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The Antifungal Occidiofungin Disrupts Morphological Switching in the Polymorphic Fungus Candida albicans

By

Aaron Albee

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Honors Thesis
in the Shackouls Honors College

Mississippi State, Mississippi

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Occidiofungin is a natural antifungal compound produced by *Burkholderia contaminans* MS14. Prior work has been shown occidiofungin to be effective against *Candida albicans* in its non-pathogenic yeast form. However, *C. albicans* is an opportunistic pathogen that requires a morphogenic switch to a filamentous form to cause disease. As previous work focusing on the non-pathogenic form has shown distinct biochemical and morphological changes with occidiofungin exposure, I hypothesis that the filamentous form of this fungi will also undergo identifiable changes that can be characterized. To this end, cells induced to undergo morphological switching in the presence of occidiofungin were analyzed by standard microbiological methods. Data showed that a lethal dose of the antifungal prevented hyphae formation while a sublethal dose slowed filament growth without obvious alterations in cell wall organization. These data adds to our understanding of occidiofungin's potential as an antifungal compound to combat the pathogenic form of *Candida albicans*.

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CHAPTER I

INTRODUCTION

Opportunistic pathogens are a large group of disease-causing organisms that most individuals encounter on a daily basis. These microorganisms can coexist with human populations without posing a danger to a healthy individual. However, if placed into the right set of circumstances such as altered temperatures, changes in resource availability, abnormal location in the body, or other situations, they can become pathogens (Kabir, Hussain, & Ahmad, 2012). Once these microbes have made the change to a pathogenic state, a wide range of potential life-threatening diseases can arise. These diseases can be especially dangerous for those individuals who are immunocompromised, surgical patients, transplant recipients, or long-term hospitalized individuals. This presents a unique problem for the medical community as some of these opportunistic pathogens are key members of the human microbiome. This makes the practice of limiting contact with these pathogens as a means of prevention impossible. This, coupled with the fact that many of these pathogens have evolved resistance to the drugs commonly used to eliminate them, makes the identification of new treatment protocols an imperative for the research community.

Opportunistic pathogens can be bacterial in origin (i.e. prokaryotic) or eukaryotic organisms and are found across a range of different genera and species. Some opportunistic pathogens are harbored in the natural environment and rarely cause infection in healthy individuals. Some common commensal bacteria such as *Streptococcus pneumoniae* and

Staphylococcus aureus can become disease causing agents under the right circumstances (Brown, Cornforth, & Mideo, 2012). *S. aureus* is capable of causing potentially fatal disease, yet many health individuals are colonized in anterior nostrils. *P. aeruginosa* is a known environmental opportunistic pathogen that can cause dangerous infections in immunocompromised patients (Sadikot, Blackwell, Christman, & Prince, 2005). Several of the eukaryotic pathogens are from the Fungi kingdom with very distinct characteristics. Fungi are ubiquitous to many environments and are contacted daily. The Center for Disease Control (CDC) estimates that there are about 300 different fungi that can cause disease including *Aspergillus*, *Cryptococcus*, *Histoplasma*, and various *Candida* species (Moore, Robson, & Trinci, 2011). *Candida albicans* is the most important opportunistic pathogen of the human gut flora. *C. albicans* exists in two different developmental morphologies: a yeast form and a hyphae form (Arkowitz & Bassilana, 2011). The yeast form exists as a common commensal microorganism of the human microbiome. In contrast, the hyphae form is known to be associated with virulence and can cause a range of diseases (Pukkila-Worley, Peleg, Tampakakis, & Mylonakis, 2009). For example, if *C. albicans* enters into the blood stream and leads to systemic spreading, this is referred to as invasive candidiasis. For immunocompromised patients this can be fatal (Pukkila-Worley et al., 2009). The exact mechanism driving virulence in *C. albicans* is not fully understood, but specialized proteins produced by cells in their hyphae form are thought to cause some disease symptoms. A lowered state of immune response can also result in more severe disease progression making this organism of medical relevance within many areas of the health care system.

While prevention of Candidiasis by limiting exposure is important, the ubiquitous nature of this organism makes complete exclusion impossible. Thus, treatment with various antifungals is a first line of defense for medical professionals. These antifungal compounds work best if their targets are unique or conserved to fungi. Antifungals of the azole family target ergosterol which is a key component of the fungal cell wall. Ergosterol is similar to cholesterol in mammalian cells and functions to stabilize the plasma membrane (Gow, Latge, & Munro, 2017). The cell wall is another key difference between fungi and mammalian cells and is also the target of antifungal drugs. The fungal cell wall is composed of proteins, chitin, and glycans and functions to protect the cell from osmotic and structural damage. Antifungals that target a fungal enzyme involved in generating the glycans present in the cell wall include those in the echinocandin family (Hawser, Francolini, & Islam, 1996). Inhibiting an enzyme required for cell wall synthesis weakens the cell wall which disrupts the cell's ability to compensate for osmotic stressors, ultimately resulting in cell death. Targeting unique properties of fungal cells is important when trying to identify/develop new antifungal treatments. The less unique the treatment target, the more chance for side effects from the treatment. Resistance to antifungals must always be a concern in the health care industry. Resistance mechanisms can vary based on the drug and organism but often involve the accumulation of mutagenic changes that allows the organism to survive higher than therapeutic doses of the drug. For example, resistance to echinocandins arise from the acquisition of amino acid mutations in a region of the target enzyme which prevents antifungal binding (Spampinato & Leonardi, 2013). The CDC has been tracking the emergence of multidrug resistant strains of *C. albicans* which is a growing concern as no new antifungal treatments have been developed for clinical treatment in many years (Cleveland et al., 2015).

The danger of resistance to antifungals has broader implications than just the health care industry. The agricultural industry must also contend with a wide range of microbial pathogens that can devastate crops. Nearly every food crop can be affected by fungal pathogens which could compromise food security. Nearly 18% of crop loss is caused by microbial agents and 70% of these are fungal in nature (Savary, Ficke, Aubertot, & Hollier, 2012). There are far more pathogenic fungal diseases than any other form of pathogens that impact crops. Wheat is one of the largest crops in the U.S. and accounts for near 15 billion dollars in annual revenues. A major fungal pathogen of wheat is Rust disease which is caused by *Puccinia graminis*. Rust disease associated with grains such as wheat has been estimated to cause up to 5 billion dollars in losses due to reduced crop yields (Savary et al., 2012). With increasing demand for sustainable food crops in developing countries, and the growing trend in the U.S. of locally sourced foods, the development of new methods to protect crops from fungal pathogens is an important focus for the agriculture industry.

In addition to fungi negatively impacting agriculture through its impact on plants, pathogenic fungi have also been linked to honey bees. As of 2006, a rapid loss of bee colonies began to occur in the United States which endangers both honey production and crop agriculture (Bromenshenk et al., 2010). While our understanding of the cause of colony collapses is not complete, fungal infection is one of the current possible causes for the decline in bee populations (Ptaszynska, Borsuk, Anusiewicz, & Mulenko, 2012). Likewise, wildlife populations are also damaged by fungal pathogens. In recent years, a disorder called “white-nose bat syndrome” (WNS) has decimated bat populations across the eastern seaboard. The cause of WNS is a fungal pathogen *Pseudogymnoascus destructans* which was likely introduced from Europe and spread

quickly through populations (Trivedi et al., 2017). Bats are also an important species for U.S. agriculture because of their role in natural pest control. Conservative estimates place the worth of this pest control at $3.7 billion dollars annually (Boyles, Cryan, McCracken, & Kunz, 2011). Bats may also have a role in human health as they control population numbers of mosquito, a pest that can harbor human pathogens.

Research has identified several new compounds to treat fungal infections. One of these new compounds was discovered at Mississippi State University and is called occidiofungin (Lu et al., 2009). This compound was found to be produced by a soil bacterium that associates with the roots of plants. Occidiofungin has a broad range of antifungal activities and is effective against both plant and animal fungal pathogens. Interestingly, occidiofungin has antifungal activity against fungi that have been shown to be resistant to current antifungal agents which makes this compound a promising agent for further investigations (Lu et al., 2009).

CHAPTER II

BACKGROUND

C. albicans as model fungal pathogen

C. albicans is a model pathogenic organism for the analysis of novel antifungal compounds in part because of the human health relevance of this pathogen. Differences in *Candida albicans* and the more commonly used budding yeast, *Saccharomyces cerevisiae*, are observed (Karathia, Vilaprinyo, Sorribas, & Alves, 2011). *S. cerevisiae* can be maintained as either a haploid or a diploid, are easily grown and maintained in a laboratory setting, have well characterized genetics, an annotated genome, and have commercially available collections of deletion and overexpression strains that makes genetic screening relatively routine. In contrast, *C. albicans* is an obligate diploid cell making genetic alteration more difficult and time consuming (Kabir et al., 2012). Often both fungal species are used when addressing questions within the antifungal field.

C. albicans is a well-studied eukaryotic fungus that is a commensal microorganism of the human gut flora (Kabir et al., 2012). However, when allowed to proliferate in areas of the body that are outside of its normal environment or in individuals who are in an immunocompromised state, growth of this organism can give rise to *C. albicans* becoming a disease-causing agent. *C. albicans* is a polymorphic yeast as it can acquire several morphologies associated with the cell's life cycle (Arkowitz & Bassilana, 2011). Laboratory cultures of this organism are commonly maintained in an oval shaped "yeast' form which reproduces by asexual

budding. This yeast form is associated with the commensal colonization of the gut flora and is typically regarded as non-pathogenic. In contrast, under environmental stressors such as elevated temperature, elevated pH, elevated CO_2/reduced O_2 levels, or limited nutrient availability, the yeast cell will undergo a morphological switch to a hyphae form (Whiteway & Bachewich, 2007). This form is marked by a long filamentous extension from the cell body. This form of *C. albicans* will undergo sexual reproduction with the diploid cells exchanging genetic material through these filamentous structures. This hyphae form is also associated with pathogenicity and biofilm formation. The exact cause of virulence in *C. albicans* is not fully understood but is linked to the cells ability to adhere to cell surfaces and to induce tissue damage with the secretion of hydrolytic enzymes (Douglas & Konopka, 2016). As these virulence traits are linked to hyphae morphology, the basic cellular mechanics for this morphology are important.

F- and G-actin

Actin is an important cytoskeletal element in eukaryotic cells. Actin is found in one of two forms: G-actin, which are monomeric actin subunits and F-actin which are composed of monomeric actin subunits that have assembled to form a two-stranded filamentous structures (Dominguez & Holmes, 2011). Actin can be found in both cortical patches and cables at different stages of cellular development. Many key cellular functions rely on actin such as vesicular transport, organization and positioning of cellular organelles, organelle segregation in mitosis, and whole cell mobility. Actin has also been identified in both the cytoplasm and the nucleus (Moseley & Goode, 2006). Key to actin function is its interaction with actin-related proteins including those involved in actin crosslinking, capping, severing, and polymerization and depolymerization events. The dynamics of actin within the cell is related to proteins that can polymerize and reorganize actin. In fungi for example, the actin binding proteins Arc35 and Sla2

are important for cellular uptake by endocytosis; Arp2/3 and Sac6 are associated with vesicle transport along actin cables; the Arp2/3 complex plays a key role in the nucleation of actin for the formation of actin patches; and Bnr1 is an essential protein for the assembly of actin cables (Amberg, 1998; Dominguez & Holmes, 2011; Galletta, Mooren, & Cooper, 2010; Moseley & Goode, 2006). Additional actin associated proteins with important cellular functions are members of the myosin family. Myo3 and Myo5 are members of the myosin type I family and have a role in endocytosis and cortical patch formation (Oberholzer, Marcil, Leberer, Thomas, & Whiteway, 2002). There are also two type V myosin proteins, Myo2 and Myo4, responsible for the transport of vesicles along actin filaments (Galletta et al., 2010). Disruption of actin, actin binding proteins, or myosin activities negatively impacts normal cellular functions and have profound effects on cell viability (Moseley & Goode, 2006).

Actin is a highly conserved protein. Actin from *C. albicans* has a 94% sequence identity to *S. cerevisiae* and 84% amino acid identity to muscle actin from *Homo sapiens* (NCBI protein BLAST). In yeast there is only one gene that codes from actin, *ACT1.* In contrast, *Homo sapiens* have six different genes coding for six isoforms of actin, all having a high degree of sequence conservation between them (Perrin & Ervasti, 2010). Two of these isoforms are found in all cell types while the four remaining isoforms are expressed only in muscle cells (Perrin & Ervasti, 2010). Although all isoforms can assemble to form actin filaments, roles for each of these isoforms may be specific to the cells where they are found.

