat of antimicrobial resistance. These host-defence peptides have broad-spectrum activity, an extensive range of targets, can be used in combination with known drugs and have been associated with a low risk of resistance development compared to conventional antimicrobial drugs (Forde *et al.*, 2018).

Os-C has been identified as an AMP that is able to inhibit planktonic as well as biofilm forms of *C. albicans*. Mbuayama *et al.* (2020) found that Os-C binds to the cell wall, accumulates on the cell membrane and translocates into the cytoplasm via an energy-dependent process. Subsequent ROS-production correlated with its antifungal activity. The aim of this study was to further investigate the mode of action of the synthetic peptide Os-C against planktonic *C. albicans* cells.

Os-C is salt sensitive (Mbuayama *et al.*, 2020) and the mode of action can thus not be evaluated in the high salt RPMI-1640 medium with assays such as the micro-broth dilution assay which is widely used for rapid and inexpensive AMP screening. Despite the inhibitory effects of salts, Os-C can still be further investigated for topical use. Therefore, the micro-broth assay was modified to determine the lowest media dilution that supports growth equivalent to the control cultures in 100% RPMI-1640. The sensitivity to AmB in the different media dilutions was determined to ensure that the drug sensitivity of *C. albicans* was not altered. Results showed that in 10% RPMI-1640/NaP medium *C. albicans* growth was equivalent to growth in 100% RPMI-1640 and the sensitivity to AmB was unchanged. With this modified micro-broth assay the MIC₅₀ of AmB, melittin and Os-C was determined as $0.50 \pm 0.06 \mu\text{M}$, $0.976 \pm 0.14 \mu\text{M}$ and $4.69 \pm 0.18 \mu\text{M}$, respectively.

The advantage of this modified micro-broth assay is that it can be used for the rapid and accurate screening of salt sensitive antimicrobial agents. Future studies could employ this assay to determine the effect of pH on the activity of Os-C. The pH on the human skin is low and the salt concentration (25 - 75 μM) of sweat is lower than the 150 mM found in physiological fluids (Proksch, 2018). This assay could also be used to assess the effect of endocytic inhibitors such as cytochalasin D (CyD), heparin, chlorpromazine and 5-(N-ethyl-N-isopropyl)-amiloride or proteases such as aspartic proteases, on the anticandidal activity of peptides such as Os-C (Li *et al.*, 2019).

This study tracked the mode of killing of Os-C from the outside of the cell zooming inward. SEM and TEM allowed the evaluation of the extra- and intracellular morphology of planktonic *C. albicans* cells exposed to concentrations equal to the MIC₂₅, MIC₅₀ and MIC₇₅ of the control, AmB and Os-C, respectively. AmB caused a collapse in cell structure, an anamorphous overall shape, deformed phenotypes, irregular cell wall, budding scars and bud formation. Changes to cellular structure confirms the mode of action described by De Kruijff & Demel (1974), Ermishkin *et al.* (1976), Matsuoka & Maruta (2003), Neumann *et al.* (2009) and Grela *et al.* (2019). Os-C caused

cytoplasmic retraction, depression in cell surfaces, cellular extravasation, the formation of buds, small vacuoles adjacent to the cell membrane and intracellular granulation. The differences between the ultrastructural effects of AmB and Os-C indicates a difference in the mode of action.

Future studies could investigate the potential of Os-C to inhibit cell wall biosynthesis or target the structural skeleton, chitin, based on the irregularities in the cell wall and overall shape observed with SEM and TEM. Studies could include ITC binding assays to determine whether Os-C inhibits β -1,3-glucan synthase by targeting the two subunits Fks1p or Rholp (Mazur and Baginsky, 1996), chitin synthase (Chaudhary *et al.*, 2013) or the glycosylphosphatidyl inositol pathway (Mutz & Roemer, 2016). Kim & Lee (2019) showed that membrane damage can be determined by measuring K^+ leakage and the K^+ voltage after treatment of fungal cells with an AMP.

