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Testing of the antifungal activity of oleylphosphocholine on *Candida albicans* planktonic cells and biofilms developed *in vitro* and *in vivo* in a subcutaneous biofilm model

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List of Abbreviations

ABC	ATP-binding cassette
ADH5	alcohol dehydrogenase
ALS	agglutinin-like sequence
AmB	amphotericin B
ATB	antibiotics
BLI	bioluminescence imaging
CSLM	confocal scanning laser microscopy
CVC	central venous catheter
DMSO	dimethylsulfoxide
Eap1	enhanced adherence to polystyrene 1
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GCA	glucoamylase
GPI	glycosylphosphatidylinositol
HGC1	hypha-specific G1 cyclin related
IP	intraperitoneal
IV	intravenous
LPL	lysophospholipase
LPTA	lysophospholipase transacylase
MFC	minimal fungicidal concentration
MFS	major facilitator superfamily
MTL	mating type locus
OPC	oropharyngeal candidiasis
PBS	phosphate buffered saline
PGE	paradoxical growth effect
PKC	protein kinase C
PLA_2	phospholipase A_2

PLB1	phospholipase B
ROI	region of interest
ROS	reactive oxygen species
SNPs	single nucleotide polymorphisms
YPD	yeast peptone dextrose

Contents

Abstract

C. albicans is a fungal, commensal organism that can cause a variety of diseases in some conditions and is one of the most important causes of nosocomial infections. *C. albicans* is known to form biofilms on both biotic and abiotic surfaces. These biofilms are associated with higher resistance to antibiotics and higher mortality rates. In current medical practice, more and more devices are implanted in a variety of locations in the human body. *C. albicans* can form biofilms on these devices. Treatment options are poor and often result in removal of the device which in turn results in increased hospital stay and costs.

Currently, three major groups of antimycotics are used for the treatment of *Candida* infections. However, all of them are associated with some sort of problem. Polyenes, amphotericin B for example, is associated with high nephrotoxicity. Azoles such as fluconazole have no activity towards *C. albicans* biofilms. Other *candida* species such as *C. glabrata,* another emerging pathogen of the *Candida* genus, are intrinsically resistant to fluconazole. Finally, echinocandins (e.g. anidulafungin and caspofungin) can only be administered intravenous. In our work we investigated the efficacy of a novel antimycotic named oleyphosphocholine (OIPC) which is based on the former anticancer drug miltefosine.

The efficacy of OIPC was first tested *in vitro*. The minimum inhibitory concentration (MIC) was between 1-4 µg/mL depending on the strain. The efficacy on polyurethane catheters and on 96 well plates (polystyrene) was also evaluated for different phases of biofilm formation: adhesion, biofilm development and mature biofilm. In all conditions no effect could be observed for adhesion phase. The most pronounced effect on catheters was observed for biofilm development. This was at 8 µg/mL. Similar effects were obtained on a polystyrene surface starting from 4 µg/mL or 8 µg/mL depending on the strain. For mature biofilm the results depend on the abiotic surface used. For polystyrene the effect was even more pronounced: inhibition started at 1 µg/mL or 4 µg/mL depending on the strain. For catheters only 8 µg/mL showed a significant effect for mature biofilms. Similar results were obtained with bioluminescence imaging, here catheter were used. However, a stronger effect for mature biofilm was observed. The results of the effect of OIPC on catheters during biofilm development was also confirmed with two visualisation techniques: confocal microscopy and SEM.

The effect of OIPC on mucosal surfaces was also evaluated. This was done *ex vivo* on mouse tongues. Surprisingly here a small increase in CFUs was observed for 13 µg/mL OIPC, Another ex vivo model showed more promising results. The effect of intraperitoneal OIPC administration in a subcutaneous mouse model was also evaluated. Five catheters infected with *C. albicans* were implanted per mouse. Administration started 24 h after implantation and 4 conditions were evaluated (sterile saline, 10 mg/kg/day, 20 mg/kg/day and 40 mg/kg/day). After 7 days the mice were killed and colony forming units (CFUs) were determined. However, no effect was observed for any given condition.

Samenvatting

Candida albicans is een veel voorkomende commensale schimmel die infecties kan veroorzaken in verschillende condities. Het is tevens ook een van de belangrijkste oorzaken van ziekhuis-opgelopen infecties. *C. albicans* kan biofilm vormen op zowel biotische als abiotische oppervlaktes. Deze bioflms zijn geassocieerd met hogere resistentie tegen antibiotica en zijn geassocieerd met een hogere mortaliteit. In de huidige geneeskunde worden steeds meer implantaten gebruikt waarop *C. albicans* biofilms kan vormen. Hiervoor zijn weinig behandelingsopties mogelijk en vaak resulteert is het verwijderen van het implantaat noodzakelijk. Hierdoor dient de patiënt langer in het ziekenhuis te verblijven en stijgen de kosten voor zowel patiënt als verzekering.

Momenteel bestaan er 3 klassen van antimycotica om *Candida* infecties te behandelen. Toch worden deze vaak geassocieerd met bepaalde problemen. De eerste klasse, de polyenen (bv. amphotericine B) zijn geassocieerd met een hogere nefrotoxiciteit. Azolen zoals fluconazole hebben geen effect op *C. albicans* biofilms. Andere *candida* soorten zoals *C. glabrata* hebben een intrinsiek resistentie mechanisme tegen deze klasse antimycotica. Verder zijn er ook de echinocandins (e.g. anidulafungin en caspofungin) die enkel intraveneus kunnen toegediend worden. In dit werk onderzochten we het effect van een nieuw antimycotica, oleyphosphocholine (OIPC). Dit geneesmiddel is gebaseerd op miltefosine, een middel dat ontwikkeld werd tegen kanker.

Eerst hebben we het effect van OIPC *in vitro* onderzocht. We vonden dat de minimum inhiberende concentratie tussen 1 –en 4 µg/mL lag, afhankelijk van de *C. albicans* stam. Het effect op polyurethaan catheters en op polystyreen 96 wel platen werd onderzocht voor verschillende fasen van biofilm vorming (adhesie, biofilm ontwikkeling en mature biofilm). In geen enkele conditie werd een effect op adhesie gevonden. Voor catheters werd het meest uitgesproken effect geobserveerd voor biofilm ontwikkeling. Dit was voor 8 µg/mL. Gelijkaardige effect werden geobserveerd in 96 well platen. Dit begon vanaf 4 µg/mL of 8 µg/mL, afhankelijk van de stam. Bij mature biofilm was het effect afhankelijk van het gebruikte oppervlakte. Voor polystyreen was het effect zelfs meer uitgesproken. Inhibitie starte vanaf 1 µg/mL of 4 µg/mL, afhankelijk van de stam. Voor de catheters werd enkel voor 8 µg/ml een significant effect geobserveerd. Bij bioluminescentie waar ook catheters gebruikt werden werd een gelijkaardig resultaat bekomen. Toch werd hier een sterker effect geobserveerd voor mature biofilm. Het effect van OIPC op biofilm ontwikkeling werd ook bevestigd via visualisatie door gebruik te maken van twee technieken: SEM en confocale microcopie.

Het effect van OIPC op mucosomale oppervlaktes werd ook geëvalueerd. Hiervoor werden muistongen gebruikt. Een kleine stijging in kolonie vormende units werd geobserveerd voor 13 µg/mL tegenover controle voor biofilm ontwikkeling. Een ander *ex vivo* model resulteerde leverde veelbelovende resultaten. Ten slotte werd het effect geëvalueerd *in vivo* in een

subcutaan muis model. Vijf *C. albicans* geinfecteerde catheters werden ingebracht in de rug van muizen. 24 h na implantatie werd gestart met intraperitoneale toediening van OIPC. 4 condities werden onderzocht (steriele zoutoplossing, 10 mg/kg/day, 20 mg/kg/day and 40 mg/kg/day). Na 7 dagen werden de muizen gedood en de infectie werd gekwantificeerd via kolonie vormende units. Spijtig genoeg werd er geen effect geobserveerd.

Introduction

1 *Candida albicans* characteristics

1.1 Taxonomy

The *Candida* genus belongs to the kingdom Funghi, phylum Ascomycota, class Saccharomycetes, order Saccharomycetales and the family of Debaryomycetaceae. Historically the Candida genus was part of the class Deuteromycetes which was described as a 'taxonomic pit', a class in which all yeast without known sexual stage or other remarkable phenotypes were thrown (Odds 1987).

1.2 General characteristics

Candida albicans is a commensal organism common in humans and some other warm blood animals. It is part of the normal flora of the digestive and genitourinary tracts (Kam & Xu 2002). It often colonizes mucosal surfaces of healthy subjects and was long considered to reproduce asexually. It is now known that mating can happen in opaque cells. Cells have to switch epigenetically from white cells to opaque cells (Meleah et al. 2013). This can be in response to a number of environmental signals but is not triggered by mating factors. Mating factors are secreted by cells with homogenous mating type locus (MTL). Two different types exist: MTLa and MTLα. MTLa/MTLa can mate with MTLα/MTLα but MTLa/MTLα cannot mate (see figure 1). Mating results in a tetraploid cells which breaks down through chromosome loss to yield recombinant diploid cells (Soll et al. 2003).

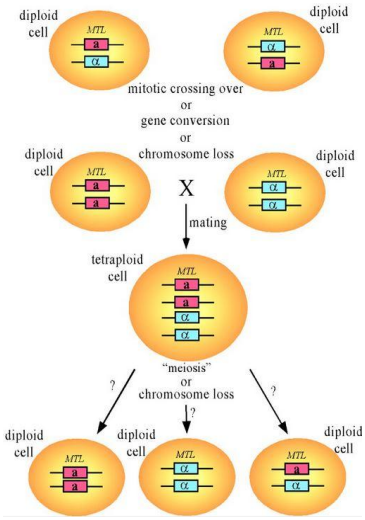


Figure 1: ***different mating types of C. albicans (adapted from Soll et. al 2003)***

1.3 Morphology

C. albicans is a polymorphic fungus, it can exist as ovoid-shaped budding yeast, as pseudohyphae (elongated ellipsoid cells with constriction at the septa), and as paralleled true hyphae (see figure 2). Other morphologies include chlamydospores (thick walled spore like structures) and white and opaque cells. Morphology switching strongly contribute to virulence and infection (see chapter 2.2). however, the role of pseudohyphae and chlamydospores are less clear. Environmental factors affect morphology of *C. albicans*. Hyphae formation is promoted under a range of

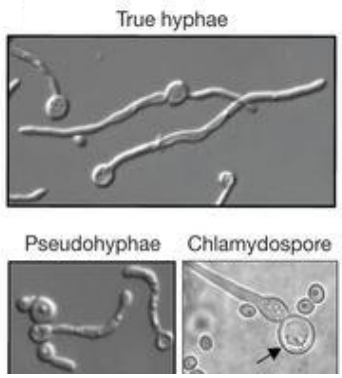


Figure 2: ***Different Candida morphologies (Source: Adapted from Meleah et al. 2013)***

conditions including high pH (>7), CO2, physiological temperature, starvation and presence of serum. Quorum sensing (another virulence factor see chapter 2.5.2), a type of cell-cell communication can also influence morphology. Low cell densities (<10^{-7} cell/mL) result in hyphal formation while high cell densities (>10^{-7} cell/mL) favour yeast growth (Mayer *et al*. 2013).

1.4 Genetic background

The standard genome of *C. albicans* is diploid, composed of eight chromosome homolog pairs. They range in size from 0.95 to 3.3 Mb (Chibana *et al.* 2000). It was the first fungal pathogen which had his genome sequenced (Jones *et al.* 2004). This was done of the SC5314 clinical isolate which showed a high degree of heterozygosity. The 32-Mb diploid genome included more than 55700 single-nucleotide polymorphisms (SNPs). *C. albicans* is able to achieve a high degree of genetic diversity by using recombination, gene replacement, chromosomal polymorphisms and cryptic mating (Chibana *et al.* 2000, Selmecki *et al.* 2010).

1.5 Epidemiology of Candidiasis

Over the last three decades, *C. albicans* has become an important pathogen. Invasive candidiasis has become a prominent problem since the early 1980s (Pfaller 1996; Pfaller & Diekema 2007). Candida is the third most common cause of bloodstream infections in the intensive care unit in America (Hidron, *et al*. 2008). Compared to bacterial infections, *Candida* infections are associated with increased mortality, higher costs and increased length of patient stay (J. Morgan, *et al*. 2005). Diagnostic is usually done with blood cutures. The downside however, is the long incubation time (Mikulska *et al*. 2010). Other diagnostic methods include antibodies against mannan antigen (ELISA and latex agglutination test), RT-PCR, MALDI-TOF and PNA-FISH (Paramythiotou *et al*. 2014).

C. albicans is still the most isolated *Candida* specie but proportions have changed over the last decades. In the 1980s *C. albicans* caused up to 76% of all *Candida* infections (Sague & Jarvis 1993). During the 1990s the distribution of species started changing. *C. glabrata* isolation increased proportionally, while *C. albicans* became less predominant (Pfaller *et al*. 1998). Currently, *C. albicans* is still the most isolated species in adults but no longer causes the majority of the invasive candidiasis. In neonates, this is no longer the case, *C. parapsilosis* is the most prevalent followed by *C. albicans* (Steinbach *et al*. 2012). For pediatric populations *C. albicans* is still the most common causal organism but the second most prevalent species is no longer *C. glabrata* but *C. parapsilosis* (Zaoutis 2010, Steinbach *et al*. 2012). The species distribution differs not only in populations but also for geographical range. The proportion of *C. albicans* for Europe and the U.S. is very similar. But the proportions of *C. glabrata* and *C.*

parapsilosis is variable depending on the country, study and time of the study. In some Asian countries *C. parapsilosis* is more prevalent compared to *C. albicans* (Falagas *et al.* 2010).

C. albicans infections are opportunistic and depend on the immune defence of the host and changes in the environment of the yeast in the organism (Hajjeh *et al*. 2004). Moreover, *C. albicans* has become a frequent cause of nosocomial infections, resulting in a serious threat for healthcare. Invasive candidiasis is often associated with high mortality rates that exceed 30% in many reports (Horn *et al*. 2009). Approximately 70% of the AIDS patients will experience oropharyngeal candidiasis (OPC) and vaginitis caused by *C. albicans* will occur in 70% in all women (Fidel *et al*. 1999).

Candidiasis is usually associated with immunocompromised patients. Healthy individuals are protected by their immune system against *C. albicans*. T-cell immune responses are important against OPC, cutaneous and vaginal infections (Calderone & Fonzi 2001). Phagocytic response involving neutrophils and mononuclear phagocytes is associated with resistance to systemic disease. AIDS patients with OPC disease progression and recurrence is often associated with a reduction in $CD4^+$ cells. Systemic infection in AIDS patients is only developed in the terminal stages of diseases. $CD4^+$ cells are possibly not associated with protection of vaginal mucosa but only with oral mucosa, suggested by the evidence that vaginitis has the same incidence in AIDS patients compared to healthy subjects (Fidel *et al*. 1999). Humoral immunity may also be important, anti-Candida antibody, if directed against key epitopes, is protective in several animal models (Han *et al*. 1998).

complement and the host innate immune system seems to play an important role as well. Disease occurs only at the site determined by the defect in the immune system (Calderone & Fonzi 2001).

2 *C. albicans* virulence factors

C. albicans can cause two major types of infections ranging from mild superficial and life threatening infections. Infections caused by *Candida* are often referred to as candidiasis. Candidiasis can occur in the oral cavity, oesophagus, gastrointestinal tract, vagina and skin (Calderone & Fonzi 2001). Most infections occur mainly in immunocompromised patients. There are several virulence factors that are required for successful colonization or invasion of host tissues and hence contribute to pathogenesis. Important virulence factors are described in details below. *C. albicans* is unique in the fact that it can infect several distinct anatomical sites in the host. Only *Pseudomonas aeruginosa* can rival *Candida* in this aspect. Immune protection is site specific, resulting in different animal models. For example, mucosal surface animal models are different than vaginitis models (Fidel, P. *et al*. 1999).

2.1 Phospolipase B

Phospholipase B is a known virulence factor in *C. Albicans*. Secretion of PLB1 is associated with survival in macrophages (Cox *et al.* 2001), production of eicosanoids, modulators of phagocytic activity and adhesion to pulmonary epithelium (Noverr *et al.* 2003). However, the structure and mechanism of action is still not understood.

2.2 Dimorphisme

Both the yeast and the hyphal form of *C. albicans* contribute to pathogenesis (see chapter 1.3) and the transition between these two forms is referred to as dimorphism. Yeast cells are usually responsible for dissemination while the hyphal form contributes to invasion of tissues. HGC1 is a gene necessary for hyphal formation and encodes a hypha-specific G1 cyclin related protein. Deletion results in fungal cells attenuated in virulence (Zheng *et al*. 2004). Hypha formation is also linked with expression of other virulence genes that are not involved in hypha formation. These include agglutinin-like sequence protein (Als3), hyphal wall protein (hwp1), secreted aspartic proteases (sap4, sap5 and sap6) and hypha associated proteins Hyr1 and Ece1 (Mayer *et al*. 2013).

2.3 Adhesins and invasins

Another factor contributing to virulence are adhesins, proteins specialized in adherence to other *C. albicans* cells, host cells and tissues, other microorganisms and abiotic surfaces. The agglutinin-like sequence (ALS) adhesins are the best studied in *C. albicans.* The *ALS* gene encodes glycosylphosphatidylinositol (GPI)-anchored glycoproteins and form a family consisting of eight members (Als1-7 and Als 9). Als3 is probably the most important for adhesion and is expressed during oral epithelial -and vaginal infections (Wächtler *et al*. 2011). Hwp1 is also important in adhesion. Hwp1 can serve as a substrate for mammalian

transglutaminases which can covalently link hyphal cells to host cells (Sundstrom *et al*. 2002). Both Hwp1 and Als3 also contribute to biofilm formation by acting as complementary adhesins (Nobile *et al*. 2008). Another important adhesion not belonging to the ALS family is Eap1 (enhanced adherence to polystyrene 1).

C. albicans can invade tissue by using two different mechanisms: active penetration and induced endocytosis (Neil *et al*. 2011). Invasins, a specialized set of proteins on the cell surface of *C. albicans* that mediate binding to host cells are needed for induced endocytosis. This binding triggers engulfment of the fungal cells in a clathrin dependent mechanism. Even heat-killed hyphae are taken up indicating that it's a passive process. So far, two invasins have been identified: Als3 (also an adhesion; Liu & Filler 2011) and Ssa1, a member of heat shock protein 70 expressed at the surface (Sun *et al*. 2010). Both bind to host E-cadherin, a host ligand present epithelial cells and induce endocytosis (Sun *et al*. 2010). Active penetration is less studied. It requires viable fungal cells and it is proposed that secreted aspartic proteases (Saps) are involved.

2.4 Secreted hydrolases

Adhesion to surfaces resulting in hyphal growth can be followed by secretion of hydrolases. They enhance the efficiency of nutrient acquisition (Naglik *et al*. 2003) and can facilitate active penetration (Saps, see section above). *C. albicans* expresses three different classes of hydrolases: lipases, phospholipases and proteases. The Sap family comprises ten members: Sap1-10. Only Sap9 and Sap10 remain cell-surface bound, all others are secreted. The large size of the family suggests redundancy between family members, but the relative contribution of each Sap is controversial. The phospholipases family consist of four classes: A, B, C and D. Only the five members of the B class (PLB1-5) are extracellular and hence can contribute to pathogenicity by disrupting host cell membranes (Mavor *et al*. 2005). Lipases are a third group of secreted hydrolases and consist of 10 members (LIP1-10). Lip8 seems to be important for virulence in an *in vivo* mouse model (Davis 2009).

2.5 *C. albicans* biofilm formation

2.5.1 Introduction

C. albicans is known to form biofilm on a variety of biotic and abiotic surfaces such as catheters, dentures, shunts and mucosal surfaces (see chapter 2.5.5). *In vitro* biofilm development can be viewed as a series of sequential events. The first step involves adherence of yeast cells to a surface (the adherence step). This is followed by the formation of elongated cells such as pseudohyphae and hyphae (initiation step). During the next step, the maturation step, extracellular matrix can accumulate. The dispersal step is the final step. This step involves the release of yeast cells to form secondary infections (Finkel & Mitchell 2010). One of the most

remarkable features of (mature) biofilms is the increased resistance to both host immune factors and antimicrobial agents (Mayer *et al*. 2013). Mature *Candida* biofilms are complex three dimensional structures. They have a typical microcolony and water channel architecture and extensive spatial heterogeneity (Ramage *et al*. 2006). The biofilm formation process is illustrated in figure 3.

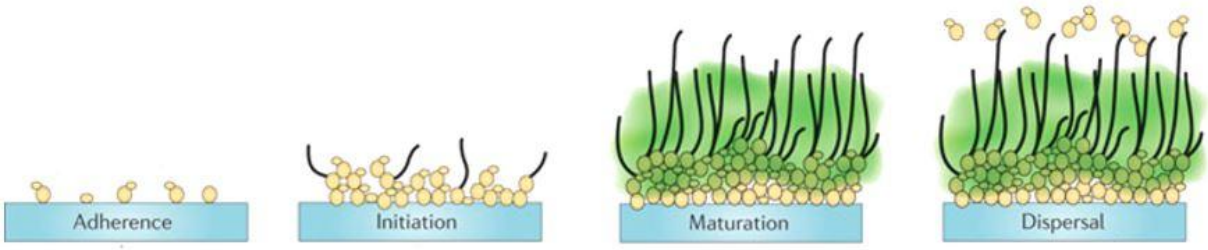


Figure 3: ***different stages of C. albicans biofilm development (Finkel & Mitchell 2010).***

2.5.2 Quorum sensing

Quorum sensing are microbial responses or behaviour which are governed by cell densities and is especially important in high density cellular communities such as biofilms. The behaviour is determined by secreting quorum sensing molecules. Farnesol is the best studied quorum sensing molecule in *C. albicans* and is an inhibitor of hyphal formation (Ramage *et al.* 2002). Only a limited amount of biofilm is formed in the presence of farnesol and are comprised of yeast cells and pseudohyphae. It can inhibit biofilm formation when exogenously administered during adherence (Ramage *et al.* 2002). Farnesol can also accumulate in the supernatans of mature biofilms which can promote biofilm dispersion (Martins *et al.* 2007).

A second well known quorum sensing molecule is tyrosol, a derivative of tyrosine. In contrast to farnesol, tyrosol stimulates hyphal formation (Alem *et al*. 2006). When administered exogenously it does not seem to have a measurable effect on biofilm development (Alem *et al*. 2006). Mating factors (see section 1.2) can also influence behaviour in white cells. They can increase cell-cell and cell-substrate adherence resulted in stimulation of biofilm formation. The white cells use a hybrid pathway to respond to mating factors compared to opaque cells (Sahni *et al*. 2009).

2.5.3 Genetic control of biofilm formation

Biofilm adherence is regulated by adhesins previously discussed (see chapter 2.3). Both Eap1 and Als1 function as initial adherence molecules. These proteins are expressed in both yeast and hyphal cells (Li *et al*. 2003). In contrast, hyphae only express Als3 (Liu & Filler 2011). In vivo adherence can carried out by both yeast cells expressing Als1 or hyphal cells expressing

Als3 (Nobile *et al*. 2008). Adherence results in activation of a gene expression response. After a few minutes drug efflux pumps are expressed (e.g. Cdr1 and Mdr1; see section 3.2.4).

During the initiation phase, yeast cell switch to hyphal cells. Bcr1 is a transcription factor important for biofilm initiation but not for morphologically normal hyphae. It is upregulated in hyphal cells and induces expression of several cell surface proteins (e.g. Als3 and Hwp1; Nobile *et al*. 2006). These hyphae are extremely sticky and promote cell-substrate and cell-cell contact. Both Als3 and Hwp1 can act as a complement in biofilm formation (Nobile et al. 2008). Additional cell surface proteins are also important (e.g. Rbt1 and Hwp2).