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Actin and hyphae formation

The most basic mechanism driving morphological switching is polarized cell growth. Fundamental to polarized growth is the organization of the actin cytoskeleton. Actin is seen in patches localized to the plasma membrane at sites of secretion (Pukkila-Worley et al., 2009). These patches are often associated with polarized growth which further supports its relation to the secretory pathway (Whiteway & Bachewich, 2007). Unlike mammalian cells which use primarily microtubules for cellular transport, yeast cells use actin-based transport along actin cables (Galletta et al., 2010). These F-actin cables direct the transport of proteins, polysaccharides, and lipids through the activity of myosin type V proteins (Kachurina, Turcotte, & Whiteway, 2012). The transport to these polarized areas of cell growth is also dependent on myosin type I proteins which mediate cortical patch formation. The control of cellular actin organization is regulated by Rho proteins which create polarized growth and maintain hyphae extension (Dunkler & Wendland, 2007). Cortical patches of actin are normally observed at the site of budding, hyphae formation, and the tip of growing hyphae. Likewise, actin cables are normally focused to the sites of polarized growth and down the growing hyphae filaments (Moseley & Goode, 2006). Two signaling cascades play an important role in the switch to filamentous growth: the Ras1p and Cyr1p signaling pathways (Sudbery, 2011). Each of these pathways relays the cell signal to turn on hyphae specific gene expression. Many aspects of initiation and maintenance of filamentous growth depend on actin function. An example of this has been shown with the Wal1 protein which in *C. albicans* is a homolog to the Wiskott-Aldrich syndrome (WASP) which is known to regulate formation of actin filaments and cytoskeletal organization (Walther & Wendland, 2004). Mutants of this protein have a disruption in cell polarization and are unable to produce hyphae under the conditions tested. Other proteins whose

loss of function are linked to hyphae defects include Efg1p, Cph1p, and Tup1p, all of which are transcription factors (Douglas & Konopka, 2016). Each of these deletion mutants have been shown to lack the ability to invade and damage host tissues. This data supports that *C. albicans* that are unable to attain a filamentous morphology have attenuated virulence, highlighting the importance of actin and the ability to reorganize actin architecture to induce morphological switching.

Antifungal compound activity in *C. albicans*

Compounds that result in fungal cell death are considered fungicidal, while those that impair fungal growth are fungistatic. Both are considered as having antifungal properties. The two most common family of antifungals used to treat *C. albicans* are azoles and echinocandins (Hawser & Islam, 1999). Azoles, such as fluconazole, are a class of drugs that inhibit the conversion of lanosterol to ergosterol by disrupting activity of fungal demethylase encoded by *ERG11* (Hawser et al., 1996). While ergosterol is an important component of the plasma membrane, azoles are fungistatic in *C. albicans*. The data also indicated that only at very high concentrations of azoles was the induction of hyphae formation blocked. The second commonly used antifungal compounds are in the echinocandin family and include Caspofungin and Mulundocandin, which noncompetitively inhibit beta-1,3-D-glucan synthase activity (Hawser & Islam, 1999). The crosslinking of glucans is key to cell wall strength. The lack of beta-glucan activity therefore reduces cellular resistance to osmotic forces that can result in cell death. This family of antifungals is fungicidal in *C. albicans*. While resistance to this family is rare, some organisms have recently developed resistance by upregulation of efflux pumps, induction of alternative glucan synthesis mechanisms, or utilizing chitin to reinforce the cell wall (Perlin,

2015). Research has shown that echinocandins will block the initiation of hyphae formation at doses well below the normal MIC for cells in their yeast form (Hawser et al., 1996).

Occidiofungin

Occidiofungin is a novel, naturally occurring secreted peptide that is extracted from liquid cultures of *Burkholderia contaminans* MS14, a bacterium that is ubiquitous in the environment (Lu et al., 2009). This peptide is not synthesized by ribosomes, rather it is produced through the activity of multiple enzymes including transferases, dehydratase, nonribosomal peptide synthetase, and polyketide synthetase (Lu et al., 2009). Occidiofungin is a cyclic molecule containing 8 amino acids, a fatty acid group, and a xylose sugar (Gu, Smith, Liu, & Lu, 2011). This compound has a broad antifungal activity against both plant and mammalian pathogens including *Candida spp.*, *Aspergillus spp.,* and *Microsporum gypseum* (Emrick et al., 2013). Interestingly, occidiofungin can target *Pythium spp.* and *Cryptococcus neoformans,* fungi that lack ergosterol and chitin or exhibit resistance to echinocandins, suggesting that occidiofungin mode of action is different than current antifungal agents (Lu et al., 2009).

In previous studies using *Saccharomyces cerevisiae*, occidiofungin was shown to induce apoptotic cell death as evidenced by the accumulation of DNA damage, reduction in cell size, and elevation in reactive oxygen species (Emrick et al., 2013). Additional work supports occidiofungin susceptibility being linked to active cellular growth as cells in quiescence were resistant to occidiofungin exposure. The addition of extracellular divalent cations also created an environment that lead to resistance (Robinson, Denison, Burkenstock, Nutter, & Gordon, 2017). Toxicologically, occidiofungin is well tolerated in a murine model at 1mg/kg dosing regimens. Though toxic effects such as organ weight loss were seen at extremely high doses (20mg/kg) (Tan et al., 2012). Overall, occidiofungin was shown to be non-toxic at doses that are consistent

with ranges for treatment of fungal infections. Occidiofungin was also shown to remain stable in low pH environments (Ellis et al., 2012) , which is important for any oral applications. These initial studies suggest that occidiofungin could be a viable antifungal treatment.

Hypothesis

An important step in determining whether a compound can function as a new form of antifungal treatment is understanding what it does to the target organism. In this project I seek to address this question by characterizing the morphological changes that result with occidiofungin exposure using the pathogenic yeast, *Candida albicans*, as the model fungi. Cells will be exposed to different doses of the compound and the effects on morphology and viability analyzed using various microbiological and cell biological assays. Given that prior work had demonstrated that the impact of occidiofungin on the yeast form of *C. albicans* could be identified by biochemical and morphological changes, (Emrick et al., 2013), I hypothesize that occidiofungin treatment of cells at the point of switching will also give rise to visible morphological defects that can be characterized.

CHAPTER III

MATERIALS AND METHODS

Yeast strain

C. albicans strain, ATCC 66027, was used for all described studies. Working cultures were generated by streaking cells from a -80˚C freezer stock to a YPD agar plate which was incubated at 30˚C for 24 hours. Cells were taken directly from this plate for the inoculation of liquid cultures.

Yeast media

For vegetative growth, *C. albicans* was maintained at 30˚C in YPD media (1% yeast extract, 2% peptone, 2% dextrose). To induce hyphae formation, cells were diluted into Spider media (1% nutrient broth, 1%mannitol, 0.2% K_2PO_4) and placed at 37˚C.

Reagents

A 2mg/ml stock of Concanavalin A (Sigma, L7647) was made in sterile water and used directly to coat glass slides for microscopy purposes. Calcofluor White (Sigma, 18909) was used without dilution to stain fixed cells for chitin. Rhodamine labeled phalloidin (Sigma, P1951) was dissolved in 100% DMSO to achieve a 3.3mM stock solution and used at 1:500 dilution for actin visualization by fluorescence microscopy. A 20mg/ml stock of Resazurin (Sigma, R7017), made in sterile water, was used at 50µg/ml in YPD or Spider media for microdilution MIC assays to identify wells with cellular activity/growth. Trypan Blue (Sigma, T8154) was used directly to monitor cell viability by dye exclusion. Vecta Shield with DAPI (Vector Labs, H-1200) was used at 50% as an antifade agent for fluorescence microscopy and nuclear DNA stain.

Antifungal compounds

All stock and working dilutions of antifungal compounds were made in 100% DMSO and stored at -20˚C until needed. Occidiofungin was purified as described ((Wang et al., 2016); a gift from Dr. Leif Smith). Stock and working solutions of occidiofungin included: 1mg/ml, 0.4 mg/ml, and 0.1mg/ml. Caspofungin was purchased from Sigma (SML0425) and dissolved in DMSO to obtain a 1mg/ml stock solution. Working solutions of 0.4mg/ml and 0.05mg/ml were made by subsequent dilution. Fluconazole (Sigma, F8929) stock was made at 200mg/ml with 100mg/ml and 8mg/ml working solutions.

CFU and spotting protocol

A saturated culture of *C. albicans* ATCC 66027 was obtained by inoculating 20mls of YPD with cells taken from a fresh streak plate and the culture maintained at 30˚C with shaking for 48hrs. The resulting cell density was determined by optical density reading at 600nm after step diluting the culture 1:40 in YPD (Sittampalam et al., 2018). To obtain a working culture, cells were diluted in Spider media to achieve a 0.05 OD_{600}/ml. The culture was divided into two equal groups; one was treated with the antifungal compound (occidiofungin at 0.25µg/ml to 1 µg/ml, fluconazole at 7.8µg/ml, and caspofungin at 0.1µg/ml) and the second was the control group treated with an equivalent volume of DMSO. Aliquots were removed from each treatment at set time points in a 0 to 6-hour window. Samples were 5-fold serially diluted in Spider media in 8-strip polypropylene PCR strips (Applied Biosystems, N8010580) to avoid cell adhesion which occurs in standard polystyrene microtiter plates. Samples (80µl) were placed in the first well of each strip and 160µl of media placed in wells 2 through 8. The serial dilution was performed by transferring 40µl across all wells in the strip. For spotting assays, 3µl volume from each well was spotted to a YPD agar plate and the plates incubated at 30˚C. All spotting was

done in duplicate for all time points. Images were taken after 24hr and 48hr of growth. For colony forming unit (CFU) determination a 50µl volume was taken from select wells of the dilution series and spread over a YPD agar plate. Three wells were selected for each sample and three 50µl aliquots were plated per well (Robinson et al., 2017). The plates were incubated for 48 hours at 30˚C. After incubation the number of colonies on each plate were counted (Heathrow Scientific eCount Colony Counter) and recorded.

Cell morphology by light microscopy

C. albicans cells were grown to saturation, diluted, and divided into experimental groups as described above. At each treatment time point, 250µl to 500µl of sample was removed and fixed with the addition of 37% formaldehyde to 3.7% final. At a later date, samples were centrifuged at 8000 xg for 10 minutes to pellet cells, the formaldehyde removed, and the cells resuspended in 30µl of 1XPBS. An aliquot of cells was combined with an equal volume of VectaShield with DAPI and 3.5µl of this mixture was placed on a concanavalin A treated glass slide. Cells were viewed using a Nikon Eclipse-50i microscope and images recorded by Q-Capture Pro software.

Samples were evaluated for morphology based on previously published metrics for *C. albicans* by Merson-Davies and F.C. Odds (Merson-Davies & Odds, 1989). All cells were placed into one of two categories: yeast form or hyphae form. Cells scored as “yeast” were spherical shaped cells that may or may not possess a bud. Cells scored as “hyphae” were spherical cells with a long-septate extension from a polarized location. Any cell that possessed a hyphae-like structure were the septum was undetached from the main cell body was binned as yeast form. Cell count numbers were recorded from images obtained using the 100X objective with a minimum of 200 cells analyzed per condition and time point.

Fluorescence microscopy of mannans, chitin, and actin

For mannan and chitin staining, cells were grown and separated into experimental groups as described in the light microcopy section above. At selected time points, 500µl to 800µl of sample was removed and fixed with the addition of formaldehyde to 3.7% final. The cells were isolated by centrifugation and the cell pellet resuspended in Vecta Shield as described above. For mannan staining, 10µl of cells were treated with 10µl of FITC labeled concanavalin A for 5 minutes at room temperature. Unbound concanavalin-A was removed with a 1XPBS wash prior to viewing on a glass slide (Hoch, Galvani, Szarowski, & Turner, 2005). For chitin staining, cells were added to an equal volume of calcofluor white and incubated at room temperature for 5 minutes prior to addition to a concanavalin A treated slide. Data was collected using a Nikon Eclipse-50i microscope with fluorescent filters for DAPI and calcofluor white (UV-2E/C) and concanavalin-A (B-2E/C FITC). Images were collected using Q-Capture Pro software with the same exposure settings.

