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Mechanism of Action of a Novel Antifungal Compound

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MECHANISM OF ACTION OF A NOVEL ANTIFUNGAL COMPOUND

By

Amber Lynne Ward

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford, Mississippi

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DEDICATION

This thesis is dedicated to my parents, Bill and Sherry Ward, for their love, encouragement and endless support. Thank you for your unconditional support with my studies, and always allowing me to pursue my dreams no matter how big or small. I am honored to have you as my parents. I love you.

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ABSTRACT

Opportunistic fungal infections have become problematic in recent years. There has been an emergence of new fungal pathogens, development of antifungal resistance, and increasing prevalence. In addition, toxicity, resistance, and lack of efficacy as a single agent limit the effectiveness of current antifungal drugs. Therefore, new drugs targeting new pathways are greatly needed. An analog derived from the marine natural product phloeodictine, analog PHL5-34A, was identified as having strong in vitro antifungal activity. In this study, its mechanism of action (MOA) was investigated in the model yeast, *Saccharomyces cerevisiae*. This study made use of a collection of 166 yeast mutants carrying deletions in transcription factors, which function as master regulators of cellular processes. Using the parent *S. cerevisiae* strain BY4742, a pilot experiment was conducted to determine the sub-inhibitory concentration of PHL5-34A that reduced cell growth on agar plates. After assay conditions were optimized, the mutant collection was screened to identify mutants with altered sensitivity to PHL5-34A. Of the 166 deletion mutants tested, 20 were found to be hypersensitive to PHL5-34A when compared to the parent. Drop-test assays were conducted and 10 mutants were confirmed to be hypersensitive to PHL5-34A. Of these confirmed mutants, 4 mutants were found to carry deletions in genes required for cell cycle functions. These 4 mutations were then analyzed by PCR to confirm that they were in the correct genes. This work has demonstrated that the cell cycle is one of the pathways affected by PHL5-34A, and it will serve as an important step in determining the exact MOA of this compound.

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LIST OF ABBREVIATIONS

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
HIV	human immunodeficiency virus
MOA	mechanism of action
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
YPD	yeast extract, peptone, dextrose

INTRODUCTION

Opportunistic fungal infections have become problematic in recent years. There has been an emergence of new fungal pathogens, development of antifungal resistance, and increasing prevalence. In addition, toxicity, resistance, and lack of efficacy as a single agent limit the effectiveness of current antifungal drugs. High rates of morbidity and mortality continue to be associated with infections that are caused by molds and yeast (Mohr et al., 2008). The advancement in medicine, surgery, and transplantology in the last three decades has caused an alarming increase in immunocompromised patients that are susceptible to fungal infections. Many patients are at a higher risk of developing mycoses, such as those suffering from HIV infection, receiving organ transplantation and intensive cancer therapy (Karkowska-Kuleta et al., 2009). With such a wide range of risk factors, the prophylactic use of antifungal therapies is one of the reasons of frequent resistance to antifungal drugs. This introductory section provides background information on the major fungal pathogens, the current antifungal agents, the value of mechanism for action studies, and the novel antifungal compound, PHL5-34A that was studied in this project.

Major fungal pathogens. *Candida* species are the most common pathogens associated with fungal disease, and *Aspergillus*, *Cryptococcus* sp and Zygomycetes account for

many fungal infections as well (Mohr et al., 2008). These fungi are very common and can be acquired from host surroundings. After antifungal treatment, the mortality rate is still very high due to the patient's immunodeficiency, late diagnosis, or fungal drug resistance (Karkowska-Kuleta et al., 2009).

There are many invasive infections associated with *Candida* species, from catheter-associated localized infections to widespread disseminated disease (Mohr et al., 2008). There are currently more than two hundred ascomycetous yeasts included in the genus *Candida*, and of these, only a few species of the genus are opportunistic pathogens of humans. *Candida albicans* are thought to be the most common cause of fungal infections in humans today (Karkowska-Kuleta et al., 2009). Identifying patients that are at a higher risk of developing *Candida* infections is an important step in determining which patients should receive treatment. Some of the treatment options may include strategies that are presumptive (there are many risk factors that raise suspicion of infection) or prophylactic (preventative measure for high-risk factors). Some of the most common risk factors that have been identified for invasive candidiasis are prolonged stay in the intensive care unit (ICU), placement of central venous catheters, prolonged treatment with broad-spectrum antibiotics, receipt of parenteral nutrition, recent surgery (particularly intraabdominal), hemodialysis, and a diagnosis of diabetes mellitus or pancreatitis (Mohr et al., 2008).