During the maturation phase extracellular matrix accumulates and growth continues. The extracellular matrix produced *in vitro* is comprised of proteins, eDNA, carbohydrates (e.g. β-1,3-glucan), phosphorus, hexosamine and uronic acid (Al-Fattani *et al*. 2006). β-1,3-glucan is almost exclusively associated with biofilm cells. The eDNA contributes to both the structure and stability of the mature biofilm (Finkel & Mitchell 2010).

Zap1, a transcription factor involved in Zn acquisition activates CSH1 and IFD6 expression, both negative regulators of biofilm matrix. Furthermore Zap1 also represses positive regulators of matrix production such as alcohol dehydrogenase 5 (*ADH5*), glucoamylase 1 (*GCA1*) and *GCA2*. *GCA1* and *GCA2* might act through soluble β-1,3-glucan release and ADH5 might have a role in quorum sensing. Zap1 is a zinc responsible gene. This can indicate a role for Zn in biofilm development. Zap1 also acts through different mechanism, it controls ergosterol biosynthesis and hexose transporters genes (Nobile *et al*. 2009).

Dispersion of yeast cells is the final stage in biofilm development. This can result in dissemination and formation of secondary sites of infections. For this to happen, hyphal cells have to undergo a reverse transition to yeast cells. Three transcription factors are important for dispersion; *Ume6*, a negative regulator and *PES1* and *NGR1*, both positive regulators (Uppuluri *et al*. 2010). Quorum sensing can result in changes in activity or expression and hence affect dispersion (Finkel & Mitchell 2010).

These released dispersed cells are distinct from 'regular' yeast cells. They have higher adherence, filamentation capacity and pathogenicity when compared to normal yeast cells (Uppuluri *et al.* 2010).

2.5.4 Antifungal susceptibility of biofilms

C. albicans biofilms are associated with higher resistance to antimicrobials. This resistance is complex and is caused by multiple mechanisms (Chandra *et a*l. 2001). The developing biofilm is associated with higher resistance. This progression in resistance is associated with increase of metabolic activity. Notably, biofilms are also heterogeneous in cell population: they differ in

growth rates. Certain subpopulation with lower growth rates can confer higher resistance of the biofilm (Tobudic, *et a*l. 2011).

Another important aspect of higher resistance in biofilms is due to the extracellular matrix (ECM). This is a physical barrier which protects the cells embedded in the biofilm community from antimicrobials. The physicochemical properties of the drug, the nature and amount of the ECM influence resistance. In one study, survival rate of *C. albicans* dropped as much as 20% when the ECM was removed. (Baillie & Douglas 1999). A certain subset of cells can survive antimicrobials well above minimum inhibitory concentration (MIC) values. They are phenotypic variants of wild-type cells and constitute 1% of the biofilm population in bacterial biofilms (e.g. *Pseudomonas aeruginosa* and *Escherichia coli*). These are dormant cells and are known to have multidrug tolerance (Keren *et al.* 2004). These so called persister cells are also found in *C. albicans* biofilms. Reinoculation of persister cells results in a new biofilm with a small subpopulation of persisters (Tobudic *et al*. 2011).

2.5.5 Clinical implications

The introduction of different devices in several locations in the body has allowed microorganisms such as *C. albicans* to colonize these devices and form biofilms. As a result they have higher protection from host defences and antimycotics. This in turn can lead to persistent infections. Colonization can also negatively affect the function of the devices.

C. albicans is a commensal organism of humans. In result, humans are the major source of contamination of implanted devices (Ramage *et a*l. 2006). After implantation these biomedical devices are often surrounded by body fluids such as urine, blood and saliva. Therefore, the devices acquire a gycoproteinaceous film covering their surface after implantation, resulting in completely different chemical characteristics of the surface (Ramage *et al*. 2006). Nonspecific factors (e.g. cell surface hydrophobicity and electrostatic forces) and specific adhesins mediate intial attachment of the *Candida* cells to the devices. These adhesins recognise specific ligands such as serum proteins (e.g. fibrinogen and fibronectin) and salivary factors in the covering film. Concomitantly, Candida cells can also adhere to bacterial cells already attached to medical devices (Chaffin *et al*. 1998).

A central venous catheter (CVC) is commonly used in current medicinal practice and will be used as an example to discuss device related *C. albicans* infections. Catheter related infections result in significant increase in hospital costs, duration of stay and patient morbidity. Moreover, intravenous catheters are the leading cause of nosocomial bloodstream infections (Ramage *et al.* 2006). Interestingly, biofilms are present on virtually all central venous catheters (Raad 1998). *Candida* species are the third leading cause of intravascular catheter-related infections and have the highest overall crude mortality. Contamination can arise at any time during hospitalization. Infection can result from extraluminar contamination originating from the skin

of either medical personnel (accounting for more than 25% of the cases) or the patient itself. Secondly, infection can also occur by hematogenous seeding from a distal infection site or by intraluminal contamination of the hub and lumen (Crump & Collignon 2000).

Catheter-related *Candida* infections are treated by removing the catheter and anti-fungal therapy for at least 4 days post positive blood culture. Non removal of the catheter is associated with higher mortality rates and poor prognosis (Viudes *et al.* 2002). However, another study suggests no outcome improvement after removal of the device (Ostrosky-Zeichner *et al*. 2010).

Other devices associated with *C. albicans* infections are implantable venous access ports, hemodialysis catheters, intrauterine catheters, urinary catheters, endotracheal tubes, intracardiac prostethic devices, pacemakers, dentures, neurological shunts, prosthetic joints, … In general, therapeutic interventions only cure the infection in rare cases. As a result, the device has to be removed. This often involves surgery which leads to higher costs and discomfort for the patient. Moreover, mortality rates associated with these infections are still unacceptable high (Ramage *et al*. 2006).

Currently, new approaches involve incorporation of antibiotics in a device or in coating on devices. Half-life of the therapeutic is an important factor. A study indicated a reduction in risk of infection by 32% when silver coated catheters are used (Karchmer *et al.* 2000).

3 Antimycotics

During the last decade, the focus of most major pharmaceutical companies has shifted to more profitable, chronic conditions. As a result, antifungal drug development has stagnated (Pierce *et al*. 2013). Moreover, like humans, fungi are eukaryotic organisms. Therefore, only a limited amount of drug targets are available. This also results in higher toxicity associated with antimycotics. As a consequence, the pipeline for antifungal drug development is essentially dry (Ostrosky-Zeichner *et al*. 2010).

3.1 Polyenes

3.1.1 Introduction

Polyenes contain alternating double and single carbon bonds. It was first isolated in 1955 in Venezuela from *Streptomyces nosodus* and is extracted on large industrial scale (Lemke *et al*. 2005). It is highly antiparasitic with a broad range activity. One of the major downsides is severe nephrotoxicity, which can resulting in kidney failure.

AmB contains a lactone ring with apolar and polar sides resulting in amphiphilic behaviour. Neighbouring polyene chains interact with each other, which allows AmB to self-associate and form aggregates starting at 0.2 µg/mL in water. In water a mixture is formed of water soluble monomers and oligomers with aggregates which are insoluble. The aggregation state of the mixture is related to its activity, toxicity and body distribution and can be determined by spectrophotometry (Torrado *et al*. 2008). The structure of amphotericin B is shown in figure 4.

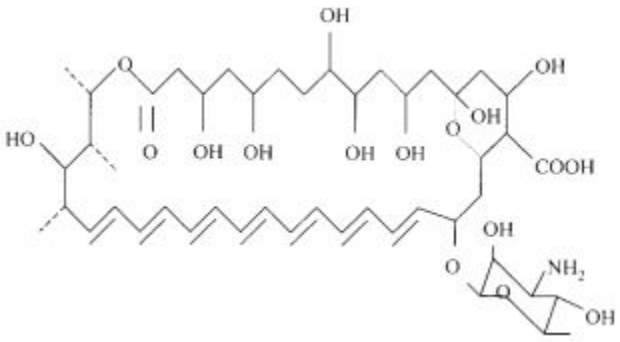


Figure 4: ***structure of Amphotericin B (Source: Torrado et al. 2008)***

3.1.2 Drug formulation

The low water solubility of AmB is one of the major drawbacks to overcome during formulation. All formulations are intravenously injected by perfusion to obtain systemic effects. The first marketed formulation is Fungizone (Life Technologies), considered the classical formulation (Kleinberg 2006). It is a micellar dispersion of AmB because it is prepared with a detergent, sodium deoxycholate (AmB sodium deoxycholate ratio is 1:2).

It has a broad spectrum activity but is often ineffective in immunocompromised patients (Veerareddy & Vobalaboina 2004). However, the major downsides are the nephrotoxic side-effects. Up to 50% of patients receiving AmB show severe renal disorders (Wingard *et al*. 1999).

Recently, formulation focus is shifted to overcome the nephrotoxic side-effects. Different lipid complexes were developed (e.g. Albecet (Sigma-tau) and Amphotec (Kadmon Pharmaceuticals)). Another, novel formulation developed is the liposomal formulatiom (AmBisome (astellas)). These new formulations are up to ten times as expensive and are mostly used for treatment of patients precluded of using Fungizone (Torrado *et al*. 2008). Next to the direct cost, the secondary cost also has to be considered. These are related to the conditions of the patient. The sicker the patient the greater the importance of the secondary costs. The question is whether the lower renal toxicity justify the higher daily cost. Currently, fungizone has been replaced by lower toxicity variants in most hospitals of developed countries (Torrado *et al.* 2008). Figure 5 illustrates amphotericin B incorporation in the phospholipid bilayer (AmBisome).

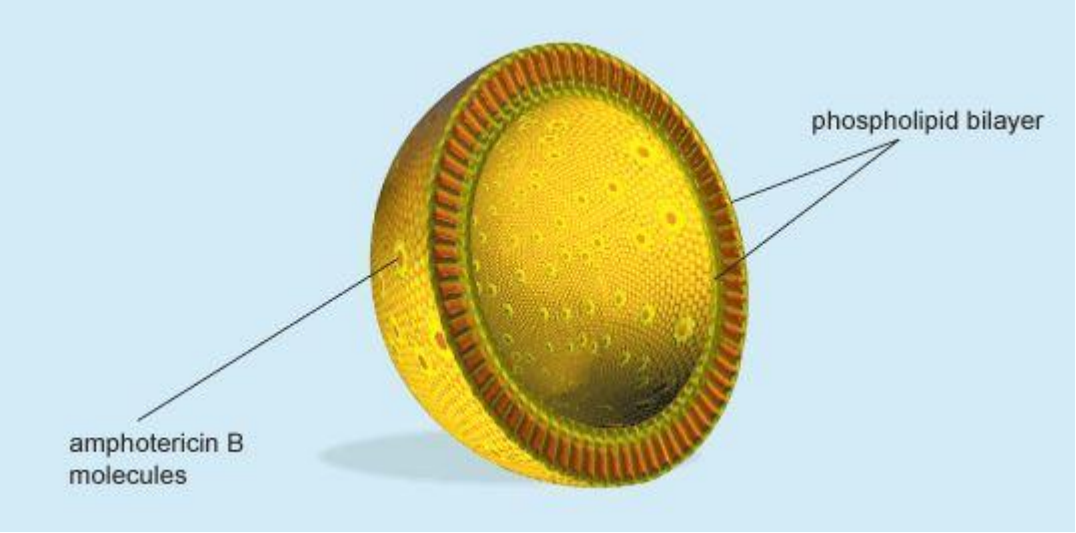


Figure 5: ***Ambisome, a special amphotericin B formulation (Source: ambisome.com)***

In AmBisome AmB is bound to the hydrophobic bilayer which consist of phosphatidylcholine and distearoylphosphatidylglycerol. These phospholipids are chosen to design a formulation stable at 37°C. Cholesterol is also added which binds AmB to increase stability. Other components are α-tocopherol, sucrose and disodium succinate hydrate. This liposomal formulation increases circulation time in the bloodstream.

3.1.3 Pharmacokinetics

Gastrointestinal uptake of Amb is minimal due to the low solubility. Still, intravenous infusion is the most used administration route. After infusion, up to 95% of the AmB binds plasma proteins (e.g. albumin, α_1 acid glycoprotein, β-lipoproteins; Bekersky *et al*. 2002).

Most of the Amb is removed in the liver and excreted with the bile. It is the unbound fraction that is excreted as an unchanged drug resulting in lower nephrotoxicity. The plasma half-life ranges between 24 and 48h, the elimination half-life is 15 days (Daneshmend & Warnock 1983). Different tissue concentrations are obtained depending on the type of carrier system. Ambisome results in higher plasmaconcentrations compared to Fungizone because they are less likely to be taken up by the reticuloendothelial system. Ambisome administration also results in lower AmB levels in lungs and kidneys and higher levels in liver and spleen (Proffitt *et al*. 1991).

3.1.4 Mode of action

After Ambisome binds the fungal cell wall, the liposome is disrupted, releasing AmB. This disruption mechanism is not yet elucidated. The release of Amb damages the fungal cell membrane. As a result the remaining liposomal fraction can penetrate through the fungal cell membrane (Menez *et al*. 2006). Next, AmB binds it cellular target: ergosterol, which is associated with the plasma and mitochondrial membranes.

Fungizone formulation does not seem to result in intracellular penetration of AmB. Binding to ergosterol leads to membrane depolarization and formation of pores resulting in leakage of monovalent ions. Evidence suggests that this is not the main mode of action. Chemical modifications of AmB result in molecules still capable of killing fungal cells but unable to form pores. This suggests that is more likely that AmB acts through sequestration of ergesterol (Palacios *et al*. 2007). It has also been shown that AmB induces reactive oxygen species (ROS) accumulation and acts through Hsp90 signalling. Furthermore, AmB addition results in expression of genes related to oxidative stress and increased mitochondrial respiration. Elevated ROS levels are associated with induction of apoptosis (Mesa-Arango *et al*. 2014).

3.1.5 Resistance

Resistance is rarely found in *C. albicans*. Resistance can be caused by a reduction in plasma membrane ergosterol concentrations. *ERG3* mutations lower the ergosterol concentrations (Cannon *et al*. 2007). Resistance of *C. tropicalis* can develop at multiple levels and are associated with decreased virulence and reduced fitness. First, modification of the drug target is possible. Also here, mutations in *ERG3* can lower the ergosterol concentration in the membrane and is also important for azole resistance (see chapter 3.2.4). Secondly, resistant yeast have no increased ROS production compared to after AmB addition. This is due to

increase in detoxification enzymes (antioxidant enzymes) such as superoxide dismutase and catalase (Mesa-Arango *et al*. 2014).

3.1.6 Activity against C. albicans biofilm

The standard formulation of amphotericin B has a reduced activity against biofilm cell compared to planktonic cells. However, liposomal formulation of amphotericin B has a similar MIC against biofilms and planktonic cells. The dispersion in to phospholipids is possibly the major cause for passing the ECM (Tobudic *et al*. 2011).

3.2 Triazoles

3.2.1 Introduction

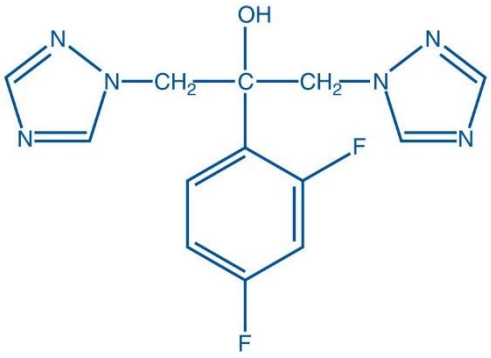


Figure 6: ***Structure of Fluconazole (source: Pfizer.com)***

Triazoles are synthetic compounds which all contain a five membered azole ring containing three nitrogen atoms. Fluconazole (see figure 6) was the first used in clinical practice and was discovered in Pfizer, UK (Richardson *et al*. 1985). It was approved fast due to the increasing need of oral active compound because of the AIDS epidemic. Currently it is used both as a prophylactic and as a curative due to its low cost and is fungistatic against Candida spp, except *C. glabrata* (Charlier et al 2006). Recently, newer triazoles (e.g. voriconazole and posaconazole) have been developed to overcome the limited efficacy against different molds, e.g. *Aspergillus* (Fera *et al*. 2009).

3.2.2 Pharmacokinetics

Fluconazole is well absorbed, has a high bioavailability (>80%) and good tissue penetration. In fasting adults plasma peak levels are reached 1-2h after administration. Only a small fraction is protein bound, the majority is excreted via the faeces. The half-life is 27-34h allowing a once a day administration in adults. Administrations of the drug can be orally, intravenously (IV) or intraperitoneally (IP) for candida peritonitis. The IV formulations are prepared in sterile water.

In adults is a dose of 200-400 mg/kg recommended for a prophylactic setting, compared to a loading dose of 800 mg/kg followed by a 400 mg/kg dose the following days for treatment of systemic candidiasis. The pharmacokinetics are different in neonates. The dose has to be doubled to reach comparable plasma levels.

Side effects occur especially with doses higher than 400 mg/kg/day. Common reported side effects include nausea, headache and abdominal pain. Other rare side-effects that have been reported include anaphylaxis, steven Johnson syndrome, hair loss and anorexia. There are no major interactions with immunosuppressive drugs, which can be important in organ transplantation were fluconazole is used as a prophylactic (Charlier *et al* 2006).

3.2.3 Mode of action

The main target of triazoles is the ergosterol biosynthesis pathway. It exerts its effect in two ways. First it inhibits 14-α-demethylase, the enzyme that removes the methyl group at the C-14 position of the substrate (e.g. lanesterol). This enzyme is encoded by the *ERG11* gene and the enzyme is referred to as erg11p in yeast. This results in accumulation of aberrant sterol intermediates on the fungal surface. Recent evidence suggests that *ERG11* expression is low in *C. albicans* biofilm and is regulated by fluconazole (Borecká-Melkusová *et al*. 2008) This in turns leads to cell cycle arrest (Lamb *et al*. 1999). Secondly, a cytochrome P450 enzyme involved in the last step of ergosterol biosynthesis is another target of triazoles, namely sterol δ^{22} desaturase (Cyp61) in yeast (Kelly *et al*. 1997). This cytochrome P450 is conserved among fungi and a homologues gene has been identified in *C. albicans* (Kelly *et al*. 1997).

3.2.4 Resistance

The azole concentration can be lowered a their site of action by the use of efflux pumps. Two classes of efflux pumps seem to be involved in antifungal resistance. First there are the *CDR1* and *CDR2* genes, both belonging to the ATP-binding cassette (ABC) transporter family. These are responsible for resistance to multiple azoles. Secondly, the *MDR1* gene which is part of the major facilitator superfamily (MFS) leads to fluconazole resistance. Resistant isolates do not always express all three, some overexpress only *MDR1* and others overexpress only *CDR1* and *CDR2* (Niimi *et al*. 2004; Fera *et al*. 2009). Influx of the azole drugs can also be important. Failure in influx can impair drug accumulation. This can be the result of lower azole penetration due to altered ergosterol levels and hence fluidity (Löffler *et al*. 2000).

Another mechanism of resistance is increased protein target levels such as erg11p. This can be the result of point mutations in the *ERG11* coding region, overexpression, gene conversion and gene amplification (Marichal *et al*. 1999). Moreover, fluconazole addition can result in up-regulation of *ERG11* (Kucharíková *et al*. 2011) Some of these can also alter the affinity of azoles for the target protein. A final mechanism can be modification (mutations) of other ergosterol biosynthesis genes. An example is $\delta^{5,6}$ desaturase encoded by the *ERG3* gene which is responsible for the accumulation of toxic intermediates after azole addition. Defects in this gene result in resistance by suppressing toxicity (Fera *et al*. 2009). Multiple mechanisms of resistance can be combined in isolates, resulting in high levels of resistance.

3.2.5 Activity against *C. albicans* biofilm

C. albicans biofilm are highly resistant against azoles. *In Vitro* grown biofilms can be up to 1000 fold more resistant compared to planktonic cells (Lamfon *et al*. 2004). These resistant mechanisms are highly phase specific. During the early phase of biofilm development efflux pumps play an important role. Reduced ergosterol levels are important for resistance during the later phases of biofilm development. Pre-treated vorinocazole *C.albicans* cells have a decreased ability to form biofilms (Katragkou *et al*. 2008).

3.3 Echinocandins

3.3.1 Introduction

Echinocandins, a type of modified lipoproteins, are derived from the fermentation broth of various fungi but are currently produced synthetically. Caspofungin is derived from pneumocandin B, produced by *Glarea lozoyensis* (Schwartz *et al*. 1989) Micafungin is derived from echinocandin B and produced by *Coleophoma empedri (*Fujie 2007). Anidulafungin is derived from echinocandin B produced by *Aspergillus nidulans* (Benz *et al*. 1985; Wiederhold & Lewis 2003). Caspofungin was the first of the echinocandins and approved by the FDA in 2001. They are effective against a wide variety of yeasts and molds and have a cidal activity against *Candida* spp. even in very low concentrations. However*, C. glabrata* is less susceptible to both caspofungin and anidulafungin when compared to *C. albicans* (Kucharíková *et al*. 2011). Figure 7 shows the structure of both caspofungin and anidulafungin.

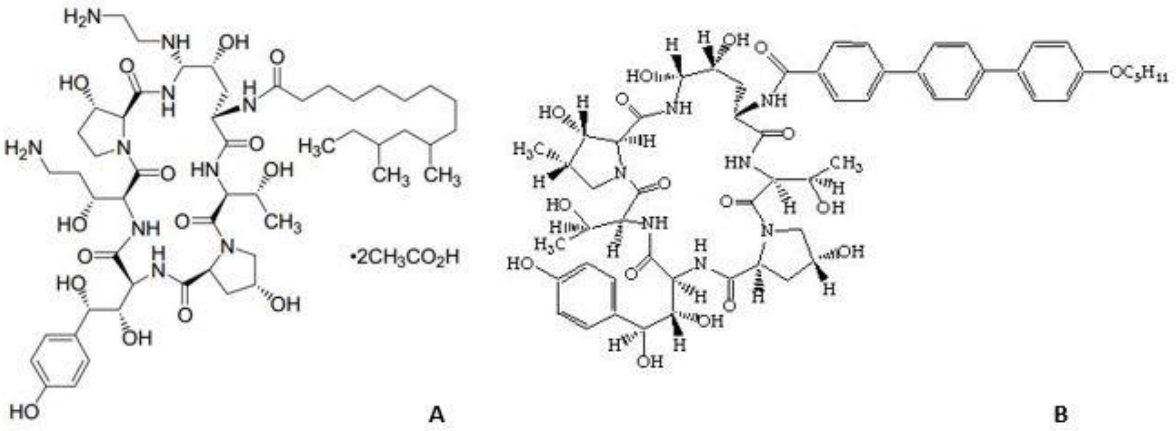


Figure 7: ***Stucture of caspofungin (A; source: Merck.com) and anidulafungin (B; source: Pfizer.com)***

One of the most remarkable characteristics of echinocandins is the paradoxical Eagle effect or paradoxical growth effect (PGE), which is characterized by induced *in vitro* growth when using higher concentrations of echinocandins and is especially pronounced for caspofungin

(Vanstraelen *et al*. 2013). While conversely, this induced growth can be overcome when the echinocandin concentration is increased even more. The Eagle effect can be seen in figure 8.