Cell staining to visualize actin was carried out as described (Ravichandran et al., 2019) with the following modifications. Samples were fixed in 3.7% formaldehyde at room temperature for 1.5 hours and cells isolated by vacuum filtration using a 0.2µm nitrocellulose filter. Cells were resuspended in 250µl of 1XPBS, permeabilized with the addition of an equal volume of 0.2% TritonX-100 in 1XPBS and stained with the addition of 1 µl of 3.3mM rhodamine-phalloidin for 30 minutes at room temperature. Cells were isolated by centrifugation at 6000 xg for 10 minutes at room temperature and the solution removed. The cell pellet was resuspended in 1XPBS and an equal volume of VectaShield with DAPI was added. Cells were visualized using the 100X objective (N.A. 1.25) with a Nikon Eclipse-50 microscope with

fluorescent filters for rhodamine (FL Y-2E/C). Images were capture using QCapture Pro software.

MIC Assay of Antifungal Compounds

A culture of *C. albicans* ATCC 66027 was grown to saturation. Cells were diluted into Spider media to achieve an OD_{600} of 0.05 (equivalent to 1.6 x 10^6 cells/ml), 1 x 10^4 cells/ml, or 1 x 10^5 cells/ml. Resazurin salt was added directly to the diluted cell cultures to achieve 50µg/ml final concentrations. Cells were placed in a 96 well plate such that well 1 had 200µl of culture and wells 2-12 had 100µl of culture. The antifungal agents were added to the cells in well 1 and then serially diluted 2-fold with the transfer of 100µl of across all wells (Ellis et al., 2012). The range of antifungals used included: occidiofungin 2µg/ml to 0.9ng/ml; caspofungin 2µg/ml to 0.9 ng/ml; and fluconazole 125µg/ml to 0.06ng/ml. Plates were incubated at 37˚C for 24 and 48 hours. Wells where the color changed from purple to pink were noted. Plate images were photographed and the 1X MIC and 0.5X MIC points recorded. Fluconazole presents in this assay with a "trailing" effect (Manavathu, Cutright, & Chandrasekar, 1998), a partial color change above the MIC value due to it being fungistatic rather than fungicidal. Only with the complete lack of metabolic activities seen with cell death does a color change not occur.

Calculations

Measurements were obtained for hyphal length from cell images using NIH ImageJ software and measured with in programs features. The size bar was used as the calibration standard. The percent cell death was calculated by the difference between average CFU per ml for treated groups and its matching untreated group as previously described (Robinson et al.,

2017). CFU calculations were based on number of colonies counted per plate multiplied by the dilution factor for the plate and corrected by the volume of culture plated to achieve 1ml.

CHAPTER IV

RESULTS

Occidiofungin activity against filamentous *C. albicans*

Occidiofungin has been shown to be an effective fungicidal agent against vegetative growing *C. albicans* (Robinson et al., 2017). However, as the virulent form of *C. albicans* is primarily when it is in its filamentous form (Pukkila-Worley et al., 2009), the efficacy of occidiofungin against this form of the yeast must be demonstrated before considering its therapeutic potential. To characterize the impact of occidiofungin against cells induced to undergo morphogenic switching, MIC assays were carried out under switching conditions. As cell numbers are not expected to significantly change under these conditions, and to ensure that wells with viable cells could be easily identified, resazurin was included in the switching media. The resazurin indicator undergoes a color change from blue to pink as a result of the irreversible reduction of resazurin to resorufin during aerobic respiration and is therefore be used to identify wells that have metabolically active cells (Elshikh et al., 2016). Under these conditions, the MIC well was identified as the well containing the lowest drug concentration that did not exhibit any change in color (Figure 4.1). At the concentration used, resazurin was shown to have no negative impact on MIC determination (data not shown). Assay results are presented in Table 4.1 and a representative MIC assay carried out in the presence of resazurin is shown in Figure 4.1.

Clinical laboratory standards for MIC determination of antifungal susceptibility are carried out using a protocol that results in an estimated cell density of ~1.0×10^4 cells/ml. As cell density significantly impacts switching efficiency of *C. albicans*, MIC assays were carried out on cells over a range of cell densities including: 1.0×10^4 cells/ml, 1.0×10^5 cells/ml, and 1.6×10^6 cells/ml. Data showed that when added at the time of switching, occidiofungin exhibited an MIC

value of 0.125µg/ml at the CLSI standard cell density of 1.0 x 10^4 cells/ml. This is 4-fold lower than the corresponding MIC for the same number of cells growing in their yeast form (Robinson et al., 2017). Not surprisingly, an increase in cell density required a higher dose of occidiofungin for full fungicidal activity (Table 4.1).

The effect of other known antifungals on morphologically switching cells has been tested by others (Hawser & Islam, 1999) and were carried out in parallel to directly compare to occidiofungin activity. Antifungals in the azole (fluconazole) and echinocandin (caspofungin) family were tested. When exposed to fluconazole under switching conditions, a “trailing effect” was observed using resazurin in the MIC assay (Manavathu et al., 1998). The “trailing” effect is due to fluconazole having fungistatic activity which results in viable, but slow growing cells that lead to a partial color change compared to no color change for a fungicidal compound. The echinocandin, caspofungin, exhibited an MIC value of 0.031µg/ml for cells at a density of $1.6x10^6$ and 1x 10^5 cells/ml. Surprisingly, cells at the lowest density tested were found to have a higher tolerance to caspofungin with an MIC value of 0.062µg/ml.

To take a quantitative approach towards measuring the impact of occidiofungin on *C. albicans* at the time of morphological switching, colony forming units (CFU)-based experiments were undertaken. Cells were grown, treated, and plated as follows. Cells from a saturated culture were diluted into fresh Spider media to achieve a cell density of 0.05 OD_{600}/ml. The culture was split, with one half of the cells receiving the antifungal at the concentration noted, and the other half receiving an equivalent volume of DMSO. Cells were placed at 37˚C to induce switching and samples removed at defined time points for serial dilution and plating. The data was first analyzed by plotting log_{10} CFU number versus time of exposure (Figure 4.3). Cells that were treated with DMSO as a control showed a short lag phase before a slight change in colony

number was detected. With an average of only a 5.4% increase in cell number over the 6-hour period (n=3) this likely reflects the ~10-15% of the cell population retained in the yeast form that entered back into the cell cycle. In contrast, cells treated with occidiofungin showed a rapid decline in colony number that is what would be expected for a fungicidal compound. The data was further analyzed to determine percent cell death at each of the time points tested (Table 4.2). Over the three experimental trials, occidiofungin treated cells showed a large increase in cell death following two-hour exposure time which continued over the 6-hour exposure time. The lack of high levels of cell death in the first two hours after exposure is an expected finding as previous experiments had shown that cells that are not actively growing are more resistant to occidiofungin (Robinson et al., 2017). The cultures used here were grown to saturation before being placed into fresh media to induce switching. As the cell numbers for both treated and untreated were similar over the first two hours, this suggests that the cells require time to activate reentry back into an actively growing phase which makes them more susceptible to occidiofungin.

Spotting assays were also run in parallel with the CFU assays to qualitatively visualize the effects of occidiofungin. Data from these assays confirmed that prior to exposure to occidiofungin, both treated and untreated samples showed an identical growth pattern (Figure 4.3). However, for all later time points a visible reduction in growth was detected for occidiofungin treated cells. This data matches the CFU and MIC assay results, supporting occidiofungin's fungicidal activity in switched *C. albicans* cultures.

Impact of 1.0X MIC dose of occidiofungin on *C. albicans* filamentation

Cell morphology has been shown to be linked to invasion and cell damage (Phan, Belanger, & Filler, 2000). As different antifungal compounds have variable effects on the cells

ability to induce filament formation, this creates a necessity to test what effects occidiofungin will have on cell morphology when used at the point of switching. To access the effects of occidiofungin on the morphology of *C. albicans* during morphological switching, cells were analyzed by light microscopy. As expected, cells that were scored immediately after being placed into switching conditions (T=0) were all in their yeast form (Figure 4.4). For the untreated group, after one hour in switching conditions ~56% of the population was in a yeast form while the remaining cells had hyphae. Over 2- and 4 hours in switching conditions, there was a continued increase in cells that had filamentous extensions with an average of 82% and 87% of the cells in the population exhibiting hyphal growth, respectively. However, by the 6-hour time point there was an increase in the population of cells in the yeast morphology (~38%) which likely represents the fact that the yeast cell populations are undergoing cell division (Figure 4.5). For cells that had been treated with occidiofungin, the population remained primarily in the yeast form over the 6 hours of the experiment; cells with hyphal extensions were rarely found (<1%). As occidiofungin was used at a concentration equivalent to 1X MIC, cells were also analyzed by live/dead assay using trypan blue to calculate the percentage of viable cells in the population. By this assay, at least 60% of the cells on each slide were viable cells for all time points. Together, this data suggests that occidiofungin exposure at the time of morphological switching interferes with a cell's ability to form a hyphal extension.

Occidiofungin at 0.5X MIC disrupts normal hyphae formation

Cells treated with antifungal compounds will commonly have cellular morphology abnormalities including changes to cell size, composition of cell wall components, and alterations to shape of cellular structures (Hawser & Islam, 1999). To determine if this was the case for occidiofungin, the impact of the compound was tested using a sublethal MIC dose for

switching cells to better characterize effects on morphology. Cells were treated at the time of switching with a 0.25µg/ml occidiofungin and collected at set times following exposure, with cells fixed for viewing by light microscopy (Figure 4.6 and 4.8). As expected, at the zero-hour time point 100% of the treated and untreated cells had the yeast morphology. In the untreated group at the one-hour time point, the majority of cells were found in their filamentous form (94%), while in the treated group, ~86% of the cells were in the yeast form with only 14% having a hyphal morphology. For the treated group the number increased to 75% of the population having filaments by 2 hours with very little change at the 4 hour and 6 hour time points (Figure 4.7). This data showed that when treated with a sublethal dose of occidiofungin, the cells could induce filamentation however fewer cells in the population were able to switch and the time needed for hyphal development required an additional hour to visually detect.

Along with the shift in time needed for switching, defects in the hyphae produced were seen at nearly every time point. The most obvious defect that was seen was a large reduction in the length of the filaments formed (Figure 4.8). The untreated group saw a steady increase in hyphal length over time, with an average length of ~23 microns at two hours and ~65 microns by four hours. This was approximately a 2.8-fold increase in length over the 2-hour period. For the occidiofungin treated group, cells have filaments of ~6 microns at two hours and ~10 microns at four hours (Figure 4.9). This represents only a 1.6-fold increase over the same period of time. This data highlights the fact that occidiofungin negatively impacts the ability of the cell to organize and grow normal hyphae structures.

The second abnormality that was identified was in the hyphal shape displayed during growth (Figure 4.10). When a normal hypha is formed there is symmetrical growth outward from the body to the hyphal tip (Sudbery, 2011). At two-hour time point, cells were identified that had

asymmetrical growth at the tip which produced a hyphae “bloat” at an average percent of 31% (Table 4.3). This abnormally began to decline by the 4-hour time point and was only seen in small numbers at six hours. However, at the six-hour time point, branching was noted in approximately 9.5% of cells (Table 4.3). The total percent of the population that presented with morphological abnormalities was determined for the 6-hour exposure time and the data presented in Table 4.3. These atypical hyphae growths suggest a disruption in the cells ability to control polarized cell growth.

Occidiofungin exposure has no effect on cell wall composition

Antifungal compounds create damage that requires changes in the cell wall composition (Hawser & Islam, 1999). Some common signs of cellular stress are upregulation of chitin and changes in mannan distribution in the cell wall. As occidiofungin was shown to upregulate chitin expression in yeast grown cells the impact of the antifungal on cells undergoing hyphal formation was determined (Emrick et al., 2013). Cells were exposed at the time of switching to 1.0X MIC dose of occidiofungin and cells were stained with calcofluor white to identify chitin or cancanavalinA-FITC to identify cell wall mannans. Unlike what was seen for yeast grown cells, no changes in the amount or localization of chitin was detected (Figure 4.13). Similar to what was observed previously (Ellis et al., 2012), no changes in mannan distribution were identified for occidiofungin exposed cells induced to undergo morphological switching (Figure 4.12).