Aspergillus fumigatus, is an ascomycetous fungus found around the world and its spores are abundant in the environment due to their small size. *A. fumigatus* conidia are frequently present in food, tap water, at home, and in office rooms (Karkowska-Kuleta et al., 2009). A definitive diagnosis of invasive aspergillosis requires a positive culture from a sterile site, or it requires histologic or radiologic evidence in a patient at high risk with compatible clinical findings. Unfortunately, when the fungi are positively identified in the body, it could be too late for treatment. Techniques for an early diagnosis, preventative strategies, and new treatment methods are needed to reduce the mortality in invasive aspergillosis patients (Mohr et al., 2008).

Cryptococcus neoformans is a saprophytic, basidiomycetous, dimorphic organism found worldwide. Its natural habitats are pigeon droppings and contaminated soil, and its small basidiospores can turn into yeast cells. The basidiospores or yeast cells may be inhaled by humans, then through the respiratory tract the pathogen can disseminate within the host causing pulmonary infections, and subsequently, due to the predilection of *C. neoformans* for the central nervous system, life threatening meningoencephalitis (Karkowska-Kuleta et al., 2009). It causes infections in both immunocompromised and immunocompetent patients. In addition, patients who have undergone organ transplantation and are receiving high-dose corticosteroids are at increased risk for development of cryptococcosis (Karkowska-Kuleta et al., 2009).

Current antifungal agents. There are currently five classes of antifungal agents that are used orally or intravenously for the treatment of fungal infections in humans. The five classes are polyenes, pyrimidine analogues, allylamines, azoles and echinocandins (Table 1). These five classes of antifungal agents each have limitations that go along with their advantages. Some of the limitations include spectrum of activity, route of administration, drug interactions, and toxicity profile (Denning and Hope, 2010). The cell membrane, DNA and RNA synthesis, and the cell wall are the three main targets of drugs that are in current use for systemic therapy of invasive mycoses. The polyenes and azoles target the cell membrane, the antimetabolite 5-fluorocytosine interferes with DNA and RNA synthesis, and echinocandins affect the cell wall (Nosanchuk, 2006).

The bacterial genus *Streptomyces noursei* produce polyenes, which are broad spectrum antifungal agents. There have been approximately ninety polyenes discovered since the 1950's, but problems with solubility, stability, oral bioavailability, and toxicity have prevented many of these compounds from being developed for clinical purposes (Denning and Hope, 2010). Amphotericin B is the polyene primarily in use therapeutically. Amphotericin B is a product of *Streptomyces nodosus*. Amphotericin B selectively and irreversibly binds fungal cell membrane sterols. The interaction of the antifungal with membrane sterols results in the formation of transmembrane pores, allowing for the leakage of ions and small molecules resulting in cellular damage or death. The major drawbacks for amphotericin B use are its significant side effects and that intravenous administration is required for treatment of invasive mycoses.

Significant toxicities include fever, chills, arrhythmia, hypotension, respiratory distress, type IV renal tubular acidosis, renal failure, and anemia (Denning and Hope, 2010).

Azoles are the second class to target the cell membrane of fungi. The major groups of azoles are the imidazoles and the triazoles. These two groups have five-membered organic rings containing either two or three nitrogen molecules. Cellular and mitochondrial membranes are both affected by azoles. The azoles inhibit cytochrome P450-dependent 14α -lanosterol demethylation, which is a critical step in the synthesis of ergosterol, an important component of fungal membranes (Denning and Hope, 2010). The mode of administration is different in imidazoles and triazoles. Imidazoles are used by topical treatment while triazoles can be administered intravenously and orally. Since the azoles affect the P450 enzyme activity, their main toxicities are due to interactions with other compounds that induce or inhibit this system (Denning and Hope, 2010). Since the azoles are fungistatic drugs, their widespread use has resulted in the development of drug resistance (Sanglard, 2002).