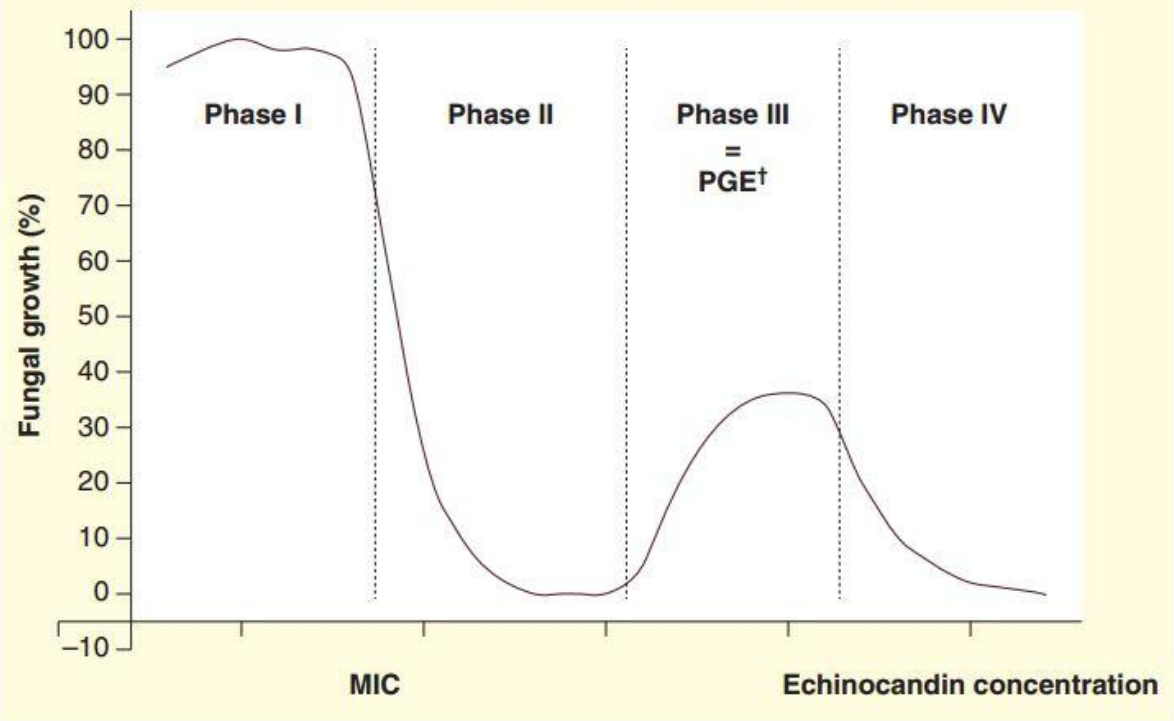


Figure 8: ***fungal growh in fuction of MIC showing the Eagle effect or PGE (Adapted from Vanstraelen et al. 2013)***

For anidulafungin and micafungin opposing results have been shown. Some research groups have shown no PGE for these echinocandins (Stevens *et al*. 2004), while others have shown a mini-PGE for anidulafungin (Fleischhacker *et al*. 2008). This can be due to strain specificity. While concomitantly, it also depends on the medium used (Vanstraelen *et al*. 2013).

3.3.2 Pharmacokinetics

It has been previously shown that HIV-positive patients, suffering from oesophageal or oropharyngeal candidiasis, treated with higher doses of micafungin (50,100 and 150 mg/kg/day, IV) for a longer period of time (14-21 days) resulted in improved treatment outcome (De Wet *et al*. 2004). Cure rates between 50 and 150 mg were significantly different. No significant side effects were observed. This seems less clear in another study (Betts *et al*. 2009). In patients with invasive candidiasis, there was no significant difference in patients treated with traditional dosed caspofungin (70 mg loading dose followed by 50 mg/day the following dose) compared to a higher dose (150 mg/day).

3.3.3 Mode of action

Echinocandins inhibit 1,3 and 1,6-D-glycan synthase, an enzyme complex involved in the synthesis of 1,3-β-D-glucan. This is a glucose polymer essential for the structure and integrity

of the cell wall. The glucan synthase complex is made up of 5 subunits; a regulatory subunit, Rho1p (a small GTPase) and 3 catalytic subunits encoded by three homologues genes *FKS1*, *FKS2* and *FKS3*. The fks1p and fsk2p are the target of the echinocandins. They have different roles but can partly substitute for each other. Changes in the 1,3-β-synthesis resulted in osmotic instability and lysis of the cell wall (Fera *et a*l. 2009).

3.3.4 Resistance

So far, reduced susceptibility and full resistance to echinocandins seems to be a rather rare event, although some researchers raised the problem of increased tolerance of *Candida* cells to echinocandins. Some amino acid substitutions have been identified and confer to reduced susceptibility. They are the result of mutations in a part of a so called hot-spot 1 region in the *FSK1* gene. The most common amino acid substitution in *C. albicans* is located at codon 645 in which a serine is replaced by proline, phenylalanine or tyrosine (Balashov *et al*. 2006). Additionally, clinical isolates with *FSK1* mutations also show reduced fitness and virulence (Ben-Ami *et a*l. 2011). *FSK2* mutations in *C. glabrata* and *C. guilliermondii* have also been reported which results in elevated chitin levels and reduced echinocandin susceptibility. However, the mechanism of action is not yet fully understood (Walker *et al*. 2008) Efflux pumps do not seem to influence echinocandin susceptibility (Fera *et a*l. 2009).

3.3.5 Activity against C. albicans biofilm

Echinocandins are known for their *in vitro* activity against *C. albicans* biofilms. They block 1,3-beta-d-glucan production which is also an ECM component. Both caspofungin (Shuford *et al.* 2006) and anidulafungin (Kucharíková *et al.* 2010) show promising results in treatment of device associated infections in an animal model. A more recent study shows a significant reduction in *C. albicans* fungal load when both caspofungin and anidulafungin are administered IV in an in vivo subcutaneous rat model. A longer treatment (10 days instead of 5 for caspofungin and anidulafungin) and higher dose (30mg/kg/day instead of 10mg/kg/day for caspofungin and anidulafungin) were needed to achieve the same result when micafungin was used (Kucharíková *et al*. 2013). This is probably due to a higher elimination in the liver of rats (Niwa *et al*. 2004) and different pharmacokinetics (Sucher *et al*. 2009) For this reason a 2-3 fold higher dose is used for anidulafungin when compared to caspofungin. Treatment with echinocandins is also more cost effective and cost saving compared to azoles and polyene drugs. This is due to the lower incidence of treatment-related side-effects (Neoh *et al*. 2014).

4 Alkylphospholipids

4.1 Miltefosine

Miltefosine (hexadecylphosphocholine) is a alkylphospholipid, first identified as potential anticancer drug. It was used as a therapy for cutaneous lymphomas (Dummer *et al*. 1993) and skin metastases of mammary carcinomas (Scherf *et al*. 1987). Miltefosine inhibits protein kinase C (PKC) in mammalian tumour cells (Geilen *et al*. 1994), inhibits biosynthesis of both sphingomyelin and phosphatidylcholine (Haase *et al*. 1991) and inhibits PI3K and akt (Dorlo *et al*. 2012). This results in increased cellular stress and induction of apoptosis mediated by reactive oxygen species (ROS; Van Blitterswijk & Verheij 2008). Figure 9 shows the structure of miltefosine.

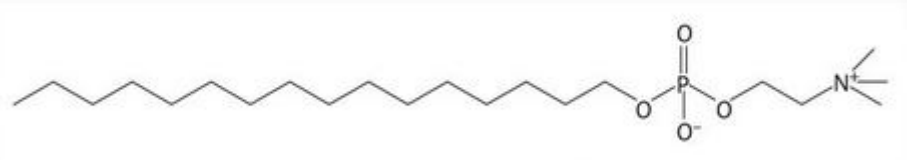


Figure 9: ***Structure of Miltefosine (Adapted from Fortin et al. 2012)***

Currently it is repositioned as a drug to treat Leishmaniosis, a zoonotic disease caused by protozoan parasites of the *Leishmania* genus and is registered in Argentina, Bangladesh, Bolivia, Colombia, Ecuador, Germany, Guatemala, Honduras, India, Mexico, Pakistan, Paraguay and Peru (Dorlo *et al*. 2012). The parasites are transmitted to humans and animals by the blood-sucking phlebotomine sandflies (Killick-Kendrick 1990). Miltefosine exhibits a good cinical and parasitological efficacy after oral administration in dogs (Mateo *et al*. 2009). So far, no single mode of action is proposed. Moreover, there are a lot of contradictory studies indicating the multitude of proposed mechanisms. Miltefosine exerts it's anti-*Leishmania* effect in mammalian cells by inhibiting phosphocholine synthesis, perturbation of ether, sterol and fatty acid metabolism, inhibition of choline uptake, and inhibition of glycosylphosphatidylinositol anchor biosynthesis and signal transduction resulting in apoptose-like celldeath. Miltefosine also affects the mitochondria and inhibits cytochrome C in *Saccharomyces cerevisiae* (Dorlo *et a*l. 2012) Another treatment for Leishmaniosis is amphotericin B (see chapter 3.2), a known antifungal (Widmer *et al*. 2006).

From the chemical point of view, the structure of miltefosine is quite simple. It is a phosphocholine ester of aliphatic long chain alcohol. This is structurally similar to phosphatidylcholine and lysophospatidylcholine, both natural substrates of the fungal phopholipase B (PLB1) enzyme (Chen *et al*. 2000), indicating a possible interaction. Indeed, Miltefosine is known to inhibit PLB1 enzyme activity and this coincides with antifungal activity in *Cryptococcus neoformans* (Widmer *et al*. 2006). PLB1 is a virulence determinant in *C. albicans* (see section x) and also produced by other fungal species (e.g. *Aspergillus fumigatus, Scedosporium prolificans, Fusarium oxysporum* and *Mucorals* sp.) resulting in a broad

spectrum antifungal effect with fungicidal concentrations comparable with amphotericin B (Widmer *et al.* 2006).

PLB1 contains three separate activities: lysophospholipase (LPL) removes the single acyl chain from lysophospholipids, lysophospholipase transacylase (LPTA) adds an acyl chain to lysophospholipids and phospholipase B (PLB) removes both acyl chains simultaneously from phospholipids. Miltefosine inhibits LPTA activity at 25 µM and all three activities at high concentrations (250 µM) but did not inhibit porcine phospholipase A_2 (PLA_2) at 25µM,indicating selectivity and not an effect of detergent-like action (Widmer *et al*. 2006). Since LPTA is involved in membrane synthesis, remodelling suggests an effect by interfering with cell membrane biochemistry. Inhibition of LPL and PLB activities can prevent detoxification and utilization of free fatty acids derived from host tissues (Widmer *et a*l. 2006).

The chemical structure of alkylphospholipids allows them to be inserted in lipid membranes and hence resist catabolic degradation. This can result in changes in membrane composition, altering fluidity and permeability. Interference with membrane proteins such as β-1,3 glucan synthase can also occur, affecting cell wall synthesis (Vila *et al*. 2012). Fluidity of the membrane depends on degree of saturation. Saturated phospholipids can be packed tightly together, decreasing fluidity. Unsaturated phospholipids are kinked and can't be packed tightly, resulting in increased fluidity. The nature of the structure allows alkylphospholipids to pass through the extracellular matrix produced by the biofilm cells, resulting in a higher activity towards biofilm structures compared to other antimycotics (Vila *et al*. 2012).

Miltefosine exhibits the most drastic effect on *C. albicans* biofilm formation *in vitro*, resulting in detachment of almost all cells. Mature biofilms are less sensitive compared to biofilm formation, but the mature biofilm have altered cells and deformed buds (Vila *et al*. 2012). Miltefosine seems to be active on both medically important surfaces biofilm cells and planktonic cells.

Miltefosine has also been tested *in vivo* against cryptococcal infection developed in a murine model. Miltefosine was administered orally for five days and this treatment of miltefosine increased survival and reduced both brain and lung cryptococcal burdens. This was achieved by using relatively low doses (e.g. 3.6 mg/kg/day and 7.2 mg/kg/day). Ten mg/kg/day resulted in serum concentrations of 110 µM in rats, which is 10-20 times the minimum inhibitory concentration (Widmer *et al*. 2006). In humans, miltefosine has a long half-life (100-200h) and a low therapeutic ratio, potentially leading to development of resistance (Soto J. & Soto P. 2006). Miltefosine also has a high haemolytic activity (HC_{50} of 38.6µM) preventing it's use in injectable form (Vila *et al*. 2012).

Side effects associated with miltefosine are diarrhea, dysorexia, nausea and self-limited vomiting. These can be diminished when combined with food but preclude it's long term use in cancer patients (Sindermann *et al*. 2003). Other reported side effects are hepatotoxicity and transient increases in liver enzymes, rashes and even Stevens-Johnson syndrome in rare

occasions. No congenital abnormalities have been reported but it has been proven to be teratogenic in high doses in rats (Sundar *et al*. 2002). Recently, miltefosine resistance has been documented in an *L. infantum* case (Cojean *et a*l. 2012).

4.2 Oleylphosphocholine (OIPC)

The long half-life of miltefosine increased the likelihood of developing drug resistance. To explore the full potential of miltefosine and guarantee it's long term use, different miltefosine analogues were generated and tested. Both alkyl chain length and the degree of saturation had an impact on potency. Among these miltefosine analogues, Oleylphosphocholine (OIPC) proved to be the most promising in an *in vitro* model against *Entamoeba histolytica* (Seifert *et al.* 2001). Figure 10 illustrates the structure of OIPC

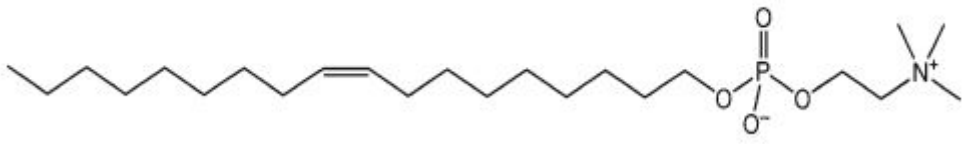


Figure 10: ***structure of OIPC (Adapted from Fortin et al. 2012).*** *Notice the double bond compared to miltefosine.*

OIPC differs from miltefosine in acyl chain length (C_{18} instead of C_{16}) and the introduction of a double bond resulting in better tolerance and bioavailability (Paulussen *et al*. 2015). This double bond is important in two aspects: first it introduces a kink in the structure, resulting in a less packed and hence higher fluidity of the membrane upon incorporation.

Secondly, the synthetic procedure can result in formation of *trans*-isomers (3-6%). These *trans*-impurities have remarkable side-effects and can negatively affect OIPC treatment (Sobottka *et al*. 1993). Therefore it is necessary that these impurities are removed. This is done by separation by reversed phase high performance liquid chromatography (Thaler *et al*. 2000).

An *in vitro* model for different *Leishmania* spp. showed comparable potency for OIPC and miltefosine suggesting they share a similar mode-of-action (Paulussen *et a*l. 2015). However, an *in vivo* hamster model with *L. infantum* revealed a higher potency for OIPC after multiple and single oral dosing (Fortin *et al*. 2012). Here they suggested that overall bioavailability was more important for killing compared to peak plasma concentrations.

Moreover, OIPC has the potential to be administered under a shorter regimen compared to miltefosine. It clears more rapidly which can be beneficial for resistance development (Fortin *et al*. 2012). OIPC was administered in both aqueous and liposomal formulation. Treatment with aqueous solution resulted in minor weight loss (e.g. 6%) but no weight loss was observed for treatment with liposomal formulation. Yet, bioavailability is considered the same, since both

formulations showed similar tolerance and efficacy. In general liposomal formulation is thought to decrease toxicity. The downside is the possible degradation of liposomal structure in the intestinal tract (Fortin *et al*. 2012). As it was already discussed in section 3.1.2, liposomal formulations are also used for amphotericin B.

Recently OIPC demonstrated activity against different *Aspergillus* species tested in an *in vitro* model and also *in vivo* against *Aspergillus fumigatus* (Paulussen *et al*. 2015). In this study, a liposomal formulation of OIPC was. OIPC showed a five times higher potency compared to miltefosine *in vitro*. Five day treatment in an *in vivo A. fumigatus* mouse model only showed a significant organ burden reduction when 50 mg/kg was administered intraperitoneal (IP) without any toxic effect. Whereas 10 day treatment resulted in 60% survival after oral administration (25 and 50 mg/kg). Potential pitfalls in this story might be the use of immunocompetent mice and a very high inoculum which could explain the limited *in vivo* activity. However, lower inoculum size resulted in less reproducible infection.

Objectives

The main aim of this master thesis was to explore the effect of a novel experimental compound - oleylphosphocholine (OIPC) on *C. albicans* biofilm development *in vitro* and *in vivo*.

C. albicans is a most common human fungal pathogen known for its ability to form biofilms on biotic and abiotic surfaces. Biofilms are associated with an increased tolerance of device-associated cells to existing classes of antifungal agents and are almost impossible to eradicate. There is only a limited number of antifungals which can reduce *Candida* biofilms. Therefore, it is crucial to search for new compounds and novel therapeutic strategies, which could prevent or diminish *Candida* attachment and subsequent biofilm formation on a device.

In this study, we first assessed the effect of the compound on biofilm formation *in vitro* using 2 different substrates, namely 96 well polystyrene plates and polyurethane catheters. Next, we examined the role of OIPC on the development of *C. albicans* biofilms on mucosal surface using an *ex vivo* oral model of infection.

In a next step we tested the effect of OIPC on *C. albicans* biofilms developed *in vivo* in a subcutaneous model of infection. This model has been successfully used to study the effect of novel and existing antifungals, such as echinocandins on *C. albicans* biofilms under *in vivo* conditions (Bink *et al.*, 2012; Kucharíková *et al*., 2010; Kucharíková *et al*., 2013).

Materials & methods

5 Materials

5.1 Buffers

5.1.1 10x phosphate buffered saline (PBS)

- 1 g KCl (VWR international)
- 40 g NaCl (Merck)
- 7.2 g Na_2HPO_4 (Merck)
- 1.2 g KH_2PO_4 (Merck)
- 450 mL Milli-Q water
- Adjust to pH 7.4 with NaOH (Merck)
- Adjust to final volume of 1l with Milli-Q water
- Autoclave at 120 °C for 20’ at 120 kPa

This buffer was diluted to a final concentration of 1x. This isotonic solution maintained fungal cells in physiological environment but arrested the cell cycle.

5.2 Media

5.2.1 Glucose 40% solution

- 400g D-glucose (Sigma-Aldrich)
- Adjust to 1l by using Milli-Q water
- Autoclave at 120 °C for 20’ at 120 kPa

Glucose was added to YP medium (final concentration of 2%) after autoclaving.

5.2.2 RPMI-MOPS, PH 7.0 solution

- 10.4 g RPMI-1640 (Sigma Aldrich)
- 34 g MOPS (3-(N-morpholino)proanesulfonic acid buffer, 99.5%; Sigma Aldrich)
- Add 950 mL Milli-Q water
- Adjust to pH 7.0 with NaOH tablets (Merck) while stirring
- Filter sterilise the solution using Fast PES filter unit (Nalgene-Thermo scientific)

This medium was used for *C. albicans* adhesion and biofilm formation in 96 well plate and on polyurethane catheters.

5.2.3 RPMI-MOPS, pH 7.0 solution + penicillin-streptomycin

- Sterile RPMI-MOPS, pH 7.0 solution is prepared as mentioned above
- 0.5 mL 10 mg/mL streptomycin-penicillin (Sigma Aldrich) is added to 49.5 mL RPMI-MOPS, pH 7.0 solution

This medium was used for *C. albicans* biofilm formation on mucosal surface during an *ex vivo* model of infection.

5.2.4 Yeast Peptone Dextrose (YPD)

- 10g Yeast extract (Merck)
- 20g Bacteriological peptone (OXOID)
- 15g Agar (Difco)
- Add 950 mL Milli-Q water
- Autoclave at 120 °C for 20' at 120 kPa
- Add 50 mL sterile 40% D-Glucose solution

This medium was used to grow *C. albicans* strains and clinical isolates. Additionally, this medium was used to enumerate *C. albicans* colony forming units.

5.3 Strains

Table x: ***An overview of all strains used in this study and their characteristics.***

Strain	Characteristics	Source	Reference
SC5314/ dpl 1000	wild type	patient with disseminated candidiasis	Gillum *et al*. 1984 Jones *et al.* 2004
TR23 CA (gLUC59)	Bioluminescence strain	Constructed from SC5314 background	Vande Velde *et al*. 2014
4.19	clinical isolate	Patient with candidemia	Blink *et al*. 2012
U-0503-18	clinical isolate	Patients with urinary cathethers	Prof. Katrien Lagrou, UZ Leuven, Belgium
U-04030-255			
U-0203-29			
U-0503-12			
U-0503-8			
U-0203-15			
HC2601-30	clinical isolate	Isolated from hemocultures	
HC0202-25			
HC2102-19			
HC1602-5			
HC0702-14			
dpl 1007	Strains with elevated MICs for caspofungin (*fks1* mutants)	Isolated from patients	Garcia-Effron *et al*. 2008
dpl 1008			
dpl 1009			
dpl 1036			
dpl 1015		Mutant SC5314 Strain	
dpl 1016			

6 Methods

6.1 OIPC stock solution

OIPC was received from Dafra Pharma, Belgium. Stock solution was dissolved in 1 mL of sterile Milli-Q water and kept maximum for one week in the fridge. Prior to each experiment working solution was prepared in RPMI-MOPS, pH 7.0. The working solutions were kept only during the day of an experiment.

6.2 *In vitro* experiments

6.2.1 Minimum inhibitory concentration (MIC)

C. albicans MIC testing was performed according to the National Committee for Clinical Laboratory Standards (NCCLS), M27-S4 protocol (2012). *C. albicans* strains and clinical isolates were grown on YPD plates, at 37 °C for 24 h. The next day, *C. albicans* cells were scraped off the plate and dissolved in 1 mL of 1x PBS. *Candida* suspension was vortexed and diluted 1:100 in a cuvette to determine OD_{600}. Next, *Candida* cells were diluted to OD_{600} 0.2 in 1x PBS. From this solution 3 µL of *Candida* suspension was added to 1 mL of pre-warmed RPMI-MOPS, pH 7.0. Next, 100 µL of this solution was added to a U-bottom 96 well plate together with 80 µL RPMI-MOPS, pH 7.0 solution and 20 µL of different concentrations of OIPC (0.5 µg/mL - 1 µg/mL – 2µg/mL – 4µg/mL – 8 µg/mL – 10 µg/mL – 13 µg/mL; see section 6.1). The 96 well plate was incubated for 24 h at 37°C. The next day, the minimal inhibitory concentrations, which inhibited the growth of *Candida* cells on 95% (MIC_{50}) was read visually.

6.2.2 Minimal fungicidal activity (MFC)

The minimal fungicidal activity was determined in order to display the concentration of OIPC, which completely abolished the growth of *C. albicans* planktonic cells. Therefore, *Candida* cells incubated in the presence of different concentrations of OIPC, during the determination of MIC according to the NCCLS protocol (section 6. 2. 1), were spotted on a square YPD plates using a metal pinner. Plates were further incubated at 37 °C, overnight. MFCs were determined when no growth was detected on YPD plate (99,9 % of inhibition).

6.2.3 Efficacy of different concentrations of OIPC on *C. albicans* adhesion, biofilm development and mature biofilm formed on polyurethane catheters

We performed this experiment to assess the effect of OIPC on the different stages of biofilm formation on catheters. *Candida* suspension and catheters preparation was identical in the following 3 experimental set ups. 1 cm long catheter pieces were FBS coated, overnight, at 37°C. *Candida* cells were adjusted to $5x10^4$ cells/mL after being counted in the Bürker chamber (section 6.2.3.1).

It is important to mention that an echinocandin: anidulafungin (Ecalta; 4 µg/mL) was used in all experiments as a reference drug. The stock solution of anidulafungin was prepared in DMSO.

6.2.3.1 *C. albicans* counting in the Bürker chamber.

