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Impact of environmental and cellular factors on the bioactivity of a novel antifungal, occidiofungin

By

Chase Alexander Robinson

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

August 2015

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Impact of environmental and cellular factors on the bioactivity

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Title of Study: Impact of environmental and cellular factors on the bioactivity of a novel antifungal, occidiofungin

Pages in Study: 65

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Occidiofungin is a novel glycolipopeptide, synthesized and secreted by *Burkholderia contaminans* MS14, demonstrating broad-spectrum antifungal activity and potential for successful clinical applications. Its mechanism of action has not yet been determined but is known to exhibit fungicidal activity via the induction of apoptosis in a manner unique from that of currently approved antifungals. As an early investigation into occidiofungin's mechanism of action, we aimed to identify environmental and cellular factors that significantly alter the susceptibility of the model organism, *Saccharomyces cerevisiae*. To that end, we have demonstrated that occidiofungin's bioactivity requires active cellular growth, that new protein synthesis is necessary to adequately respond to occidiofungin exposure, and that alterations in transcriptional regulation in response to glucose and phosphate deprivation have synergistic and antagonist consequences, respectively, on occidiofungin's effectiveness. Together, this data provides a foundation on which occidiofungin's mechanism of action can be illuminated.

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CHAPTER I

INTRODUCTION

Invasive Fungal Infections

An Invasive Fungal Infection (IFI) is the infection of blood, normally sterile body fluids, tissues, and organs by fungal organisms. These are typically classified as being either endemic or opportunistic in nature, and their classification is an indication as to how the infection was acquired (Pfaller and Diekema 2007). Endemic IFIs are associated with exposure to a fungal pathogen in its natural environment, thus the causative organisms vary according to geographical region. In the United States, endemic IFIs are most commonly caused by *Histoplasma capsulatum* and *Blastomyces dermatitidis* in southern and midwestern states, and by *Coccidioides immitis* in southern and western regions (Chu, et al. 2006). The organisms of endemic IFIs capably infect immunocompromised patients, but differ from their opportunistic counterparts in their propensity to infect healthy hosts. One population-based study, conducted on a national database of hospital inpatient stays in 2002, found that 13% of patients who died from endemic IFIs were likely already at an increased risk of mortality due to serious underlying conditions, while the remaining 87% were healthy and immunocompetent prior to infection onset. This study also found a relatively low rate of mortality for endemic IFIs, with crude mortality rates for children and adults at 5% and 7%, respectively (Chu, et al. 2006). The same was not found for opportunistic IFIs.

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Opportunistic IFIs, most commonly caused by *Candida* spp., *Cryptococcus neoformans*, *Pneumocystis jirovecii*, and *Aspergillus* spp., are of particular concern because almost all are Healthcare Associated Infections (HAIs) (Pfaller and Diekema 2010). The Centers for Disease Control and Prevention (CDC) defines an HAI as "a localized or systemic condition resulting from an adverse reaction to the presence of an infectious agent(s) or its toxin(s) that was not present on admission to the acute care facility" (The Centers for Disease Control and Prevention 2014). In order to more accurately classify HAIs for epidemiological purposes, these infections are further divided into two subclasses, Community-Onset (CO) and Healthcare Facility-Onset (HO; formerly termed "nosocomial"), based on the period of time between a patient's admission to a healthcare facility and the onset of infection (before and after 72 hours post-admission, respectively) (National Healthcare Safety Network (NHSN) Overview 2012).

The Epidemiology of Opportunistic Invasive Fungal Infections

The true presence of fungal infections is difficult to quantify, but improvements in hospital reporting practices and the culmination of data from numerous studies has provided a glimpse into their increasing epidemiological significance. One such study is the National Hospital Discharge Survey (NHDS), a database compiled by the National Center for Health Statistics consisting of inpatient data from roughly 500 nonfederal acute care hospitals representatively distributed across all geographic regions in the U.S. An analysis of this database found that, between 1979 and 2000, the rate of sepsis caused by fungal organisms increased by 207%, greater than that of sepsis caused by gram-positive or -negative organisms (Martin, et al. 2003). In 1996, the incidence of

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hospitalization for invasive mycoses was 306 per million (Wilson, et al. 2002), and the overall incidence of such infections has continued to rise (Alangaden 2011; Oren and Paul 2014; Pfaller and Diekema 2010). Further, opportunistic IFIs are generally associated with high mortality rates.

Numerous factors complicate resolving true case-fatality ratios. This is due to the tremendous variations in patient age, health, prior exposure to risk factors, and underlying conditions. However, studies using case-control methods are more accurately able to determine the mortality rates due to IFIs by matching relevant background information of patients with and without fungal infections and comparing the patients' outcomes. Using this method, attributable mortality rates for candidemia (also called invasive candidiasis [IC]), cryptococcosis, and invasive aspergillosis (IA) have been estimated at between 10-49%, 21%, and 58%, respectively (Pfaller and Diekema 2010). Even this method of analysis is inaccurate on some level, however, as a 2014 study analyzing data from 11 single-center, multi-center, and nationwide reports on nearly 200,000 autopsies found that, of the 9,187 autopsies indicating the presence of IFIs, only 46% were diagnosed pre-mortem. This investigation revealed that much of the epidemiological data based on the pre-mortem diagnosis of IFIs could be grossly underestimated (Dignani 2014).

Regardless of the true epidemiological presence of IFIs, it is generally accepted that this trend is – in part – due to, rather than in spite of, the development and more widespread application of advanced medical and surgical procedures (Weinstein and Fridkin 2005). Though these practices generally improve patient welfare, many of these advances have increased the number of groups at-risk for the development of

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opportunistic IFIs. The largest of these high-risk groups include those with previous exposure to broad-spectrum antibiotics (due to a disruption of the host's natural flora, opening up formerly competitive growth environments to pathogenic organisms), recipients of immunosuppressive therapy (particularly for cancer, hematopoeitic stem cell transplants [HSCT] and solid organ transplants [SOT]), major surgery (especially those involving the gastrointestinal [GI] tract), those with AIDS, neoplastic diseases, advanced age, and premature birth (Procop and Roberts 2004; Weinstein and Fridkin 2005; Pfaller and Diekema 2010; Alangaden 2011). These risk factors often leave the host extremely vulnerable to pathogens that would otherwise be defeated by a healthy immune system.

With the increasing incidence of fungal infections, their association with high mortality rates, and the growing size of populations at-risk, the fact that IFIs are a tremendous financial burden on the U.S. healthcare system is not surprising. In 1998 alone, attributed costs for the treatment of systemic fungal infections were estimated to be $2.6 billion, averaging $31,200 per patient and making up approximately 0.24% of the total U.S. health expenditures for that year, an expense incurred by only 0.03% of the total U.S. population. Further investigation found that the extended length of stay required for many fungal infections was responsible for 47% of these costs, drug expenses for 17%, laboratory tests 11%, and the remaining amount accounted for by other factors (Wilson, et al. 2002). With such a significant portion of healthcare expenditure dedicated to the treatment of fungal infections, the characterization of fungal pathogens for use as model systems is an essential foundation for the development of safer and more effective antifungals.

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Candida and Candidemia

Of the approximately 600 fungal species known to be human pathogens, *Candida* species are a valuable prospect for the characterization of fungal pathogenicity (Mayer, Wilson and Hube 2013). This is largely due to the high mortality rate and frequency of infections by *Candida* spp. in comparison to other fungal pathogens. As shown in Table 1.1, the incidence of infections by *Candida* spp. is comparable only to that of *Cryptococcus* species – a pathogen with a relatively low case-fatality ratio – and far exceeds the incidence of fungal infections with analogous mortality rates (Rees, et al. 1998). *Candida*'s identity as the most important cause of opportunistic mycoses is supported by numerous other studies.

For example, studies evaluating the epidemiology of IFIs throughout various time periods have consistently reported Candidemia (the presence of *Candida* species in the blood) as the most prevalent of all systemic fungal infections, and for the last two decades *Candida* species have remained the fourth leading cause of HO Bloodstream Infection (BSI) in the United States, making up between 8-10% of all such BSIs (Edmond, et al. 1999; Wisplinghoff, Bischoff, et al. 2004). Given that 10% of HO infections are BSIs, and that a conservatively estimated 8% of those are candidemia, Wenzel et al postulated that the annual number of HO candidemia cases ranges from 7,000-28,000. Considering that – according to data from the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) – the crude mortality rate of candidemia is 40%, Wenzel and colleagues estimated that 2,800-11,200 deaths per year are associated with HO candidemia (Wenzel and Edmond 2001). Therefore, based on the assumption that two-thirds of all *Candida* BSIs are HO, the number of candidemia cases

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in the U.S. could range from 10,500-42,000 infections per year (Pfaller and Diekema 2007).

Table 1.1 Incidence and case-fatality ratios for selected fungal infections, San Francisco Bay Area counties, 1992 – 1993 [a]

Pathogen	Incidence (no. cases/million/yr) [b]	Case-fatality ratio (%)
Candida species	72.8	33.9
C. albicans	37.1	38.1
Non-*albicans*	35.7	29.5
Cryptococcus	65.5	12.7
Coccidioides	15.3	11.1
Aspergillus	12.4	23.3
Histoplasma	7.1	21.4
Zygomycetes	1.7	30.0
Other	3.5	< 0.2
Total	178.3	22.4

[a] Data adapted from Rees et al. 1998.
[b] Based on cases known to be the patient's first episode of the infection.

The high occurrence of *Candida* infections thus places a much greater burden on the U.S. healthcare system than other IFIs. Though the incidence of other major systemic fungal infections – such as Invasive Aspergillus (IA) – appear to be decreasing, the frequency of Invasive Candidiasis has remained steady over the past decade; and recent data revealed that, with respect to IC, the risk of death during hospitalization is no lower now than in the 1980s and early 90s (Pfaller and Diekema 2007). Furthermore, hospitalization due to IC has been shown to increase the length of stay by an average of 14 days, burdening hospitals by limiting the space and personnel resources available to care for other patients. The incidence, high mortality rates, and extended length of stay

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for treatment associated with IC (Table 1.2) translated to an estimated total cost of $1.7 billion in 1998 (65% of the total costs for systemic fungal infections in that year), at an average per-patient additional cost of $14,804 (Wilson, et al. 2002). This financial burden has continued to rise, with more recent studies indicating that the costs of IC average $39,331 in additional expenses for adults and $92,266 for pediatric patients (Zaoutis, et al. 2005).

These aforementioned factors have obligated clinical investigators to place great emphasis on finding ways to reduce the burden of IC and other IFIs. The primary tactic used is similar to that of the prevention of other HAIs: improved training of health care workers (HCW) (Alangaden 2011). This strategy includes three simple, "low-tech" practices: maximizing conformity to existing hand hygiene recommendations, improving adherence to guidelines for the insertion and maintenance of central venous catheters, and the rigorous enforcement of responsible antimicrobial application (Pfaller and Diekema 2007; Alangaden 2011). However, as indicated by the persistent incidence of IC compared to other IFIs, reducing the infection rate of *Candida* spp. has proven particularly difficult.

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Table 1.2 Incidence and associated costs of candidiasis and other common invasive fungal infections [a]

	NHDS 1996				
	N	Rate per million US population	Incremental costs[b]	LOS[c] (days)	In-hospital mortality rate per admission
Candidiasis	61,680	228.19	$14,804	14	6.3%
Aspergillosis	9,261	34.26	$36, 867	19	3.9%
Cryptococcosis	7,987	29.55	$6,328		16%
Histoplasmosis	3,681	13.62	$329		3.4%
Total	82,608	305.62	$15,813		6.8%

[a] Wilson 2002.
[b] average per-person hospitalization costs projected to 1998 dollars.
[c] LOS – Length of Stay (excess hospitalization due to infection).

One reason for this resilience is that some *Candida* species, particularly *Candida albicans*, are natural constituents of human microbial flora; thus, infections frequently arise when these natural residents of the gastrointestinal tract gain an advantage over the immune system of their host (Nucci and Anaissie 2001; Alangaden 2011). In fact, a review of 203 published candidemia studies found 21 that evaluated specific sources for *Candida* infections, and an analysis of these identified the gut as the primary endogenous source for candidemia (Nucci and Anaissie 2001). For the remaining infections of exogenous origin, the culprit could be any number of things, as *Candida* spp. have been isolated from environmental cultures of various items in health care facilities such as floors, countertops, other inanimate surfaces, and even food (Vazquez, Sanchez, et al. 1993; Vazquez, Dembry, et al. 1998). This seemingly omnipresent residency status in the host and healthcare environments makes IC prevention understandably difficult. Thus,

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although the continued implementation of HAI prevention strategies is important, the search and development of novel antifungal therapies through the use of established model fungi is an essential step towards improving patient outcomes.