Flucytosine is a pyrimidine analogue, and it has been available since 1972. Flucytosine is the only antimetabolite available for the treatment of systemic fungal infections (Denning and Hope, 2010). Flucytosine is a fluorine analogue of cytosine that functions as an inhibitor of thymidylate synthetase. It is only efficacious when administered in combination with amphotericin B. The major toxicity problems for flucytosine include bone marrow suppression, myocardial suppression, myocardial toxicity, and renal

failure. The spectrum of activity is rather limited with flucytosine, but it is active in *Candida* spp., *C. neoformans*, and some black molds (Denning and Hope, 2010).

The only allylamine in clinical use today is terbinafine. Terbinafine was discovered as a derivative of the topical antifungal naftifine, which was the original compound of the allylamine class (Denning and Hope, 2010). Terbinafine has limited activity for treatment of invasive fungal diseases, and is mainly effective against fungi such as dermatophytes. It can be combined with voriconazole for treatment of infections caused by *Scedosporium prolificans* (Denning and Hope, 2010).

The echinocandins are the newest agents clinically available for use. They are water-soluble large amphipathic polypeptides that are only available in intravenous forms. The drugs inhibit 1,3- β -glucan, the major glucan in many fungi (Denning and Hope, 2010). Organisms such as *C. neoformans*, which have mainly 1,6- β -glucan and α -glucans, are resistant. The major toxicities with this include drug interactions, phlebitis, and fever (Denning and Hope, 2010).

Table 1: Currently Available Antifungal Agents

Class and Compound	Target	Spectrum	Limitations
Polyene antibiotics Amphotericin B Lipid formulation of amphotericin B	Cell membrane (interaction with ergosterol, increased membrane permeability)	Broad spectrum against most invasive fungal infections	<ul style="list-style-type: none"> • Nephrotoxicity • High cost of lipid formulations
Nucleoside analog Flucytosine	DNA, RNA synthesis	<i>Candida</i> spp., <i>Cryptococcus</i> spp., and some filamentous species	<ul style="list-style-type: none"> • Not very effective alone • Resistance problems
Antifungal azoles Fluconazole	Cell membrane (ergosterol biosynthesis)	<i>Candida</i> spp., <i>Cryptococcus</i> spp., and some filamentous species	<ul style="list-style-type: none"> • Narrow spectrum • Resistance problems
Allylamines Terbinafine	Cell membrane (ergosterol biosynthesis)	Dermatophytes	<ul style="list-style-type: none"> • Useful only in fungal nail infections • Not of use in invasive infection
Echinocandins Caspofungin	Cell wall [β -(1,3)glucan synthase]	<i>Candida</i> spp including azole-resistant species, and some filamentous species	<ul style="list-style-type: none"> • Modest efficacy as first line agent • Narrow spectrum

Mechanism of action studies on new antifungal compounds. As described above, the antifungal drugs that are being used today have several limitations including toxicity, resistance, lack of efficacy as a single agent, a limited spectrum of activity, and high cost. In addition, their targets are mainly restricted to the cell membrane and cell wall. Therefore, the need for new drugs targeting new pathways is greater than ever. The identification of the molecular targets of new antifungal drugs and understanding how these drugs work in fungal cells is important in the antifungal drug discovery process (Agarwal et al., 2008). Compounds that have a distinct mechanism of action (MOA) are at a higher demand because they can be used for combinatorial or chemical modifications. It is also important to determine whether a novel antifungal drug is inhibiting a pathway that is targeted by currently available drugs or if it is targeting a

new pathway. If a compound inhibits a novel pathway, it can serve as a useful tool for further exploring the targeted biological pathway and understanding its regulation. When testing a novel antifungal compound, it is also important to predict the toxic effects of the compound early in drug development. Finally, identifying pathways that lead to drug resistance, which are sometimes related to the MOA of a drug, would provide further information useful to the drug discovery process.