For an AMP to be effective it needs to be able to locate and attach to the pathogen. The cell wall and membrane have been identified as specific AMP targets, where ionic interactions between the anionic surface molecules and cationic AMPs are required before AMPs can enter fungal cells (Zasloff, 2002). Previously, Mbuayama *et al.* (2020) showed that Os-C activity is partially inhibited by laminarin suggesting that Os-C binds to laminarin to transverse the cell wall to reach the membrane. To better understand this mechanism, the binding of Os-C to laminarin and mannan was investigated using ITC. In addition, the ability of Os-C to cause cell membrane depolarization compared with melittin was evaluated using DiBAC₄(3) and the modified micro-broth assay. Using ITC, Os-C was found not to bind laminarin or mannan although the modified micro-broth dilution assay showed that Os-C binds laminarin but only at very high concentrations deemed physiologically irrelevant. In conclusion, the polysaccharides might not be a primary target for Os-C binding, but high concentrations thereof could have an effect on the fungicidal activity of the Os-C peptide by obstructing the peptide from the cell membrane. Compared to melittin, Os-C caused negligible cell membrane permeabilization and did not affect the membrane potential.

AMPs interact with the cell wall and membrane in several ways. In future, the interaction of Os-C with other cell wall components such as chitin, glycoproteins and receptors, can be determined with ITC and the modified micro-broth assay, the RDA or other methods such as the pre-incubation, centrifugation and ELISA- based detection method described by Osmond *et al.* (2001). Van der Weerden *et al.* (2010) and Hayes *et al.* (2018) studied the plant defensin NaD1 that has a similar mode of action to Os-C. NaD1 binds to the fungal cell surface and then enters the cytoplasm by endocytosis (Hayes *et al.*, 2018) before killing the fungal cell via the generation of ROS and permeabilization of the cell membrane. Future studies could include Western blot analysis using a fluorescently labelled anti-Os-C antibody to determine the ability of Os-C to interact with fungal cell wall extracts. In addition, selective enzymatic removal of specific cell wall components could identify molecular motifs required for Os-C binding and activity.

Besides being membrane active, Os-C was shown to localize within the planktonic *C. albicans* cytosol (Mbuayama *et al.*, 2020) and cause intracellular damage (Figure 3.5). This is a positive attribute that distinguishes Os-C as a multifunctional peptide with more than one target and reduces the risk for resistance development. To narrow down the possible intracellular targets this study first focused on the fungal vacuoles. CLSM and flow cytometry revealed that Os-C is able to enter and successfully carry the fluorescent tag 5-FAM into planktonic *C. albicans* cells.

The use of drug delivery vehicles to advance pathogen recognition and quick delivery is a prospective objective in improving antimicrobial therapeutics (Gong & Karlsson, 2017). The fact that Os-C could deliver the fluorescent 5-FAM tag to the interior of the *C. albicans* cells, makes it a CPP that can be considered as a candidate for drug delivery. Future studies should consider the potential of Os-C as a drug delivery vehicle by using transcription-factor delivery assays (Kang *et al.*, 1998) or by visualizing true cytoplasmic-delivery of proteins at biologically relevant concentrations using the green fluorescent protein (GFP) split-complementation endosomal escape (SEE) assay described by Milech *et al.* (2015).

The CLSM and flow cytometry further showed that Os-C was also able to enter the vacuoles and cause moderate vacuolar break down independent of concentration. It is concluded that vacuole degradation from MIC₂₅ to MIC₇₅ treatments happens too slow for the yeast vacuoles to be a primary target for Os-C but vacuole damage could be due to oxidative stress caused by ROS.

In order to facilitate the development of Os-C as a therapeutic agent future studies should also focus on tracking vacuolar damage by including more dilutions of Os-C and a time-study. Further exploration of ROS generation caused by Os-C can be undertaken as in the study of Kim and Lee (2019). These researchers reported that the porcine myeloid antibacterial peptide (PMAP-23) induces apoptosis of *C. albicans* at low concentrations by disrupting Ca²⁺ homeostasis and altering the NAD⁺/NADH ratio resulting in increased ROS production and mitochondrial damage. The accumulation of Ca²⁺ due to treatment with Os-C can be measured using an intracellular specific dye Fura-2 AM. Altered NAD⁺/NADH ratios can be determined with the NAD⁺/NADH cycling assay and increased levels of oxidized glutathione can be measured with the glutathione assay. The major site of ROS generation is the mitochondria. The change in Ca²⁺ levels in the mitochondria can be measured with a mitochondrial specific dye Rhod-2 AM as described by Kim and Lee (2019). Mitochondrial Ca²⁺ overload results in ROS production.