Two Yeasts in a Pod: Candida albicans and Saccharomyces cerevisiae

Of the more than 200 described *Candida* species, just 5 are responsible for the majority of candidemia cases. As part of the ARTEMIS DISK Global Antifungal Surveillance Study, data from 127 medical centers in 39 countries indicated that *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei* were responsible for 90-95% of cases worldwide (Pfaller and Diekema 2007; Warnock 2007; Guinea 2014). Of the 1,890 HO BSI *Candida* isolates identified in the SCOPE project between 1995-2002, *C. albicans* accounted for 54% of cases, with *C. glabrata* ranking second at 19%, followed by *C. parapsilosis* and *C. tropicalis* at 11% each, and *C. krusei* at 2% (Wisplinghoff, Bischoff, et al. 2004). Its prevalence makes *C. albicans* an exceptional candidate for use as a model fungal pathogen and, as a result of such studies, much has been discovered about its pathogenicity mechanisms and complex host-pathogen relationship (Mayer, Wilson and Hube 2013). In addition to its prevalence in healthcare, other factors have also strengthened its nomination as a model for IFIs. Particularly notable is its similarity to one of the most extensively studied eukaryotic model organisms, the budding yeast, *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae, or Baker's Yeast, has long been an integral part of the continued pursuit to discover and understand the eukaryotic cell. Its single-cellular nature, relatively quick replication cycle, and ease of handling/manipulation have reinforced its continued use as a model eukaryote. From the early characterization of

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eukaryotic organelles and cellular trafficking, to the descriptions of interactions between proteins and the mapping of entire regulatory cascades, the molecular techniques perfected in *S. cerevisiae* ushered in entire new fields of study like "functional genomics" and "systems biology". Specifically, in April 1996, *S. cerevisiae* became the first eukaryotic organism to have its entire genome sequenced. Like many studies before it, what began in yeast has now expanded to more complex organisms, strengthening the foundation of our current understanding of eukaryotic cell biology (Botstein and Fink 2011). The published sequence of its 16 chromosomes, encoding 6,604 open reading frames, has enabled comparisons of functional genomics and evolutionary biology studies between *S. cerevisiae* and numerous other organisms. The addition of this genetic perspective has not only refined current phylogenetic tree models but also given a "head-start" to the genomic characterization of other organisms, particularly *C. albicans* (Botstein and Fink 2011; Scannell, Butler, and Wolfe 2007). In fact, such studies on *S. cerevisiae* and *C. albicans* have revealed that over 80% of the genes are similar between both organisms (Kabir, Hussain and Ahmad 2012).

Both *S. cerevisiae* and *C. albicans* are members of the Saccharomycetaceae family, characterized by reproduction via budding, within the Ascomycota (sac-fungus) phyla of Fungi (Scannell, Butler and Wolfe 2007). These two yeasts share many qualities including rapid growth, easy handling, dispersed cells, and replica plating. Additionally, they frequently share certain aspects of many cellular signaling cascades. For example, the Hog1 Stress-Activated Protein Kinase (SAPK) involved in sensing osmotic and oxidative stresses is homologous between the two species, while the putative K^+ transporter Kch1 involved in the ER stress response of both yeasts are merely orthologs

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(Smith, Morgan, and Quinn 2010; Stefan and Cunningham 2013). Because of this close relationship, many molecular technologies that have been discovered and perfected in *S. cerevisiae* are directly transferrable to *C. albicans* after only small procedural modifications (Kabir, Hussain and Ahmad 2012). These congruencies have continued to support the role of *C. albicans* as a model for IFI pathogenicity. However, despite its overarching prevalence in healthcare and likeness to *S. cerevisiae*, there are significant limitations to using *C. albicans* that make *S. cerevisiae* more appropriate for initial studies aimed at characterizing the Mechanism of Action (MoA) of novel antifungals. For example, one key difference between the two organisms is the genetic complexity of *C. albicans*.

Researchers in *S. cerevisiae* labs first began working with *C. albicans* around the time when its identification as a microbial pathogen became increasingly common in the 1970s and 1980s, and even more began to study it in the '90s; but the chromosomal instability and diploid nature of *C. albicans* greatly slowed the pace at which genetic manipulation became readily feasible. Even with the publication of the complete genome sequence of the *C. albicans* in 2004, progress was considerably slower than that of *S. cerevisiae*, primarily due to the work required to make the *C. albicans* genome amenable to manipulation. Hence, genomic studies have since experimentally verified the function of only 1,403 genes (22.97% of its genome). Another 77.03% (4,705) of its genes' functions have been assigned via comparative sequence analysis, but the remaining 152 genes/ORFs have yet to be characterized at all (Kabir, Hussain and Ahmad 2012). Although select homozygous and heterozygous deletion mutants and regulated expression strains are available for *C. albicans*, these libraries are incomplete

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(McCluskey, Wiest and Plamann 2010). To the contrary, a complete library of unique deletion mutants for every nonessential *S. cerevisiae* gene exists and is readily available. This mutant library is a vital asset for studies on the bioactive mechanisms of antifungal agents. Thus, the abundant similarities between the two organisms, combined with the comprehensiveness of the *S. cerevisiae* mutant library, make it a comparable subject for the wide-scale susceptibility testing required for antifungal MoA determination. Its successful history as a research tool for other drugs also supports its continued use in this regard (Cardenas, et al. 1999).

After the publication of the complete genome sequence of *S. cerevisiae* in 1996, the scientific community's understanding of conserved cell biology began improving considerably. Of particular importance was the realization that the baker's yeast has far more in common with mammalian cells than previously thought. Though there are expected differences, a surprising number of partially or completely conserved gene and protein functions were discovered to exist between mammals and this yeast. In fact, at least 31% of *S. cerevisiae*'s genes have homologs in humans (Botstein, Chervitz and Cherry 1997). In instances where homologs do not exist, the ability to heterologously express mammalian genes in *S. cerevisiae* has proved particularly useful. As such, it has commonly (and successfully) been used to study genetic components of various human diseases and the mechanisms of a wide array of drugs, including immunosuppressants (cyclosporine A, FK506, and rapamycin) and steroid receptor antagonists (tamoxifen) (Cardenas, et al. 1999). As discussed previously, *S. cerevisiae* also shares many features with the pathogenic yeast, *C. albicans* (Goldstein and McCusker 2001). Its unique relationship to both mammalian and fungal cells offers a unique opportunity, as *S.*

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cerevisiae appears to stand in the middle ground between two distinct yet frequently cohabitating organisms (Cardenas, et al. 1999). Using this concept as an advantage, it is possible to identify cellular pathways in *S. cerevisiae* that, when altered, have an impact on antifungal susceptibility. This information can subsequently serve as the groundwork for describing an antifungal's MoA. Additionally, characterizing the activity of antifungals could highlight novel, fungi-specific targets and potentially open doors to the development of entirely new classes of drugs.

Modern Antifungal Therapies

With such a significant impact on healthcare worldwide, fungal infections surely deserve attention with regards to the research and development of effective antifungal treatments. Unfortunately, antifungal research is a relatively young field and, consequently, severely lacking in the variety of clinically available therapies. The first antifungal agent, griseofulvin, was isolated in 1939 from a culture of *Penicillium griseofulvum dierckx*, but its antifungal activity was not described until 1955, when it was discovered to possess activity *in vitro* against pathogenic skin fungi. In 1958 it was found that only oral administration was effective for the treatment of dermatomycoses. Unfortunately, its activity was limited to this fungal group, making griseofulvin unsuitable for treating systemic infections (Flint, Forsey and Usher 1959). Most of the systemic antifungal drugs in use today were introduced after 1990, but the history of these antifungals began with the discovery of amphotericin B in the 1950s (Nett and Andes 2012). Amphotericin B's potent and broad-spectrum activity led to its widespread use against serious invasive fungal infections, but the dose-limiting toxicities associated with its use illuminated the need for safer alternatives (Lewis 2011). Since, considerable

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progress has been made in antifungal therapy and there are now four classes of antifungal drugs available for the treatment of systemic and invasive fungal infections. These include Polyenes, Pyrimidine Analogs, Azoles, and Echinocandins. Their respective cellular targets, spectrums of activity, bioavailability, toxicities, pharmacological characteristics, and potentials for resistance development vary between and even within classes, therefore all characteristics need to be considered when comparing the clinical effectiveness (Nett and Andes 2012). Each class with their respective drugs and characteristics are described in further detail below.

The Polyene Class

In the polyene drug class, amphotericin B (AmB) is the only molecule approved for treating systemic fungal infections and is clinically available in four formulations (Nett and Andes 2012). Its discovery in 1953 was a result of the broad screening of *Streptomycete* cultures for antifungal activity. AmB possessed remarkable antifungal activity but, in its pure form, had limited solubility in aqueous solutions at physiological pH. It was determined that clinical use of AmB would require its association with another molecule to facilitate clinical bioavailability, so a complex of AmB and sodium deoxycholate was constructed that allowed for successful treatment by means of intravenous infusion (Dutcher 1968; Laniado-Laborin and Cabrales-Vargas 2009). Amphotericin B primarily kills yeast by binding ergosterol, but also has a secondary mechanism wherein it permeabilizes the membrane by forming discrete channels through which ions freely pass. This secondary effect is not required for AmB's fungicidal activity; instead, it is believed to increase the drug's potency and the rate of fungal cell death (Gray, et al. 2012). This dual-threat mode of action is a key factor in its broad

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spectrum of activity. Because ergosterol is such a ubiquitous component of fungal cell membranes, AmB possesses activity against a variety of fungi, including many pathogenic yeasts, molds, and dimorphic species (White, Marr and Bowden 1998). However, AmB's partial affinity for cholesterol (ergosterol's mammalian homolog) can cause serious infusion-related side effects, most notably renal toxicity. This led to the development of three AmB lipid conjugates in the late 1990s that, although less severe, still possess similar infusion-related toxicities. Thus, lipid-based AmB preparations are considered first-line treatment options and AmB deoxycholate as an alternative choice (Nett and Andes 2012).

The Pyrimidine Analog Class

The Pyrimidine Analog class also contains just one approved drug. Flucytosine (5-fluorocytosine; 5-FC) is a fluorinated cytosine analog first synthesized in 1957 as a potential candidate for anti-tumor therapy (Duschinsky, Pleven and Heidelberger 1957). Though its effectiveness as a cancer drug was limited (Heidelberger, et al. 1958), it was soon discovered to possess activity against *Candida* spp., *Cryptococcus neoformans*, and fungi causing chromoblastomycosis (a fungal infection of the skin) (Benson and Nahata 1988). Flucytosine is approved for use as an oral capsule, with bioavailability of the drug remarkably high (between 80-90%) (Schönebeck, et al. 1973). Interestingly, 5-FC possesses no antifungal activity in its native form; rather, fungal uptake of the molecule by cytosine permease allows for subsequent intracellular conversion to 5-fluorouracil (5-FU) via cytosine deaminase. This molecule is then further converted to two additional active metabolites that inhibit RNA and DNA synthesis (Waldorf and Polak 1983). There are multiple factors limiting 5-FC's successful use as an antifungal. First, dependence on

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cytosine deaminase for 5-FC sensitivity is a major limiting factor in its spectrum of activity, as fungi lacking this enzyme are obviously unable to convert the inactive 5-FC to its active metabolite (A. Polak 1977). Further, this limitation cannot be circumvented through the direct use of 5-FU because of its severe toxicity to mammalian cells and limited uptake of 5-FU by fungi (Polak and Grenson 1973). Second, the intrinsic resistance of many strains and the frequent occurrence of resistance during treatment further complicate the use of 5-FC. Consequently, 5-FC is not typically utilized as a monotherapeutic agent, but rather co-administered with other antifungals such as AmB (Vermes, Guchelaar and Dankert 2000).

The Azole Class

Azoles are the most abundant class of antifungals, with at least 9 different drugs approved for the treatment of either topical or systemic fungal infections. These drugs are characterized by the presence of a five-membered heterocyclic ring that contains one or more additional atoms (either nitrogen, sulfur, or oxygen). In the case of antifungal azoles, they contain either two or three nitrogens and are termed imidazoles or triazoles, respectively (Sheehan, Hitchcock and Sibley 1999). The imidazoles (apart from ketoconazole) are only used as superficial antifungal therapies. Triazoles, on the other hand, are used to treat a variety of superficial and systemic fungal infections and also show greater specificity for fungal cells versus those of the host, making them much safer in comparison. Regardless of their structure or effective use, azole antifungals function by interfering with the synthesis of ergosterol via inhibition of the enzyme, lanosterol demethylase. This enzyme, also called 14-sterol demethylase (encoded by the *ERG11* gene), is a cytochrome P450-dependent component of the ergosterol synthesis pathway,

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and its inhibition results in the depletion of total ergosterol and a buildup of the enzyme's usual substrate, 14-methylated sterols. Ergosterol is both the major sterol of the fungal cell membrane (and therefore essential for membrane integrity and fluidity) and a vital component in nutrient transport and chitin synthesis. Further, trace amounts of ergosterol are required, as it serves a "sparking" function to allow progression through the cell cycle (Sheehan, Hitchcock and Sibley 1999). Because their MoA results in inhibited growth and altered membrane structure and function, azoles are merely fungistatic in their activity. Thus far, four mechanisms of azole resistance have been described in *Candida* species. These mechanisms involve the induction of efflux pumps that reduce intracellular drug concentrations, point mutations in the *ERG11* gene that reduce drug affinity, upregulation of lanosterol 14-alpha-demethylase (thereby increasing the azole concentration required for effect), or the development of bypass pathways in which the membrane-disruptive effects of azoles are circumvented. Strains resistant to azoles may possess one or more of the above mutations (Pfaller 2012). Like most antifungals, azoles are typically administered in combination with other therapeutic agents to improve therapy effectiveness and reduce the likelihood of resistance development.