PHL5-34A, a novel antifungal compound. PHL5-34A is a novel marine compound that was studied in this project. PHL5-34A is a synthetic compound based on the phloeodictine class of compounds found in marine sponges such as *Pellina eusiphonia* (Li et al., 2011). These compounds have a bicyclic tetrahydropyrrolopyrimidinium core with an aliphatic side chain at C-6 (Figure 1). A novel analog of one of these compounds, PHL5-34A was synthesized chemically at the National Center for Natural Products Research at the University of Mississippi.

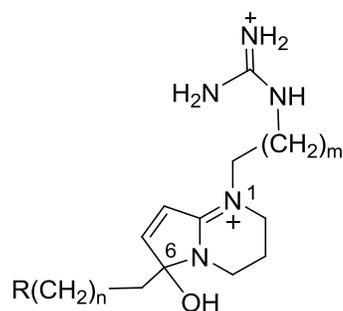


Figure 1. Structure of phloeodictine class of compounds.

This compound was shown to possess potent antifungal activity against the three major opportunistic fungal pathogens *C. albicans*, *C. neoformans*, and *A. fumigatus*. Table 2 shows PHL5-34A compared to amphotericin B. The first half of the table shows the MIC, which is the minimum inhibitory concentration against three different fungal organisms. This is the lowest concentration that inhibits growth. Amphotericin B has MIC below 2 µg/ml and PHL5-34A has MIC below 3 µg/ml. Thus, PHL5-34A is comparable in its *in vitro* antifungal activity to amphotericin B. The right side of the table shows the IC₅₀ of the two compounds in mammalian cells, which is a measure of their cytotoxicity. The IC₅₀ is the concentration that gives 50% inhibition. In this case, the higher the IC₅₀, the lower the toxicity levels. As can be seen, PHL5-34A has an IC₅₀ higher than that of amphotericin B showing that it is much less toxic in this laboratory *in vitro* test. Therefore, due to its strong antifungal activity and low toxicity, this marine compound was selected for mechanism of action studies in this project.

Table 2. *In vitro* Antifungal Activity and Cytotoxicity of PHL5-34A

Compound	Antifungal Activity [MIC ^a (MFC ^b), µg/mL]			Cytotoxicity (IC ₅₀ ^c , µg/mL)	
	<i>C. albicans</i>	<i>C. neoformans</i>	<i>A. fumigatus</i>	Vero ^d	HepG2 ^e
PHL5-34^a	2.5 (3.75)	0.47 (0.47)	2.5 (2.5)	26.0	18.5
Amphotericin B	0.63 (0.63)	0.94 (1.25)	1.25 (1.25)	7.5	12.0

^a Minimum Inhibitory Concentration (lowest concentration that allows no detectable growth)

^b Minimum Fungicidal Concentration (the lowest concentration that kills the fungus)

^c 50% Inhibitory Concentration

^d African green monkey kidney cells

^e Human hepatic carcinoma

MATERIALS AND METHODS

Strains, media, and reagents. YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) was used in all experiments. The medium was buffered with 0.165 M 3-[N-Morpholino]propanesulfonic acid (MOPS) and the pH was adjusted with NaOH to 7.0. For preparation of agar medium, YPD broth (MOPS buffered, pH 7.0) was aliquoted into a glass bottle, agar was added to a final concentration of 2%, and autoclaved at 121°C for 30 minutes. The wild-type *S. cerevisiae* BY4742 strain and the 166 transcription factor mutant strains were obtained from Open Biosystems (Huntsville, AL). PHL5-34A was synthesized at NCNPR, and dimethyl sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO). All stock solutions of PHL5-34A were prepared in DMSO.

Determining drug concentration for mutant screen. A six-well assay was performed to determine the drug concentration to be used for screening a collection of *S. cerevisiae* transcription factor mutants. PHL5-34A was added to the YPD (MOPS buffered, pH 7.0) agar at different concentrations. The concentrations were as follows: 0 µg/mL, 0.625 µg/mL, 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, and 10 µg/mL. The medium was then poured into a 6-well plate, and a single colony of the parent yeast strain (BY4742) was streaked on each well of the plate. This plate was incubated at 30°C for two days. An image of the plate was obtained using the CCD camera available in the Bio-Rad Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