C. albicans SC5314 was grown on a YPD plate at 37 °C, overnight. The next day, a cell suspension was prepared by dissolving *C. albicans* cells in 1 mL of 1x PBS. *Candida* cells were diluted 1:100 and counted using the Bürker chamber (Brightline). *Candida* suspension was adjusted to $5x10^4$ cells/mL in pre-warmed RPMI-MOPS (pH 7.0).

6.2.3.2 *C. albicans* adhesion on polyurethane catheters in the presence/absence of OIPC

An experimental timeline displaying the period when the OIPC was added to *C. albicans* cells is shown in figure 11.

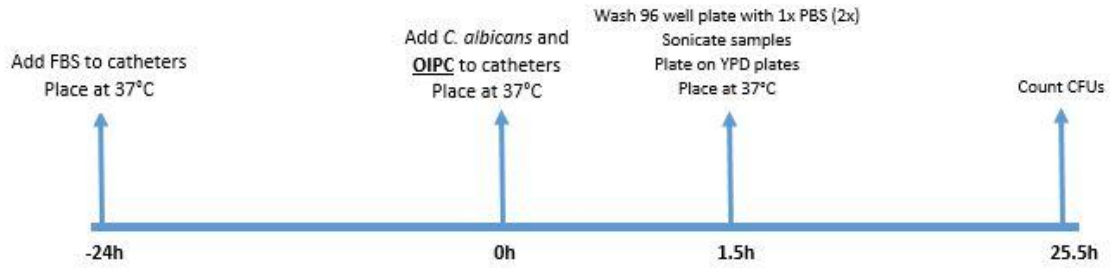


Figure 11: ***Timeline of the effect of OIPC on C. albicans cells during the period of***

After the steps described above, the catheters were infected with the *C. albicans* cells together with the different OIPC concentrations. OIPC – treated catheters were submerged in 900 µL of *Candida* suspension together with 100 µL of different concentrations of OIPC (2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL). Catheters challenged only with *Candida* cells (900 µL of suspension + 100 µL of clean media) were considered as controls or used to quantify fungal growth without OIPC in a separate experiment. Anidulafungin – treated catheters were supplemented with 900 µL of *Candida* suspension together with 100 µL of anidulafungin (4 µg/mL). The effect of OIPC on adhesion was followed at 37 °C, 90 min. Afterwards, non-adhering cells were removed by washing steps (twice, always using 1 ml of 1 x PBS). The amount of *Candida* adhered cells was quantified by colony forming unit counts (CFUs) as described in section 6.2.3.5.

6.2.3.3 *C. albicans* biofilm development on polyurethane catheters in the presence/absence of OIPC

The experimental timeline of the effect of OIPC on *C. albicans* biofilm development on polyurethane catheters is demonstrated in figure 12.

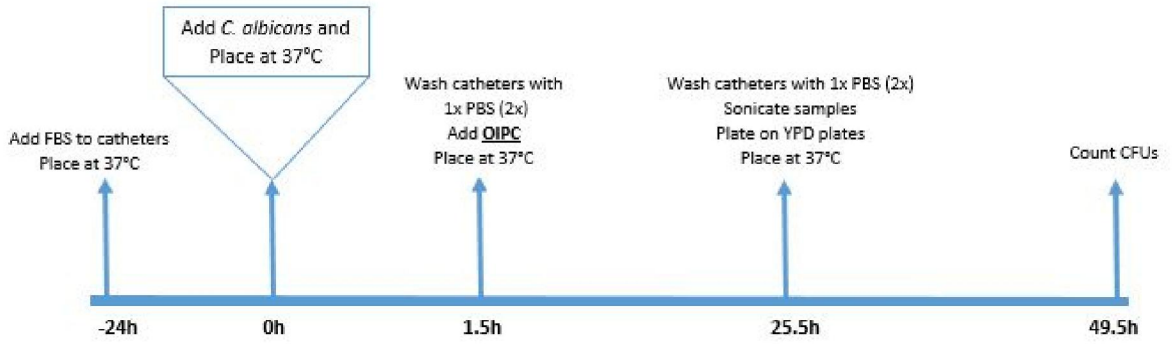


Figure 12: ***Timeline of the effect of OIPC on C. albicans biofilm development on catheters****.*

In this experimental set up *Candida* adhesion on polyurethane catheters was performed in clean RPMI-MOPS, pH 7.0 (37 °C, 90 min). Afterwards, non-device associated cells were removed by washing steps. Subsequently, catheters were submerged in 900 µL of fresh RPMI-MOPS, pH 7.0 with the different concentrations of OIPC (2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL). Catheters supplemented with RPMI-MOPS, pH 7.0 without OIPC were considered as controls. Additional control catheters were used in an independent experiment to quantify *C. albicans* biofilm development in the absence of OIPC. Anidulafungin – treated catheters were supplemented with 900 µL of *Candida* suspension together with 100 µL of anidulafungin (4 µg/mL). The effect of OIPC on biofilm development was performed at 37 °C, for 24 h. After that, non-biofilm forming cells were removed by washing steps (twice, always using 1 ml of 1 x PBS). The amount of remaining *Candida* cells was quantified by colony forming unit counts (CFUs) as described in section 6.2.3.5.

6.2.3.4 *C. albicans* mature biofilm on polyurethane catheters in the presence/absence of OIPC

The timeline below indicates all actions performed during 'the effect of OIPC on *C. albicans* 'mature biofilm' experiment.

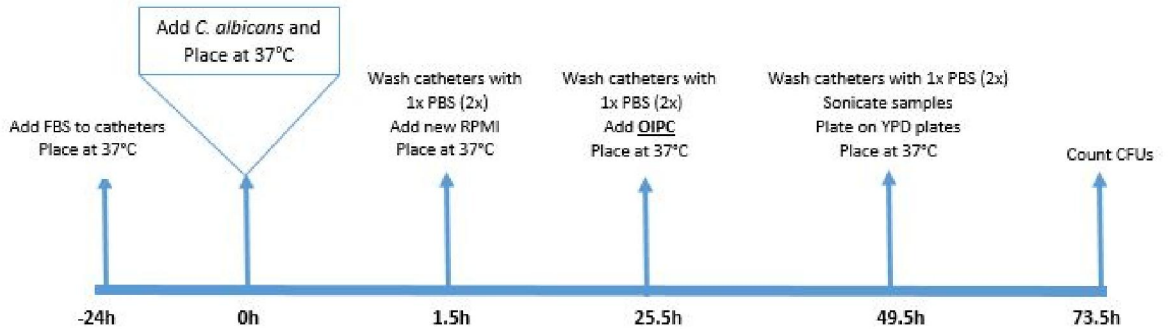


Figure 13: ***Timeline of the effect of OIPC on C. albicans mature biofilm on catheters****.*

Following the steps in section 6.2.3 the serum pre-coated catheters were supplemented with 1 mL of *C. albicans* cell suspension. The next 90 minutes the 24 well plate was placed in the 37°C incubator to allow the *Candida* cells to adhere to the catheters. Next, non-adhered cells were removed by washing the catheters twice using 1x PBS. The catheters were then transferred to a new 24 well plate and were submerged in 1 mL clean RPMI-MOPS, pH 7.0 solution. Finally, 24 well plate was placed back in the 37°C incubator for another 24 h to allow biofilm development of the *C. albicans* cells.

Two wash steps were performed the next day. The catheters coated in *C. albicans* biofilm were placed in a new 24 well plate. Controls were submerged in 1 mL clean RPMI-MOPS, pH 7.0 solution. In contrast, 900 µL RPMI-MOPS, pH 7.0 solution and 100 µL OIPC solution of different concentrations (1 µg/mL - 2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL) were added to the other catheters. Positive controls were also included: here 900 µL RPMI-MOPS, pH 7.0 solution and 100 µL Anidulafungin (Ecalta; 4 µg/mL) solution were supplemented to the catheters as internal controls. This was followed by maturation phase (24 h, 37°C). The next day, The catheters were washed twice with 1x PBS and the amount of *Candida* biofilm cells was quantified by colony forming unit counts (CFUs) as described in section 6.2.3.5.

6.2.3.5 Quantification of adhered- and biofilm-forming cells by Colony forming unit counts (CFUs)

After the last washing step, the catheters were transferred to Eppendorf tubes containing 1 mL of 1x PBS and immediately placed on ice. The samples were then sonicated for 10 min, followed by vortexing for 30 s. Original samples and a dilution series (1:10 and 1:100) prepared in PBS were plated on YPD plates. Every dilution was plated in duplicate. YPD plates were incubated at 37°C for 24 h. CFUs were determined by enumeration of colonies grown on YPD plates.

6.2.4 XTT assay

6.2.4.1 Concept

Mitochondrial enzymes of *C. albicans* can transform the XTT dye 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide to formazan. The colour changes of the substrate due to the formazan formation can be measured spectrophotometrically at OD_{590}. In this way, only metabolic active cells were quantified. XTT solution was supplemented with menadione in order to induce the formation of formazan.

6.2.4.2 Common steps

200 µL FBS solution was added to each well of the 96 well plate to pre-coat the wells. Subsequently, the plate is placed in the 37°C incubator for 24 h. Two *C. albicans* strains were

used in this experiment: SC5314 (wild type) and 4.19 (clinical isolate). Both strains were counted with the Bürker chamber as in section 6.2.3.1 but were adjusted to 1 x10^7 cells/mL.

6.2.4.3 Effect on adhesion

All major steps off 'the effect of OIPC on adhesion in 96 well plates' experiment is shown in the figure below.

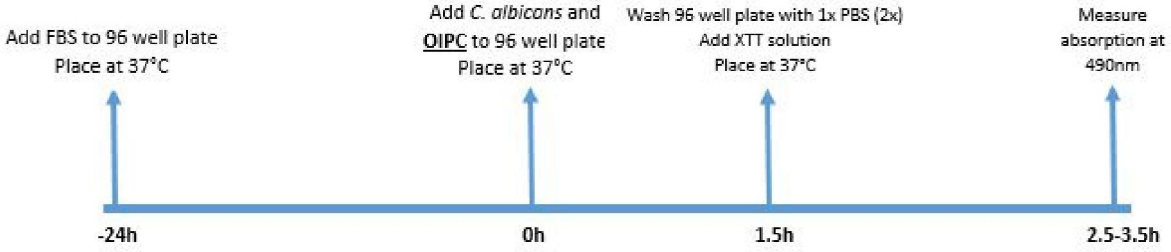


Figure 14: ***Timeline of the effect of OIPC on C. albicans adhesion to 96 well plates***

Following the common steps as described above, 200 µL of the *C. albicans* suspension was added to the FBS-coated wells for the control. In the other wells 100 µL of the *C. albicans* suspension, 80 µL pre-warmed RPMI-MOPS, pH 7.0 solution and 20 µL OIPC solution of different concentrations (0.5 µg/mL - 1 µg/mL - 2 µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL) were added. Here, no internal control was added because plenty of literature of other antimycotics is available on the subject. This was followed by period of adhesion (90 min, 37°C). Subsequently, The wells were washed twice with 200 µL 1x PBS. Quantification of the *C. albicans* cells was done as described in section 6.2.4.6.

6.2.4.4 Effect on biofilm development

Subsequent events in the biofilm development experiment are shown in figure 15.

Figure 15: ***Timeline of the effect of OIPC on C. albicans biofilm formation to 96 well plates***

During 90 minutes C. albicans cells (as prepared in 6.2.4.1) were allowed to adhere to FBS-coated wells in 37°C. This was followed by wash step with 1 x PBS . Only clean pre-warmed RPMI-MOPS, pH 7.0 was added to the control wells. The other wells were supplemented with

100 µL of the *C. albicans* suspension, 80 µL RPMI-MOPS, pH 7.0 solution and 20 µL OIPC solution of different concentrations (0.5 µg/mL - 1 µg/mL - 2 µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL). The 96 well plate was incubated for another 24 h at 37°C. The following day, we measured the metabolic activity as discussed in section 6.2.4.6

6.2.4.5 Effect on mature biofilm

The figure below provides an overview of the experiment.

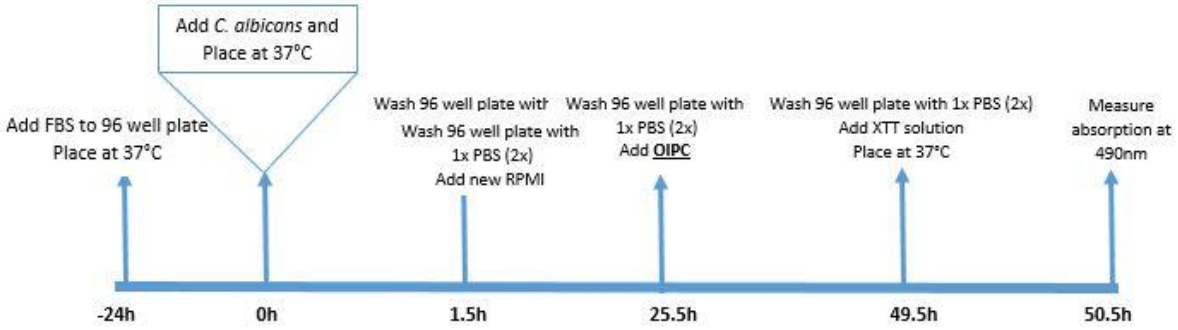


Figure 16: ***Timeline of the effect of OIPC on C. albicans mature biofilm to 96 well plates***

After common steps (section 6.2.4.2), FBS coated wells were supplemented with *C. albicans* suspension followed by period of adhesion (90 min, 37°C). Next, 1x PBS was used to wash the wells twice. Subsequently, 200 µL of clean, pre-warmed RPMI-MOPS, pH 7.0 solution was added to each well followed by biofilm development (24 h, 37°C). The next day, The RPMI-MOPS, pH 7.0 solution was removed and each well was washed twice with 200 µL 1x PBS. Controls had only 200 µL clean pre-warmed RPMI-MOPS, pH 7.0.added to their wells. The other wells were submerged in 20 µL of different OIPC concentrations (0.5 µg/mL - 1 µg/mL - 2 µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL), 80 µL RPMI-MOPS, pH 7.0 solution and 100 µL of the *C. albicans* suspension. Finally, the wells were incubated for another 24 h at 37°C. *C. albicans* biofilms were quantified the next day by measuring the metabolic activity as described in section 6.2.4.6.

6.2.4.6 Measurement of metabolic activity

100 µL of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) solution (1 mg/mL) supplemented with 1 µM menadione was added after the final wash step. This solution was made in the absence of light and was used to quantify metabolic activity of the *C. albicans* cells. The 96 well plates were then placed back in the 37°C incubator for 1-2h. Finally, the colometric changes were measured with the SPECTRAmax plus 384 (Molecular Devices) at 490 nm.

6.2.5 Bioluminescence imaging (BLI) assay

6.2.5.1 Concept

In bioluminescence light is produced from chemical energy by using enzymes. These enzymes are called luciferases, the substrates luciferins. We used the pLUC59 strain (see 5.3) were *Gaussia princeps* luciferase is used which is coupled to a membrane protein. The substrate is coelenterazine which is converted by the luciferase to coelenteramide which is bioluminescent and can be measured. After addition of the substrate a short flash of high intensity can be measured, these are the so called 'flash kinetics'.

6.2.5.2 Protocol

This experiment was performed to quantify the effect of OIPC on the different stages of *C. albicans* biofilm formation in a different way than CFUs. Here, the *C. albicans* cells are quantified by measuring bioluminescence (P/s). For every stage (adhesion - biofilm development - mature biofilm) of biofilm formation, the protocol was the same as in section 6.2.3 until the last wash step. Instead of washing the catheters with 1x PBS, the catheters were dipped twice in 1x PBS. Therefore, *C. albicans* cells also loosely adhered to FBS-coated catheters are quantified. Another difference with the protocol of 6.2.3 is the use of other catheters (Certofix Trio S730 BBraun). These catheters do not interfere with the BLI as the polyurethane catheters do.

After dipping the catheters in 1x PBS the C. albicans cells had to be quantified. This was done by first preparing a solution of the luciferase substrate (coelenterazine). Water-soluble coelenterazine (Nanolight) was dissolved in water to prepare a stock solution (500 µg/mL). This stock solution was diluted (1:1000) in 1x PBS. BLI measurements were done in the MoSAIC facility of the KU Leuven. Here, 500 µL coelenterazine was added to the catheters in the new 24 well plate. Next, the 24 well plates were placed in the IVIS 100 system (Caliper LS, Almeda, USA) and the plates were imaged in a 20 cm field of view (FOV). The exposure time (5-15 seconds) was variable to avoid saturation of the detector. The images were analysed using the Living Image Software (Xenogen, CaliperLS). Only the signal retrieved from the catheters called Region of interest (ROIs) was used to calculate bioluminescence.

6.2.6 Confocal laser scanning microscopy: biofilm development

The effect of OIPC on *C. albicans* biofilm development on FBS coated catheters was visualised using confocal microscopy. This allowed us to look inside the biofilm structure. Biofilm development was used because here the effect of OIPC was the most pronounced. FBS-coated catheters were prepared as in section 6.2.3.3 until the last wash step. In this experiment, the catheters were washed by gently dipping them in 1 mL of 1x PBS solution. Subsequently, the catheters were cut with sterile scissors and were transferred to a 24 well

plate containing 0.5 mL 1x PBS. Two dyes were added: Concavalin A conjugated to Alexa fluor 488 (Invitrogen) and Texas Red® Conjugated to concavalin A (Invitrogen). Concavalin A is a lectin which binds specifically to α-D-mannosyl and α-D-glucosyl groups. Alexa fluor 488 stains green, texas red stains red. Therefore, mannosylated and glycosylated proteins in the cell wall were stained. 5 µL of the stock solution of each dye was added to each catheter (in the dark to avoid photobleaching) followed by staining (15 min, 37°C, 100 rpm).

6.2.7 Scanning electron microscopy (SEM): biofilm development

Another visualisation technique was used to visualize the effect of OIPC on biofilm development on FBS-coated catheters. Biofilm development was used because here the effect of OIPC was the most pronounced. This time SEM was used to visualize the surface of the C. albicans biofilm. Again, FBS-coated catheter were prepared as in section 6.2.6.2 until the last wash step. Subsequently, the catheters were washed by gently dipping them in 1 mL of sterile 1x PBS. Next, the catheters were cut with sterile scissors and were transferred to an empty 24 well plate and were left to dry. Finally, the samples were coated using Platina (Pt) to improve imaging of the samples.

6.3 *Ex vivo* experiments

6.3.1 Interference assay

Because antibiotics will be used to sterilise the tongues in the next experiment, an exploratory experiment was performed to assess the effect of antibiotics (penicillin-streptomycin) on OIPC efficacy and *C. albicans* biofilm formation. This was done with an XTT assay biofilm development phase similar to section 6.2.4.4. Biofilm development was used because here the effect of OIPC was the most pronounced on catheters. Therefore the effect of OIPC on mucosal surfaces (see next section) will also be on biofilm development. In this experiment RPMI-MOPS, pH 7.0 + penicillin-streptomycin solution was used instead of RPMI-MOPS, pH 7.0 for biofilm development. Tow conditions were tested: RPMI-MOPS, pH 7.0 + penicillin-streptomycin alone and one concentration of OIPC (8 µg/mL). We also included two internal controls: RPMI-MOPS, pH 7.0 alone and 8 µg/mL with just RPMI-MOPS, pH 7.0.

6.3.2 Efficacy of OIPC on *C. albicans* biofilms formed on mucosal surface

The experimental timeline indicates all subsequent events of the experiment.

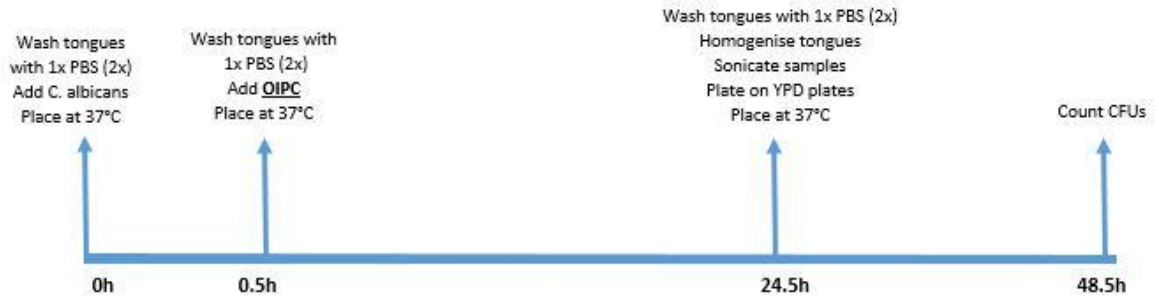


Figure 17: ***Timeline of the effect of OIPC on C. albicans biofilm formation on tongues.***

To test the efficacy of OIPC on mucosal surfaces mouse tongues were used. This was tested *ex vivo*. Mucosal surfaces are a completely different surface compared to abiotic surfaces. This murine infection model was adapted from Peters *et al*. 2010. For this experiment fresh mouse tongues were cut and stored in the fridge in 1 mL of RPMI-MOPS, PH 7.0 + penicillin-streptomycin solution. These antibiotics are necessary to remove potential bacterial contaminations. Next, the tongues were dipped in 1 mL of 1x PBS twice and the weight of each tongue was determined.

A fresh plate of *C. albicans* was prepared. The following day, *C. albicans* cells were scraped off the plate and dissolved in 1 mL 1x PBS. The OD_{600nm} was determined of the *Candida* suspension and the suspension was diluted to OD_{600nm} 1 in 1x PBS. Tongues were transferred to a 24 well plate and supplemented with 1 mL of the *C. albicans* suspension (as prepared above). This was followed by period of adhesion (30 min, 37 °C, 100 rpm). Here, only 30 min are used for period of adhesion because *C. albicans* adheres better to mucosal surfaces compared to an abiotic surface. Subsequently, the tongues were dipped twice in 1x PBS to remove non-adhered cells. Next, the tongues were transferred to a fresh 24 well plate. 1 mL and 900 µL of RPMI-MOPS, pH 7.0 + penicillin-streptomycin solution was added to the control and other tongues, respectively. 100 µL of both OIPC (13 µg/mL) and Histatin 5 (100 µg/mL; Sigma Aldrich) were added to the appropriate tongues. Histatin 5 is part of human salivary and is fungicidal. This is used as an internal control. The plate was placed back in the stationary 37°C incubator for another 24 h to allow biofilm development.

The next day, two wash steps were performed with 1x PBS. Next, the tongues were transferred to cryovials on ice and 1 mL of 1x PBS was added. Finally, the tongues were homogenised. In between, the homogeniser was sterilised with 100% ethanol and flushed with sterile milli-Q water. Following homogenisation, similar as in section 6.2.3.5 the CFUs were determined.

Another version of the model was also used. Here, period of adhesion (30 min, 37°C, 100 rpm) was performed in RPMI-MOPS, pH 7.0 + penicillin-streptomycin instead of 1x PBS as in the

model mentioned above. In this model, RPMI-MOPS, pH 7.0 was also from a different brand (Gibco). The tongues were also dipped in 1x PBS three times (compared to twice in the model mentioned above) and biofilm development was done in the shaking incubator (24 h, 37°C, 100 rpm). Finally, homogenisation was done for a longer time period to allow better homogenisation of the samples. No internal control was included in this model because not enough tongues were left. The effect of OIPC was also visualised on these tongues by using confocal microscopy. This was done similar to catheters (see section 6.2.6) but only Concavalin A conjugated to Alexa fluor 488 (Invitrogen) was used.

6.4 *In vivo* experiments

6.4.1 Subcutaneous mouse model

For an overview of the subcutaneous mouse model see timeline below.