In conclusion, based on the findings of the present and other studies (Prinsloo *et al.*, 2013; Taute *et al.*, 2015; Malan *et al.*, 2016; Ramafoko, 2018 & Mbuayama *et al.*, 2020) a mode of action for Os-C can be proposed and is schematically presented in Figure 5.1. The typical *C. albicans* cell is enveloped by a thick cell wall and a double layer membrane. The cell content is distributed evenly with an electron dense nucleus, large vacuole and cytoplasm containing membrane bound organelles. When *C. albicans* cells are treated with Os-C, the peptide does not bind laminarin or mannan but accumulates on the cell membrane, before translocating into the cytoplasm via ATP-

mediated endocytosis and does not enter due to membrane depolarization or increased permeability. Once inside, it accumulates within the cytoplasm, causes moderate vacuole degradation and may bind DNA. Os-C induces the production of an overwhelming amount of ROS which causes oxidative stress. ROS generation leads to membrane and protein damage observed as changes in the shape of the cell wall and membrane structure, moderate membrane permeabilization and pore formation, the accumulation of small vacuoles along the inside of the cell membrane, degradation of intracellular organelles and the formation hyphal filaments. All these effects will eventually lead to cell death probably due to apoptosis.

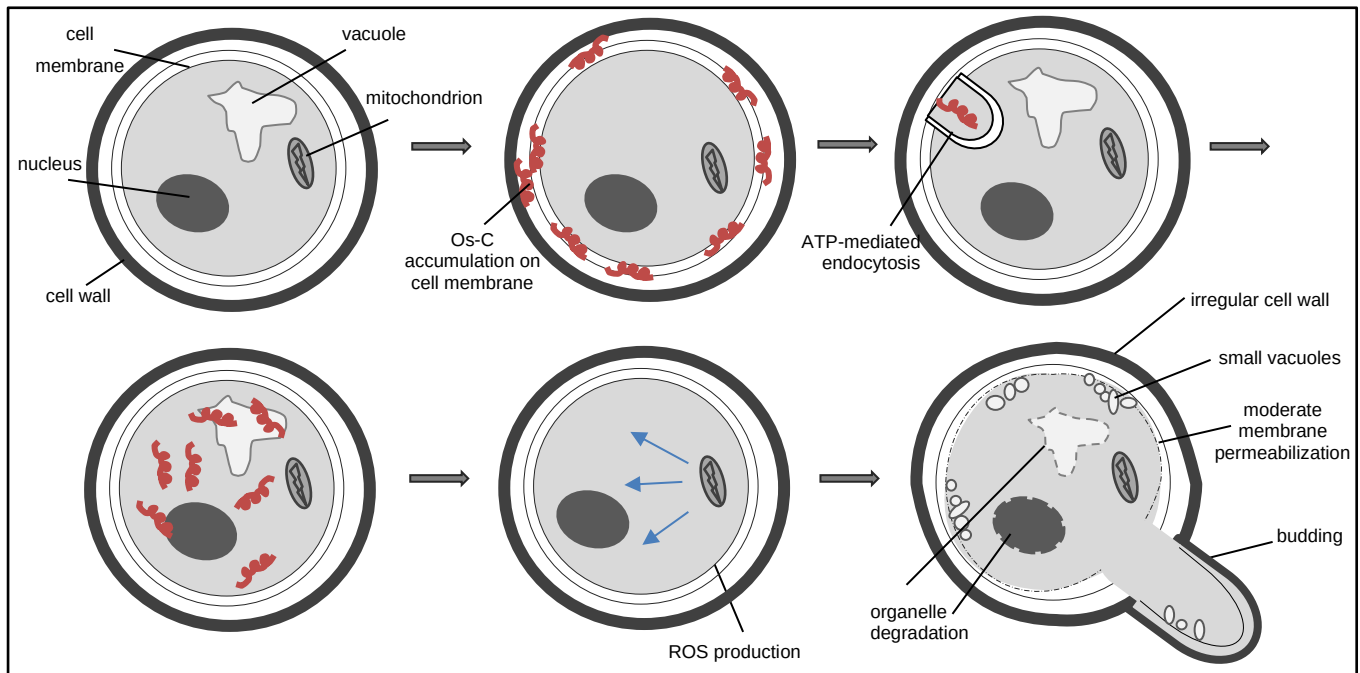


Figure 5.1: A proposed mode of action of Os-C against planktonic *C. albicans* cells. This simplified schematic is based on the results presented in this, as well as the Mbuayama et al. (2020) study. It is depicted that Os-C crosses the cell wall, accumulates on the cell membrane, translocates via ATP-mediated endocytosis, fills the entire intracellular space, induces ROS-production, causes collapse in cell structures, membrane permeabilization, organelle degradation and bud and hyphal cell formation.

CHAPTER 6: REFERENCES

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APPENDIX A: Os-C HPLC and MS data

Sample Name :Os-C
 Sample ID :U7284ED080-3
 Time Processed :16:55:01
 Month-Day-Year Processed :04/23/2019

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
 Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
 Total Flow:1 ml/min
 Wavelength:220 nm
 <<LC Time Program>>

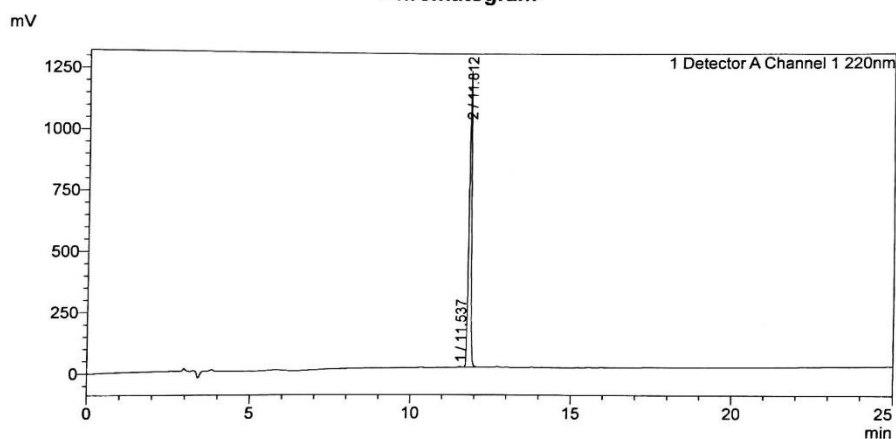
Time	Module	Command	Value
0.01	Pumps	Pump A B.Conc	5
25.00	Pumps	Pump A B.Conc	65
25.01	Pumps	Pump A B.Conc	95
32.00	Pumps	Pump A B.Conc	95
32.01	Pumps	Pump A B.Conc	95
40.00	Pumps	Pump A B.Conc	5
40.00	Pumps	Pump A B.Conc	5
	Controller	Stop	