C. albicans cells exposed to 1.0X MIC dose at the time of switching disrupts actin organization

Actin is a key structural component of the cell responsible for several steps involved in hyphae formation (Sudbery, 2011). Actin reorganization into cortical patches is a major step in establishing cell polarity for hyphae growth and the formation of F-actin cables is needed for

proper growth of the filamentous structures (Dominguez & Holmes, 2011). Finally, actin is a key part of the Spitzenkorper, a formation in the tip of the hyphae responsible for polarized elongation, which must be maintained for proper symmetrical growth (Sudbery, 2011). To look at the impact that occidiofungin has on cellular actin organization, cells were treated with a 1.0X MIC dose of occidiofungin at the point of switching and Rhodamine labeled phalloidin was used to visualize actin in cells at various stages of occidiofungin exposure. In untreated cells, actin could be detected as small actin patches generally distributed throughout the unbudded cells. After 1hour, the small actin patches started to organize into brighter patches (Figure 4.14 A). These patches become the concentrated area for filament formation by 2 hours and finally formed a bright actin patch at the tip of the hyphae (Figure 4.14 C). In contrast, cells that were exposed to occidiofungin never reorganized their actin having only small punctate patches through the cell body (Figure 4.14 B). This lack of any reorganization to the actin architecture is likely the reason behind the absence of hyphae formation in these cells.

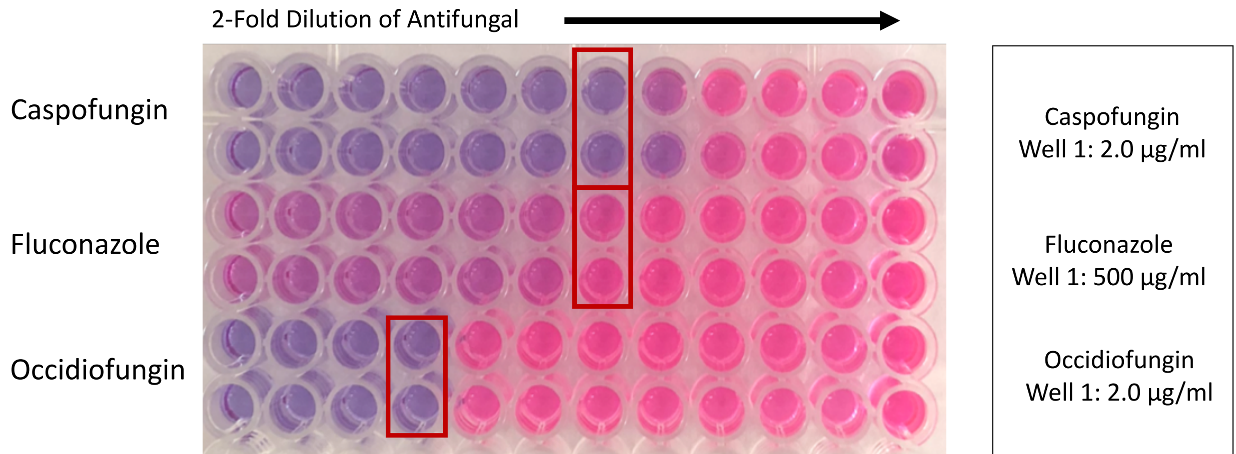

Figure 4.1 Resazurin MIC Assay

Concentration dependent antifungal activity of Occidiofungin, Caspofungin, and Fluconazole. Assay performed in YPD for 48 hours at 37˚C with resazurin metabolic activity indicator. A change in color from blue to pink indicates metabolic activity associated with viable cells. The MIC value is the well that has the lowest dose of antifungal that retains its blue color. Red boxes indicate the 1.0X MIC dose.

Table 4.1 Antifungal Sensitivity

Antifungal	10^4 cells/ml	10^5 cells/ml	10^6 cells/ml
Occidiofungin	0.125μg/ml	0.25μg/ml	1.0μg/ml
Caspofungin	0.062μg/ml	0.031μg/ml	0.031μg/ml
Fluconazole	3.9mg/ml	7.8mg/ml	>12.5mg/ml

Antifungal sensitivity is cell density dependent. Cells from a 48hr saturated culture were diluted into Spider media to achieve $1x10^4$, $1x10^5$, or $1.6x10^6$ cells/ml for use in a microtiter-based MIC assay. Resazurin was included to identify wells with viable cells. The MIC value was identified as the well with the lowest concentration of antifungal that inhibited growth. Data from 3 independent experiments are shown.

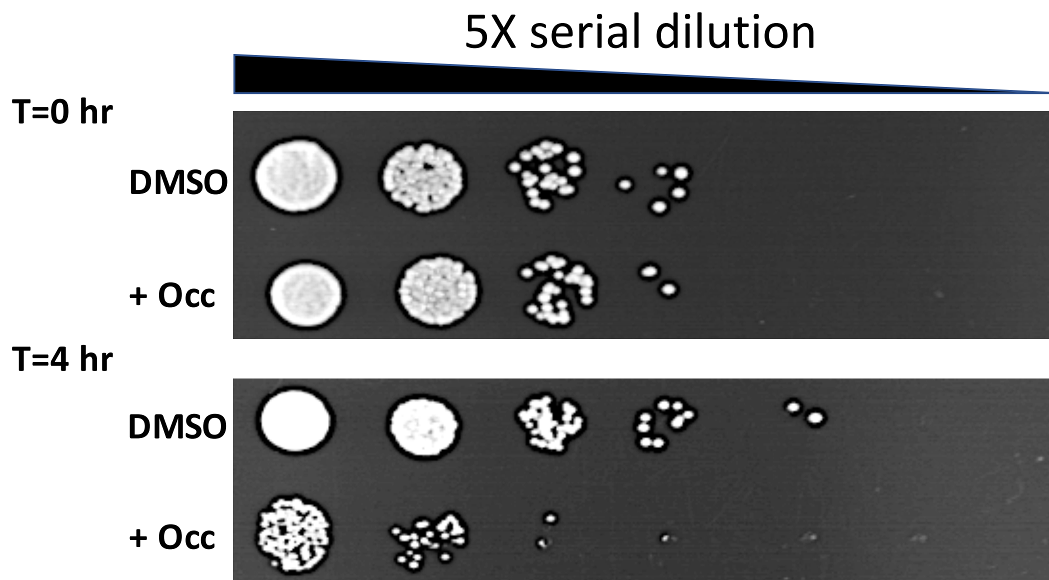

Figure 4.2 Spotting Assay for Cell Growth

Cells from a saturated culture of *C. albicans* were diluted into Spider media with occidiofungin (1.0X MIC) or vehicle control (DMSO) and placed at 37˚C. Samples were removed at the indicated time points, 5-fold serially diluted, and spotted to YPD. Photos were taken after 24hr-48hr of growth at 30˚C. A representative image is shown.

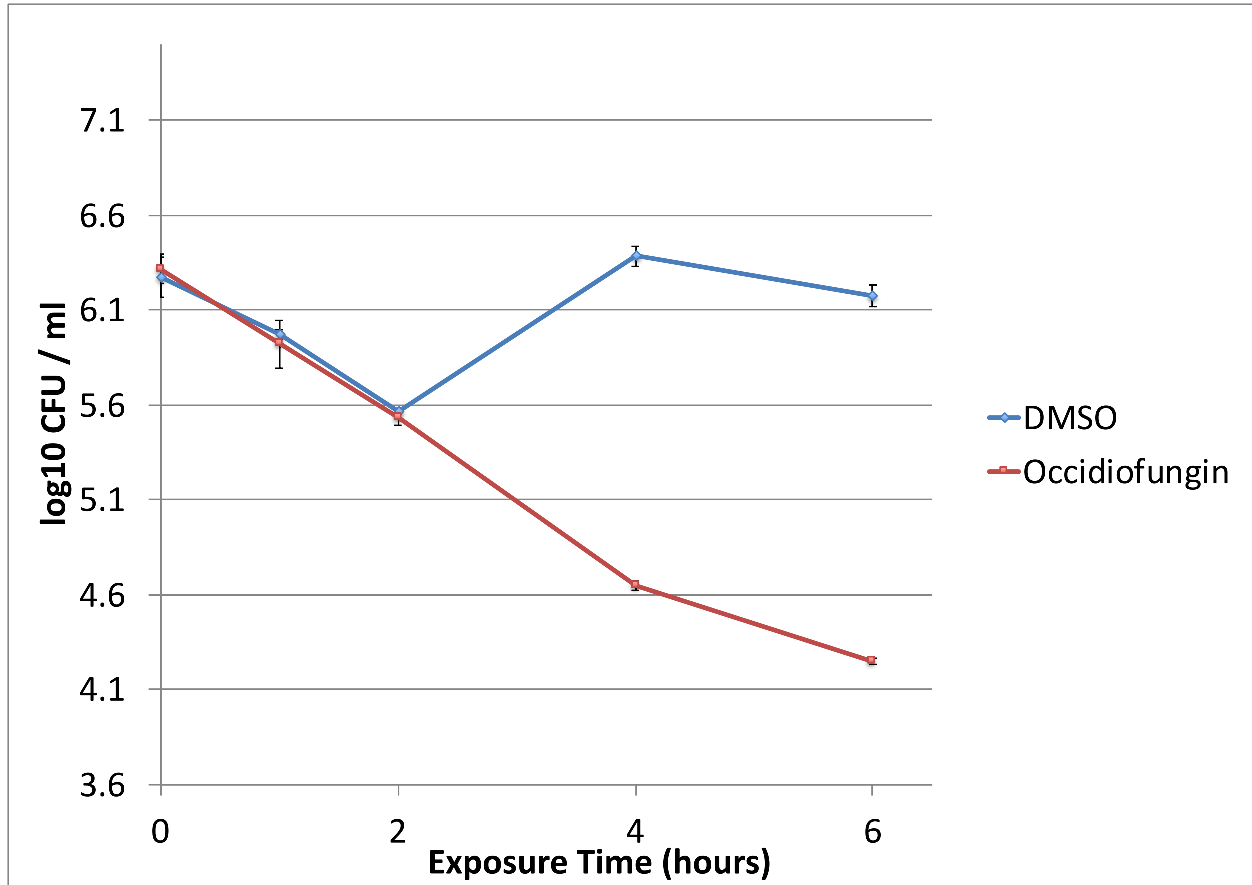

Figure 4.3 Cell Growth Analysis by Colony Forming Unit Assay

Cells were placed in switching media to achieve a density of 0.05 OD_{600}/ml. DMSO or occidiofungin at 1.0X MIC (1.0µg/ml) was added T=0. Samples were removed at defined time points over the 6hr period, serially diluted, and select sample dilutions spread onto YPD plates (3 plates/well; 2-3 wells/treatment regime). Following 48hr of growth at 30˚C the number of colonies per plate were determined. The colony forming units per ml was calculated and the data plotted for each time point analyzed.

Table 4.2 Percent Cell Death

Time (hr)	
0	-0.11 ± 0.58 (n=3)
1	4.82 ± 3.74 (n=3)
2	1.69 ± 1.09 (n=3)
4	18.32 ± 8.23 (n=3)
6	31.75 ± 9.53 (n=3)

Percent cell death induced by occidiofungin exposure. Values determined based on the accumulated CFU data, collected as described in Figure 4.3 for 3 independent experiments.