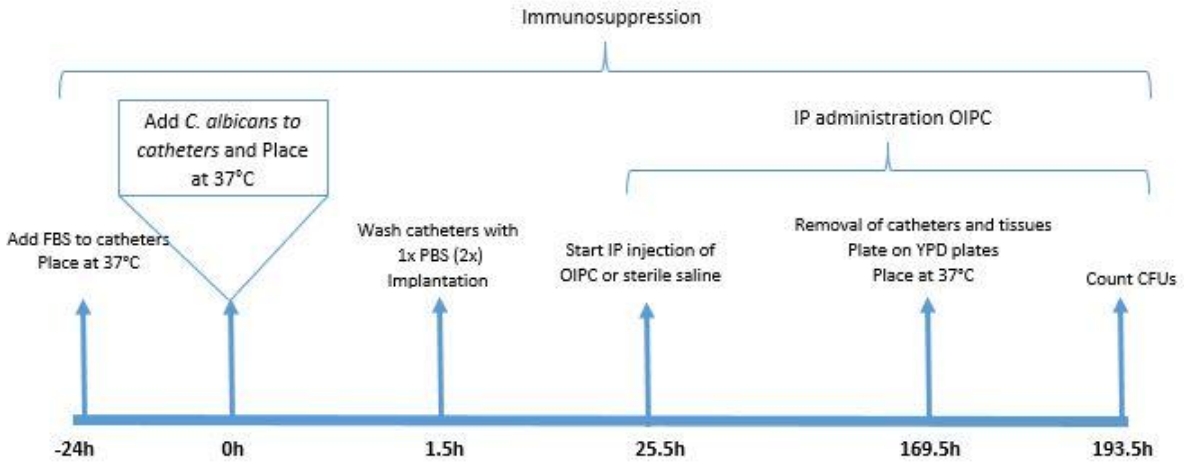


Figure 18: ***Timeline of the subcutaneous mouse model***

All animal experiments were approved by the bioethics committee of the KU Leuven and were in compliance with European and national regulations. The subcutaneous mouse model was adapted from the subcutaneous rat model developed by Řičicová *et al*. 2009. For this experiment female Balb/C mice were used. They were kept in filter top cages, with 3 mice per cage. The mice were immunosuppressed by adding dexamethasone (10 mg/mL) to their drinking water. Concomitantly, ampicillin (0.5 mg/mL) was also added to their drinking water to avoid bacterial infections. Both ampicillin and dexamethasone were supplemented to their drinking water for the entire experiment.

Infected catheters for implantation were prepared similar as the controls in section 6.2.3.2. however, only three catheters were used to determine CFUs of the FBS catheters at time point of implantation. The other catheters were stored on ice until implantation. Next the mice were anaesthetised by injecting 60-80 µL per 10 g bodyweight IP of a mixture of ketamine (Ketamine1000) and medetomidine (domitor). This mixture was prepared by mixing 75 µL of

ketamine (100 mg/mL) with 100 µL metetomidine (1 mg/mL) and 825 sterile saline. Before injection, the mice were sedated with isoflurane in the induction chamber to allow the injection. Right before the surgery terramycin/ polymyxin-b ophthalmic ointment (Pfizer) was applied on the eyes of the mice to prevent them from drying/infecting.

Next, the backs of the mice were shaved, disinfected with chlorohexidini alcoholicus 0.5% (Cedium) and a small incision was made with sharp scissors (approximately 1 cm above the tail). With blunt scissors, a wide cavity was created to place 5 catheters next to each other. Sterile tweezers were used to place the catheters in the back of the mice.

The wound was closed with 3 staples and linisol 2% (Braun) drops were applied to the wound to anaesthetize the animal locally at the wound. Finally, 300 µL of an atipamezole (antisedan) solution was injected IP to reverse the anaesthesia. The atipamezole solution was prepared by adding 50 µL atipamezole (5 mg/mL) to 4.95 mL sterile saline. Remark that the mice were kept on a heatpad during surgery to avoid hyperthermia and that the surgical tools were disinfected with 100% ethanol.

Starting day 2 of the experiment, the mice were injected IP with either sterile saline (control group), 10 mg/kg/day, 20 mg/kg/day, 40 mg/kg/day of OIPC depending on the group for the following 6 days. Each group consisted of 6 mice with 5 catheters each. The OIPC solution was prepared in dimethylsulfoxide (DMSO). At the end of the experiment, the mice were sedated with isoflurane in the induction chamber and were then sacrificed with cervical dislocation. Both the tissues surrounding the catheters and the catheters themselves were removed. The tissues were then weighted, placed in 1x PBS, homogenised and plated to determine the CFUs as was done in section 6.3.2 for the tongues. The catheters were also washed twice with 1x PBS and CFUs were determined as in section 6.2.3.5.

6.5 Statistical analysis

Statistical analyses was done by using the Prism software (Graphpad). The data were analysed with Mann whitney U test. In this test, the data are assumed to be unpaired because mean and standard deviation of each condition (concentration) are determined, therefore each dataset is assumed as separate from each other. It is a non-parametric test of the null hypothesis in which the null hypothesis states that two samples come from the same population. This test can only be used to compare 2 groups at the time. In our work we always compare control (0 µg/mL OIPC) with a given concentration of OIPC. When $p \leq 0.05$ we can assume that the null hypothesis is rejected with 95% accuracy.

Results

The majority of the experiments were performed with a wild type strain *C. albicans* SC5314 and some additional experiments were performed with clinical isolate *C. albicans* 4.19. Experiments including clinical isolates from Prof. K. Lagrou (UZ Gasthuisberg, KU Leuven, Belgium) and from Prof. D. Perlin (New Jersey Medical School, USA) are summarized at the end of the results chapter.

7 *In vitro* experiments

7.1 Minimum inhibitory concentration (MIC)

Clinical isolate 4.19 and wild type (SC5314) were grown in 96 well plates together with different concentrations of OIPC (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL). After 24 h, the MIC_{50} was read visually. This is the concentration of OIPC at which 50% of *C. albicans* growth is inhibited. We observed the MIC_{50} of 2 µg/mL for both the wild type (SC5314) and clinical isolate 4.19.

7.2 Minimal fungicidal concentration (MFC)

The content of the 96 well plates was plated on YPD plates in order to determine the minimum fungicidal concentration (MFC). The MFC represents the lowest concentration of the drug where no growth is detected. Similar to MICs we also determined the MFCs of 2 µg/mL for both wild type (SC5314) and clinical isolate 4.19.

7.3 *C. albicans* biofilm formation on catheters without OIPC

Prior to any biofilm susceptibility testing, we first characterized the ability of *C. albicans* SC5314 to adhere and to form biofilms on FBS-coated polyurethane catheters. Adhesion period was determined after 90 min, whereas biofilm formation was characterized after 24 h. The amount of adhered and biofilm-forming cells retrieved from different catheters is displayed in figure 19.

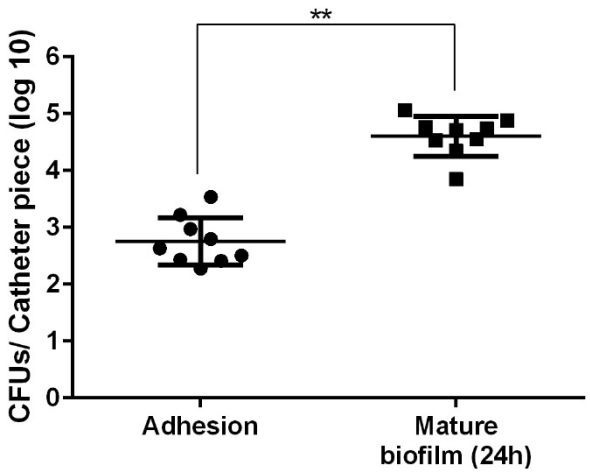


Figure 19: ***C. albicans adhesion and biofilm formation.*** *Each dot represents an individual CFU value retrieved from catheters after the period of adhesion (90 min) and after the biofilm development (24 h). Horizontal line represents average of attached and biofilm-forming cells per catheter piece. A statistical difference between the period of adhesion and the biofilm development was observed (**p<0.05). The figure shows the results of two independent experiments*

As it can be seen in figure 19, *Candida* cells colonized devices corresponding to an average of 2.75 ± 0.42 Log_{10} cells attached per catheter. After 24 h (biofilm development) the amount of biofilm forming cells significantly increased (4.60 ± 0.35 Log_{10} cells/catheter piece) in comparison with the adhesion period (p<0.05). These observations indicate that polyurethane catheters were favourable substrates for *C. albicans* adhesion and further biofilm development.

7.4 Efficacy of OIPC on different stages of *C. albicans* biofilm developed on polyurethane catheters

7.4.1 Effect of OIPC on adhesion

In order to analyse the effect of OIPC on different stages of biofilm development, we first assessed the efficiency of this drug on the first stage of biofilm formation – adhesion. *C. albicans* cells were allowed to adhere to serum-coated catheters during the period of adhesion (37 °C, 90 min). At the same time they were treated with the different concentrations of OIPC (2µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL). Non-OIPC treated *Candida* cells were considered as control samples. Additionally, anidulafungin (AND, 4 µg/mL) was used as a reference drug. The effect of OIPC and anidulafungin on *C. albicans* adhesion is displayed in figure 20.

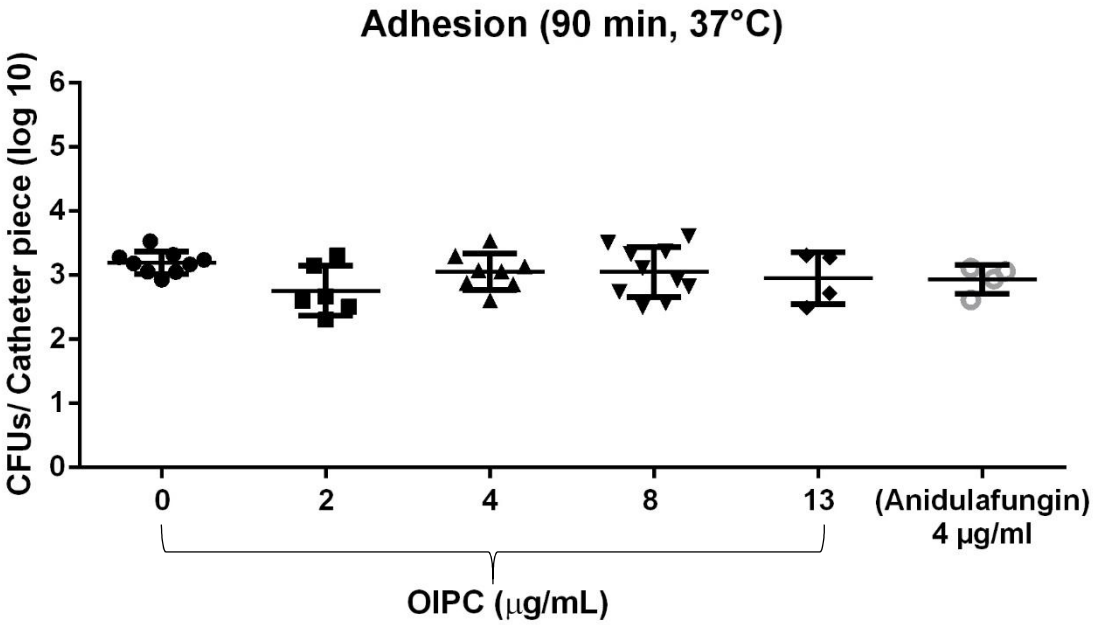


Figure 20: ***The effect of OIPC and anidulafungin on C. albicans adhesion on polyurethane catheters.*** *The amount of Candida CFUs retrieved from catheters treated with the different concentrations of OIPC (*2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL*) and anidulafungin (*4 µg/mL*) during the period of adhesion (90 min, 37 °C). Candida cells incubated in the drug-free medium were considered as controls. Anidulafungin was added as a positive control at a concentration of 4 µg/mL. Horizontal line represents average of attached cells per catheter piece. These experiments were repeated four times independently always using 2 catheters per condition.*

Figure 20 displays no effect of OIPC and also anidulafungin any effect on *C. albicans* adhesion ($p>0.05$).

7.4.2 Effect of OIPC on biofilm development

As it was demonstrated above, OIPC did not display any effect on *C. albicans* adhesion. Therefore, we further investigated the effect of OIPC on the next stage of biofilm formation – biofilm development. *C. albicans* cells were allowed to adhere to serum-coated polyurethane catheters during the period of adhesion (90 min, 37°C) in RPMI-MOPS, pH 7.0. Non-adhered cells were removed by washing and subsequently *C. albicans* infected catheters were incubated in the presence of different OIPC concentrations (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL) during biofilm development (24 h, 37°C). Non-treated *Candida* cells were considered as control samples. Additionally, anidulafungin (4 µg/mL) was used as a reference drug. The effect of OIPC and anidulafungin on *C. albicans* biofilm development is displayed in figure 21. 13 µg/mL is the highest possible OIPC concentration, at higher concentrations OIPC can start acting as a soap. Therefore, we did not include any higher concentrations.

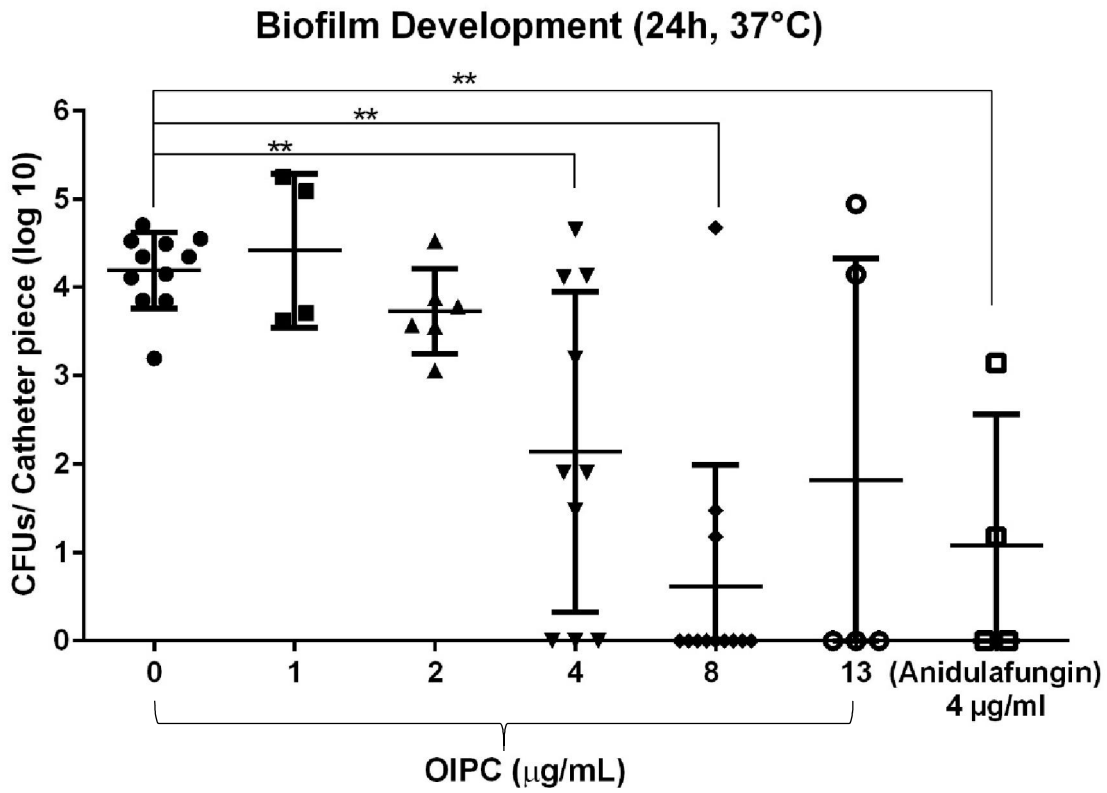


Figure 21: ***The effect of OIPC and anidulafungin on C. albicans biofilm formation on polyurethane catheters.*** *The amount of Candida CFUs retrieved from catheters treated with the different concentrations of OIPC (1 µg/mL* – 2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL*) and anidulafungin (*4 µg/mL) *during biofilm development (24 h, 37 °C). Candida cells incubated in the drug-free medium were considered as controls. Anidulafungin was added as a positive control at a concentration of 4 µg/mL. A statistical difference could be observed for two concentrations of OIPC (e.g. 4 µg/mL and 8 µg/mL) and for anidulafungin (**p≤0.05). Horizontal line represents average of attached cells per catheter piece. These experiments were repeated three times independently, except for OIPC 13 µg/mL and anidulafungin (4 µg/mL), which was performed only once.*

As it is demonstrated in figure 21 the amount of *Candida* CFUs retrieved from catheters treated with 4 µg/ml and 8 µg/ml was significantly lower in comparison to non-treated samples ($p \leq 0.05$). A considerable amount of completely sterile catheters (7 out of 10) was observed upon treatment with 8 µg/ml of OIPC. Surprisingly, when higher concentration of OIPC (13 µg/mL) was used we did not observe any significant difference in biofilm development. However, 2 out of 4 catheters were sterile. It is important to mention that experiment including 13 µg/mL was performed only once, therefore no major conclusion can be made. Additional experiments including 13 µg/mL will be performed in the near future. As it was expected, anidulafungin (4µg/mL) significantly reduced *C. albicans* biofilm development in comparison with the control ($p \leq 0.05$).

7.4.3 Effect on mature biofilm

Next, we were curious whether OIPC would have an effect on 24 h old (mature) biofilms. *C. albicans* adhesion, as well as 24 h old biofilm development were formed in drug-free medium. Mature biofilms (24 h old) were washed and submerged in the different concentratons of OIPC (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL). Catheters were incubated at 37 °C for 24 h. Anidulafungin (4 µg/mL) was used as a reference drug. The effect of OIPC and anidulafungin on mature *C. albicans* biofilm is displayed in figure 22.

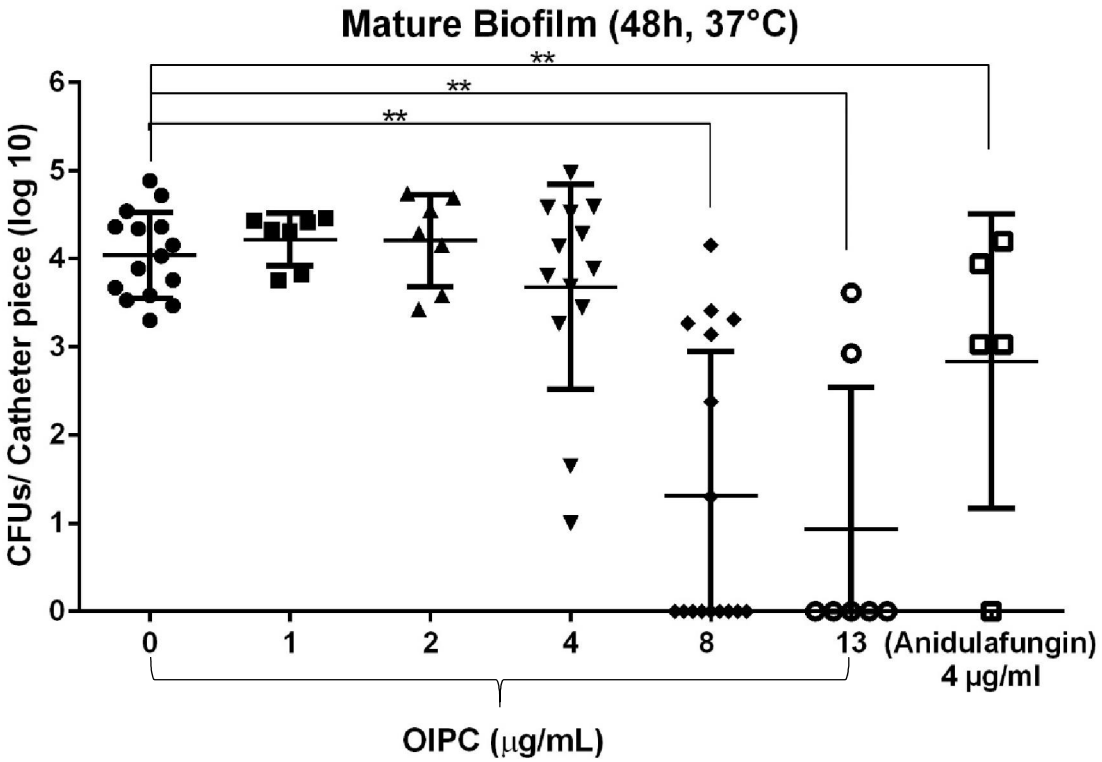


Figure 22: ***The effect of OIPC and anidulafungin on C. albicans mature biofilm on polyurethane catheters.*** *The amount of Candida CFUs retrieved from catheters treated with the different concentrations of OIPC (1 µg/mL* – 2 µg/mL – 4 µg/mL – 8 µg/mL – 13 µg/mL*) and anidulafungin (*4 µg/mL*) during mature biofilm (48 h, 37 °C). No OIPC was addd to the controls. Anidulafungin was added as a positive control at a concentration of 4 µg/mL. A statistical difference could be observed for both 8 µg/mL and 13 µg/mL of OIPC and for anidulafungin (**p≤0.05). Horizontal line represents average of attached cells per catheter piece. These experiments were repeated five times independently, except for OIPC 13 µg/mL and anidulafungin (4 µg/mL), which was performed twice.*

Figure 22 demonstrates the amount of *Candida* CFUs retrieved from catheters treated with OIPC. Both 8 µg/mL and 13 µg/mL had significantly lower CFUs in comparison with non-treated samples ($p \leq 0.05$). Similar to biofilm development, a considerable amount of completely sterile catheters (9 out of 16) was observed upon treatment with 8 µg/mL of OIPC. In contrast

to biofilm development, a higher concentration of OIPC (13 µg/mL) showed a significant difference for mature biofilm. Moreover, 5 out of 7 catheters were sterile. Although smaller a smaller reduction is observed compared to biofilm development, anidulafungin (4 µg/mL) again significantly reduced reduced *C. albicans* mature biofilm in comparison with the control ($p \leq 0.05$). Overall, these data indicate that the effect of OIPC on *C. albicans* biofilm formation on catheters is biofilm specific.

7.4.4 Bioluminescence imaging of *C. albicans* biofilms developed on polyurethane catheters treated with OIPC

In the following experiments we employed bioluminescence imaging (BLI) to quantify biofilms formed on polyurethane catheters. In comparison with other existing methods for biofilm quantification, such as CFUs count and XTT, herewith *Candida* cells produced light, which can be measured. Therefore, in these experiments we used a *C. albicans* bioluminescent strain, derived from a wild type *C. albicans* SC5314, called SKCA23-*gLUC*. This strain was engineered in our laboratory. Briefly, *C. albicans* SC5314 was transformed with Clp10::Act1p-gLUC59 plasmid containing a luciferase gene from *Gaussia princeps*. In this particular strain *gLUC* was fused to the endogenous *PGA59* gene (cell wall protein) under the control of *ACT1* (actin) promoter (Vande Velde *et al*., 2014). It important to mention that this promoter is constitutively expressed, therefore it is active in the yeast, as well as hyphal stage of fungal growth. Importantly, for the induction of reaction and production of light a substrate coelenterazine must be added to *Candida* cells.

C. albicans biofilm development and mature biofilms were formed on polyurethane catheters in the presence of different concentrations of OIPC (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL - 13 µg/mL). We studied also the effect of OIPC on adhesion, but 1 µg/mL and 2 µg/mL of OIPC were not tested. Additionally, anidulafungin (4 µg/mL and 8 µg/mL) was used as a reference drug. *Candida* cells incubated in RPMI-MOPS, pH. 7.0 medium were considered as controls. After addition of coelenterazine to adhered or biofilm forming cells, the photon flux (p/s) was measured. Regions of interest (ROIs) were selected to determine the photon flux of the attached and biofilm-associated cells on the catheters. An example of a 24 well plate containing catheters with selected ROIs is shown in figure 23.