The Echinocandin Class

Echinocandins are the newest and final class of antifungal drugs approved for the treatment of systemic infections. The FDA approved the first agent, caspofungin, in 2002, and two additional class members, micafungin and anidulafungin, were subsequently approved in 2005 and 2006, respectively. Echinocandins are semisynthetic, cyclic hexapeptides that are N-linked to a fatty acyl side chain (Perlin 2011). While polyenes and azoles target the cell membrane, echinocandins possess concentration-dependent

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antifungal activity via strong inhibition of ß-1,3-D-glucan synthase, which synthesizes the cell wall biopolymer ß-1,3-D-glucan (Pfaller 2012). This enzyme is composed of two subunits, Fks and Rho. The Rho subunit is a GTP-binding protein and regulates ß-1,3-glucan synthase activity. The catalytic activity of enzyme is contributed by Fks (encoded by the *FKS1*, *FKS2*, and *FKS3* genes) and is the target of echinocandins. Though the exact mechanics of the echinocandin–glucan synthase interaction are unclear, studies have indicated that enzymatic removal of the aliphatic tail results in the drug's inactivation. It is also unknown whether the activity of echinocandins is dependent upon transport into the cell, but it is hypothesized that the tail inserts itself into the lipid bilayer of the cell membrane and carries out its inhibitory affects from the extracellular side (Perlin 2011). Regardless of the exact mechanism, echinocandins have rapidly become the preferred treatment against a number of fungal pathogens (Perlin 2011). This is partly due to their specificity for impact on the fungal cell wall – a target conveniently absent from mammalian cells – making it a relatively low-risk therapy. This, combined with a lack of cross-resistance potential with other antifungals and their activity against susceptible *Candida* spp. and *Aspergillus* spp., equates to a favorable therapeutic index. Though echinocandins are fungicidal against yeast and even azole-resistant *Candida* strains, their activity against molds like *Aspergillus* spp. is fungistatic. Although clinical failure as a result of echinocandin resistance in susceptible species like *Candida* is unusual, isolates with reduced susceptibility to these drugs are being identified with increasing frequency (Perlin 2011). Resistance is typically associated with mutations in two highly conserved regions of *FKS1* and/or *FKS2* that result in amino acid substitutions

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in the Fks subunit; these mutations generally impart cross-resistance among the echinocandin class (Perlin 2011).

The Need for Novel Antifungal Therapies

With the limited treatment diversity, various associated toxicities, and increasing occurrences of antifungal resistance in already-prevalent fungal strains, great emphasis has been placed on the discovery of novel antifungal agents to fill the gaps in, and improve, current therapy options.

Occidiofungin, a Novel Antifungal

Occidiofungin is a novel cyclic glycolipopeptide demonstrating great potential for clinical antimycotic application as a broad-spectrum antifungal agent. The soil bacterium *Burkholderia contaminans* MS14 synthesizes the peptide via nonribosomal peptide synthetases and secretes it into the surrounding environment, thus it was first recognized to have antifungal activity as a result of the bacteria's suppression of a fungal turf grass disease, Brown Patch (Lu, et al. 2009). Structural analysis revealed two closely related variants, occidiofungin A (1199.55 Da) and B (1215.55 Da). Both possess a cyclic structure composed of eight amino acids, one of which is ß-hydroxy tyrosine and another is an 18-carbon novel fatty amino acid. This novel fatty amino acid contains a small acyl group and functions as an attachment point for a xylose sugar. The two variants differ only in the addition of an oxygen atom to occidiofungin B's asparagine (forming ß-hydroxy asparagine), which explains the slight difference in mass.

A 56kb genomic DNA region containing 16 genes, now termed the occidiofungin gene (*ocf*) cluster, is responsible for occidiofungin's biosynthesis in *B. contaminans*

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MS14 and has been described (Gu, Smith and Liu, et al. 2011). Further analysis of this genetic and biochemical map demonstrated that the *ocfC* gene, which encodes a glycosyltransferase, is responsible for the addition of xylose to occidiofungin. After examining the antifungal activity of the *ocfC* gene mutant, MS14KC1, it was determined that presence of xylose is not important for occidiofungin's bioactivity (Chen, et al. 2013).

Additional research conducted on the chemical stability and *in vitro* activity have revealed that occidiofungin retains the full potency of its activity after exposure to not only extreme pH ranges (2-9) and high temperatures (100°C), but also after incubation with gastric proteases. These findings suggest that occidiofungin may be suitable for oral administration, as these parameters are representative of the environments the drug will encounter in the GI tract. This is significant because the only currently available oral therapies are azole-class antifungals, to which a growing number of *Candida* spp. strains are acquiring resistance (Ellis, et al. 2012). Also supporting occidiofungin's potential for clinical use are recent studies conducted on its toxicity in a mammalian system. These studies revealed that a single dose as high as 20 mg/kg in mice did not affect hematological or serum biochemistry, and additional experiments using lower, repeated dosing returned similar results. This indicates that occidiofungin administration may not result in substantial alterations in organ function. Though initial examinations reveal a low potential for toxicity, the researchers did note that further investigation was vital to completely characterize the range of occidiofungin's pharmacological effects (Tan, et al. 2012).

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Although its exact mechanism of action is not fully understood, studies indicate that occidiofungin possesses broad-spectrum activity against fungi and exhibits its effects via a mechanism unique from currently available antifungals. Thus far, Minimum Inhibitory Concentration (MIC) assays have determined that occidiofungin's activity is not reliant on disruption of the plasma membrane or other lytic pathways, the binding of ergosterol, or the activity of β-1,3-glucan synthase. Further, occidiofungin exposure does not appear to impact cell cycle progression (Emrick, et al. 2013). Occidiofungin does, however, impart morphological changes on fungal cells at subinhibitory concentrations, as cells analyzed after exposure to such doses demonstrated reduced coat (manno) proteins on the outer cell wall, possessed intracellular inclusions, and had an enhanced distribution of chitin (primarily at emerging bud tips) in daughter cells (Emrick, et al. 2013). Additionally, occidiofungin appears to induce damage to the cell wall, as indicated by the activation of the Cell Wall Integrity pathway as soon as 10 minutes after exposure. However, occidiofungin's fungicidal activity is primarily via apoptosis due to the increased production of reactive oxygen species (ROS), double stranded DNA breakage, and the externalization of phosphotidylserine that occurs upon exposure to lethal occidiofungin concentrations. Further, under anaerobic conditions (an environment known to reduce the production of ROS), the MIC of the wild type and mitochondrially dysfunctional yeast was double that of typical conditions, suggesting ROS may play a significant role in supporting occidiofungin's MoA. Further supporting an apoptotic mechanism of action is the two-fold increase in the occidiofungin concentration required to inhibit growth in the *YCA1* gene mutant. This gene encodes a caspase-like cysteine protease responsible for, among other things, regulating apoptosis in response to high

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levels of reactive oxygen species (Emrick, et al. 2013). The role of another apoptosis-associated gene, *NDE1* (encoding for a mitochondrial external NADH dehydrogenase), was assessed via MIC analysis and the deletion strain was found to be 2-fold more sensitive than the wild type. Other apoptotic mutants exhibiting resistance to occidiofungin include those deleted for *RNY1*, a gene encoding a vacuolar RNase that promotes apoptosis under oxidative stress, and *CSG2*, a calcium regulatory protein involved in the biosynthesis of sphingolipids. Due to the considerable amount of cross-talk between apoptotic and autophagic pathways, various autophagic mutants were also assessed via MIC, but none suggested a role for autophagy in occidiofungin-induced cell death. Thus, apoptosis appears to be the cause of death in occidiofungin exposure.

Objectives and Experimental Design

In this study, our goal is to further characterize occidiofungin's bioactivity by comparing the susceptibility of yeasts to occidiofungin under a variety of environmental conditions. In this way, we hope to identify cellular pathways that, when their regulation is altered in response to these conditions, have an impact on occidiofungin's bioactivity. By categorizing pathways as having a positive, negative, or neutral impact on susceptibility, we will establish a number of directions in which research on this novel antifungal can confidently progress.

To accomplish this, we will utilize Colony Forming Unit (CFU) assays using sub-lethal concentrations of occidiofungin. Though the Minimum Inhibitory Concentration (MIC) assay is the standard tool for determining antifungal resistance in the clinical setting, we have found that its results have limited utility with regards to detecting subtle variances in susceptibility. By design, the MIC assay allows for the determination of the

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minimum concentration of an antimicrobial agent required to inhibit the visible growth of a microorganism. This is in contrast to a Minimum Lethal Concentration (MLC) assay, in which the data reported indicates the minimum concentration required to kill a microorganism. In the context of occidiofungin with regards to MIC and MLC values, occidiofungin's fungicidal activity renders the two values equal, meaning that yeasts are either killed by the drug or survive unscathed. Additionally, both assays report results after overnight incubation. Thus, a single yeast surviving exposure to a particular concentration of occidiofungin in these assays may be able to proliferate to detectable levels, therefore impacting the reported values. Consequently, for our purposes, MIC/MLC values are insufficient in reporting whether certain strains or environmental conditions lend yeasts more or less vulnerable to occidiofungin. The CFU assay, therefore, has been selected due to its increased sensitivity and ability to monitor cell viability over a period of hours instead of days. Additionally, our goal of detecting subtle variances in susceptibility led us to conduct CFU assays using sub-lethal concentrations of occidiofungin, as lethal concentrations would leave no surviving cells and lend our assay no more useful than an MIC.

Cellular Pathways Examined in This Study

Due to our interest in using altered environmental conditions to induce transcriptional changes in the yeast cell, it is important to establish which pathways are responsible for sensing and responding to these altered conditions. In this regard, important pathways involved in nutrient sensing are described below.

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Quiescence

Like most living cells, yeast are capable of exiting the cell cycle and entering an alternative resting state called quiescence. Early studies described quiescence as a single G_0 state of the cell cycle, initiated by the prolonged deprivation of nutrients regardless of the specific environmental conditions in which the cell was placed. These studies were conducted on cells grown to saturation in rich media, and the results described particular characteristics that came to define quiescence, namely resistance to heat-shock and oxidative stress, thickened cell walls, and altered transcriptional profiles (Gray, et al. 2004). Recent work by Klosinska et al, however, has indicated that only a small subset of genes is consistently associated with quiescent cells independent of the specific nutrient for which the cell is starved. The remaining transcriptional changes appear to reflect compensation for the declining availability of specific nutrients. For instance, the upregulation of genes involved in vacuolar transport and autophagy allows the cell to recycle existing proteins and organelles upon starvation for nitrogen in order to reallocate its limited resources (Klosinska, et al. 2011). Based on these findings, it was proposed that quiescence is not a single, distinct phase of the cell cycle, but rather a limited set of discrete cellular programs tailored to improve survival under specific stresses encountered by the cell. Further, it was illustrated that most of the stress-resistant properties associated with quiescence are simply extensions of those found in slow growing cells (Klosinska, et al. 2011). Additionally, apart from the detection of a carbon source (the presence of which is a primary and sufficient stimulant for cell cycle reentry) quiescent yeast are also capable of responding to changes in their environment, such as irradiation, heat shock, oxidative stress, and exposure to certain chemicals and toxins, and

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do so by altering the transcription of genes just as actively proliferating yeast do (Gray, et al. 2004). For these reasons, it is desirable to assess the bioactivity of occidiofungin in quiescent cells, as establishing whether active cellular growth or normal physiological processes are required for its activity could be an important indicator as to its mechanism of action.

Nutrient Signaling and the Cellular Response to Starvation Conditions

Nutrient sensing and signaling mechanisms related to nutrient-controlled cellular regulation have been well characterized in yeast. Most of these pathways are regulated using the nutrient itself as an indicator, and these nutrients have been shown to modulate numerous signaling cascades associated with their transport and metabolism. For example, glucose is the preferred fermentable carbon source for S. cerevisiae and serves as the key signaling molecule in a variety of cellular pathways. Some of these pathways are involved in catabolic repression and serve to conserve resources by suppressing alternative metabolic pathways when a more favorable energy source, like glucose, is available (Conrad, et al. 2014). The presence of glucose also regulates other pathways, such as those involved in repressing stress tolerance mechanisms and stimulating cell proliferation. By using glucose as the central signal for these regulatory cascades, the cell is able to mount a whole-cell transcriptional response appropriate for the availability of glucose. This theme is central to other nutrient-dependent cellular responses, such as the phosphate-regulated PHO pathway (Conrad, et al. 2014).

Additionally, although the deprivation of particular nutrients impacts regulatory pathways specific to their reacquisition, broader signaling cascades responsible for synchronizing cellular processes to nutrient availability are also activated. For example,

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the Target of Rapamycin (TOR) kinases play an important role in regulating the transcriptional response to nutrient availability (Aronova, et al. 2007). As the name suggests, it was first discovered as the target of the inhibiting drug, rapamycin. Upon exposure to rapamycin, cells exhibited significant alterations in physiology similar to the starvation response, thus providing the first indication of TOR's role in the cell. Saccharomyces cerevisiae have two TOR kinases, Tor1p and Tor2p, which associate with other proteins to form the TOR1 Complex (TOR1C). This complex is responsible for regulating cellular growth in response to the availability of extracellular nutrients, and its inhibition by rapamycin mimics the native response to nutrient deprivation (Aronova, et al. 2007). Although the exact manner in which nutrient levels are communicated to TOR remains to be described, the varying branches of the TOR pathway provide an opportunity to identify transcriptional profiles in the cell that, when altered, might have an impact on the bioactivity of antifungals. Though our early studies using MIC assays indicated that the deletion of tor1 had no impact on occidiofungin bioactivity (data not shown), characterizing occidiofungin activity as it pertains to nutrient-specific transcriptional responses could reveal further paths to pursue.

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CHAPTER II

MATERIALS AND METHODS

Strains, Media, and Reagents

All yeast strains used in this study are derivatives of *Saccharomyces cerevisiae* BY4741, obtained from Thermo Fisher Scientific, and are listed in Table 2.1. The *CIT2:HA3::HIS3* strain was constructed as reported previously by Ünlü and colleagues (Ünlü, Narayanan and Gordon 2013). All media preparations (Table 2.2) were conducted using previously published protocols (Sherman 1991). A working dilution of occidiofungin at 0.4mg/mL in 100% DMSO was prepared from the 1mg/mL stock solution. Rapamycin (Sigma) was prepared at 1mg/mL in 100% ethanol. Cycloheximide (Sigma) was prepared at 10mg/mL in sterile distilled water. Chloramphenicol (Sigma) was prepared at 34mg/mL in 100% ethanol.