Transcription factor mutant screen. To determine the sensitivity of a collection of 166 *S. cerevisiae* transcription factor deletion mutants against PHL5-34A, an agar-based screen was conducted. Each mutant was first streaked from a frozen glycerol stock on YPD agar plates containing 200 µg/ml of G418 to obtain single colonies. G418 is an antibiotic that allows selective pressure for growth of the mutant strains, and allows prevention of contaminant growth by non-mutant strains. Single colonies of each mutant were streaked on YPD (MOPS buffered, pH 7.0) agar plates containing either DMSO or 3.0 µg/ml PHL5-34A. At this concentration, PHL5-34A caused partial growth inhibition of the parental strain BY4742. One hundred and sixty-six mutants and the parental strain BY4742 were streaked on each screening plate using sterile inoculating loops. Plates were incubated at 30°C for 2 days. Strains were identified that showed a marked difference in growth when compared with the parental strain on agar plates with PHL5-34A but not on plates with DMSO. Images of the plates were obtained using the Bio-Rad Gel Documentation System.

Confirming PHL5-34A hypersensitivity of 20 transcription factor mutant strains. To confirm the PHL5-34A hypersensitivity of the 20 strains identified in the mutant screen, agar-based drop-test assays were performed. The 20 strains were re-streaked from frozen glycerol stocks on YPD + G418 agar plates. Overnight cultures of each strain were started from single colonies in 2 ml of YPD broth (MOPS buffered, pH 7.0) containing G418, and incubated at 30°C for 22 h. The parent BY4742 strain was also grown in a

similar manner, except no G418 was added to the medium. The cell density of each culture was monitored by measuring the optical density (OD) at 600 nm using a 2100 Pro UV-Vis Spectrophotometer (GE Healthcare, Piscataway, NJ). Each culture was then diluted to an OD₆₀₀ of 3.0 in YPD broth (MOPS buffered, pH 7.0). From this diluted culture, 1:5 serial dilutions were prepared in the same medium, resulting in 6 total dilutions. Cells from each dilution were then spotted in 3 µl amounts on YPD agar plates +/- 3.0 µg/ml PHL5-34A. Plates were incubated for 2 days at 30°C. Images of the plates were obtained using the Bio-Rad Gel Documentation System.

PCR confirmation of 4 transcription factor mutant strains. Deletion mutant strains were streaked for single colonies on YPD + G418 plates. Colony PCR was performed by denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. Platinum Taq DNA Polymerase (Invitrogen Corporation), a hot-start enzyme, was utilized in all reactions to increase specificity and sensitivity. Each PCR reaction was analyzed by agarose gel electrophoresis, and an image of the gel was obtained using the Bio-Rad Gel Documentation System. Each deletion mutant was confirmed using the "A," "B," "C," "D," "KanB," and "KanC" primers as described on the *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). The "A" and "D" primers are located upstream and downstream of the coding region respectively,

while the "B" and "C" primers are located within the coding region. The "KanB" and "KanC" primers are located in the KanMX gene used to replace the target gene in each deletion strain. PCR products are obtained in the "A"/ "KanB" and "D"/ "KanC" primer combinations, while the "A"/ "B" and "C"/ "D" primer combinations result in no PCR products.

RESULTS AND DISCUSSION

Strategy for evaluating the MOA of PHL5-34A. The strategy for evaluating the mechanism of action (MOA) of PHL5-34A was to identify hypersensitive mutants from a collection of yeast mutants that have deletions in transcription factors. The deleted genes in the mutants provide clues about the MOA of the drug. Using deletion mutants in a MOA study is an important strategy. For example, ketoconazole is an antifungal drug that inhibits ergosterol biosynthesis by targeting the *ERG11* gene in the ergosterol synthesis pathway. If a normal wild type cell is treated with ketoconazole, it will show sensitivity to ketoconazole since the pathway will be inhibited at the step catalyzed by *ERG11*. If there is a deletion in the *ERG8* gene, which catalyzes a step upstream of the *ERG11* step, this mutant (*erg8Δ*) will show hypersensitivity to ketoconazole because the pathway will be inhibited at two steps in this mutant (Hillenmeyer et al., 2008). Similarly, a mutant with deletion in the *ERG9* gene (*erg9Δ*), and a mutant with a deletion in the *ERG4* gene (*erg4Δ*), both of which catalyze various steps in the pathway, is hypersensitive to ketoconazole (Hillenmeyer et al., 2008). Thus, if a novel antifungal compound is identified and if *erg11Δ*, *erg8Δ*, *erg9Δ*, and *erg4Δ* mutants are hypersensitive to it, then that would suggest that the novel compound is an ergosterol synthesis inhibitor.