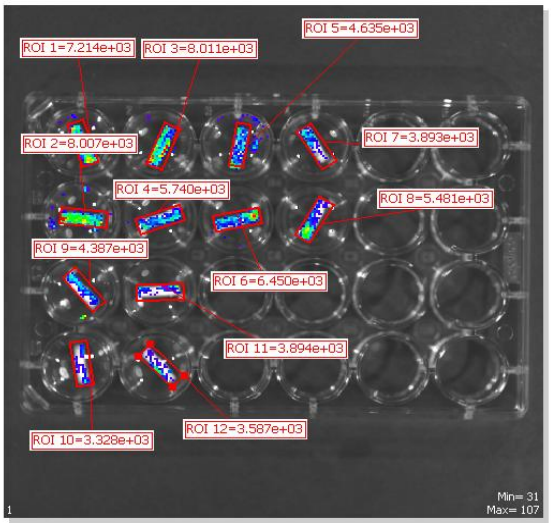
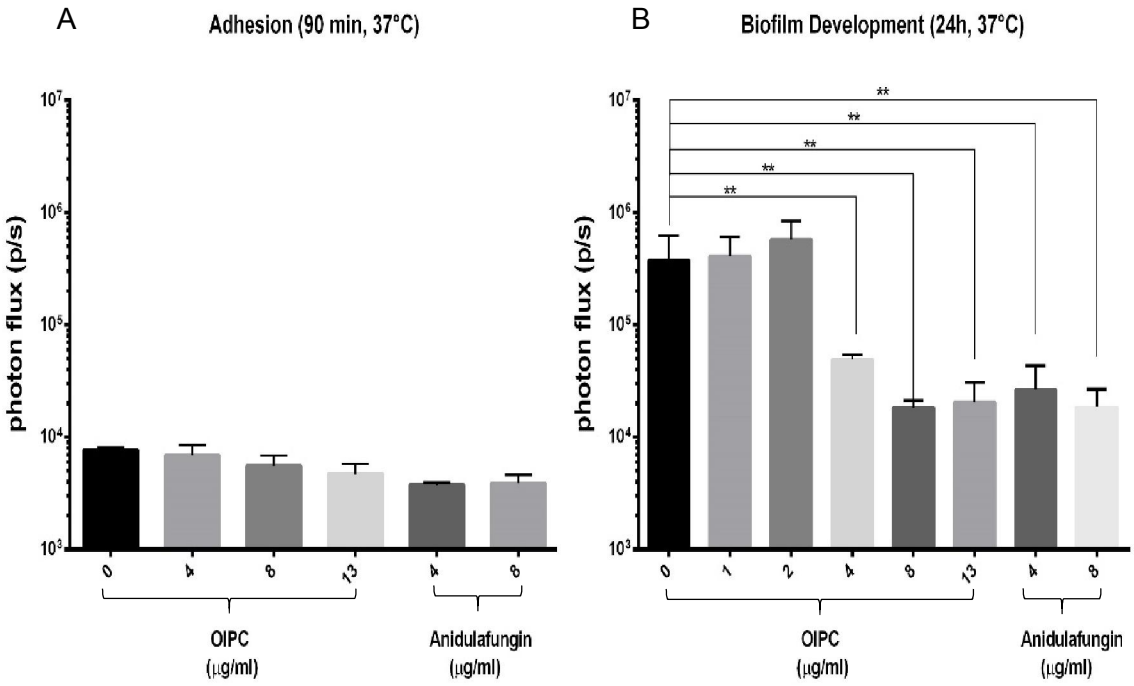


Figure 23: ***A 24 well plate showing selected catheters (ROIs) to quantify the effect of OIPC on period of adhesion (90 min, 37°C)****. The ROI values are indicated in the figure. All catheters show similar values. ROI 1 and 2 are controls, ROI 3 and 4 are 4 µg/mL OIPC, ROI 5 and 6 are 8 µg/mL OIPC, ROI 7 and 8 are 13 µg/mL OIPC, ROI 9 and 10 are 4 µg/mL AND and ROI 11 and 12 are 8 µg/mL AND.*

The results of the BLI experiment of the three different stages can be seen in the figure below.



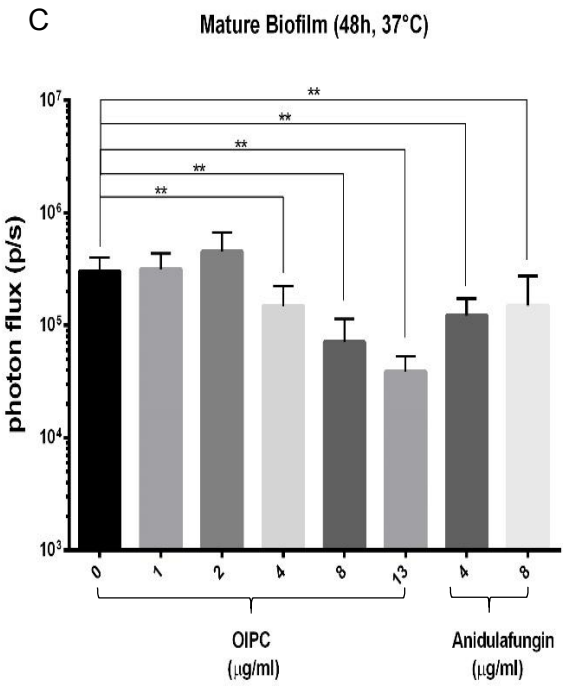


*Figure 24**: C. albicans wild type adhered and biofilm forming cells treated with OIPC were measured with bioluminescence (p/s).** Photon flux was measured to quantify bioluminescence of the attached and biofilm forming cells on the catheters. Different OIPC concentrations* (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL - 13 µg/mL) *were added to three different stages of biofilm formation. **A**: OIPC was added during the period of adhesion (90 min, 37°C). **B**: OIPC was added during biofilm development (24 h, 37°C). **C**: OIPC was added to mature biofilm (48 h, 37°C). Anidulafungin was included as a reference drug. This experiment was performed only once. Columns indicate the average per condition. Statistical significance when **p≤0.05.*

As can be seen on figure 24, A OIPC has no effect on period of adhesion (90 min, 37°C). For both biofilm development (B; 24 h, 37°C) a significant reduction in photon flux can be seen for 4 µg/mL, 8 µg/mL and 13 µg/mL and both anidulafungin concentrations (4 µg/mL and 8 µg/mL). For mature biofilm (C; 48h, 37°C) a lower reduction is observed but this is still significant for 4 µg/mL, 8 µg/mL and 13 µg/mL. This is also the case for anidulafungin were a reduction is observed for both concentrations (4 µg/mL and 8 µg/mL).

7.4.5 Confocal scanning laser microscopy (CSLM)

Confocal microscopy allows us to visualize three-dimensional *C. albicans* biofilm architecture. The efficiency of different concentrations of OIPC (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL) on *C. albicans* SC5314 biofilm development on polyurethane catheters (24 h, 37°C) was documented by CLSM. *C. albicans* substrate-associated cells were stained with concavalin A conjugated to Alexa fluor 488 and concavalin A conjugated to Texas Red. Concavalin A is a lectin, which binds mannose and glycosyl residues in the cell wall and extracellular matrix.

Alexa fluor 488 stains the cells green, whereas Texas Red stains red. Confocal images are displayed in figure 25.

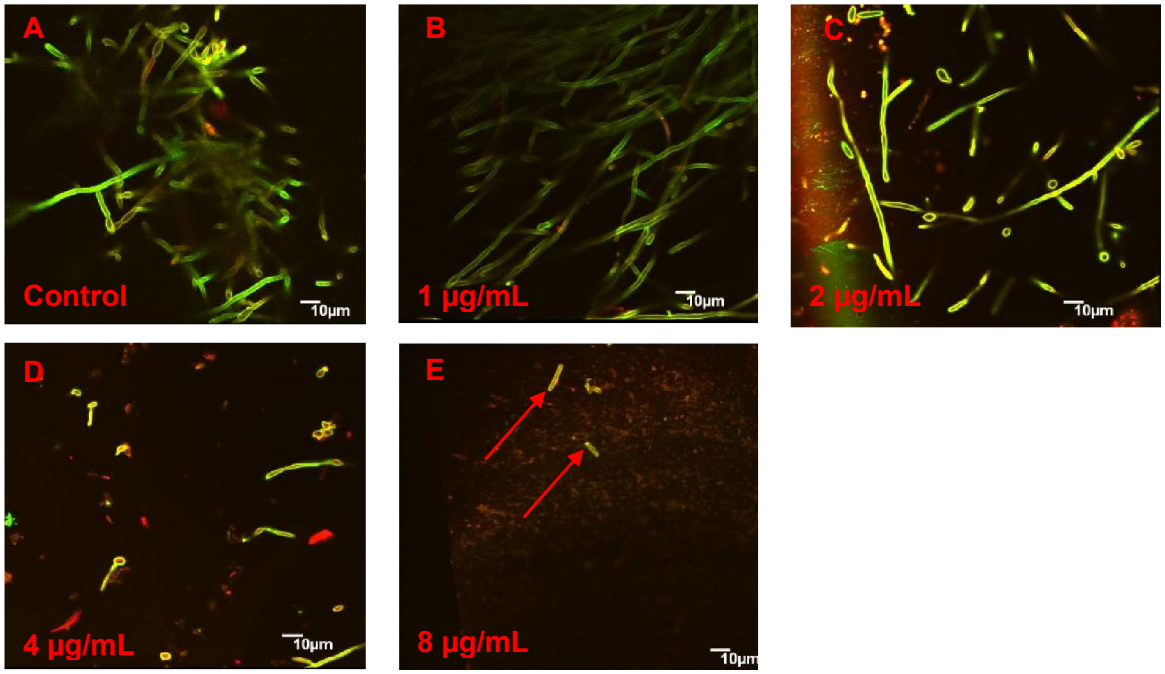
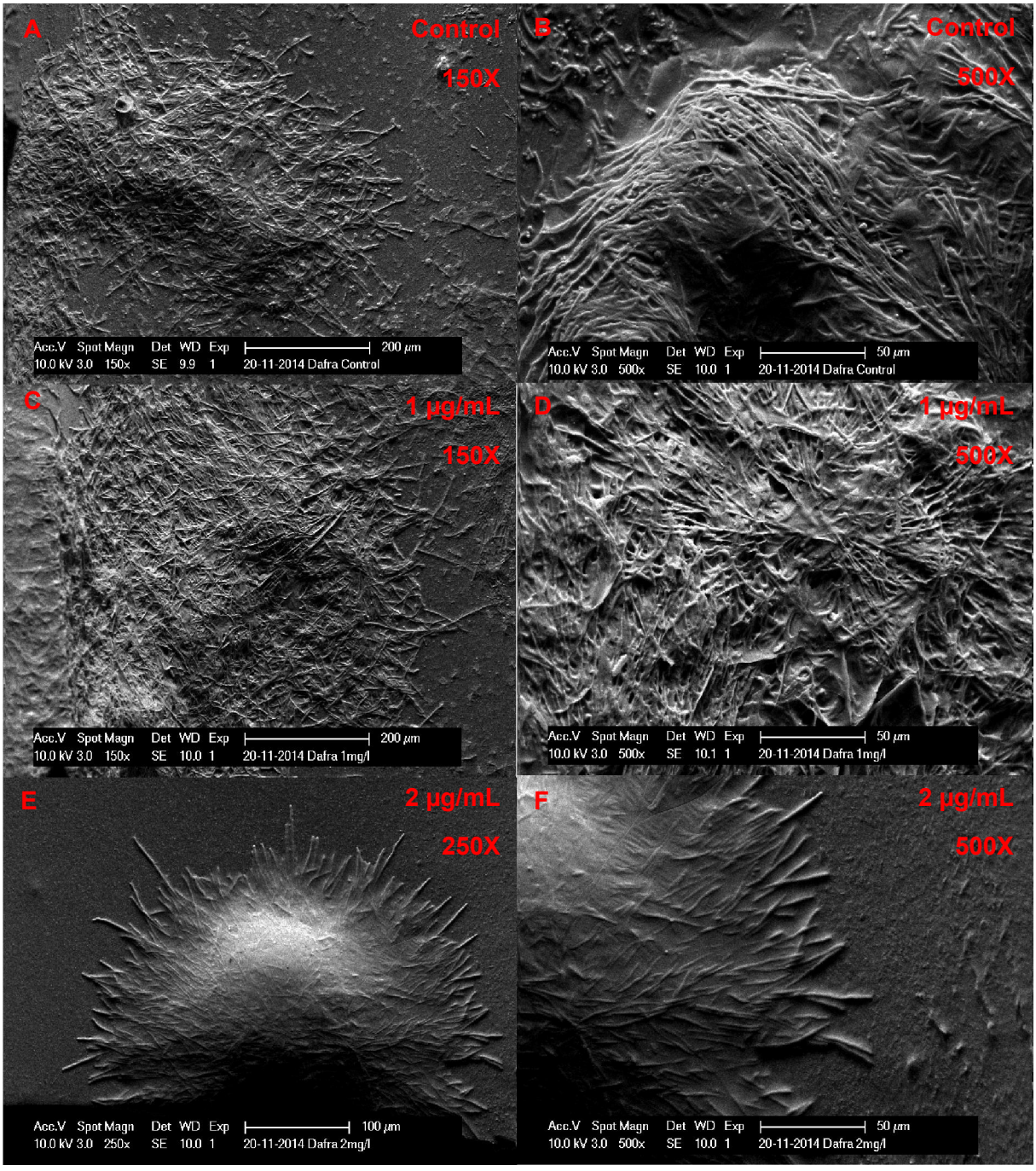


Figure 25: ***Concavalin A Alexia fluor 488 (green) and Concanavalin A conjugated to Texas Red (red) stained C. albicans wild type biofilms developed in the presence of different concentrations of OIPC****. **A**: Control (non-OIPC treated cells). **B**: 1 µg/mL OIPC. **C**: A reduction in biofilm can be observed for 2 µg/mL. **D**: 4 µg/mL, no biofilm structure could be observed. **E**: Only, a few remaining cells (indicated by the arrows) were left for 8 µg/mL.*

As it is shown in figure 25 A, B, non-OIPC treated cell and cells treated with 1 µg/mL exhibited thick biofilm architecture. At 2 µg/mL (c) cells did not form biofilms although quite high portion of attached cells could be still observed. At higher concentrations of OIPC (e.g. 4 µg/mL, D and 8 µg/mL, E) only a scattered amount of cells was documented. Moreover, both live and dead cells were stained so these cells might be dead. The C. albicans cells at these higher concentrations also tend to be smaller compared to control. These data support our findings from the CFUs quantification upon treatment with higher concentrations of OIPC.

7.4.6 Scanning electron microscopy (SEM)

We used SEM to visualise the surface of the *C. albicans* biofilms formed on polyurethane catheters in the presence of OIPC. This was done similar to CSLM where we used biofilm development (24 h, 37°C). Both control (no OIPC added) and different concentrations of OIPC (1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL) were visualised. Samples were Platina (Pt) coated to improve imaging of the sample. SEM pictures can be seen below.


A
Control
150X
Acc.V Spot Magn Det WD Exp 200 µm
10.0 kV 3.0 150x SE 9.9 1 20-11-2014 Dafra Control
B
Control
500X
Acc.V Spot Magn Det WD Exp 50 µm
10.0 kV 3.0 500x SE 10.0 1 20-11-2014 Dafra Control
C
1 µg/mL
150X
Acc.V Spot Magn Det WD Exp 200 µm
10.0 kV 3.0 150x SE 10.0 1 20-11-2014 Dafra 1mg/l
D
1 µg/mL
500X
Acc.V Spot Magn Det WD Exp 50 µm
10.0 kV 3.0 500x SE 10.1 1 20-11-2014 Dafra 1mg/l
E
2 µg/mL
250X
Acc.V Spot Magn Det WD Exp 100 µm
10.0 kV 3.0 250x SE 10.0 1 20-11-2014 Dafra 2mg/l
F
2 µg/mL
500X
Acc.V Spot Magn Det WD Exp 50 µm
10.0 kV 3.0 500x SE 10.0 1 20-11-2014 Dafra 2mg/l

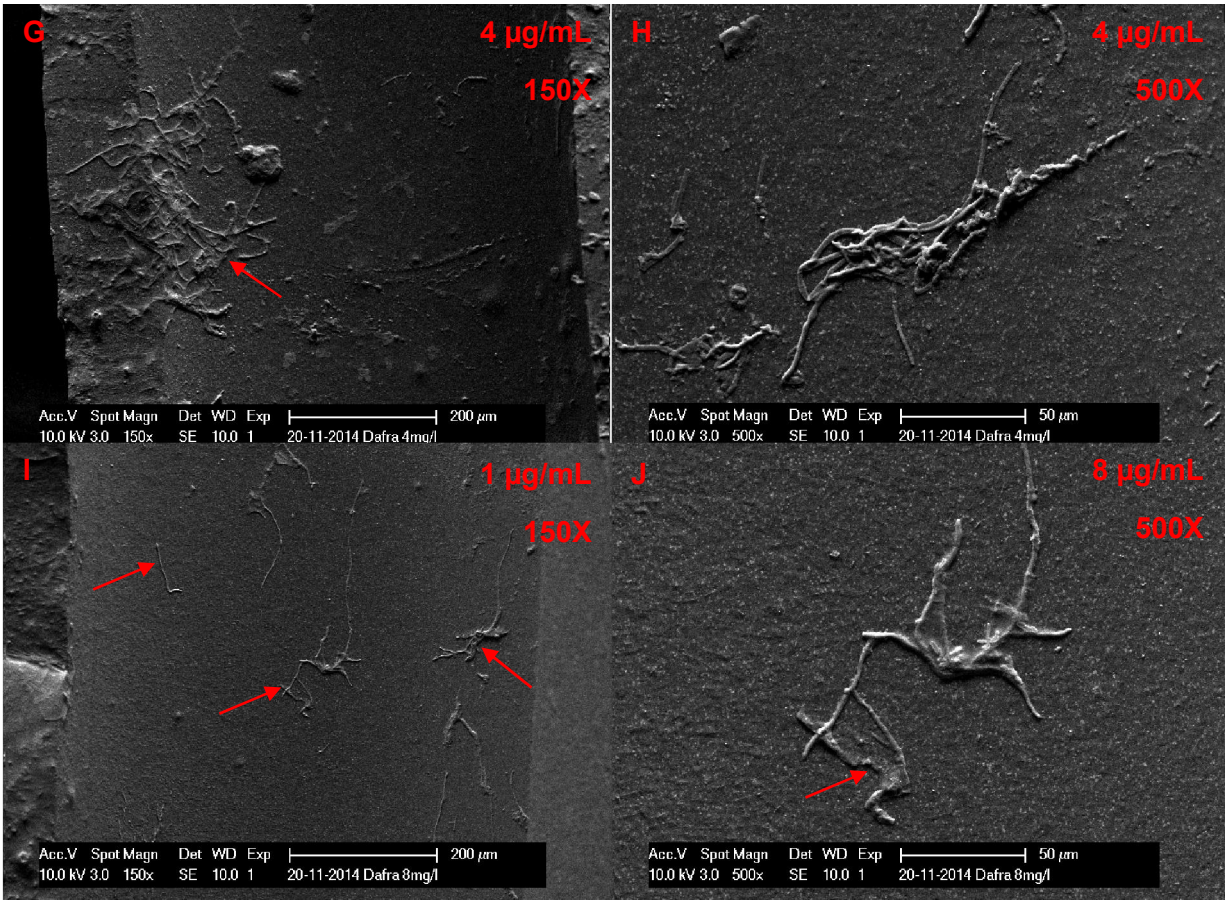


Figure 26: ***SEM pictures of Pt coated C. albicans wild type biofilm development stage (24 h, 37°C), incubated with different OIPC concentrations.*** *Different concentrations (1 µg/mL – 2 µg/mL – 4 µg/mL – 8 µg/mL) of OIPC were added to C. albicans infected polyurethane catheters during biofilm development (24 h, 37°C). Pictures on the left were 150X enlarged (except 2 µg/mL, 250X), pictures on the right were 500X enlarged.* ***A****: Control (150X). A thick biofilm can be observed.* ***B****: Control (500X). A close-up of the biofilm structure can be observed.* ***C****: 1 µg/mL (150X). A similar biofilm structure can be observed.* ***D****: 1 µg/mL (500X). Again a close-up of the biofilm structure.* ***E****: 2 µg/mL (250X). A smaller biofilm structure is observed.* ***F****: 2 µg/mL (500X). A close-up of the biofilm structure.* ***G****: 4 µg/mL (150X). No real biofilm structure could be observed. However, remaining hyphae with extracellular matrix can be observed (arrow).* ***H****: 4 µg/mL (500X). A few remaining hyphae are observed.* ***I****: 8 µg/mL (150X). Only, a few remaining cells were left (indicated by the arrows).* ***J****: 8 µg/mL (500X). Close-up of the hyphae cells, some appear to be dead (arrow).*

As can be seen in figure 26, similar results were obtained for SEM as for the confocal microscopy which also correspond to the CFU data. Again, thick biofilm structure is observed for control (A and B), 1 µg/mL (C and D) and 2 µg/mL (E and F). The biofilm structure formed in 2 µg/mL OIPC conditions is smaller and has increased extra cellular matrix. A strong reduction in cells could be observed for 4 µg/mL (G) and the cells are hypae (H). Only a few hyphae were observed for 8 µg/mL OIPC (I). Here, some hyphae appear to be dead (J).

7.5 The effect of OIPC on *C. albicans* adhesion, biofilm development and mature biofilms developed inside 96-well polystyrene plates.

7.5.1 Experimental set up with a WT strain *C. albicans* SC5314

Next, we assessed the effect of OIPC on the different stages of *C. albicans* biofilm formation on a different substrate, namely 96-well polystyrene plates. These plates are considered as favourable substrate used for a fast screening, for example, of novel compounds, which may be potent against biofilms. In comparison with polyurethane catheters, 96-well plates allow to study biofilm development on the bottom and on the sides of the wells. In these assays, planktonic cells are inoculated directly inside the wells (usual volume is between 100 µl – 200 µL) and further incubated at the desired temperature for a certain period of time. From the quantification point of view, we used XTT reduction assay, instead of CFUs quantification, to measure the metabolic activity of adhered and biofilm forming cells.

In this study, we used 96-well plates to explore the effect of the different concentrations of OIPC (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL) on adhesion, biofilm development and mature biofilms of *C. albicans* SC5314. *Candida* cells incubated only in RPMI-MOPS, pH 7.0 medium were considered as controls. In order to mimic the situation on serum-coated catheters, we pre-incubated the wells with serum at 37 °C, overnight. Subsequently, *Candida* cells were introduced to serum-coated wells and OIPC was added. The results demonstrating the metabolic activity of adhered and biofilm forming cells upon OIPC treatment effect on adhesion period, biofilm development and mature biofilms of a WT strain are shown in figure 27 A, B, C, respectively.