Table 2.1 *Saccharomyces cerevisiae* strains used

Mutant Strain *	Systematic Name
Δ*tor1::Kan*^{R}	YJR066W
CIT2:HA3::HIS3	YCR005C
Δ*pho4::Kan*^{R}	YFR034C
Δ*pho80::Kan*^{R}	YOL001W
* all strains are derivatives of *S. cerevisiae* BY4741, having the genetic background MAT**a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	

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Table 2.2 General preparation of media

Media	Components
YPD	+ 10g Bacto™ Peptone + 5g Bacto™ Yeast Extract ^ distilled water to 475mL Autoclave 28 minutes @ 121°C + 25mL 40% Glucose
YPD agar plates	+ 10g Bacto™ Agar + 10g Bacto™ Peptone + 5g Bacto™ Yeast Extract ^ distilled water to 475mL Autoclave 28 minutes @ 121°C + 25mL 40% Glucose
Depleted YPD	A 25mL culture of wild-type yeast were grown to saturation in YPD at 30°C with shaking for 5 days. Depleted YPD media was obtained by passing the culture through a 0.2µm filter to remove yeast prior to use.
Synthetic Defined (Minimal Complete)	+ 0.85g Yeast Nitrogen Base (w/o amino acids and ammonium sulfate) + 10g Glucose + 2.5g Na_2SO_4 + 2.5g $(NH_4)_2SO_4$ + 0.01g Histidine + 0.04g Leucine + 0.01g Methionine + 0.01g Uracil ^ distilled water to 500mL Autoclave 23 minutes @ 121°C
SD –Glucose	SD media was prepared as previously described, without the addition of glucose.
SD –Nitrogen	SD media was prepared as previously described, without the addition of ammonium sulfate

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Table 2.2 (continued)

SD –Phosphate	+ 0.85g Yeast Nitrogen Base (w/o amino acids and ammonium sulfate) + 2.5g Na_2SO_4 + 2.5g $(NH_4)_2SO_4$ + 5mL 1M $MgSO_4$ + 10mL 29% NH_4OH ^ distilled water to 500mL • Mix 1 hour @ RT • Filter 2X through Buchner Funnel + 10g Glucose + 0.01g Histidine + 0.04g Leucine + 0.01g Methionine + 0.01g Uracil • Autoclave 23 minutes @ 121°C (Kaneko, Toh-e and Oshima 1982)
SD Complete +Glutamate	+ 0.85g Yeast Nitrogen Base (w/o amino acids and ammonium sulfate) + 10g Glucose + 2.5g Na_2SO_4 + 2.5g $(NH_4)_2SO_4$ + 0.05g Glutamic Acid + 0.01g Histidine + 0.04g Leucine + 0.01g Methionine + 0.01g Uracil ^ distilled water to 500mL Autoclave 23 minutes @ 121°C
SD +2% Raffinose	SD Media was prepared as previously described, with the addition of 10g raffinose substituted for glucose.

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Colony Forming Units (CFU) and Spotting Assays

For each condition tested, unless otherwise noted, cells were removed from a culture in mid-log phase of growth (~0.5 OD_{600}) via centrifugation (13,000 x *g* for 8 minutes at 4°C) and resuspended in the appropriate media at a final cell density of 0.5 OD_{600}, or approximately 1.67 x 10^7 cells/mL. At indicated time points, 200µL of cells were transferred to the first column on a 96-well microtiter plate. Each sample was then 5-fold serially diluted 7 times by transferring 40µL cells from one column into the next well containing 160µL of the same media. Depending on the viscosity of the media, between 3.0 – 4.0µL from each well was spotted in profile onto a 150mm YPD plate and then incubated at 30°C. Multiple images were recorded between 24 and 48 hours of incubation. To determine colony-forming units (CFU), 50µL from select dilutions were spread, in duplicate, onto 100mm YPD agar plates. Colonies were counted after 48 hours of incubation at 30°C. Only data from plates that had between 30 and 300 colonies were used in determining CFUs. The CFU value for each sample was calculated using the following formula:

$$\text{CFU} = N \times 20 \times 5^{D-1} \qquad \text{(Equation 2.1)}$$

where N = # colonies on plate and D = column # of the corresponding dilution

For all samples, the Mean CFU at each indicated time point was determined, and its value was plotted as the Log_{10} (Average CFU) versus time (hours).

For instances in which samples were treated with occidiofungin, cells were treated with 1µg/mL occidiofungin, indicated by "+occ", and that sample's counterpart treated with an equivalent volume of vehicle control, indicated by "Ø" (*theta*). Samples were

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treated immediately after the dilutions at $T_{0\ hours}$ and then incubated at 30°C with shaking for the indicated duration of the experiment.

Where indicated, samples were pre-treated with either 25µg/mL cycloheximide or 60µg/mL chloramphenicol, or their vehicle controls (sterile distilled water or 100% ethanol, respectively). In these instances, all samples were prepared simultaneously and those which were selected for pre-treatment (as indicated) received one of the pharmacological agents while untreated samples received the appropriate vehicle control(s). All samples were incubated at 30°C with shaking for 30 minutes followed select samples (as indicated) being treated with one of the drugs immediately prior to the T_0 CFU dilution. Appropriate samples were subsequently treated with occidiofungin or its control, as indicated and described above.

Preparation of Quiescent Cells and Verification of the Quiescent Cell State

A quiescent culture was obtained by growing wild type cells in 25mL YPD at 30°C with shaking for 4 days. To verify quiescence, resistance to oxidative stress and elevated temperatures was tested as previously described (Klosinska, et al. 2011). Briefly, to assess resistance to oxidative stress, 1mL aliquots of cells were subjected to 0mM, 1mM, 5mM, 10mM, and 50mM hydrogen peroxide for 1 hour at 30°C. Cells were subsequently diluted in YPD (unless otherwise noted) to 1.67×10^7 cell/mL and a 200µL aliquot from each sample was 5-fold serial diluted in YPD in a 96-well microtiter plate in the same manner as described in "Colony Forming Units (CFU) and Spotting Assays". 3.75µL of each dilution was spotted, in profile, onto a 150mm YPD plate. A similar treatment and dilution protocol was followed in parallel for cells from an

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exponentially growing culture. Plates were incubated at 30°C and images recorded between 24 and 48 hours to compare cell viability.

To test resistance to elevated temperatures, 1mL aliquots of both quiescent and exponentially growing cells were subjected to temperatures of 50°C, 53°C, and 55°C for 0, 5, 10, and 20 minutes. Cells were diluted to 1.67 x 10^7 cells/mL in distilled water and a 200µL aliquot from each sample were 5-fold serial diluted in YPD in a 96-well microtiter plate, as previously described. 3.75µL of each dilution was spotted, in profile, onto a 150mm YPD plate. Plates were incubated at 30°C and images recorded between 24 and 48 hours.

Percent Cell Viability with Exposure to Elevated Temperatures

A mid-log culture was prepared as outlined above. As described above, a quiescent culture was prepared and split into a control and fresh media group. Samples of each group were prepared simultaneously by pelleting via centrifugation (13,000 x *g* for 8 minutes at 4°C) and subsequent resuspension in media to a cell density of 0.5 OD_{600}. The mid-log and quiescent fresh media samples were resuspended in fresh YPD while the quiescent control sample was resuspended in Depleted YPD. At the start of the experiment (T_{0hours}), 1mL of cells from each culture was exposed to 53°C for 10 minutes and a volume equivalent to 1x10^5 cells was diluted with YPD in a microtiter plate, as previously described. Following the same protocols outlined above, Spotting and CFU Assays were carried out. Cultures were incubated at 30°C with shaking for the duration of the experiment and the assays repeated after 0.5, 1, and 2 hours of exposure to the media. Percent cell viability was calculated at the indicated time points by dividing each sample's post-heat treatment CFU value by the untreated CFU value of the same sample.

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Western Blot Analysis

Total cellular protein was extracted by alkaline cell lysis followed by TCA precipitation and then solubilized in SDS-PAGE loading buffer via bath sonication (Riezman, et al. 1983). For western blot analysis, protein samples equivalent to 0.1 OD_{600} of original cell culture were separated by SDS-PAGE and transferred to nitrocellulose. After staining the membrane with amido black to visually confirm equal loading, the membrane was blocked with 1X TBS-Tween (20mM Tris pH 7.4, 200mM NaCl, 0.1% Tween-20) supplemented with either 2% BSA or 5% non-fat dry milk. Protein detection was conducted using murine primary antibodies at 1:5,000 (HA_3; Covance) and 1:20,000 (Pgk1p; Invitrogen) dilutions and an HRP-conjugated anti-murine secondary antibody (GE Healthcare) at a 1:8,000 dilution. Pierce enhanced chemiluminescence detection system from Thermo Scientific was used for detection.

Antifungal Preparation

The occidiofungin used in this study was a gift from James Leif Smith, Department of Biology, Texas A&M University and was isolated as previously described (Gu, Smith and Wang, et al. 2009). In summary, *Burkholderia contaminans* strain MS14 was cultured in potato broth at 28°C for 7 days without shaking. The culture extract (cell free) was precipitated using 50% weight/volume ammonium sulfate and the resulting pellet resuspended in 35% acetonitrile (ACN):water (volume/volume) and further purified by Reversed-Phase High Performance Liquid Chromatography using a 4.6 × 250mm C18 column (Grace-Vydac). Aliquots of lyophilized occidiofungin were resuspended in 100% DMSO to generate a 1mg/mL stock.

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CHAPTER III

RESULTS

Quiescent Yeast Exhibit Resistance to Occidiofungin

Due to the similarities between quiescent and slow growing yeast, it was desirable to characterize the susceptibility of quiescent cells to occidiofungin exposure. To verify that the yeasts had reached a quiescent state by the four day incubation period, their resistance to oxidative stress and elevated temperatures was assessed. As shown in Figure 3.1, exponentially growing cells were markedly more susceptible to oxidative stress than quiescent yeast. A similar sensitivity profile was obtained when elevated temperature was used as the stressor (Figure 3.2). Therefore, retention of cell viability for the quiescent culture after exposure to oxidative stress and elevated temperatures confirmed that these cells were in quiescence.

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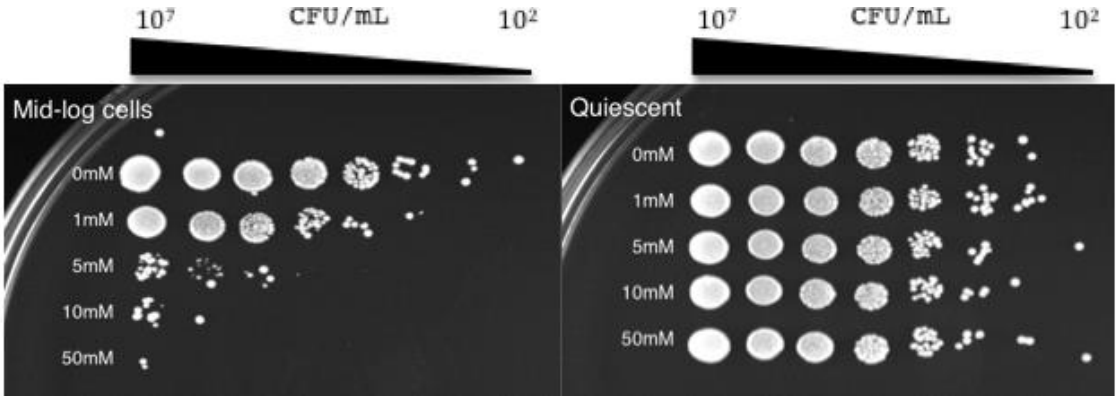

Figure 3.1 Quiescent cells exhibit resistance to oxidative stress

Depiction of differences between mid-log and quiescent cells in resistance to oxidative stress. An aliquot of cells from mid-log and quiescent cultures were incubated at 30°C in 0mM, 1mM, 5mM, 10mM, and 50mM H_2O_2 for 1 hour prior to 5-fold serial dilution and spotting. Representative image shown (n=2).

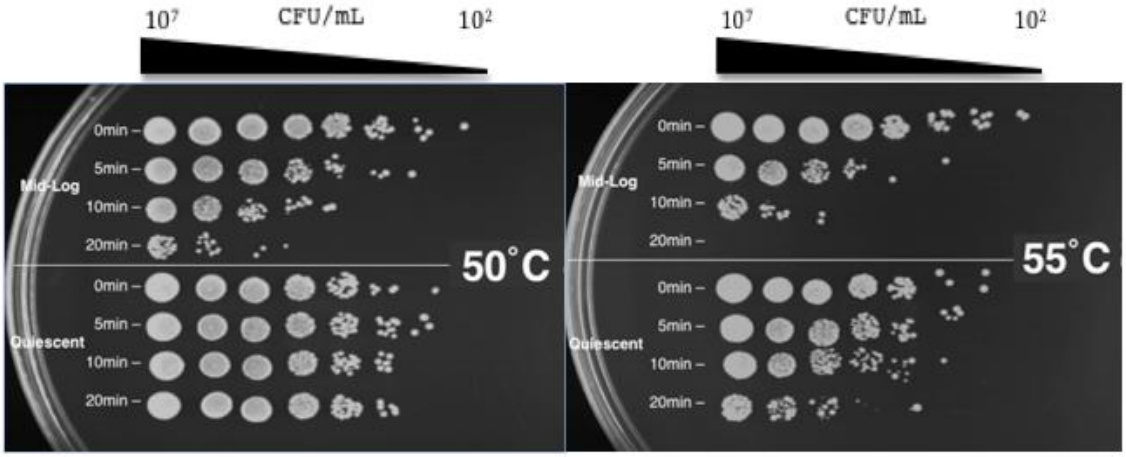

Figure 3.2 Quiescent cells exhibit resistance to elevated temperatures

Depiction of the differences between mid-log and quiescent cells in resistance to elevated temperatures. Aliquots of 1mL cells from mid-log and quiescent cultures were subjected to 50°C and 55°C for 0, 5, 10, and 20 minutes. 200µL from each was five-fold serial diluted 7 times and 3µL were spotted, in profile, onto a 150mm YPD agar plate. Representative image shown (n=2).