<<Column Performance>>

<Detector A>

Column : AlltimaTM C18 4.6 x 250 mm
 Equipment:ZJ17010508

<Chromatogram>

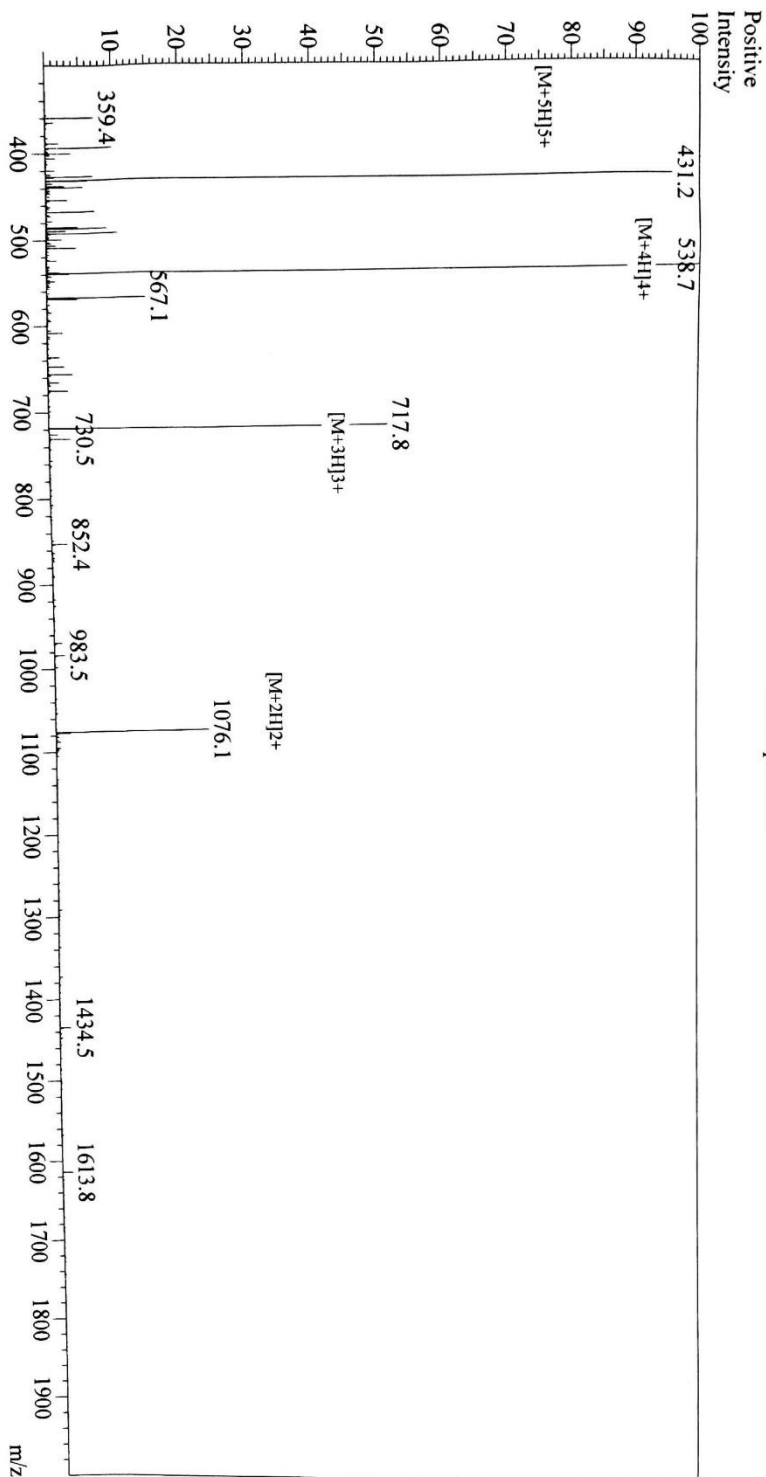


<Peak Table>

Detector A Channel 1 220nm

Peak#	Ret. Time	Area	Height	Area%
1	11.537	15935	3440	0.243
2	11.812	6554556	1226601	99.757
Total		6570491	1230041	100.000

Mass Spectrum



Sample Information
 Acquired by : Gary
 Month-Day Processed : 04/24/19
 Time Processed : 03:58:19 PM
 Injection Volume : 0.4
 Sample Name : Os-C
 Sample ID : U7284ED080-3
 Theoretical MW : 2150.49
 Observed MW : 2150.8

Interface
 Nebulizing Gas Flow : 1.5 L/min
 CDL Temp : 250
 Block Temp : 200
Equipment
 Interface Bias : +4.5 kV
 Drying Gas Flow : 0.2 ml/min
 T.Flow : 50%
 B.conc : 40% MeOH

: GK11010007