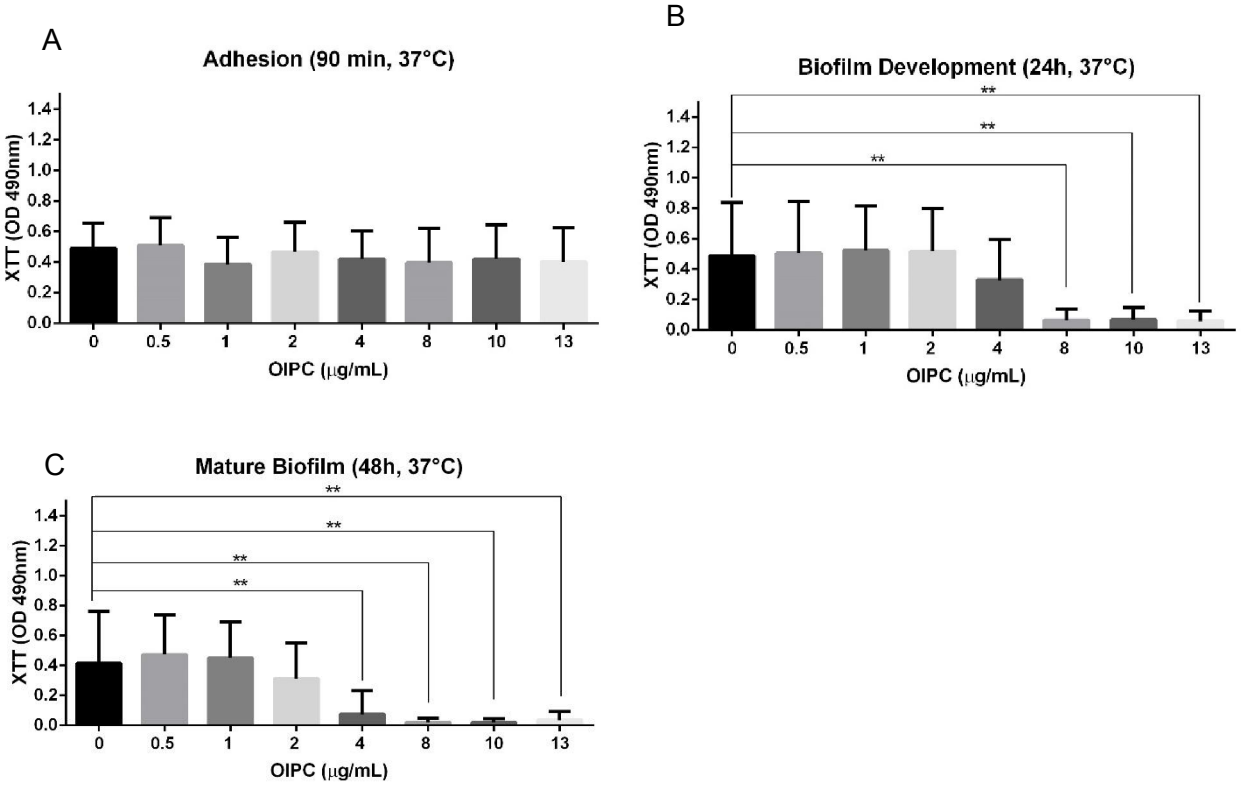


Figure 27: ***Metabolic activity of C. albicans SC5314 biofilm forming cells upon treatment with OIPC.*** *The colorimetric change of metabolically active adhered and biofilm-forming cells was measured by spectrophotometer at 490 nm. Different OIPC concentrations* (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL) *were added to three different stages of biofilm formation.* ***A****: OIPC was added during the period of adhesion (90 min, 37°C).* ***B****: OIPC was introduced after the period of adhesion for the next 24 h (biofilm development).* ***C****: OIPC was added to 24 h old mature biofilms for the next 24 h. Columns indicate the average of measurements obtained from three independent experiments. Statistical difference was considered when **p≤0.05.*

As it can be seen in figure 27 A, the different concentrations of OIPC did not display any effect on *C. albicans* SC5314 adhesion in comparison with non-treated cells. In contrast, 8 µg/ml, 10 µg/ml and 13 µg/ml of OIPC significantly reduced *C. albicans* SC314 biofilm development in comparison with the wells where no OIPC was added ($p \leq 0.05$; Figure 27 B). In order to demonstrate the survival of *Candida* cells surrounding the biofilms and to prove that OIPC may have a fungicidal effect on the cells associated with the substrate, the planktonic cells were plated on YPD plates prior to the washing steps. Strikingly, we did not observed any growth at 4 µg/mL, 8 µg/mL, 10 µg/mL and 13 µg/mL, indicating that OIPC completely abolished the ability of *C. albicans* to develop biofilms (data not shown). Next, we observed that 4 µg/ml, 8 µg/ml, 10 µg/ml and 13 µg/ml of OIPC almost completely abolished mature biofilms in comparison with the control ($p \leq 0.05$; Figure 27 C). Similarly to biofilm development, we also plated the planktonic cells surrounding the mature biofilms. Noteworthy, no *Candida* growth was observed at 4 µg/mL, 8 µg/mL, 10 µg/mL and 13 µg/mL ,suggesting that OIPC has a potential to combat *C. albicans* mature biofilms (data not shown).

7.5.2 Clinical isolate 4.19

Additionally, the same experiment was performed with the clinical isolate 4.19 (Katrien Lagrou, KU Leuven, Belgium). Again, the effect of different OIPC concentrations (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL) on adhesion, biofilm development and mature biofilm was investigated on serum pre-coated 96 well plates. The results of the effect of OIPC on the different stages of biofilm formation for clinical isolate 4.19 are shown in figure 28.

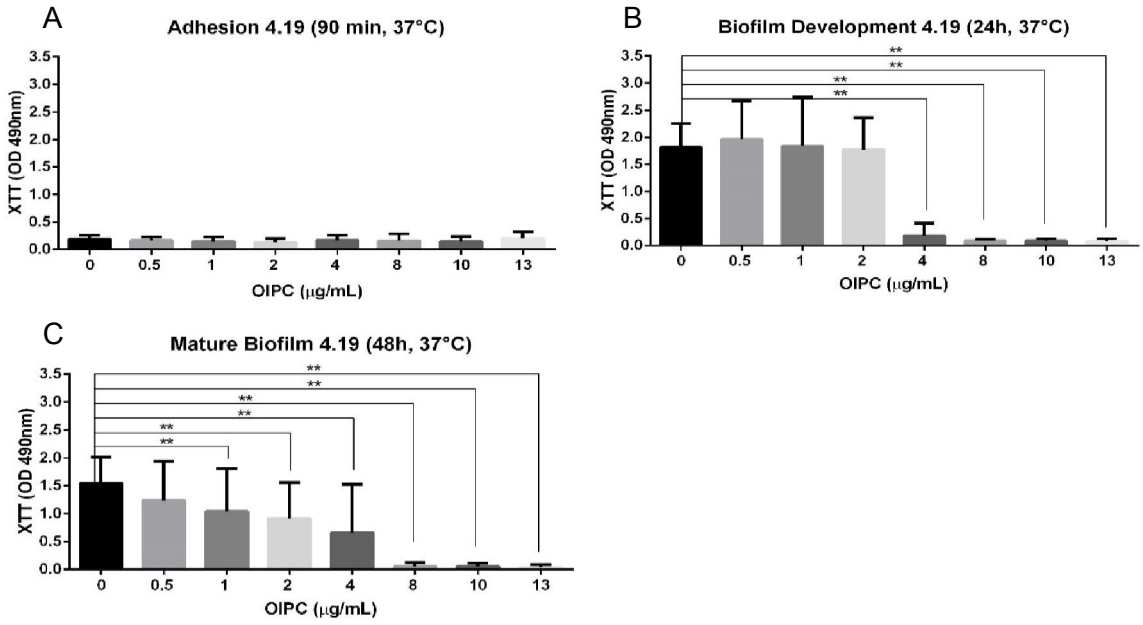


Figure 28: ***Metabolic activity of C. albicans clinical isolate 4.19 biofilm forming cells treated with OIPC.*** *Absorbance (490nm) was measures to quantify metabolic activity for C. albicans 4.19 in 96 well plates. Different OIPC concentrations* (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL) *were added to three different stages of biofilm formation.* ***A****: OIPC was added during period of adhesion (90 min, 37°C), no effect can be observed.* ***B****: OIPC was added during biofilm development (24 h, 37°C).* ***C****: OIPC was added to mature biofilm (48 h, 37°C). These experiments were repeated two times independently. Columns indicate the average per condition. Statistical difference is marked with ** (p≤0.05)*

Similar to the results obtained for wild type (SC5314), no effect was observed for period of adhesion (figure 28, A). 4.19 appears to be slightly more susceptible to OIPC for biofilm development (B) and mature biofilm (C). For biofilm development, a statistical significant difference ($p \leq 0.05$) compared to control was observed for 4 µg/mL, 8 µg/mL, 10 µg/mL and 13 µg/mL. For mature biofilm a dose-dependent effect could be observed starting from 1 µg/mL. Both 1 µg/mL, 2 µg/mL 4 µg/mL, 8 µg/mL, 10 µg/mL and 13 µg/mL show a statistical significant difference ($p \leq 0.05$) compared to control. These results are slightly different compared than wild type. Also, absorbance (490 nm) is higher compared to wild type. It is known that formazan production is strain dependent which can result in higher absorbance.

8 Efficiency of OIPC on the development of *C. albicans* biofilms on mucosal surface using an *ex vivo* oral model of infection

In the following step we explored the potential effect of OIPC on *C. albicans* biofilms developed on a mucosal surface using tongues extracted from sacrificed BALB/c mice. It is important to mention that during this assay we encountered bacterial contamination occurring on the tongues. Although tongues were obtained from pathogen-free BALB/c mice, the housing of

animals and the food are not pathogen-free, therefore it was expected that tongues were colonized. It has been previously shown that *Candida* may express different characteristics in the presence of several bacterial species (references from Mary Ann) and therefore, this fact could have an influence not only on the growth of *C. albicans* but also on the activity of the OIPC. Hence, we sought for a solution to eliminate the bacterial contamination. For this purpose, extracted tongues were stored in RPMI-MOPS, pH 7.0 supplemented with penicilin-streptomycin cocktail. Further, we wanted to use the medium supplemented with antibiotics to test the efficiency of OIPC on *C. albicans* biofilms developed on a mucosal surface, but we were not sure whether penicillin or streptomycin would interfere with *Candida* growth or with the activity of OIPC. Because of this reason, we first performed an exploratory experiment to analyse the effect of OIPC on *C. albicans* (SC5314) biofilm development on 96-well polystyrene plate in the presence of both antibiotics (ATB). Additionally, a control condition (no OIPC or ATB) and 8 µg/mL OIPC with regular RPMI-MOPS, pH 7.0 were included. In total, four different conditions were investigated (control; 8 µg/mL OIPC, ATB alone; 8 µg/mL + ATB). Biofilms were quantified with an XTT reduction assay (Figure 29).

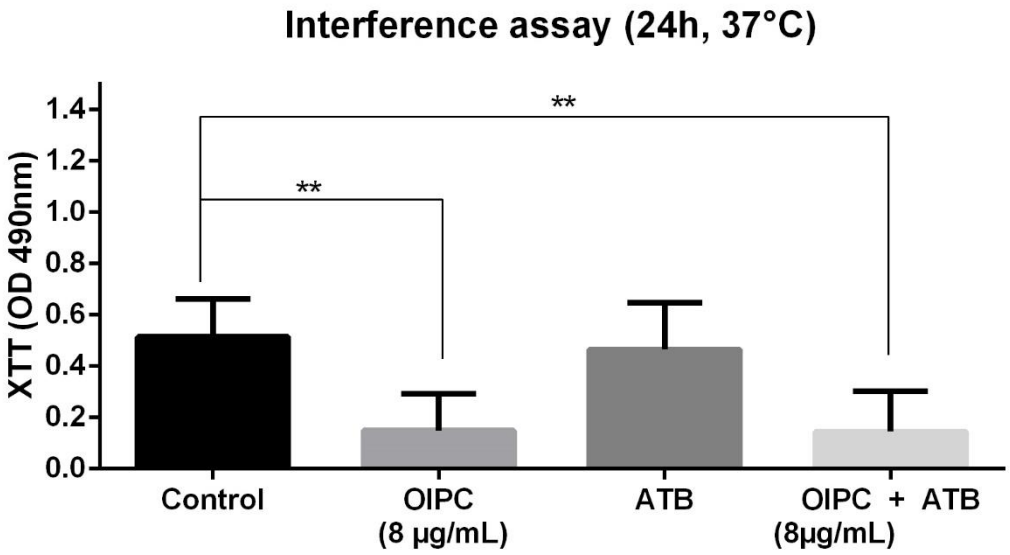


Figure 29: ***The effect of antibiotics and OIPC on biofilm C. albicans development (24 h, 37°C)****. C. albicans biofilm forming cells were quantified with XTT. Four different conditions were evaluated: Control with RPMI-MOPS, pH 7.0, 8 µg/mL of OIPC with RPMI MOPS solution, ATB: control with RPMI-MOPS, pH 7.0 + penicillin-streptomycin and 8 µg/mL of OIPC with RPMI-MOPS, pH 7.0 with penicillin-streptomycin. The difference between control and OIPC (8 µg/mL) alone or in the presence of antibiotics was statistically significant (**p≤0.05). This experiment was performed only once, using 6 wells/condition.*

As it is shown in figure 29, there was no effect of the antibiotics observed on C. albicans growth alone or on the efficiency of OIPC. Therefore, the RPMI-MOPS, pH 7.0 medium supplemented with antibiotics could be used for the experiment using tongues.

The *ex vivo* model of oral infection caused by *C. albicans* was adapted from Peters *et al*. (2010). In this experiment *Candida* cells prepared in 1 x PBS solution were allowed to adhere to tongues during the period of adhesion (30 min, 37°C). To determine the amount of adhered cells/tongue, 2 tongues were homogenised right after the adhesion phase. Afterwards, remaining tongues were washed 3 x with PBS and submerged in RPMI-MOPS, PH 7.0+ATB medium containing OIPC (13 µg/mL). Non-treated *Candida* infected tongues were considered as control samples. Tongues were incubated for the next 24 h at 37°C (biofilm development). Only OIPC was validated, this is the highest possible OIPC concentration, at higher concentrations OIPC can start acting as a soap. Additionally, histatin 5 was used as a positive control. Histatin 5 is a component of human saliva with known fungicidal activity (Puri & Edgerton 2014). Two tongues were supplemented with the medium mentioned above containing 100 µg/mL of histatin 5. This concentration is within physiological limits and it was tested before in this model (Peters et al. 2010). The effect of OIPC and Histatin 5 on *C. albicans* biofilm development on mouse tongues is displayed in figure 30.

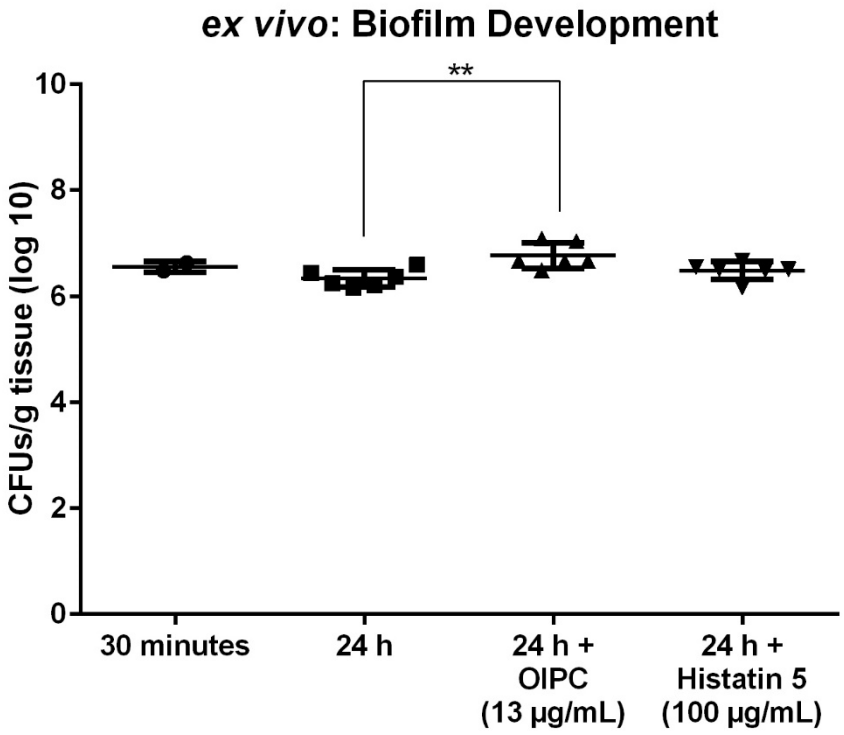


Figure 30: ***The effect of OIPC and Histatin 5 on C. albicans biofilm development on mouse tongues.*** *Four different conditions of the murine infection model are shown on the figure. No effect was observed between after 30 minutes compared to 24 h (control). A statistical significant difference could be observed for 24 h (control) and 13 µg/mL, with 13 µg/mL CFUs being higher (**p≤0.05). No effect was observed for Histatin 5 (100 µg/mL) and control. The data shown are the result of two independent experiments. Horizontal line represents average of attached and biofilm forming cells per g tissue.*

Figure 30 demonstrates that higher CFUs were obtained for 13 µg/mL (6.77 ± 0.24) compared to 24 h old biofilm (control; 6.33 ± 0.16). In contrast, similar CFUs were obtained after adhesion (30 min; 6.55 ± 0.10) compared to control. We would expect an increase in CFUs for 24 h old biofilm as can be seen for the catheters. Additionally, no effect between Histatin 5 (6.48 ± 0.17) and control was observed. Furthermore, we concluded that the model was not optimal to investigate the effect of OIPC efficacy on *C. albicans* biofilm development on mouse tongues.

Next, we performed additional experiment with a brief modification. *C. albicans* adhesion to tongues was performed in RPMI-MOPS, pH 7.0 medium instead of PBS. During the adhesion and also infection process the tongues were gently shaking (150 rpm), whereas in the set up mentioned above tongues were incubated under the static conditions. Similarly to the experiment above, *Candida* cells were treated with OIPC (13 µg/mL) during the biofilm development (24 h at 37°C).

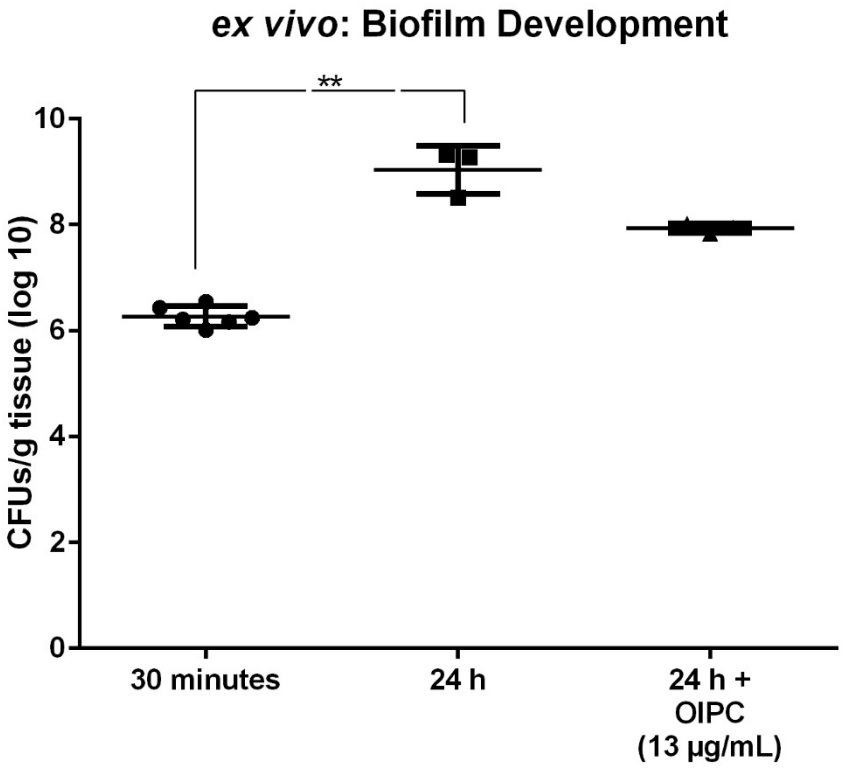


Figure 31: ***The effect of OIPC on C. albicans biofilm development on mouse tongues in an ex vivo murine infection model.*** *The amount of Candida cells retrieved after the period of adhesion (30 min, 37 °C) and after 24 h of biofilm development. Mouse tongues treated with OIPC (13 µg/mL) show a non-significant reduction in CFUs compared to non-treated (24 h). A statistical significant difference can be noticed when 30 minutes is compared to 24h (**p≤0.05). Horizontal lines represent an average of attached and biofilm forming cells per g tissue. These experiments were performed twice independently, always using at least 2 tongues/condition.*

Figure 31 shows that *C. albicans* cells attached quite rapidly to the tongue tissue (6.26 ± 0.19) and they proliferated during the period of biofilm development (9.03 ± 0.45). The difference between the amount of attached cells and biofilm forming cells was statistically significant ($p \leq 0.05$). Importantly, *Candida* biofilms developed on the tongues in the presence of OIPC (7.92 ± 0.09) were not significantly reduced in comparison with the non-treated tongues, although there is an obvious reduction in the amount of biofilm-forming cells

Additionally, after OIPC treatment, some tongues were visualised with CSLM. *Candida* cells remaining on the surface of the mucosal tissue were stained with concanavalin A conjugated to Alexa fluor 488 and the surface of the tongue was examined with confocal microscope (figure 32).

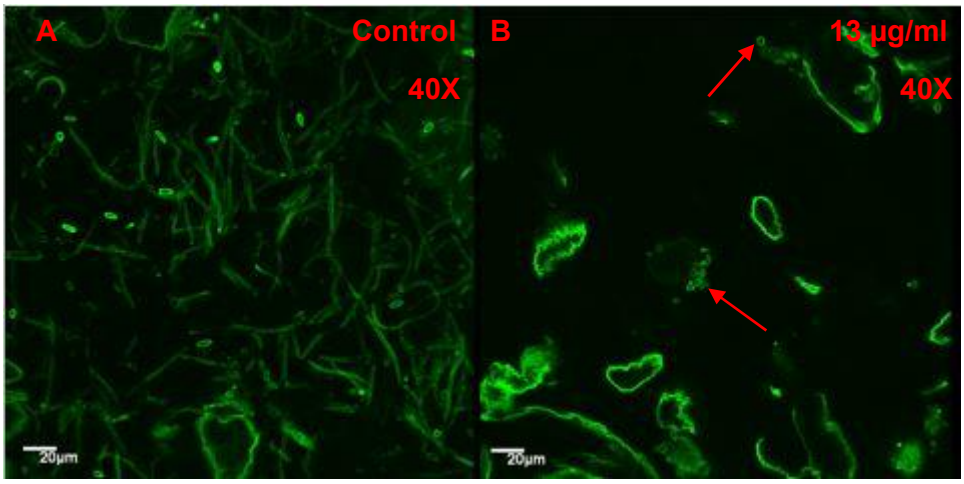


Figure 32: ***Concavalin A Alexia fluor 488 (green) stained C. albicans Adhered to mouse tongues.*** *Mouse tongues were incubated with C. albicans cells to allow biofilm development (24 h, 37°C). Two conditions were visualised: control and 13 µg/ml. the big green cells are tongue tissue cells* ***A****: Control. A thick biofilm with lots of hyphae can be observed.* ***B****: 13 µg/mL OIPC. Only some planktonic cells can be observed (indicated by red arrows). Magnification 40 x.*

As can be seen in figure 32 confocal images clearly displayed a strong reduction in the amount of *Candida* cells found on the surface of the tongues treated with OIPC. No hyphal cells were observed on the treated tongues.

9 *In vivo* experiments

9.1 Subcutaneous mouse model

All *in vitro* experiments demonstrated the potential of OIPC to treat or to prevent *C. albicans* biofilm development. Therefore, in our next experiments we examined its potential activity against *in vivo* mature *C. albicans* biofilms. A subcutaneous mouse model was used to investigate the effect of OIPC *in vivo*.. Serum-coated polyurethane catheters were challenged with *Candida* SC5314 cells during the period of adhesion (90 min, 37°C). After adhesion three catheters were washed and plated to determine the amount of attached cells/catheter piece. Additional catheters were implanted in the backs of immunosuppressed mice (5 catheters per mouse). After 24 h, six mice/group were injected daily for 7 days with the different concentrations of OIPC, namely 10 mg/kg of body weight/day, 20 mg/kg of body weight/day and 40 mg/kg of body weight/day. Mice injected with sterile saline were considered as controls. After 7 days of treatment mice were sacrificed by cervical dislocation. Catheters were removed, washed twice with 1 x PBS and plated on YPD plates. The amount of *Candida* cells retrieved from each individual catheter is presented in figure 33.