To determine if these differences in cell viability impact susceptibility to occidiofungin, the sensitivity of quiescent cells was compared to that of mid-log phase

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cells by Colony Forming Unit determination (CFU; Figure 3.3) and Spotting Assay (Figure 3.4). The data confirm that quiescent cultures were resistant to occidiofungin compared to actively growing cells during the first hour of exposure. However, cells maintained in culture for a longer period of time showed a decline in viability, likely due to cells exiting their quiescent state and reentering the cell cycle in response to newly available carbon source.

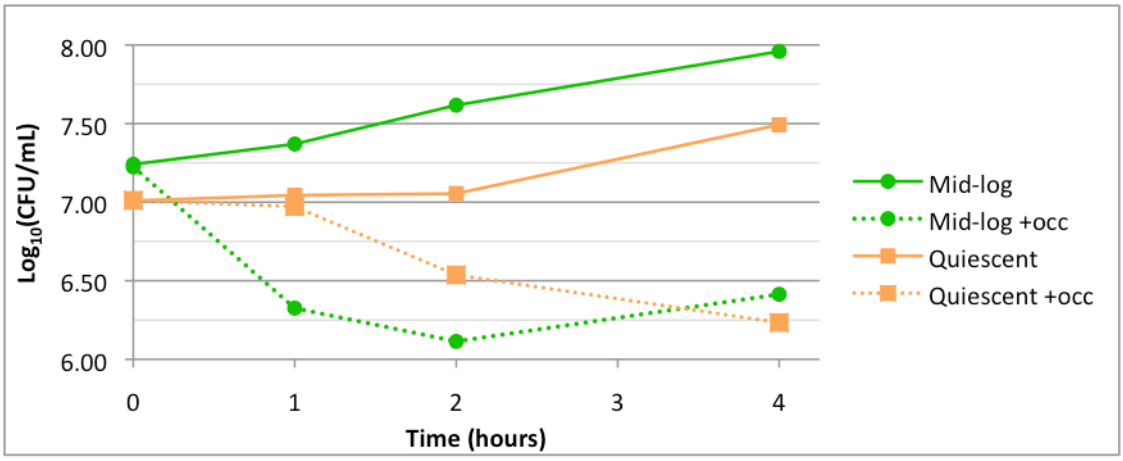

Figure 3.3 Quiescent cultures exhibit temporary resistance to occidiofungin in fresh YPD

Graph illustrating the temporary resistance of quiescent cells compared to mid-log in YPD with 1.0µg/mL occidiofungin (+occ). Representative graph shown (n=2).

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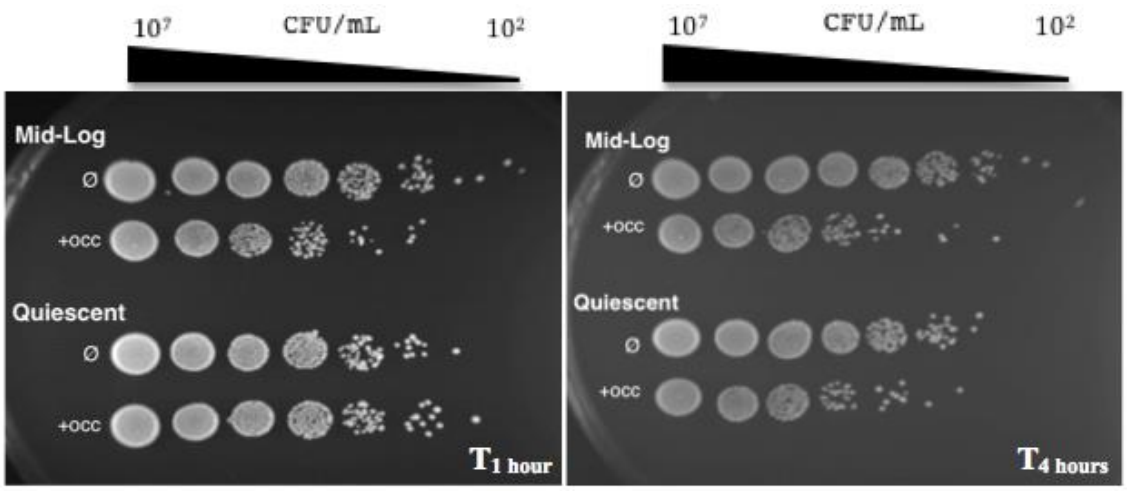

Figure 3.4 Quiescent cells exhibit temporary resistance to occidiofungin in fresh YPD

Mid-log and quiescent cells – after treatment with 1.0µg/mL occidiofungin (+occ) or an equivalent volume of 100% DMSO control (Ø) for the indicated time – were 5-fold serially diluted in YPD in a microtiter plate and 4.0µL from each dilution were spotted, in profile, onto a YPD agar plate. Representative image shown (n=2).

To further characterize the initial resistance and subsequent decline in cell viability, the same experiment was conducted with the addition of "depleted" YPD media (see "Materials and Methods") to determine the effects decreased nutrient availability had on occidiofungin bioactivity in both cell types. As seen in Figure 3.5, quiescent cell viability trends in fresh YPD were similar to previous findings: cells exhibited an initial resistance to occidiofungin followed by a decline in cell number. Quiescent cultures in depleted media, however, maintained almost complete resistance to occidiofungin up to 4 hours post-exposure. Interestingly, mid-log phase cells were significantly more susceptible to occidiofungin in depleted YPD than in the fresh media control.

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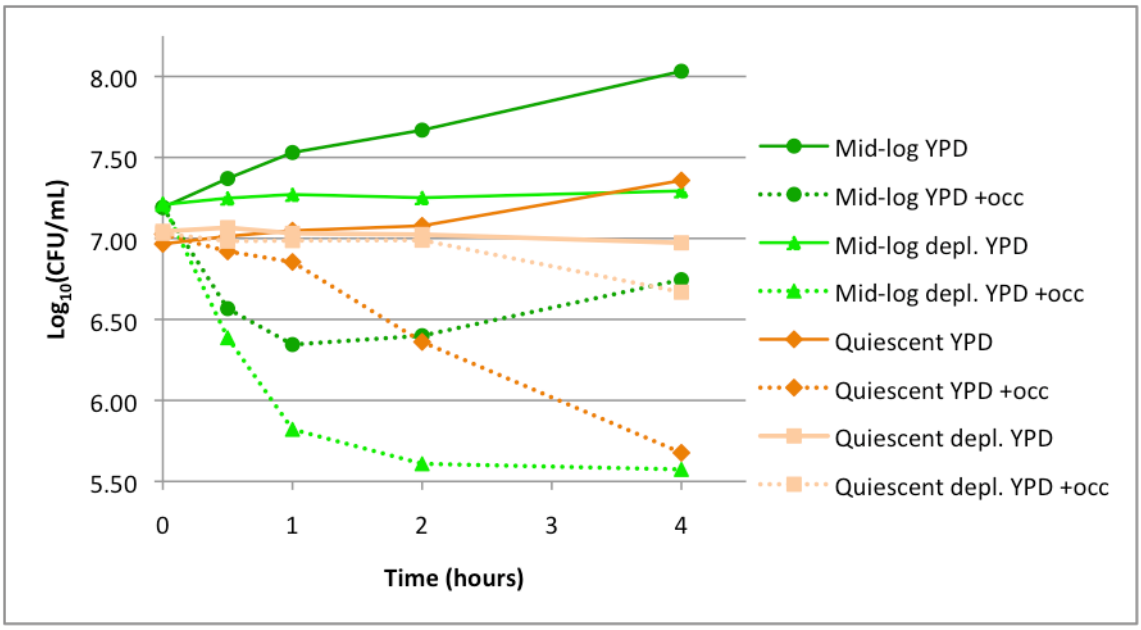

Figure 3.5 Nutrient availability has a significant impact on susceptibility to occidiofungin

Graph illustrating mid-log and quiescent cells in new and depleted (depl.) YPD with 1.0µg/mL occidiofungin (+occ). Representative graph shown (n=2).

The delayed susceptibility of quiescent cells to occidiofungin is of particular interest. Given that the biological target of occidiofungin has yet to be identified, it is important to characterize the shift from resistant to susceptible that occurs when quiescent cells are exposed to occidiofungin in fresh media, as it may aid in uncovering the mechanism by which occidiofungin induces cell death. To monitor the transition from a quiescent to actively growing state, the assay comparing % Cell Viability after exposure to elevated temperatures was repeated with the addition of a quiescent cell sample placed in fresh YPD media (as described in the Materials and Methods). As shown in Figures 3.6 and 3.7, quiescent cell resistance to high temperatures declined over time when cells were introduced to fresh media and approached that of actively growing cells within 2 hours.

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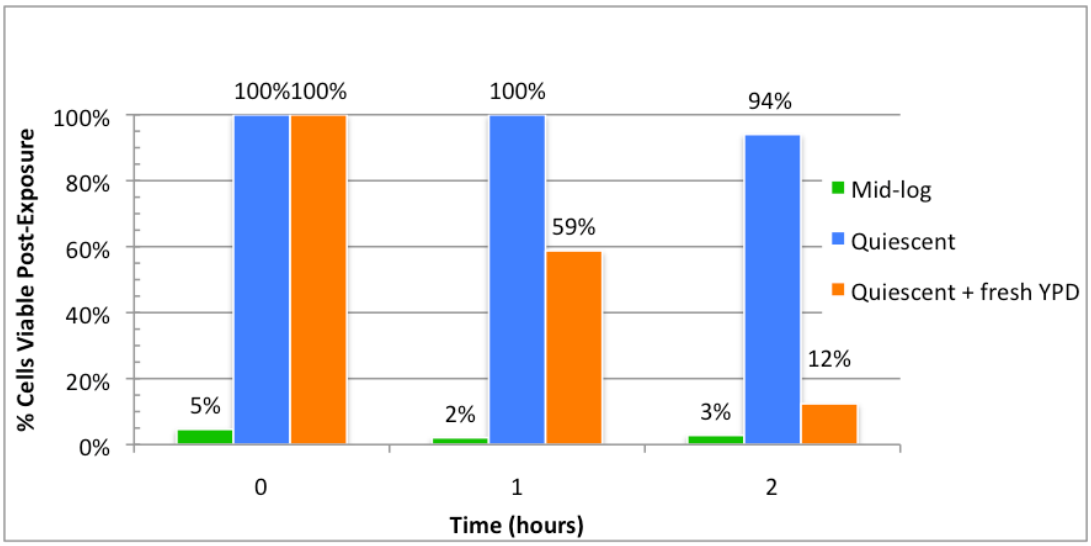

Figure 3.6 Quiescent cells approach mid-log levels of sensitivity to elevated temperatures (53°C) in fresh YPD with respect to time

This graph indicates the percentage of viable cells remaining after 10 minutes of exposure to 53°C, relative to unexposed cells, with respect to the indicated time maintained in fresh YPD ("mid-log" and "quiescent + fresh YPD" samples) or depleted YPD ("quiescent" sample). Representative graph shown (n=3).

The loss of resistance to elevated temperatures is indicative of cells exiting their quiescent state and resuming proliferative growth. This is in support of the notion that yeasts are protected from occidiofungin as long as they remain in quiescence.

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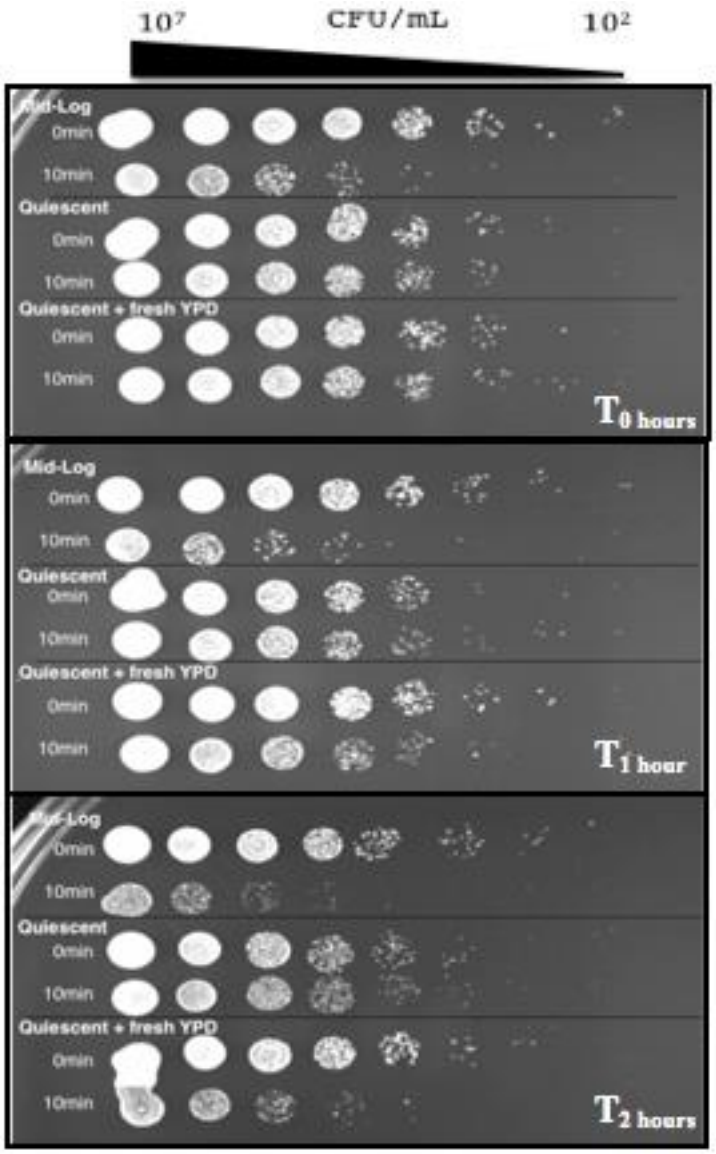

Figure 3.7 Quiescent cells placed in fresh YPD lose their resistance to elevated temperatures (53°C) over time

Spotting Assay depicting cell viability of mid-log cells in YPD, quiescent cells in their culture medium, and quiescent cells in fresh YPD for 0, 1, and 2 hours prior to exposure to 53°C for 10 minutes. Representative image shown (n=3).