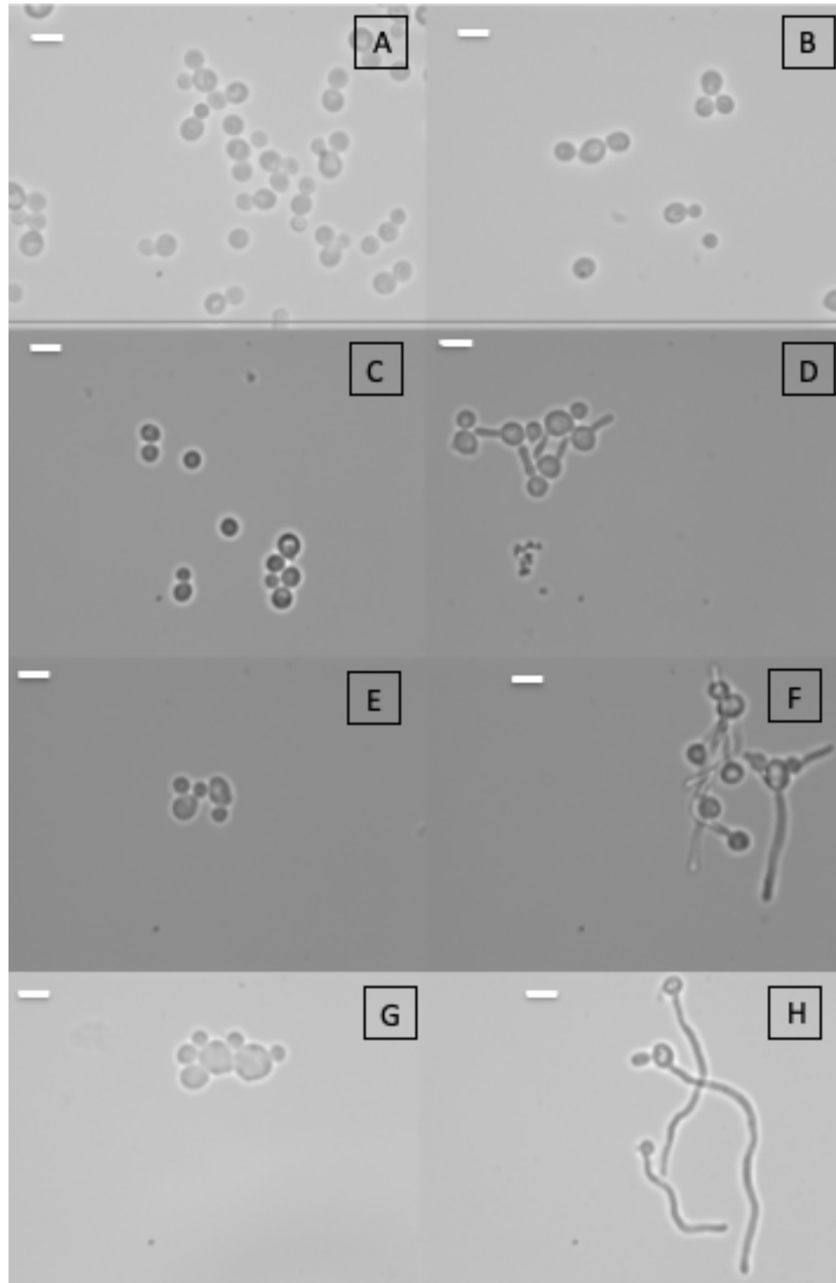

Figure 4.4 Morphology of *C. albicans* by Light Microscopy

Cells from a saturated culture were diluted into fresh media to achieve a cell density of 0.05OD_{600}/ml. Occidiofungin (A, C, E, and G) at 1X MIC dose (1.0 µg/ml) or DMSO (B, D, F, and H) was added and samples incubated at 37˚C to induce switching. Aliquots were removed at 0hr (A, B), 1hr (C, D), 2hr (E, F), and 4hr (G, H) post switching for visualization by 100X light microscopy. Size bar= 5µm

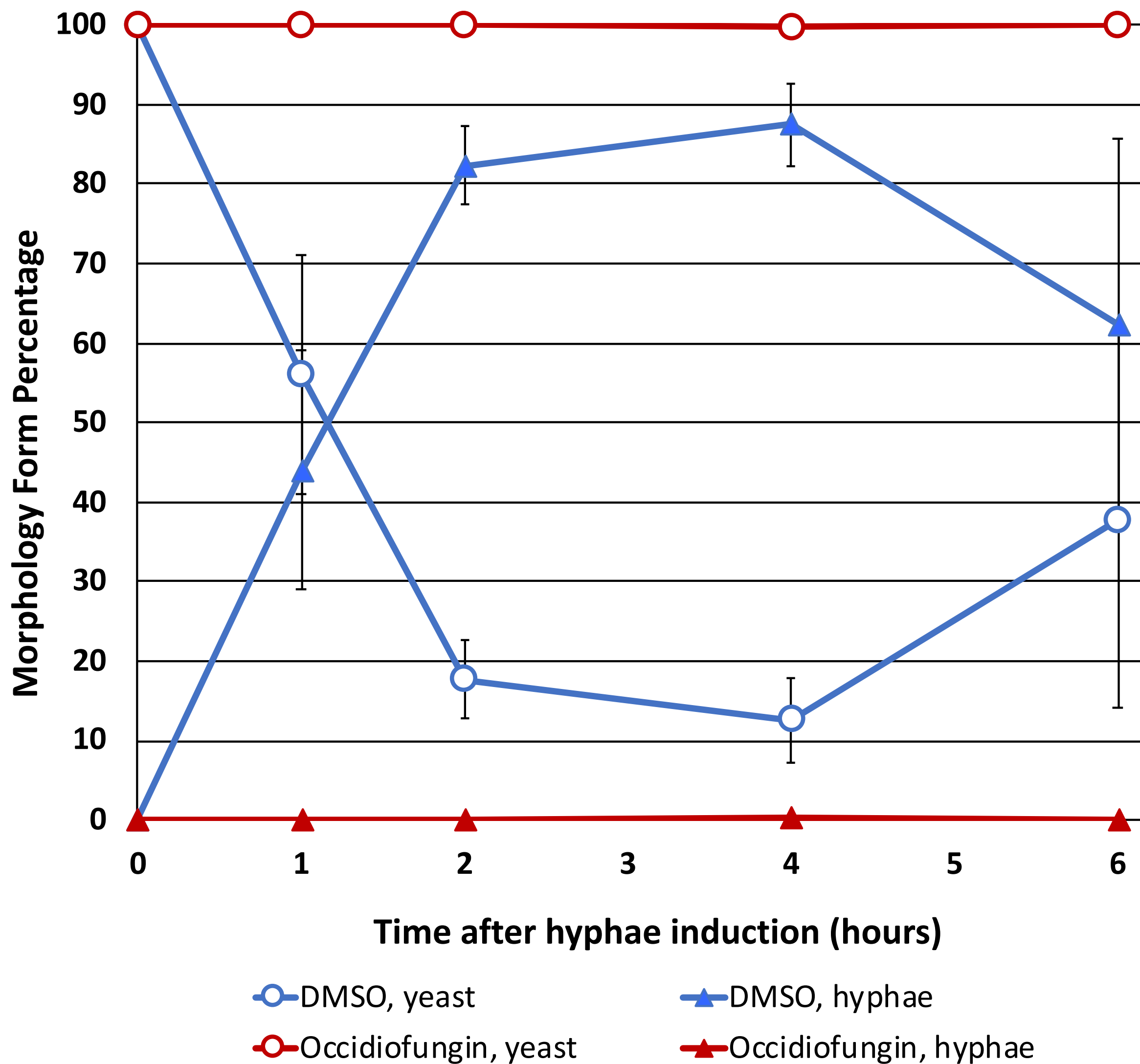

Figure 4.5 Percent Morphology 1.0X MIC

Occidiofungin prevents morphological switching of *C. albicans*. Cells were prepared as described in Figure 4.4. The percentage of cells in the population having either a yeast (open circles) or hyphae morphology (filled triangles) were calculated. At least 200 cells were counted for each time point. The average and standard deviation for data collected from 4 independent experiments is shown.

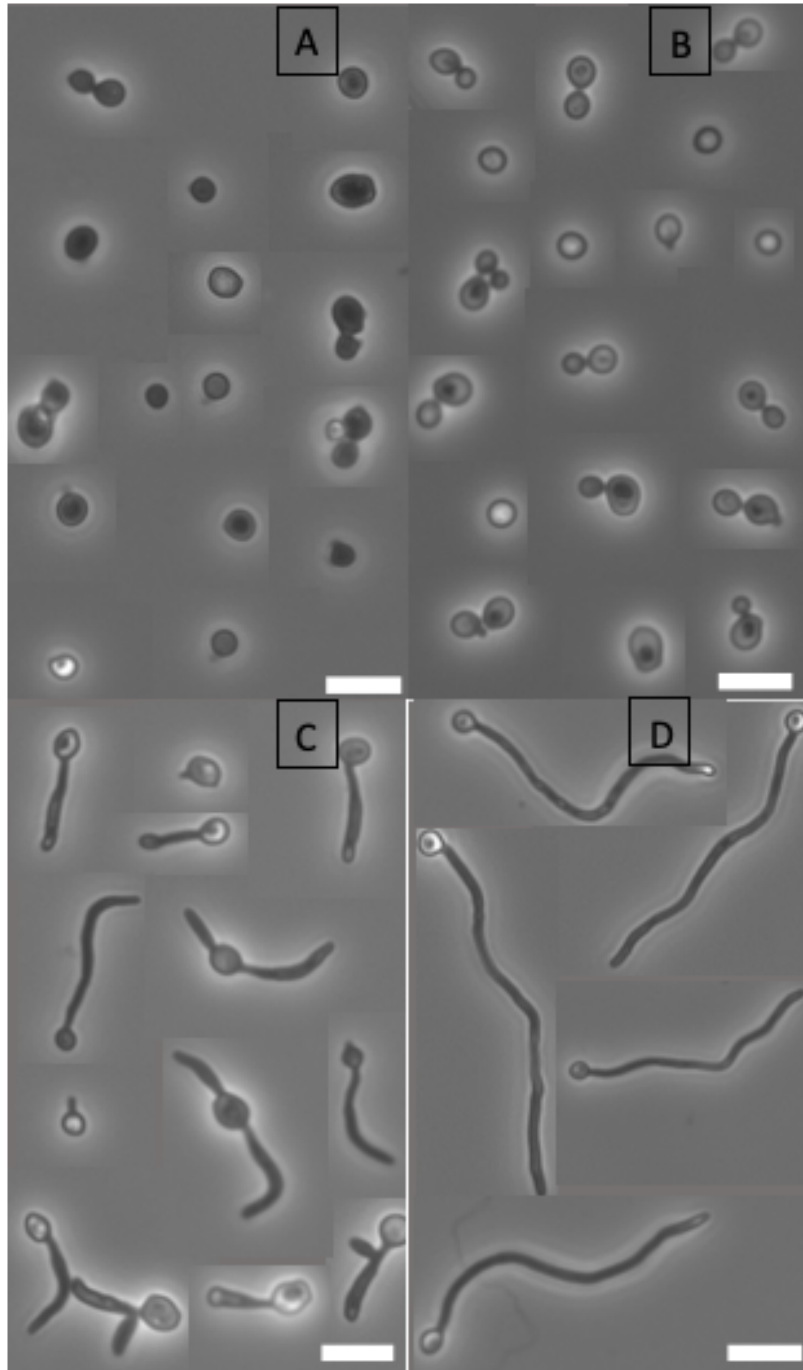

Figure 4.6 Percent Morphology of *C. albicans* 0.5X MIC

Cells from a 1 x 10^5 cells per ml culture were induced to switch with fresh media and incubated at 37˚C with samples taken at hour-based time points. A montage where A) T=0 hours untreated, B) T=0 hours, 0.5X MIC occidiofungin treatment, C) T=4 hours, untreated, D) T=4-hour, 0.5X MIC occidiofungin treatment. Size bar = 5µm

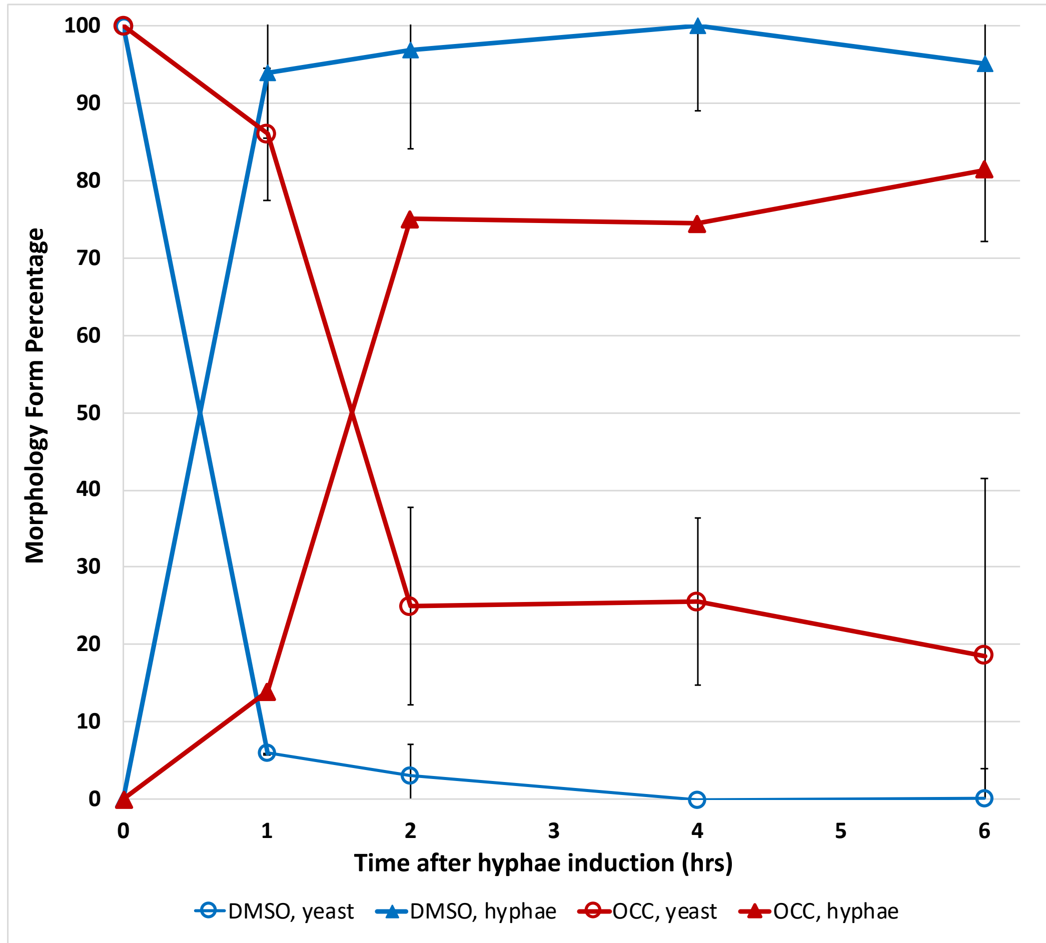

Figure 4.7 Percent Morphology 0.5X MIC

Occidiofungin delays morphological switching. At a sublethal dose, cells were prepared as described in Figure 4.4 except the occidiofungin was used at 0.5X MIC 0.25µg/ml and culture are 1 x 10^5 cell/ml. The percentage of cells in the population that were yeast (open circles) or hyphae (filled triangles) were determined. At least 200 cells were counted for each time point per group. The average and standard deviation for data collected from 4 independent experiments is shown.