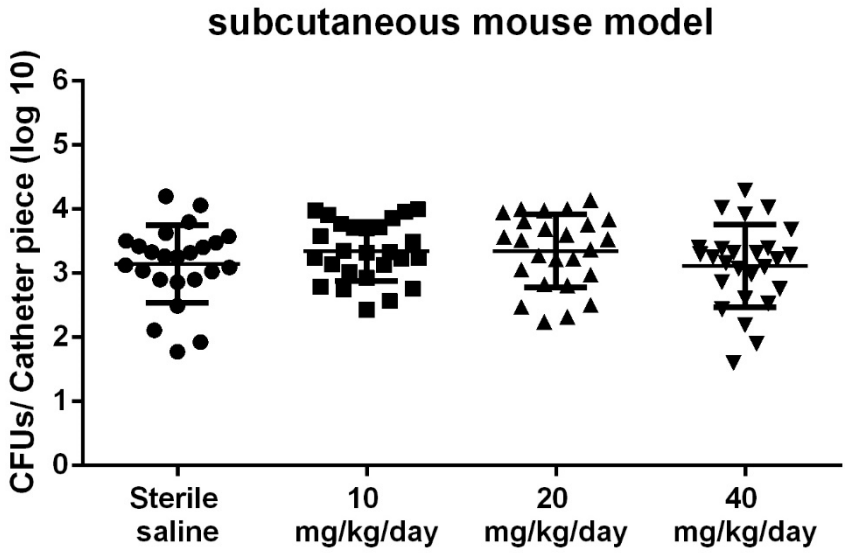


Figure 33: ***The efficacy of OIPC on C. albicans biofilms developed in a murine subcutaneous biofilm model.*** *Four different conditions of the subcutaneous mouse model are shown on the figure. Non-treated mice were injected with sterile saline. No effect can be observed between non treated mice and mice treated with 10 mg/kg/day, 20 mg/kg/day or 40 mg/kg/day.*

As it is shown in figure 33, OIPC at different concentrations tested did not reduce *C. albicans* biofilm formation. This model was originally adapted from Řičicová *et al*. (2010). The fungal load of the catheters after adhesion (before implantation) was also determined (data not shown). The average of CFUs/ catheter piece was 3.0 ± 0.14. This amount of cells was similar

to the number of *Candida* cells retrieved from sterile treated catheters (3.34 ± 0.46) after 7 days, indicating that *Candida* cells did not proliferate inside the mice.

The CFUs of the tissues surrounding the catheters were also plated to check the effect of OIPC on dissemination of *C. albicans* in surrounding tissues. The resulting CFUs are shown in the figure below.

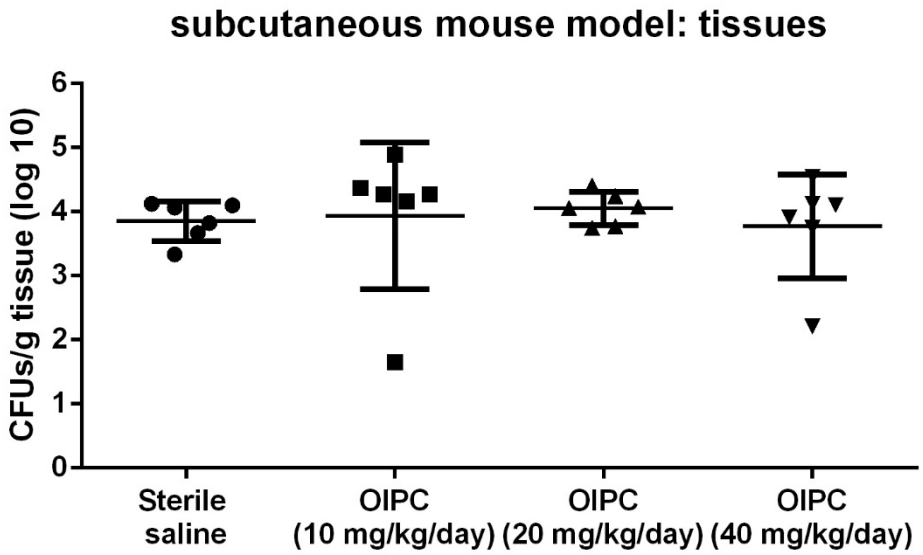


Figure 34: ***The efficacy of OIPC was analysed in an in vivo subcutaneous mouse model, CFUs of Tissues are shown****. Tissues surrounding the catheters were plated. No effect was observed for mice treated with OIPC compared to non-treated mice (sterile saline).*

As can be seen on figure 34 similar to the CFUs obtained from the catheters, no effect is observed for the tissues surrounding the catheters when treated mice are compared to non-treated mice.

10 The effect of OIPC on planktonic cells of *C. albicans* clinical isolates isolated from patients with urinary catheters and from hemoculture

10.1 Minimum inhibitory concentration (MIC)

Different clinical isolates derived from either patients with urinary catheters or from hemoculture were grown in 96 well plates together with different concentrations of OIPC (0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL). After 24 h, the MIC_{50} was read visually. This is the concentration of OIPC at which 50% of *C. albicans* growth is inhibited. The strains starting with U are strains isolated from people with urinary catheters. The strains starting with HC are hemocultures derived strains. Additionally, clinical isolate 4.19 was also tested.

*Table x:**Different MIC_{50} values for C. albicans clinical isolates.** Different clinical isolates were grown in the presence of different OIPC concentrations ((0.5 µg/mL - 1 µg/mL - 2µg/mL – 4 µg/mL – 8 µg/mL – 10µg/mL - 13 µg/mL). Some strains show reduced MIC values (U-0403-255/U-0203-29/U-0503-12/U-0203-15).*

Strain	HC2601-30	HC0202-25	HC2102-19	HC1602-5	HC0702-14	4.19
MIC_{50}	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL
Strain	U-0503-18	U-04030-255	U-0203-29	U-0503-12	U-0203-15	
MIC_{50}	2 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	

These strains have the same MIC_{50} as the wildtype. Other strains retrieved from patients with urinary catheters and have slightly reduced MICs (e.g. U-0403-255,U-0203-29,U-0503-12 and U-0203-15).

10.2 Minimum fungicidal concentration (MFC)

The content of the 96 well plate is also plated on YPD plates to determine the minimum fungicidal concentration (MFC). The minimum fungicidal concentration is the lowest concentration of drug were no growth was detected on the plate. The result are shown in the table below.

*Table x:**Different MFC values for C. albicans clinical isolates.** The content of the 96 well plate was plated to determine MFC values. Similar results were obtained as MIC_{50}.*

Strain	HC2601-30	HC0202-25	HC2102-19	HC1602-5	HC0702-14	4.19
MFC	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL
Strain	U-0503-18	U-04030-255	U-0203-29	U-0503-12	U-0203-15	
MFC	2 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	

MFC values were similar as MIC_{50} values.

11 The effect of OIPC on planktonic cells of *C. albicans* strains with elevated Mics to caspofungin.

11.1 Minimum inhibitory concentration (MIC)

Similar to the clinical isolates, the MIC_{50} of the perlin strains was determined (dpl strains). Different different OIPC concentrations (0.06 µg/mL – 0.12 µg/mL – 0.25 µg/mL – 0.5 µg/mL – 1 µg/mL – 2 µg/mL - 4 µg/mL and 8 µg/ml) or caspofungin concentrations (0.25 µg/mL – 0.5 µg/mL – 1 µg/mL – 2 µg/mL - 4 µg/mL - 8 µg/ml - 16 µg/mL – 32 µg/mL) were used to determine MICs. The dpl strains are strains with increased MIC_{50} values for caspofungin. These strains

can be used to test for cross-resistant between OIPC and caspofungin. MIC values are shown in table X.

*Table x:**Different MIC_{50} values for C. albicans perlin strains with elevated MICs for caspofungin.** Different perlin strains were grown in the presence of different OIPC concentrations (*0.06 µg/mL – 0.12 µg/mL – 0.25 µg/mL – 0.5 µg/mL – 1 µg/mL – 2 µg/mL - 4 µg/mL and 8 µg/ml) or in the presence of caspofungin *(*0.25 µg/mL – 0.5 µg/mL – 1 µg/mL – 2 µg/mL - 4 µg/mL - 8 µg/ml - 16 µg/mL – 32 µg/mL*). Two strains (dpl 1008 and dpl 1009) also show higher MIC_{50} values for caspofungin. Next to the wild type (dpl 1000) all the strains show elevated MIC_{50} values for caspofungin.*

Strain	dpl 1000	dpl 1007	dpl 1008	dpl 1009	dpl 1015	dpl 1016	dpl 1036
MIC_{50} OIPC	2 µg/mL	2 µg/mL	4 µg/mL	4 µg/mL	2 µg/mL	2 µg/mL	2 µg/mL
MIC_{50} Caspofungin	0,5 µg/mL	2 µg/mL	8 µg/mL	2 µg/mL	4 µg/mL	>32 µg/mL	>32 µg/mL

As can be seen in table x two strains also have increased MIC_{50} for OIPC (e.g. dpl 1008 and dpl 1009) compared to the wildtype strain (dpl 1000/SC5314). All other strains show increased MIC values for caspofungin. Moreover, two strains even have MIC_{50} values more than 64x MIC_{50} compared to wild type.

11.2 Minimum fungicidal concentration (MFC)

Similar to clinical isolates, the contents of the 96 well plate was plated to determine the MFC. The table below summarizes all MFC for the perlin strains.

*Table x:**Different MFC values for C. albicans perlin strains.** The content of the 96 well plate was plated to determine MFC values. Similar results were obtained as MIC50. However, two strains have lower MFCs compared to MIC50 (e.g. wild type/dpl 1000 and dpl 1016). In contrast, dpl 1007 had increased MFC for caspofungin compared to MIC_{50}.*

Strain	dpl 1000	dpl 1007	dpl 1008	dpl 1009	dpl 1015	dpl 1016	dpl 1036
MFC OIPC	2 µg/ml	2 µg/ml	4 µg/ml	4 µg/ml	2 µg/ml	2 µg/ml	2 µg/ml
MFC Caspofungin	0,25 µg/mL	2-4 µg/mL	8 µg/mL	2 µg/mL	4 µg/mL	16 µg/mL	>32 µg/mL

Table x shows the MFC values for both caspofungin and OIPC. The values for OIPC were similar to MIC_{50}. In contrast, some MFCs deviate from MIC_{50} values. Both dpl 1016 and dpl 1000 have reduced MIC_{50} values and dpl 1007 has a slight increase.

Discussion

C. albicans is commensal fungal organism that can cause infections in some conditions. Several risk factors have been identified to develop *Candida* infection and include diabetes mellitus, immunosuppression, duration of intensive care unit (ICU) stay and central venous catheter (Paramythiotou *et al*. 2014).

Different medical devices can be implanted in a variety of locations in the body and microorganisms such as *C. albicans* can colonize these devices (Ramage *et a*l. 2006). Moreover, *C. albicans* is known to form biofilms on biotic and abiotic surfaces. Mature biofilms have a unique architecture and have increased resistance to immune factors and antimicrobial agents (Mayer *et al*. 2013). Catheter related *Candida* infections are associated with high mortality and increased hospital stay and costs. In most cases removal of the device is necessary.

Currently, Three major classes of antimycotics exist. The first, the polyene class is associated with high nephrotoxicity (Wingard *et al*. 1999). The second class, the triazoles, is associated with another problem. *C. albicans* biofilms are intrinsically resistant to fluconazole (Lamfon *et al*. 2004), the most common used antimycotic of the triazole class. The last class are the echinocandins. This class is known to kill *C. albicans* biofilms but can only be administered IV (Paramythiotou *et al*. 2014).

In our work, the efficacy of a novel antifungal, oleylphosphocholine (OIPC), was tested. This is a drug based on a repurposed anticancer drug called miltefosine and the intellectual property is currently owned by Daphra Pharma, Turnhout, Belgium. A variety of mode of actions have been proposed and it has been proposed that miltefosine and OIPC have a similar mode of action (Paulussen *et al*. 2015).

Our first experiment was to determine the minimum inhibitory concentration (MIC) and minimal fungicidal (MFC) concentration of OIPC on *C. albicans*. This turned out to be 2 µg/mL for the wild type (SC5314). Miltefosine showed an MIC of 2-4 µg/mL to *C. albicans* biofilms (Vila *et al*. 2012). This is slightly higher to what we obtained.

When the MIC_{50} values are compared to other antifungals this is relatively high. Another study (Lemos *et al.* 2009) shows MIC values ≤ 1 µg mL for Amphotericin B and caspofungin. However, echinocandins and especially caspofungin are known to cause the Eagle-effect. They show (paradoxal) growth at increased caspofungin concentration (Vanstraelen *et al*. 2013). We did not observe this for OIPC even at high concentrations.

We also were able to identify some different strains with higher (Perlin strains; 4 µg/mL) and lower (strains isolated from urinary catheters; 1 µg/mL) MIC_{50} values. A study (Pfaller *et al*. 2003) showed a lack in cross resistance for fluconazole resistant isolates when caspofungin was used. We also showed that caspofungin resistant strains (Perlin strains) have similar or

slightly higher MICs and MFCs to wild type, indicating the lack of cross resistance between caspofungin and OIPC. Caspofungin works by inhibiting β-1,3 glucan synthase (Fera *et a*l. 2009). *Vila et al*. 2012. suggested β-1,3 glucan synthase as a possible drug target for miltefosine. Moreover, as stated above, Paulussen *et al*. 2015 proposed a similar mode of action for OIPC. However, no cross-resistance is observed for between caspofungin and OIPC. This indicates a different mode of action.

We also evaluated the efficacy of OIPC on polyurethane catheters with *C. albicans* biofilms. We can conclude that OIPC has no effect on adhesion. In contrast, an effect on biofilm development and mature biofilm can be observed for OIPC. The effect was more pronounced for biofilm development than mature biofilms. This is also the case for miltefosine (Vila *et al*. 2012). We also visualised the effect of OIPC on biofilm development formed on polyurethane catheters by using two different techniques. Both confocal microscopy and SEM confirmed the results.

It is known that mature biofilms are associated with increased resistance to antimicrobials (Mayer *et al*. 2013). For biofilm development 8 µg/mL showed the biggest effect. However, 13 µg/mL showed the highest effect for mature biofilm. This might indicate that the effect of OIPC on *C. albicans* biofilm formed on catheters is biofilm specific. 13 µg/mL is the highest possible concentration we can use. Higher concentrations of OIPC are associated with a soapy effect (Daphra pharma). All experiments show a relative high standard deviation.

Polyurethane is thought to be a lesser substrate for formation of C. albicans biofilms formed in vitro when compared to polystyrene (Schinabeck *et al*. 2004). Therefore, the efficacy of OIPC was also tested on polystyrene 96 well plates. Here biofilm formation was quantified with XTT. Vila *et al*. 2012 investigated the effect of miltefosine on C. albicans biofilms in polystyrene wells. They only found an effect on biofilm development at 16x MIC values for miltefosine. However, they used two other strains (44A and ATCC 10231). We used 4.19 and SC5314 (wildtype). In our work a decrease in metabolic activity can be observed starting from 8 µg/mL (SC5314) and 4 µg/mL (4.19). This is 3x MIC and 2x MIC, respectively. This confirms that OIPC has a higher efficacy *in vitro* compared to miltefosine. Similar effects were observed by Vila *et al*. 2012 for mature biofilm. Moreover, they also showed that miltefosine had a better activity than amphotericin B. Again, OIPC had a better activity compared to miltefosine for mature biofilm. However, it is known that different strains metabolize the XTT at different rates making it hard to compare the strains (Kuhn *et al*. 2003). This can also explain the difference observed between wild type and 4.19. ANIDULAFUNGIN

Bioluminescence imaging (BLI) is another technique to quantify C. albicans biofilms. Our *in vitro* BLI results again confirm the results observed by our other experiments. These data need to be interpret with caution: Vila *et al*. 2012 showed that miltefosine had an effect on the cell wall in planktonic cells. This can interfere with the luciferase enzyme which was targeted to the

cell wall. Next, this could potentially alter biofilm architecture or difference in extracellular matrix. It is known that extra cellular matrix can potentially sequester the substrate. While, concomitantly an increase of organic matter in the extracellular matrix might stimulate substrate auto-oxidation (Vande Velde et al. 2014). This could result in a decrease in photon flux at higher OIPC concentrations.

We also tested the effect of OIPC on mucosal surfaces. we used mouse tongues to investigate this effect. This model was adapted from Peter *et al*. 2010. Histatin 5 was used a positive control. Histatin 5 acts by causing ionic imbalance triggered by osmotic stress but the exact mechanism is under debate (Puri & Edgerton 2014). We could not confirm this fungicidal activity of Histatin 5. Remark that in our model, Histatin 5 from Sigma Aldrich was used. In the model by Peter *et al*. 2010 the Histatin was synthetized by another lab. They also added Histatin 5 during period of adhesion. In contrast, we added Histatin 5 and OIPC after period of adhesion. In this model, an increase in CFUs was observed for *C. albicans* biofilm development cells were grown in the presence of 13 µg/ml OIPC.

When the other *ex vivo* model was used, no statistical difference was observed. But here, a reduction in CFUs could be observed. Moreover, when the tongues were visualised with confocal microscopy a reduction in cells could be noticed. Also, these were planktonic cells. This could possibly explain why we saw no increase after period of adhesion and an increase for 13 µg/mL OIPC. Hyphae are stronger attached to each other compared to planktonic cells. Therefore, one CFU could correspond to multiple hyphae cells, resulting in an underestimation in CFUs. However, this was only done once and should be repeated.

In vivo data are the most important pre-clinical data for the development of novel antibiotics. We used a subcutaneous mouse model adapted from the subcutaneous rat model Řičicová *et al*. 2009. We did not observe an effect in for the different concentrations. However, we did not see an increase in CFUs after implantation compared to adhesion. In the model of Řičicová *et al*. 2009 CFUs were $\log_{10}$ 4.5. In our model, CFUs $\log_{10}$ were only 3 on average, indicating too high immunosuppression.

Recently, Paulussen et al. 2015 investigated the effect of OIPC on *Aspergillus fumigatus*. Here, they used 25 mg/kg/day and 50 mg/kg/day of OIPC and injected it IP. Only 50 mg/kg/day showed a significant reduction in organ burden in a five day treatment regime. They also tested oral administration. Here they saw 60% survival in a 10 day treatment. They did not see any toxic effect. This might indicate that OIPC is more potent *in vivo* when orally administered.

In general we observed a strong effect of OIPC on biofilm development and- depending on the substrate on mature biofilm. The effect on mucosal tissues should be repeated but the results look promising. In contrast, no effect was observed *in vivo* when administered IP.

Future perspectives

We focused only on *C. albicans* in our work. Other *Candida* species such as *C. glabrata* and *C. parapsilosis* are currently on the rise (Steinbach *et al*. 2012). Therefore the efficacy on these species can be investigated. Kucharíková *et al*. (unpublished) determined the effect on *C. glabrata.* OIPC is not fungicidal but fungistatic towards *C. glabrata*. Moreover, mixed species biofilms together with Staphylococcus sp. are associated with higher mortality and the effect of OIPC could be investigated on mixed species biofilm in the future.

Another aspect which could be investigated is the combinatory effect with a checkerboard assay. Amphotericin B could be a strong candidate because miltefosine enhances gastrointestinal membrane permeability *in vitro* by increasing the monomeric proportion of amphotericin B. (Menez *et al*. 2006).

It is also known that miltefosine kills mammalian cells in an apoptose-like manner (Van Blitterswijk & Verheij 2008). Therefore, *C. albicans* cells could be stained with annexin V and PI staining similar to Hao *et al.* 2013 to determine if OIPC kills by causing necrosis or apoptosis. Next, the mode of action of OIPC could be investigated by using a deletion collection of *C. albicans* or by RNA sequencing.

Finally, we will also investigate the effect of OIPC in a subcutaneous rat model as it was developed by Řičicová *et al*. 2009. In this model OIPC would be administered orally as this would result in a better bioavailability (Daphra Pharma). Here we would start administration right after implantation of the catheter. This is similar to biofilm development which shows the highest activity towards *C. albicans*.

The subcutaneous mouse model is more related to biofilm infections developed in joint or voice prostheses (Kucharíková *et al*. 2013). Other models such as central venous catheters model could also be tested to assess the effect of OIPC under different environmental -and flow conditions. In other models different host niches could be used to test the *in vivo* effect of OIPC. Which can result in different immune responses and different drug accessibility.

Promising *in vivo* data (of at least two independent research groups) are needed to move on to the next stage of antibiotics development, clinical trials on humans. OIPC is currently already under development as an anti- leishmaniasis drug and has just successfully finished phase I. Here the effect on healthy volunteers was assessed. No toxic side effects were observed. If promising in vivo data are obtained for OIPC it can immediately move on to phase II for the development as novel antimycotic.

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Addendum

Risk analysis

Researcher related risks

1) Risk related to chemical substances

- Dexamethasone is not classified as a dangerous substance but should be used with caution. It can irritate eyes, skin and respiratory tract. It is advised to wear a lab coat and gloves.
- Isoflurane is not listed as dangerous. Leakage might cause oxygen shortage. Therefore, the supply system should be checked regularly.
- Menadione is hazardous when swallowed. It irritates skin, eyes and respiratory tract. It is poisonous for all water organisms therefore disposal in the sink is forbidden. Protective clothing should be worn and the work space should be ventilated sufficiently. The skin should be washed thoroughly with water and soap when contact with the substance occurs.
- NaOH can cause severe burns because it is an corrosive substance. Therefore, contact with the eyes should be avoided and protective clothing such as lab coat and gloves should be worn.
- RPMI-1640 powder is not listed as a dangerous substance. However, the use of lab coat and gloves is advised.
- XTT is not classified as dangerous but both protective clothing and should be worn when it is used.

2) Risk related to flammable substances

- Ethanol is highly flammable. Therefore caution should be applied when working with fire and ethanol to sterilise certain objects.

3) Risks related to organisms

- *C. albicans*: this fungus is classified as a class 2 organism for both humans and animals. It should be manipulated under the laminar flow at all times and has to be removed with 70% ethanol or 5% dettol immediately after spilling. Working in a L2 lab is advisable. Clinical isolates in this work were used. These are highly virulent and should be handled with caution. Especially the perlin strains with elevated MIC values for caspofungin. Containers which have conta

- ined C. albicans should be autoclaved before discarding. Hands should be washed immediately when *C. albicans* was manipulated. Moreover antisepsis should be used on the hands and surfaces should be disinfected.
- Mice: the mice in this study were pathogen free. Therefore they have a reduced risk of zoonosis. However, they should still be handled with care. Gloves should be worn and washing of the hands should be washed every time the mice were handled. Moreover, exposure to faeces should be avoided and a mask can be worn to prevent allergenic reactions. A lab coat should also be worn at all times.

4) Risks due to physical harm

- Needles should never be recapped to avoid injury. Specific needle container should be used to dispose the needles.
- Sharp objects such as scalpels should be handled with caution to reduce the risk of injury.

<u>Risks for the experiment</u>

1) Contamination associated risks

- Gloves and lab coat should be worn to avoid contamination
- All used materials and surfaces should be disinfected prior to using them with 2% Dettol and/or 70% ethanol. All glass work, solutions, etc. should be autoclaved or UV sterilised when possible.
- The laminar flow should be used to reduce the risk of external contamination.
- When mice are used, special attention should be applied on contamination. The mice are pathogen free when they enter the facility. Therefore, all cages and bedding should be sterilised, a specific lab coat should be used for the animal room, etc.

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