Occidiofungin Bioactivity and Nutrient Deprivation

To expand on the impact nutrient availability had on occidiofungin's bioactivity, mid-log cells were exposed to occidiofungin in synthetic defined media lacking glucose,

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nitrogen, or phosphate. As seen in Figures 3.8 and 3.9, cell viability after occidiofungin exposure varied greatly depending on the starvation conditions in which cells were placed. Mid-log cells exposed to glucose-deprivation conditions appeared to be more susceptible to occidiofungin, while those in SD media lacking phosphate were resistant. Nitrogen availability did not appear to impact occidiofungin bioactivity.

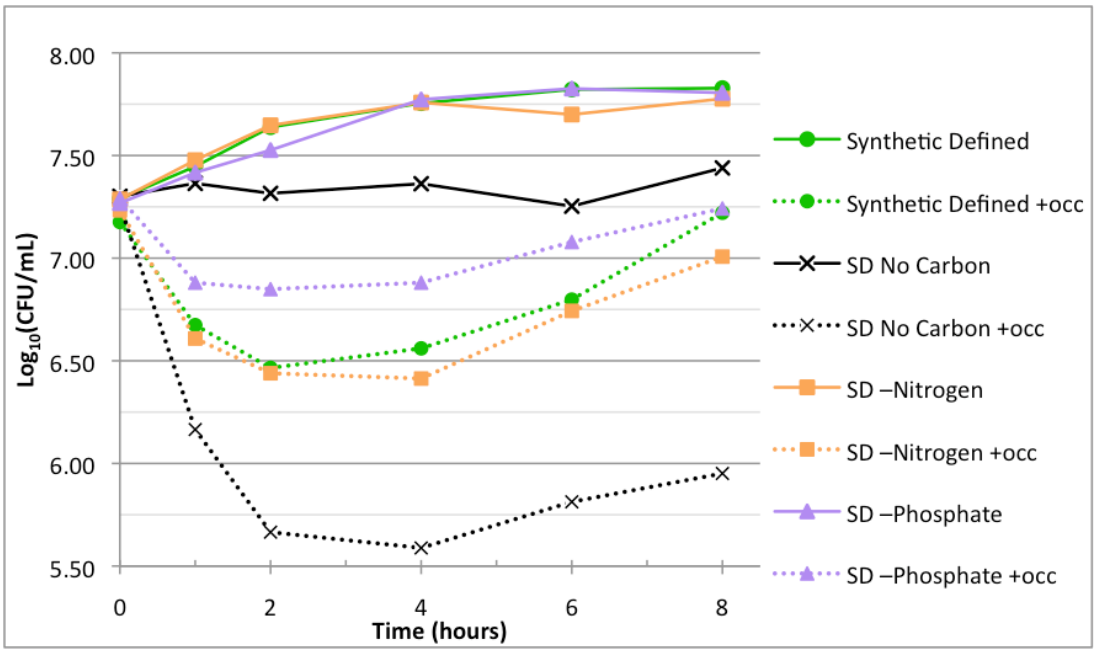

Figure 3.8 Cellular response of mid-log cells to occidiofungin is dependent on nutrient availability and is nutrient-specific

CFU Assay depicting cell viability of mid-log cells post-exposure to 1.0µg/mL occidiofungin (+occ) in Synthetic Defined (SD) media as well as SD media lacking Glucose (No Carbon), Nitrogen, or Phosphate. Representative graph shown (n=2).

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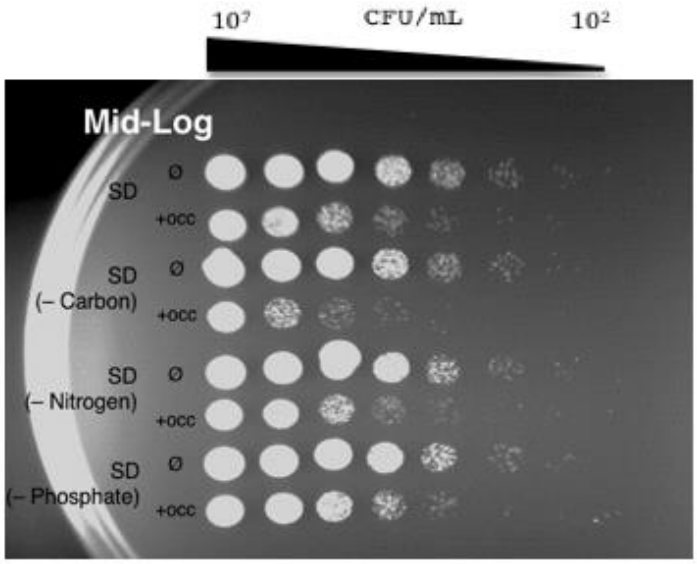

Figure 3.9 The susceptibility of mid-log cells varies in normal and starvation conditions

Spotting assay depicting differences in susceptibility at 2 hours post-exposure to 1µg/mL occidiofungin (+occ), or vehicle control (Ø), under normal and various starvation conditions. SD; Synthetic Defined. Representative image shown (n=2).

Glucose Starvation and Rapamycin-Induced Inhibition of the Nutrient Sensing Complex, TOR1C

The largest difference in susceptibility was the response to glucose-starvation conditions. The stark decrease in viable cells seen in the absence of glucose may be due to the abrupt inhibition of protein synthesis seen in cells whose extracellular glucose source is removed (Ashe, De Long and Sachs 2000). To evaluate whether this translation-inhibiting response to the absence of glucose is what resulted in such drastic differences, we compared the cellular response to occidiofungin under a chemically induced starvation response with the response to the absence of glucose.

To further characterize the cellular response to occidiofungin in glucose-starved cells, we took advantage of rapamycin's TOR inhibiting capabilities in a CFU assay utilizing media both with and without glucose in the presence of rapamycin and

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occidiofungin. To initially confirm that rapamycin blocked TOR signaling, we measured changes in Cit2p levels by Western blot analysis. Previous work has shown that rapamycin induces expression of *CIT2*, a finding that was repeatable (Giannattasio, et al. 2005). As shown in Figure 3.10, mid-log cells exposed to 0.1µg/mL rapamycin showed a significant increase in Cit2p levels within 20 minutes of addition. Occidiofungin is unlikely to interfere with signaling downstream of Tor1C, as Cit2p expression was still increased in the presence of both rapamycin and occidiofungin (data not shown).

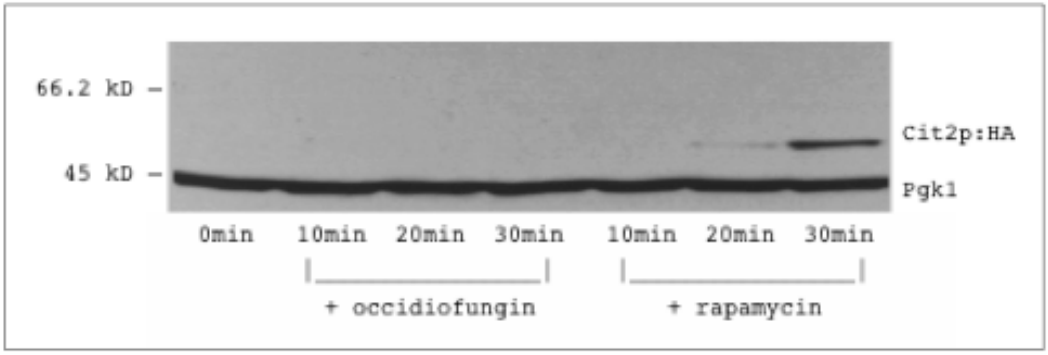

Figure 3.10 Expression of Cit2p:HA_3 increases upon exposure to 0.1µg/mL rapamycin but not after exposure to 1.0µg/mL occidiofungin

Expression of Cit2p:HA_3 increased within 20 minutes of exposure to rapamycin. Total cell protein extracts (equivalent to 0.1 OD_{600}) were separated by SDS-PAGE. Protein detection by western blot was conducted using anti-HA to detect Cit2p and anti-Pgk1p to detect Pgk1p. Pgk1p was included to confirm equal loading. Representative image shown (n=2).

The resulting data (Figure 3.11) holds strong implications for the role of the cell's response to nutrient availability in occidiofungin's varying bioactivity. Cells exposed to both rapamycin (at 0.1µg/mL) and occidiofungin (1.0µg/mL) experienced a much greater decline in cell viability in both media types than with occidiofungin alone, though this effect was more pronounced in media lacking glucose.

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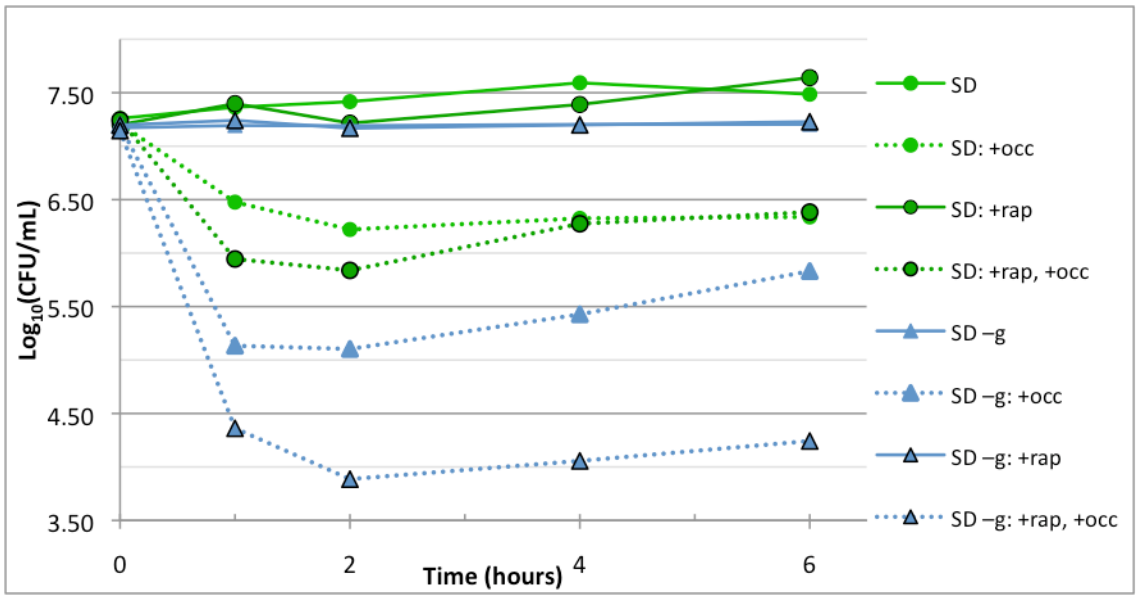

Figure 3.11 Rapamycin enhances the effects of occidiofungin

CFU data of mid-log cells exposed to 0.1µg/mL rapamycin (+rap) and 1.0µg/mL occidiofungin (+occ) in the presence (SD; synthetic defined) and absence (SD-g) of glucose. Representative graph shown (n=2).

Inhibition of Cytosolic and Mitochondrial Protein Synthesis

In addition to rapamycin, cycloheximide was also used to pharmacologically assess occidiofungin bioactivity. We subjected mid-log cells to 25µg/mL cycloheximide and 1.0µg/mL occidiofungin and found a marked decrease in the ability of cultures to recover from occidiofungin exposure (Figure 3.12). However, because cells exposed to cycloheximide experience no growth, it remained unclear if the difference was because the cells' inability to complete the cell cycle simply revealed total cell death unmasked by the usual culture recovery, or if they are due to other factors.