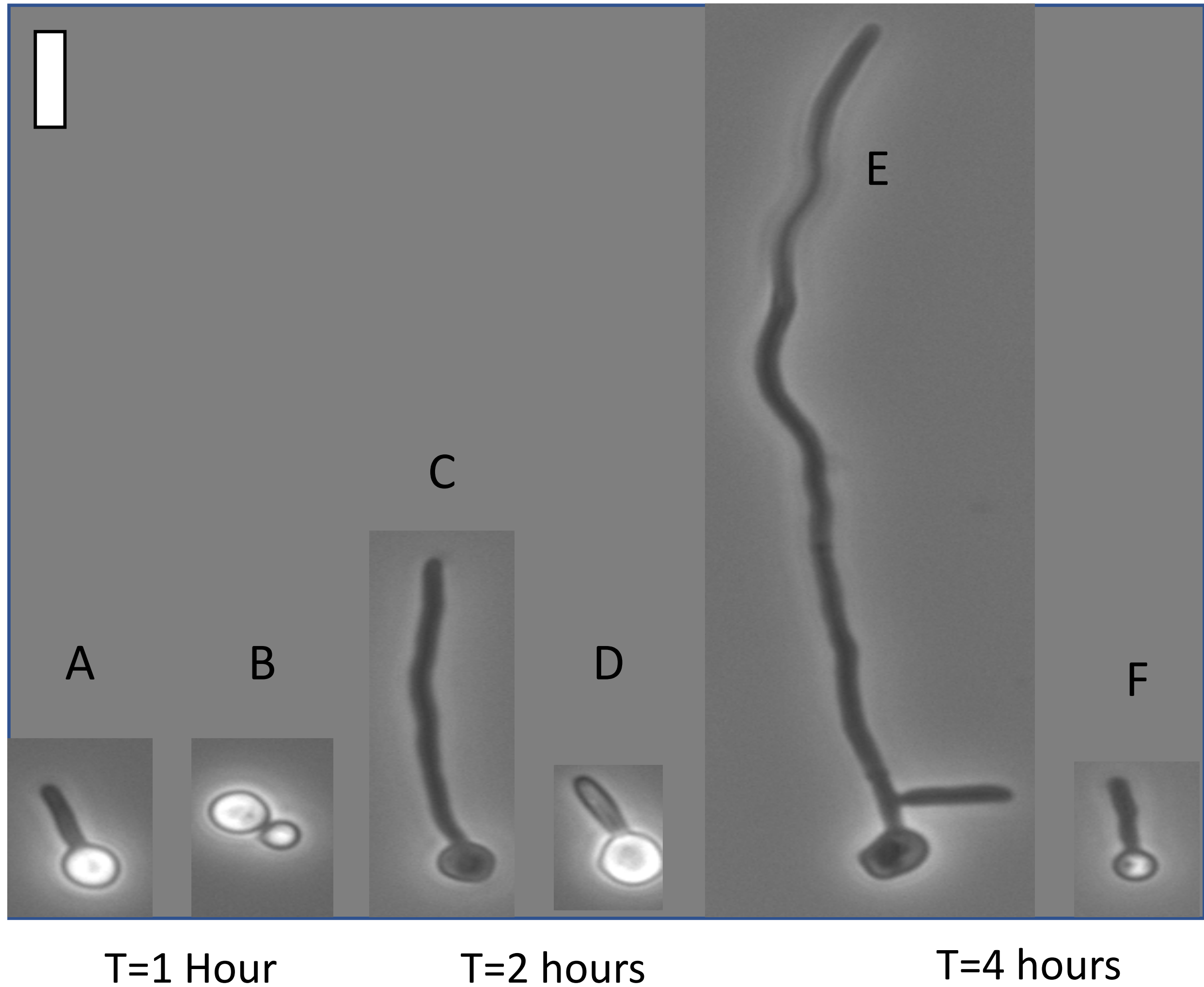

Figure 4.8 Hyphae Length Comparison 0.5X MIC

Cells from a saturated culture were diluted into Spider media to achieve a density $1x10^5$ cells/ml. The culture was split with one sample receiving occidiofungin at a dose of 0.25µg/ml (0.5X MIC) and the other an equivalent volume of DMSO. Cells mounted with Vecta shield on glass slides and viewed by 100X light microscopy. An example image characteristic of cells at the indicated time points are shown for untreated (A, C, and E) and occidiofungin treated (B, D, and F) cells at 1, 2, and 4 hours post switching. Size bar = 10 µm.

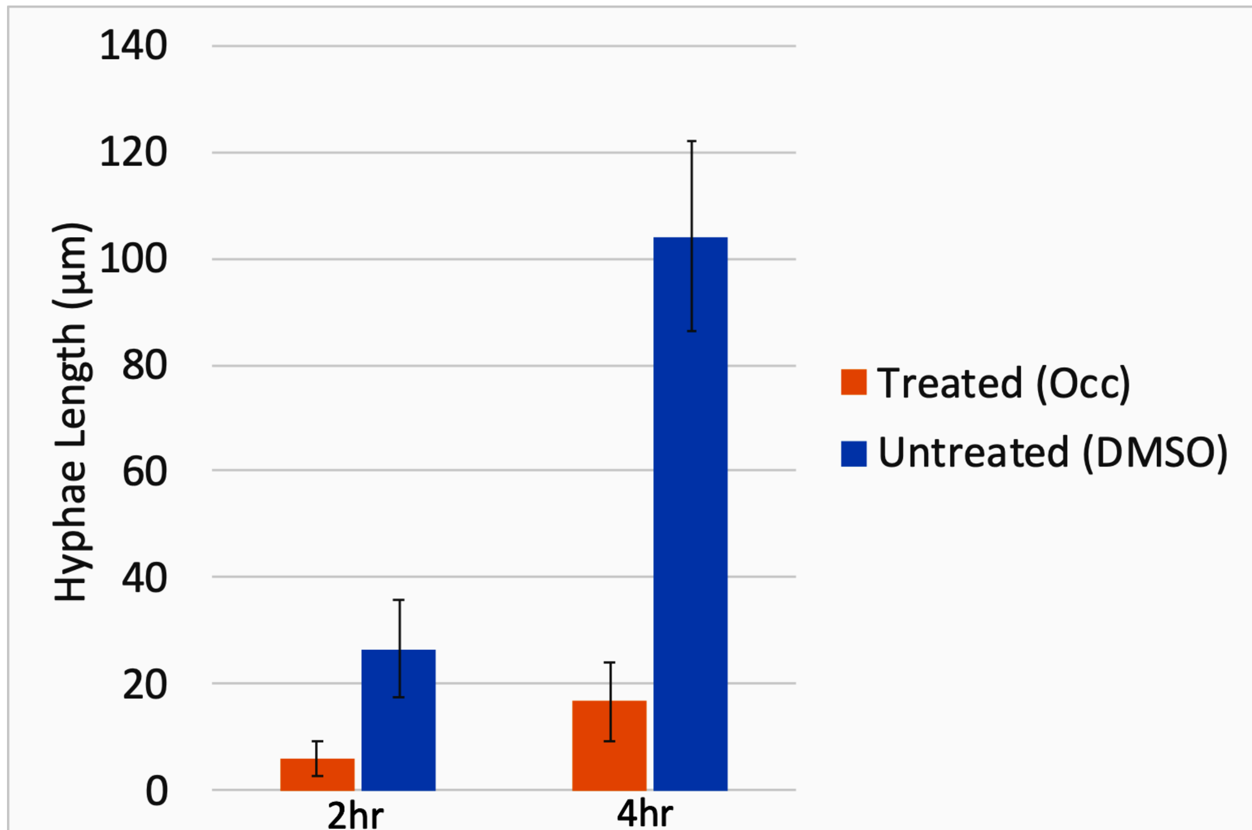

Figure 4.9 Hyphae Length Graph 0.5X MIC

A sublethal dose of occidiofungin slows hyphae formation. Hyphae length in untreated and 0.5X MIC occidiofungin treated cells were determined for cells processed as described in Figure 4.8. Hyphal lengths were measured using NIH ImageJ with a minimum of 50 cells per time point per sample. Data for the average and standard deviation from 2 independent experiments are shown.

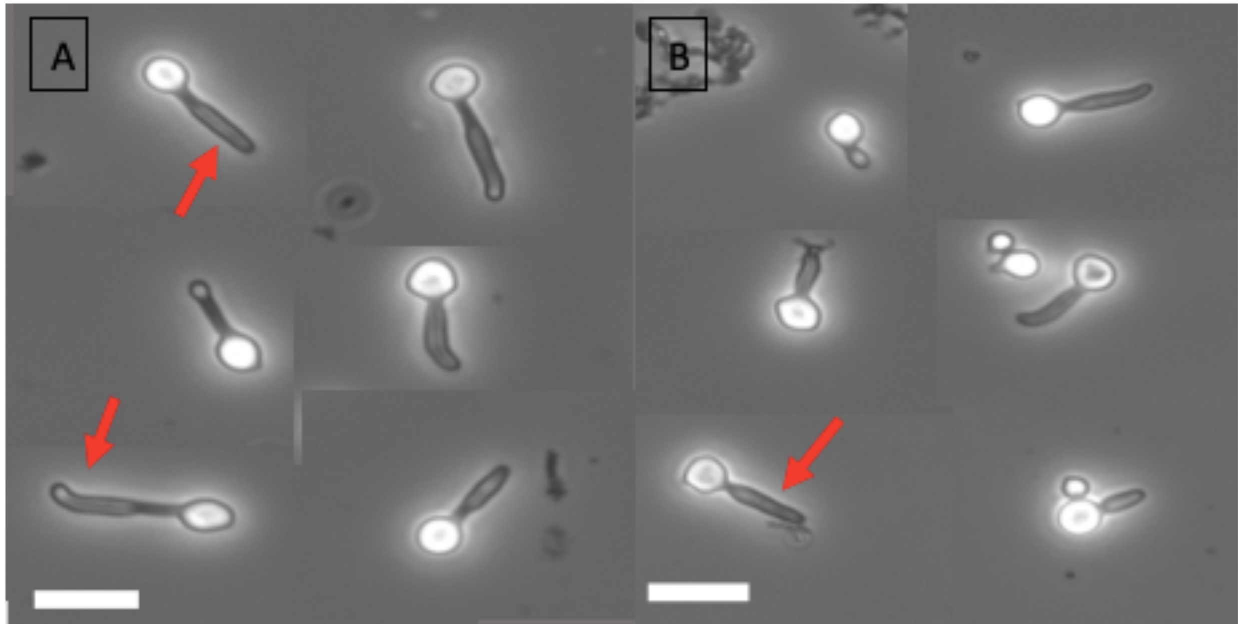

Figure 4.10 Morphological Defects 0.5X MIC

C. albicans cells treated with a sublethal dose of occidiofungin as viewed by light microscopy. Cells from a saturated culture were diluted into Spider media to achieve a density $1x10^5$ cells/ml. The culture was treated with 0.25µg/ml occidiofungin (0.5X MIC) for 2 hours. A montage of cell that exhibited loss of polarized tip growth resulting in “hyphal tip bloat” are shown. Cells with these morphological defects make up ~30% of the population at this time point. Size bar=10 µm.

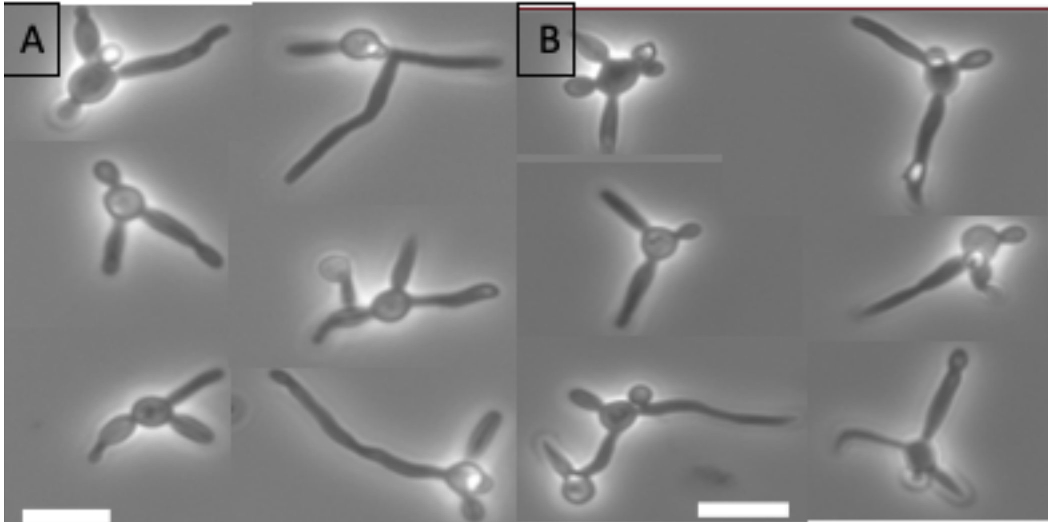

Figure 4.11 Morphological Defects 0.5X MIC

C. albicans cells treated with 0.25µg/ml occidiofungin (0.5X MIC) at the time of switching as viewed by light microscopy. Cells from a saturated culture were diluted into Spider media to achieve a density $1x10^5$ cells/ml and maintained in the presence of the antifungal compound for 6 hours. Cells exhibiting a loss of polarized hyphal growth which includes hyphal bloat and the formation of multiple filaments are shown. These morphological defects make up ~3% and ~9% of the cell population, respectively. A montage of images from 2 independent experiments are shown (A and B). Size bar=10 µm.

Table 4.3 Total Morphology Defects 0.5X MIC

Time Point	**11-11-18**	**11-18-18**	**Average(n=2)**
T=1 hr	Hyphae Bloat: 3 Abnormal Hyphae Branching: 0	Hyphae Bloat: 3 Abnormal Hyphae Branching: 0	Hyphae Bloat: <0% Abnormal Hyphae Branching: 0%
T=2 hr	Hyphae Bloat: 106 Abnormal Hyphae Branching: 0	Hyphae Bloat: 67 Abnormal Hyphae Branching: 0	Hyphae Bloat: 31% (+/- 15%) Abnormal Hyphae Branching: 0%
T=4 hr	Hyphae Bloat: 1 Abnormal Hyphae Branching: 11	Hyphae Bloat: 26 Abnormal Hyphae Branching: 7	Hyphae Bloat: 5% (+/- 6%) Abnormal Hyphae Branching: 3.5% (+/-1%)
T=6 hr	Hyphae Bloat: 1 Abnormal Hyphae Branching: 45	Hyphae Bloat: 18 Abnormal Hyphae Branching: 18	Hyphae Bloat: 3% (+/-4%) Abnormal Hyphae Branching: 9.5% (+/- 4%)

Total cell counts: 11-11-18 T=1 (135); T=2 (255); T=4 (258); T=6 (354)
11-18-18 T=1 (493); T=2 (330); T=4 (259); T=6 (293)

Hyphae abnormalities seen by light microscopy in *C. albicans* cells treated with 0.5X MIC occidiofungin. Cells processed as described in Figure 4.10. The total number of abnormality types identified, and their average percent of the total cell population are presented. Data acquired from 2 independent experiment; standard deviation.

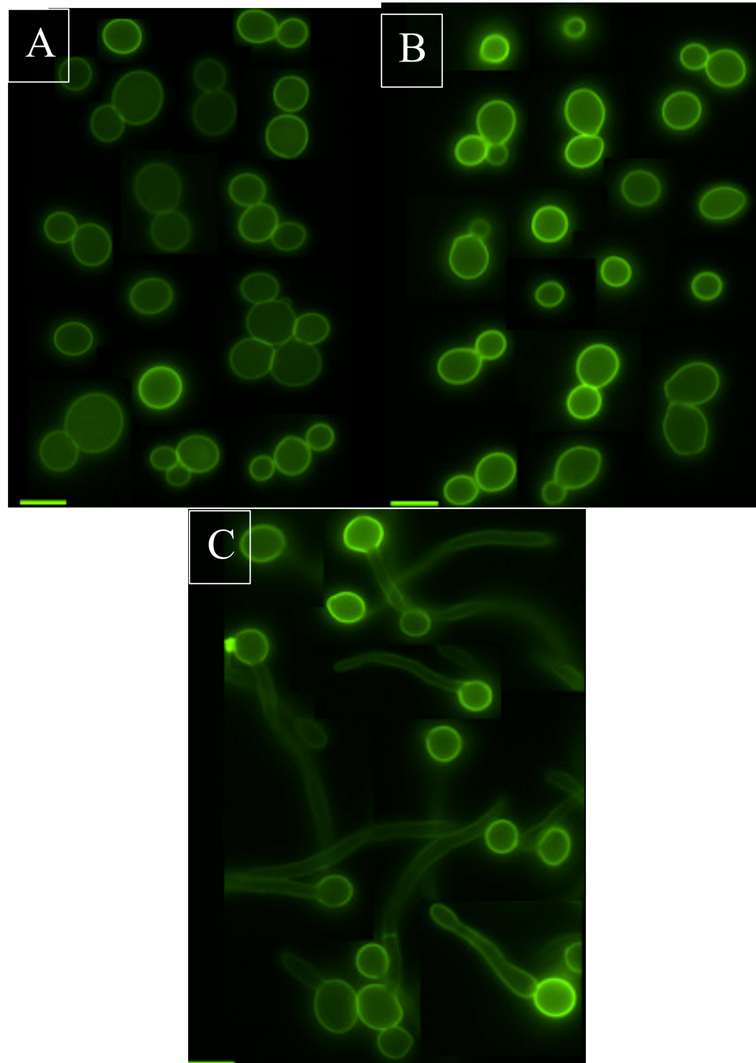

Figure 4.12 Mannan Distribution 1.0X MIC

C. albicans cells induced to undergo morphological switching visualized by concanavalinA-FITC. Cells diluted into Spider media to promote hyphae formation were treated with occidiofungin (1X MIC 1.0µg/ml) in a culture of 0.05 OD_{600} (B) or with an equivalent volume of DMSO (A, C). Samples were collected at T=0hrs (A) and 4hrs (B, C), fixed in formaldehyde, followed by treated with concanavalinA-FITC. Cells were visualized by immunofluorescence microscopy using 100X magnification. Size bar = 5 µm.

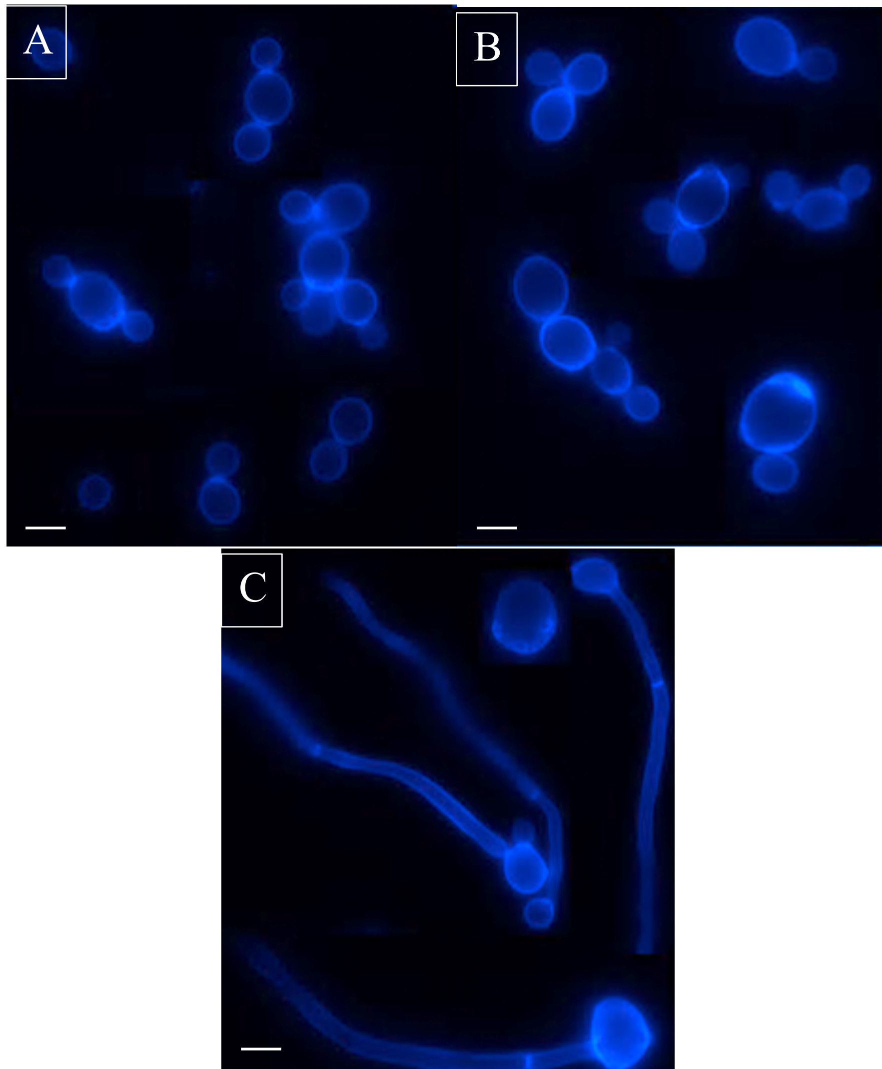

Figure 4.13 Chitin Distribution 1.0X MIC

Fluorescence microscopy of cells stained by calcofluor white. *C. albicans* cells induced to undergo switching were treated with occidiofungin (1.0X MIC 1.0 µg/ml) in a culture 0.05 OD_{600} (B) or DMSO, the vehicle control (A, C). The distribution of chitin was determined for untreated cells at T=0hrs and cells exposed to occidiofungin (B) or DMSO (C) for 4 hours. Size bar=5 µm.