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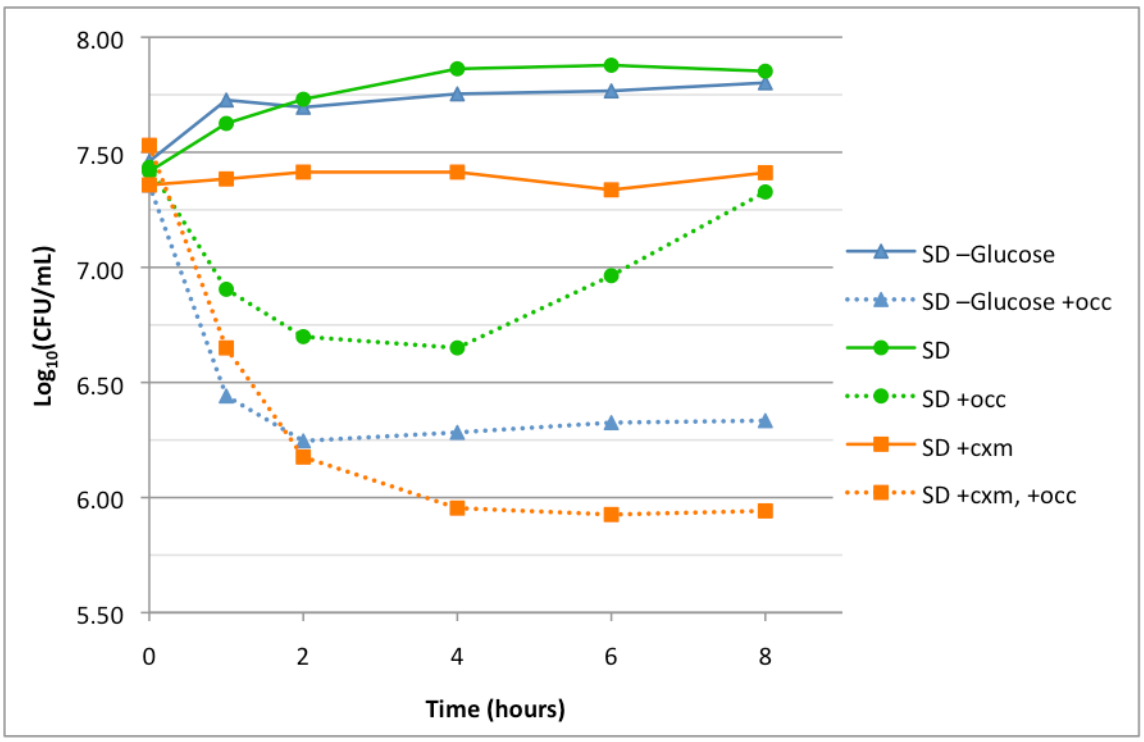

Figure 3.12 Cycloheximide addition inhibits the culture's recovery from occidiofungin

Graph depicting the effect of 25µg/mL cycloheximide (+cxm) on the susceptibility of cells to 1.0µg/mL occidiofungin (+occ) in SD (synthetic defined) media as compared to occidiofungin treatment in SD-glucose media. Representative graph shown (n=2).

To this end, it was decided that pre-treating cells with cycloheximide for 30 minutes would aid in elucidating the cause of this effect, as the effects of translation inhibition would be established in pre-treated cells. Our data (Figure 3.13) demonstrates that pre-treatment with cycloheximide actually provides a protective effect against occidiofungin, as opposed to the synergistic effects seen when cells were concurrently exposed to both drugs.

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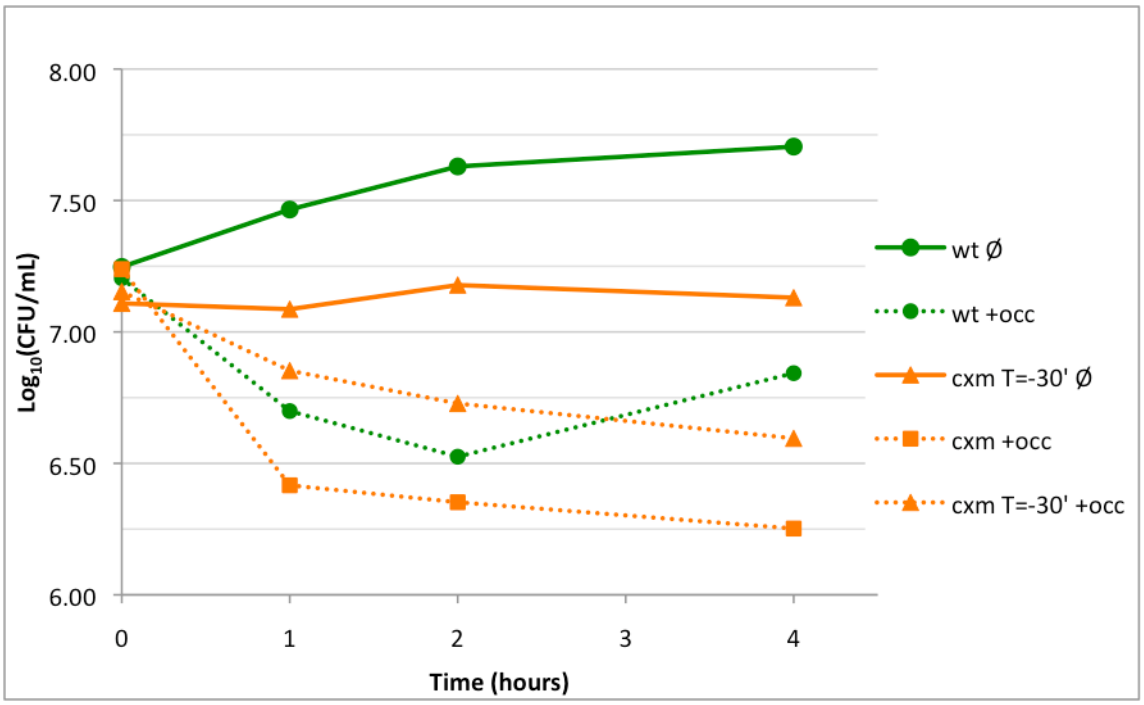

Figure 3.13 Pre-treatment with cycloheximide is protective against occidiofungin, while simultaneous treatment has synergistic effects

Graph illustrating the protective effects of 30 minutes of pre-treatment (T=-30') with 25µg/mL cycloheximide (cxm) and the synergistic impact of simultaneous treatment (n=5) with 1µg/mL occidiofungin (+occ). Representative graph shown (n=3).

In addition to cycloheximide, the effects of pre- and concurrent treatment with chloramphenicol were also evaluated with respect to occidiofungin. As the mitochondrion is a significant producer of ROS in the cell, we hoped that utilizing this mitochondrial translation inhibitor might aid in elucidating whether mitochondria might play a role in occidiofungin-induced cell death. Further, due to the mitochondrial repression effects induced by glucose, we were also interested in investigating the effects of chloramphenicol when mitochondrial activity was not repressed (Mian, Küenzi and Halvorson 1973). In this regard, the impact of chloramphenicol was tested in synthetic

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media containing 2% glucose and compared to the same media containing 2% raffinose as the carbon source (Figure 3.14).

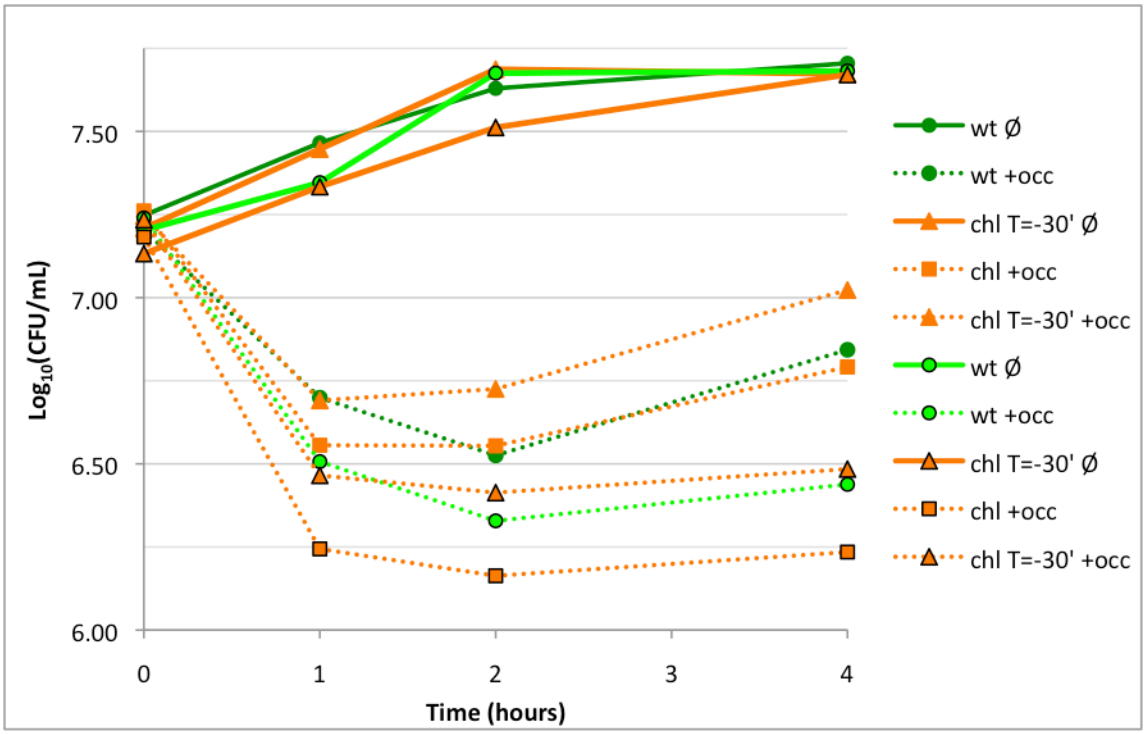

Figure 3.14 Pre-treatment with chloramphenicol (chl) had little impact on occidiofungin bioactivity, but is synergistic when added simultaneously with occidiofungin

Graph demonstrating the effect of pretreatment (T= -30') with 60µg/mL chloramphenicol (chl) versus concurrent treatment with 1.0µg/mL occidiofungin (+occ) in synthetic media containing glucose (no marker outlines) as compared to synthetic media containing raffinose (outlined markers) as a carbon source. Representative graph shown (n=2).

Our results indicate that chloramphenicol treatment at 60µg/mL in conjunction with 1.0µg/mL occidiofungin treatment appears to exhibit synergistic effects in synthetic media with glucose as well as in media with raffinose as a carbon source. In both media types, pre-treatment of cells with chloramphenicol may have slight protective effects, though this effect appears to be minimal.

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Phosphate Deprivation and Occidiofungin Activity

With a small number of factors identified that increase susceptibility, we shifted our focus to the resistance demonstrated in phosphate deprivation conditions. We selected two primary PHO pathway deletion mutants, Δ*pho4* and Δ*pho80*, to aid in characterizing the resistance response that occurs under phosphate starvation conditions. As shown below in Figures 3.15 and 3.16, the Δ*pho4* mutant was resistant to occidiofungin compared to both the wild type and Δ*pho80* cells under phosphate replete and deplete conditions, while Δ*pho80* exhibited only slightly greater susceptibility than the wild type.

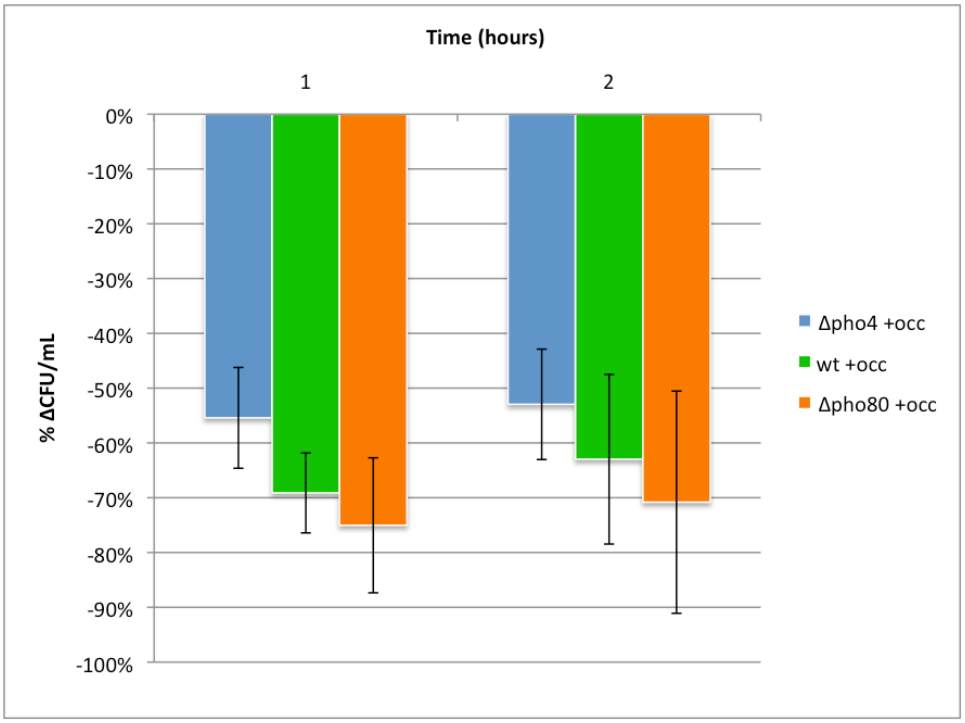

Figure 3.15 Occidiofungin is more effective against *pho4* mutants than *pho80* mutants and the wild type in SD media

Values for the average % ΔCFU (n=3) after occidiofungin exposure in SD media for *pho4* and *pho80* mutants were calculated by taking the mean change in the CFU value between T_0 and subsequent time points. The average of this value over 3 trials was then used to represent the average % change in CFU values for each strain after occidiofungin exposure, with respect to time.