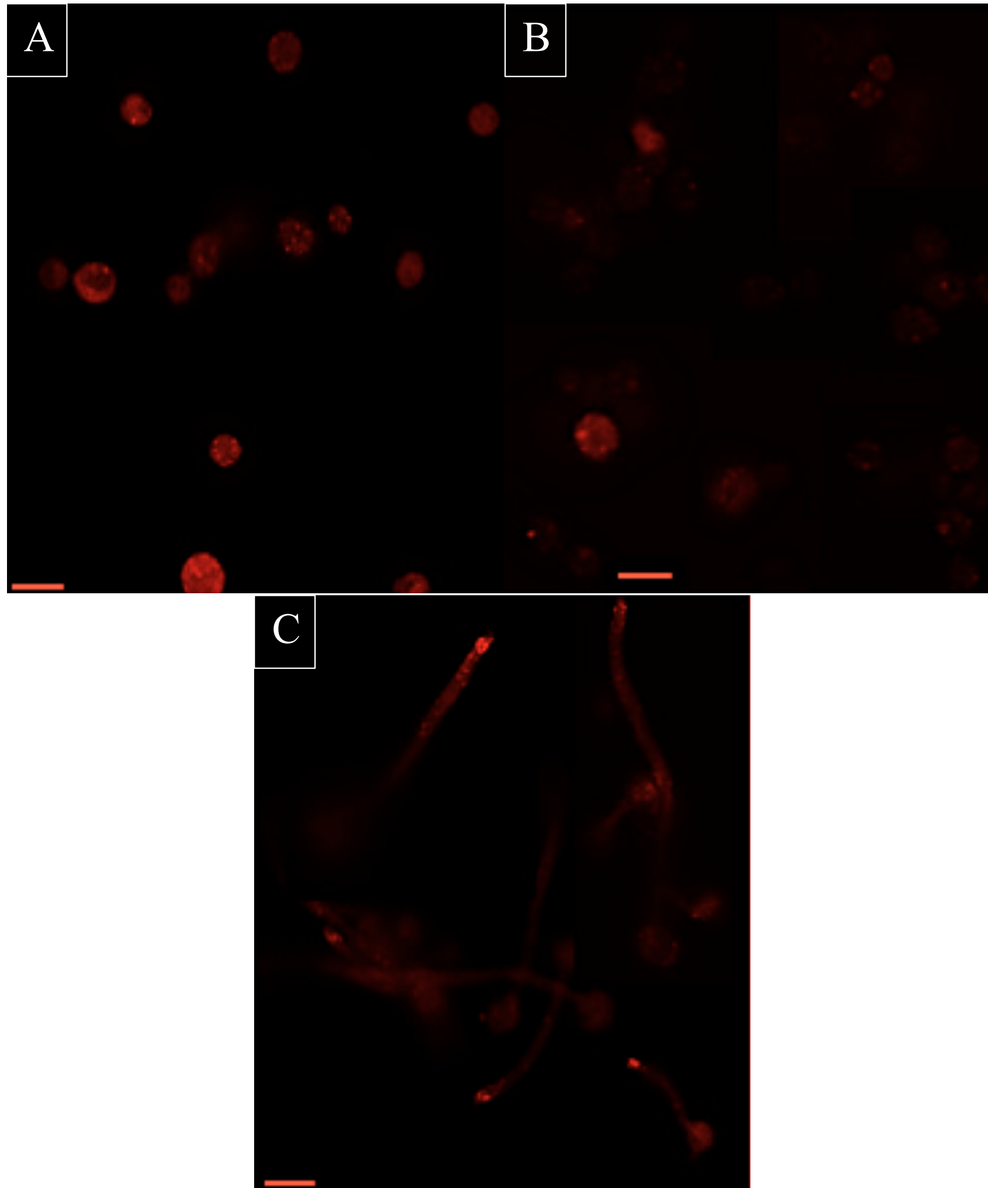

Figure 4.14 Actin Distribution 1.0X MIC

Fluorescence microscopy of actin treated and untreated cells by rhodamine-phalloidin staining. *C. albicans* cells induced to undergo switching were treated with occidiofungin (1.0X MIC 1.0 µg/ml) in a culture 0.05 OD_{600} (B) or DMSO, the vehicle control (A). The distribution of actin was determined for untreated cells at T=0hrs and cells exposed to occidiofungin (B) or DMSO (C) for 4 hours. Size bar=5 µm.

CHAPTER V

CONCLUSIONS/DISCUSSION

The collection of data from these experiment helps establish occidiofungin as a potential antifungal compound for the treatment of candidiasis. Opportunistic pathogens, such as *C. albicans*, as natural components of the human microbiome pose a unique set of concerns. When patients undergo a medical treatment that leaves their immune system in a compromised state, limiting potential exposure to environmental pathogens can be accomplished. However, when the pathogen is part of the natural flora it becomes impossible to simply limit exposure to prevent infection. Having reliable choices in the treatment of these kinds of infections is an answer to this problem.

The first important step in evaluating potential treatments is to determine how effective the compound is at eliminating the target pathogen. Previous work has shown that the antifungal compound, occidiofungin, was effective against the yeast form of the nonpathogenic fungi, *S. cerevisiae*, as well as various *Candida* species (Emrick et al., 2013; Robinson et al., 2017). For *C. albicans* to invade and damage host tissue it must undergo a morphological switch from the yeast form into a filamentous morphology (Phan et al., 2000). This fact formed the basis for these studies.

Multiple approaches demonstrated that occidiofungin was effective against cells that were induced to undergo morphological switching. Specifically, MIC assays revealed occidiofungin has dose- and cell density-dependent fungicidal activity. Most importantly, the data found that the dose needed for antifungal activity was lower for cells undergoing switching than that of cells in yeast form. CFU assays established that fungicidal activity occurred when cells were in switching media for two hours. When this data is compared to data showing that

yeast cells that are in quiescence show a higher resistance to occidiofungin (Robinson et al., 2017), a link between cell growth and occidiofungin effectiveness can be made. Cells placed into fresh media following growth to saturation likely require time for cells to activate cellular activities to resume an active growth phase. This suggests a number of possibilities with regards to occidiofungin bioactivity. It is possible that its cellular entry, which is still unidentified, is mediated by the mechanics of cellular growth. It is also possible that the compound's target is only present, or only required for viability, when the cell is actively growing. When a cell has switched to a filamentous morphology growth is polarized and focused to the tip of the hyphae (Sudbery, 2011). A key part of the sustained growth in both filamentous and yeast morphology is the recycling of material by endocytosis (Galletta et al., 2010). This increase in the amount of material being taken up from outside could be a point of entry for occidiofungin. Thus, when only minimal uptake or no uptake by endocytosis takes place, a reduced amount of compound would gain entry to the cell resulting in a reduction in the effective action of the compound. A return to active growth would lead to enhanced endocytosis and a lower dose of the compound would be needed for fungicidal activity. The second possibility is that the target for occidiofungin is only present or essential for viability when the cell is growing. For example, some signaling pathways are only triggered by stimuli under specific conditions such as the switch from yeast to hyphae morphology. This signaling pathway activates a specific set of hyphae-related genes that may not be expressed otherwise (Sudbery, 2011). This ensures that a cell is not expending energy on production of components that are not necessary or waste the materials needed to synthesize them. In the case of occidiofungin, the fact that cells can be targeted as yeast and hyphae suggests that the target would be present in both cell types.

Having established that occidiofungin is effective against cells undergoing hyphae switching, we next analyzed the impact on switching morphology. When exposed to a lethal dose of occidiofungin, treated cells never formed hyphae over the 6-hour time frame of analysis. Trypan blue staining confirmed ~60% of cells at 6hrs of exposure were viable, which means the cells had the opportunity to undergo a morphological switch but were unable to. This data suggests that the target within the cell for occidiofungin is essential for hyphae morphology. While there is more than one target that meets both the active growth and hyphae formation requirements, there is one very important element that is necessary for both: actin and its associated proteins. Prior published work indicates that occidiofungin specifically targets actin, binding to actin in *in vitro* assays and disrupting actin-based activities in *in vivo* studies (Ravichandran et al., 2019). One of the key features of cellular growth is the delivery of material to growing cell surfaces. Actin is a major player in both the uptake of extracellular materials for growth and the delivery of those materials to the polarized areas of growth (Arkowitz & Bassilana, 2011). When a cell exits quiescence, actin must be reorganized to both form the cortical patches for endocytosis and provide the pathway for movement of materials by vesicle transport. Thus, any disruption of the actin organization or protein movement along actin will block the cell from the formation of a filament. This suggests that if occidiofungin blocks the transition into filaments of even cells that are not killed by the initial dose, these remaining cells will be unable to invade and damage tissues (Pukkila-Worley et al., 2009). From a treatment standpoint, this would mean that less damage would occur in the time needed to resolve the infection making the compound more attractive as a therapeutic.

Prior work had demonstrated that *S. cerevisiae* cells have a loss of F-actin cables when exposed to occidiofungin (Ravichandran et al., 2019). Based on these findings, the impact of occidiofungin on *C. albicans* during morphological switching was analyzed in this study. Normally when a cell begins filamentous growth, actin is reorganized from small cortical patches that are evenly spread thought the cell surface to a central polarized patch (Sudbery, 2011). From that patch, F-actin cables form that run the length of the filament with the central patch of actin at the tip of the growing hypha. In cells exposed to a 1X MIC dose of occidiofungin, this reorganization from small cortical patches to a central polar location failed to take place even after six hours. This data confirms that actin organization is disrupted with occidiofungin exposure. In cells exposed to a sublethal dose of occidiofungin data indicated that the timing of switching (as determined by morphology) was delayed by around an hour. Whereas untreated cells had a switchover (50% filament/50%yeast) 30 minutes following switching induction, in cells exposed to sublethal levels of occidiofungin this switchover occurred approximately two hours after placement in switching inducing conditions. In addition to altering the timing of switching, fewer cells formed hyphae (80% versus 95-100% in untreated). This data suggests that occidiofungin partially inhibited cells from making the switch to filamentous growth. This may be because the lower dose allows some cells to compensate for the disruption in actin dynamics before the damage is permanent. However, this compensation does not come without consequences for the growing cells as *C. albicans* cells acquired morphological defects during filament formation. This was identified as a loss of polarized tip growth which generated tip "bloat". This may be due to loss of polarized cell growth with addition occurring on the sides of the hyphae as well as the tip. As discussed before, actin is a key element in the polarized delivery to the growing filament tip (Sudbery, 2011). The loss of proper actin structures could result in

either improper location of growing cell surface materials or the buildup of materials within the hyphae without addition to the growing cell surface. Both of these events would result in multidirectional expansion of the filament as opposed to polarized growth. This abnormality was found to decrease over time which may suggest that these cells have rescued polarized growth within this time frame. An additional morphological abnormality appeared which happened to coincide with the reduction in the "bloat" effect. This was abnormal branching of the formed filaments. Several of the filaments had a range of branching issues such as too many filaments formed at the cell body and filaments growing from abnormal locations on an existing filament. These abnormalities represented around 10% of filaments visualized at six hours after exposure. The formation of a polarized actin patch is a key step in the proper formation of filaments. This finding adds more support to the idea that occidiofungin disrupts the normal cellular actin dynamics as proper filament formation to a polarized location requires actin and actin associated proteins. The final abnormality identified was a profound reduction in the length of all filaments formed with occidiofungin exposure. Untreated cells formed filaments that were around 100 micrometers in length by six hours after switching. The average length of filaments in the sub-lethal dosing group were ~18-19 micrometers which is a close to a 3-fold reduction in the length of the filaments. This data suggests that while hyphae initiation may be recovered, the normal growth of the resulting filament is severely impacted. This collection of data on sub-lethal dosing points to irreversible damage that is done to normal filament formation when exposed to lower concentrations of occidiofungin. When looking for treatment options for a pathogen whose virulence is dependent on this morphology, occidiofungin may represent a promising option. If treatment of sublethal dosing of occidiofungin creates damage to filaments but allows for the survival and function of the yeast form, occidiofungin may reduce the damage done to the

beneficial flora. This could reduce the amount of side effects seen with chronic antifungal treatments that many patients must endure. More experiments into the nature of these defects, whether *C. albicans* with these morphological defects have altered virulence, and morphological effects of occidiofungin when used at even lower doses are called for to better explore this data.

The combined findings of this study have shown that occidiofungin is effective as an antifungal compound against *C. albicans* cells that are undergoing morphological switching. This antifungal activity results in the death of cells and the inhibition of surviving cells to switch into the morphology that is considered virulent. This is likely mediated through its impact on altering the organization of actin in these cells, possibly generating an inhibition or delay in switching events that alter cell morphology. When these findings are combined with previous work showing acceptable toxicity in mouse models, tolerance to low pH (important for oral applications), and a broad range of fungal species effected, the potential applications for occidiofungin could be numerous (Ellis et al., 2012; Tan et al., 2012).

There are a number of potential therapeutic uses for an antifungal compound with the properties exhibited by occidiofungin. For example,

1. When applied to the site of dental implants a reduction in post implant candidiasis may result.
2. Use as a pretreatment for IV lines susceptible to contamination by fungal colonization might lower hospital acquired fungal infections.
3. Inoculation of plants with the bacteria that naturally produces occidiofungin could reduce crop loss from fungal pathogens without the need for more potentially toxic pesticides.

4. Treatment of difficult fungal diseases in domestic animals such as blastomycosis. A potentially fatal fungal disease where the current treatments can have harsh and even fatal side effects, could have improved outcomes with occidiofungin.

Continued research into occidiofungin is necessary to discover just how many of these applications, if any, are viable. To this end, work must continue to determine the effects occidiofungin has on cells post switching. The structural changes in actin organization in cells treated with a sublethal dose of occidiofungin, and the impact of occidiofungin on biofilm formation remain to be determined. These and many more questions must be asked to achieve a true understanding of how occidiofungin will fit into its role as a future antifungal agent. We believe that the findings in this study warrant the continued pursuit of research endeavors to answer all of these questions.

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