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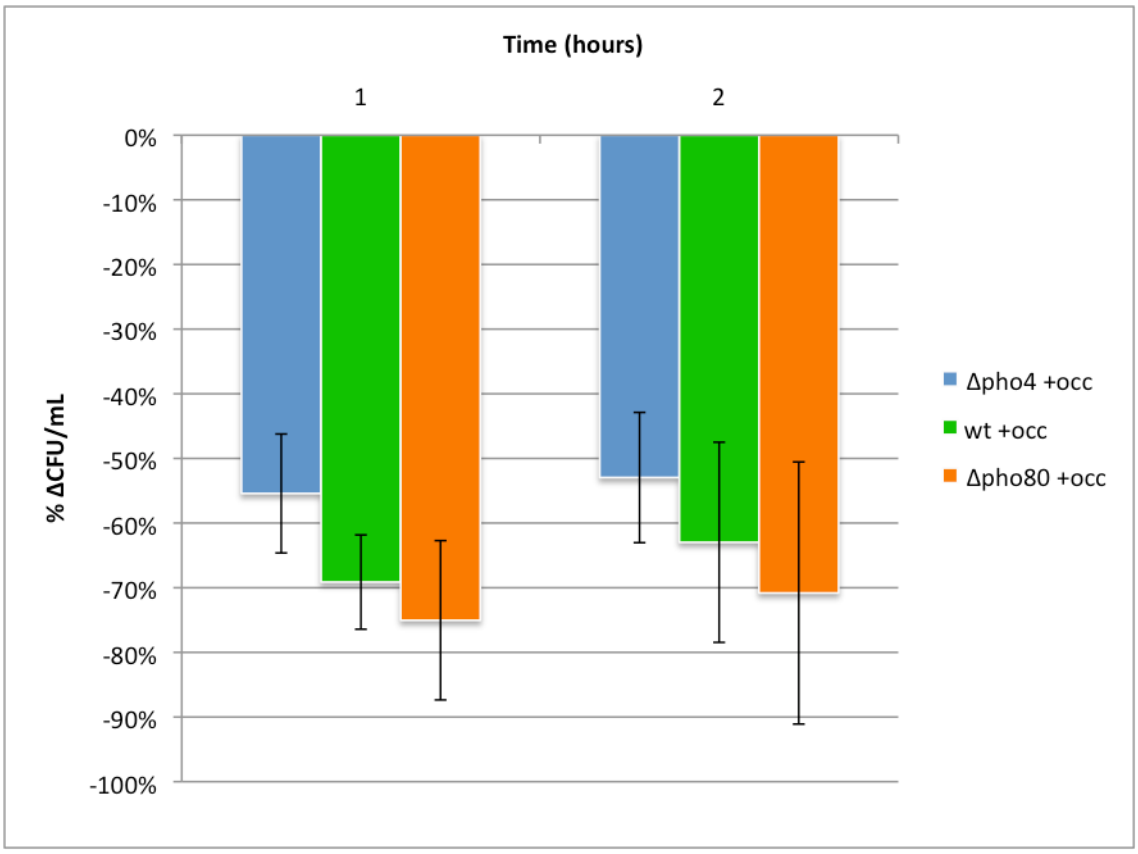

Figure 3.16 Occidiofungin is more effective against *pho4* mutants than *pho80* mutants and the wild type in SD media lacking phosphate

Values for the average % ΔCFU (n=3) after occidiofungin exposure in SD media lacking phosphate for *pho4* and *pho80* mutants were calculated by taking the mean change in the CFU value between T_0 and subsequent time points. The average of this value over 3 trials was then used to represent the average % change in CFU values for each strain after occidiofungin exposure, with respect to time.

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CHAPTER IV

SUMMARY

Discussion of Results

The need for novel antifungal therapies has never been more apparent. The increasing significance of IFIs in healthcare and rising prevalence of resistance development in fungal pathogens makes the discovery and characterization of novel antifungal therapies ever important. Occidiofungin's unique and broad-spectrum activity against fungi, limited toxicity to mammalian tissue, and chemical stability support its candidacy for further investigation as a useful antifungal in clinical therapy. Though its mechanism of action is not fully understood, we have identified a number of cellular and environmental factors that either enhance or reduce occidiofungin's effectiveness against *Saccharomyces cerevisiae*.

Unfortunately, the nature of conducting CFU assays using dose-dependent drugs makes statistical analysis of the data difficult. Even slight variations in starting cell culture density appear to have an impact on the degree to which differences in sensitivity are detected. Thus, graphs exemplifying general trends in susceptibility consistent across multiple trials were used to illustrate results.

We have demonstrated that yeast in a starvation-induced quiescent state are resistant to the effects of occidiofungin and retain this resistance until environmental stimuli (specifically, the availability of a carbon source) induce their reentry into the cell

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cycle, after which they become susceptible to the drug. This suggests that occidiofungin may be less effective in quiescent cells or that susceptibility may require active growth. Further, this sustained resistance allows us to deduce that occidiofungin must require active transport processes in order to exhibit its effects. If occidiofungin were able to interact with the quiescent yeast in depleted media in a manner that promoted its eventual import into the cell (via receptor binding or non-specific interactions with the outer cell wall), these cells are likely to have succumbed to occidiofungin upon transfer to YPD agar and the data would have reflected cell death.

The potential for genes involved in quiescence to be effective targets of antifungals seems unlikely. However, these findings are significant due to the metabolic characteristics that quiescent yeast share in common with slow growing cells, which are often found within biofilms, and provides support for the notion that active cellular processes may be required for occidiofungin's import and activity. For drugs that rely on active cellular processes to exhibit their activity, one would expect their efficacy to decrease as cell metabolism slows. This has been demonstrated in one study aimed at characterizing the contribution of slow growth rates to amphotericin B and azole resistance in fungal biofilms. Though it was found that the antifungal resistance of biofilms is not solely attributable to the slow growth of its cells, their findings did indicate that planktonic cells at very low growth rates (a trait the authors noted was also found in the innermost cells of fungal biofilms) exhibited similar resistance (Baillie and Douglas 1998). For these reasons, it was decided that the retained ability to respond to stimuli and abundant characteristics of slow growth found in quiescent cells may suffice as a preliminary model to aid in determining whether

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occidiofungin's activity is reliant on active cellular processes. However, our results are not conclusive with regards to the impact of growth rate on occidiofungin susceptibility, and these findings should be confirmed through the use of a chemostat to manipulate growth conditions before characteristics on the susceptibility of slow growing planktonic cells can be reported.

Further, we have shown that transcriptional responses to starvation for specific nutrients have variable impacts on occidiofungin bioactivity. The absence of glucose, the key signal in regulating normal cellular processes (particularly gene expression as it relates to growth), drastically increases susceptibility to occidiofungin (Ashe, De Long and Sachs 2000). This effect is presumably due to the starvation-induced alteration of the cell's transcriptional profile and abrupt cessation of translation, and not due specifically to the absence of glucose. Knowing this, the possibility arises that the increased susceptibility to occidiofungin in the absence of glucose may be due the cells' inability to synthesize the proteins necessary to respond to and repair cellular damage caused by occidiofungin. This would result in more rapid cell death than in cultures able to compensate for some of the stresses.

This hypothesis is supported by the synergistic effects seen under rapamycin-induced TOR1C inhibition (falsely interpreted by the cell as starvation), as these effects were observed in both the presence and absence of glucose. With regards to previously published research concerning the concurrent use of rapamycin and antifungals, the data is inconclusive. One report by Dannaoui, et al. indicated that synergistic effects between rapamycin and the antifungals amphotericin B, itraconazole, posaconazole, and ravuconazole were present in 70%, 50%, 40%, and 30% of the 10 zygomycete isolates

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tested, respectively, while antagonistic effects were observed with itraconazole in 20% of isolates and with ravuconazle in 10% (Dannaoui, Schwarz and Lortholary 2009). These results are in contrast to data reported later by Narreddy, et al., which demonstrated "consistent and significant antagonism" with rapamycin and posaconazole in most of the 28 clinical zygomycete isolates tested. This research group attributed these contrasting findings to the different endpoint readings utilized in the two studies, as Narreddy, et al. used the CLSI M38-A2 standard of 100% inhibition as the endpoint, while Dannaouri, et al. used an endpoint of 50% inhibition (Narreddy, et al. 2010). The utility of rapamycin as a candidate for combination therapy is therefore unresolved, and additional research will be necessary to definitively characterize these effects.

Notably, a mutant strain lacking *TOR1* showed no change in MIC value compared to a wild type strain (data not shown), a result seemingly contrary to data reported here. We suspect this is likely due to sensitivity differences between MIC and CFU assays discussed previously and not an indication of conflicting results, as these discrepancies have been demonstrated in our lab before (data not shown). As CFU assays are a more accurate analysis of cell number as it relates to susceptibility, we suggest reevaluating the Δ*tor1* strain using a CFU assay. Regardless, there is significant evidence for the role of glucose starvation-induced changes in transcriptional regulation in the increased susceptibility we demonstrated.

We also demonstrated seemingly conflicting trends in the effects of cycloheximide-mediated inhibition of cytosolic translation and chloramphenicol-induced inhibition of mitochondrial translation. Cycloheximide (cxm) is a standard tool used in molecular biology as it inhibits the elongation phase of eukaryotic translation by

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interfering with the translocation step. This is useful for the general analysis of mRNA translation and ribosome profiling, but was also helpful in our case, as it allowed us to determine that active translation is necessary for surviving occidiofungin exposure (Gerashchenko and Gladyshev 2014). Chloramphenicol (chl), in contrast, is a bacteriostatic translation inhibitor that functions by inhibiting the peptidyl transferase activity of prokaryotic and mitochondrial ribosomes, thereby preventing protein chain elongation. Chloramphenicol does not interfere with cytosolic translation. We demonstrated that concurrent addition of these inhibitors with occidiofungin resulted in greater cell death than samples that received occidiofungin alone. This suggests that translation may be required to abrogate cellular damage due to occidiofungin exposure. When cells were exposed to occidiofungin after being pre-treated with these drugs, however, the effects were opposite: cells were somewhat protected. In the case of cycloheximide pre-treatment, it is possible that the lack of growth under such conditions could play a role in this antagonistic effect. Given that occidiofungin activity appears to be reliant on active cellular processes, it is also possible that, while cells enduring cycloheximide-induced translation inhibition are indeed still active, either occidiofungin's transport into the cell or its mechanism of inducing cell death may be dependent on processes heavily influenced by the rate of new protein synthesis. In support of the latter conclusion, previous research has indicated that cycloheximide-induced translation inhibition prevents both apoptotic chromatin condensation and DNA fragmentation. It was demonstrated that treatment with 15µg/mL cycloheximide, a concentration sufficient to reduce the rate of cytosolic translation by >92%, for 30 minutes prior to hydrogen peroxide exposure provided protective effects

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against oxidative-stress, thereby preventing the cell from inducing its own cell death (Madeo, et al. 1999). The lack of growth seen in the cycloheximide pre-treatment control (not exposed to occidiofungin) not only supports the active growth requirement for occidiofungin's activity, but also indicates a potential disadvantage of combination therapy using cycloheximide and occidiofungin, as any number of factors impacting the utility of the two compounds could drastically alter occidiofungin's effectiveness.

With regards to mitochondrial translation inhibition, pre-treatment with chloramphenicol had little impact on cell death due to occidiofungin, while simultaneous treatment resulted in increased cell death. One implication of these results is that the mitochondria may not play a direct or significant role in occidiofungin's bioactivity. Rather, it is likely that the production of ROS due to normal or increased mitochondrial activity may only enhance the degree to which the cell experiences oxidative stress, potentially hastening an apoptotic response. The resistance to occidiofungin seen in cells under anaerobic conditions supports this conclusion (Emrick, et al. 2013).

Finally, we demonstrated that cells exhibit resistance to occidiofungin under phosphate-deprivation conditions, and examined this resistance further by evaluating two phosphate signaling pathway mutants and their respective susceptibilities to occidiofungin. Phosphate signaling in budding yeast has been well characterized and is mediated by a subset of genes in what is known as the PHO pathway. This pathway utilizes the phosphorylation of the transcription factor Pho4p to control its localization in the cell and therefore its access to its effector genes in the nucleus. In

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the presence of phosphate, the Pho80p-Pho85p complex phosphorylates Pho4p, preventing its entry into the nucleus. Under phosphate deprivation conditions, this complex is unable to phosphorylate Pho4p, which consequently results in its translocation to the nucleus where it upregulates the expression of phosphatase and phosphate scavenging genes (Mouillon and Persson 2006). Due to the role of Pho80p in phosphate signaling, one would have expected the Δ*pho80* mutant to display a resistance profile similar to that of cells placed into phosphate starvation conditions, as the Pho80p-Pho85p complex would no longer exist to phosphorylate Pho4p, resulting in a perpetual cellular response to phosphate starvation regardless of actual nutrient availability. On the other hand, the Δ*pho4* mutant in phosphate starvation conditions would be expected to behave as the wild type did in SD complete media due to the inability of the strain to respond to phosphate deprivation. Our results, however, were in direct contrast to our hypothesis of the impact of occidiofungin on these PHO pathway mutants. With regards to the *pho4* mutant, research by Hu et al identified a number of genes whose transcriptional regulation was altered in strains deleted for *pho4* (Hu, Killion and Iyer 2007). We suspect these transcriptional changes may well be responsible for the resistance effects seen in our study and that genes within this dataset would be interesting candidates for future pursuit. Regardless, we did identify the deletion of *PHO4* as a contributor to occidiofungin resistance, the mechanism of which could be resolved through an analysis of the transcriptional changes that occur in the cell upon deletion of this transcription factor.

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Directions for Future Research

The role of occidiofungin in the arsenal of modern antifungal therapies has yet to be determined, as there is much to be discovered about its activity in the fungal cell. Through our studies, we have identified a number of potential avenues in which information about occidiofungin's mechanism of action could be further characterized. First, altered transcriptional profiles in response to starvation conditions are clear effectors of occidiofungin bioactivity. An investigation of the literature associated with starvation response and TOR1C inhibition, and the inclusion of a CFU analysis of yeast deleted for TOR1, could yield promising indicators of where this novel antifungal functions in the cell. Further, as we have established that active cellular processes may be required for its import and activity, studies evaluating the effects of slow growth rates and the role of various transport mechanisms of yeast on occidiofungin's bioactivity could prove worthy of inquiry.

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