Using mutants with deletions in transcription factors is also an important strategy in MOA studies. A transcription factor is a protein that binds to a specific DNA sequence in

a gene and regulates its expression. For example, Upc2 is a transcription factor that regulates the expression of the *ERG11* gene (Vik and Rine, 2001). Upc2 also regulates several other ergosterol synthesis genes (e.g., *ERG2*, *ERG3*, *ERG9*, *ERG10*, *ERG24*, *ERG26*) making Upc2 a master regulator of genes involved in ergosterol biosynthesis (Teixeira et al., 2006). Interestingly, the Upc2 deletion mutant is hypersensitive to ketoconazole (Agarwal et al., 2003). Thus, if a novel antifungal compound is identified and if *upc2Δ* mutant is hypersensitive to it, then that would suggest that the novel compound is an ergosterol inhibitor. In MOA studies, one can analyze the Upc2 transcription factor mutant instead of all of the mutants with deletions in individual ergosterol synthesis genes.

Determining drug concentration for the mutant screen. First, an experiment in a six-well assay was used to determine the drug concentration to be used in the mutant screen. PHL5-34A was added to YPD agar at different concentrations. A no-drug control was included which consisted of 1% DMSO since DMSO was the solvent used to dissolve the drug. The medium was then poured into a 6-well plate, and the parent yeast strain was spread on the medium. The plate was incubated at 30°C for two days, and photographs were taken after the incubation period. As can be seen in Figure 2, the left bottom well did not have any drug, and as you move to the right and up, the drug concentration increased. If the growth of the yeast cells is observed, there is maximum growth without drug, and the growth decreases as the drug concentration increases.

Based on this assay, 3 $\mu\text{g}/\text{ml}$ was chosen as the drug concentration because at this sub-inhibitory concentration, cell growth would be inhibited, but not very severely.

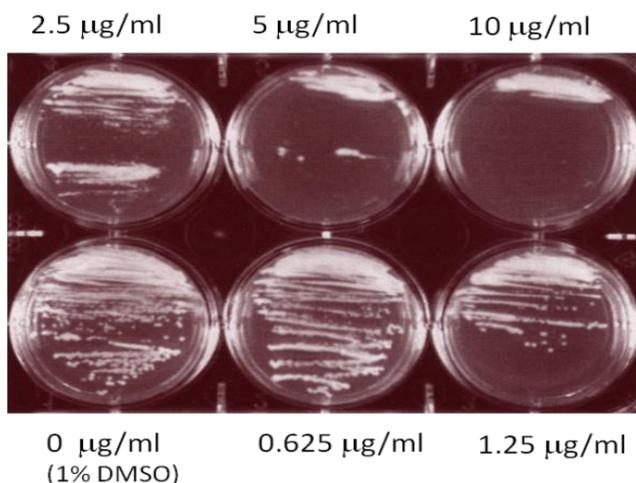


Figure 2. Determining PHL5-34A concentration for the mutant screen. A 6-well assay was performed at various concentrations of PHL5-34A using the parent strain. Image taken after 2 days of incubation is shown.

Transcription factor mutant screen. Once the drug concentration was determined, a collection of 166 yeast mutants with deletions in transcription factors was screened against PHL5-34A. PHL5-34A was added to agar at a concentration of 3 $\mu\text{g}/\text{ml}$, and 1% DMSO was added to the agar as a control. The medium was then poured into petriplates, the parent and the 166 yeast mutant strain was spread onto the plates. These plates were then incubated at 30°C for two days. With this screen of 166 yeast mutants, twenty mutants were identified as hypersensitive. Figure 3 shows images of 4 representative mutants that were hypersensitive to PHL5-34A.

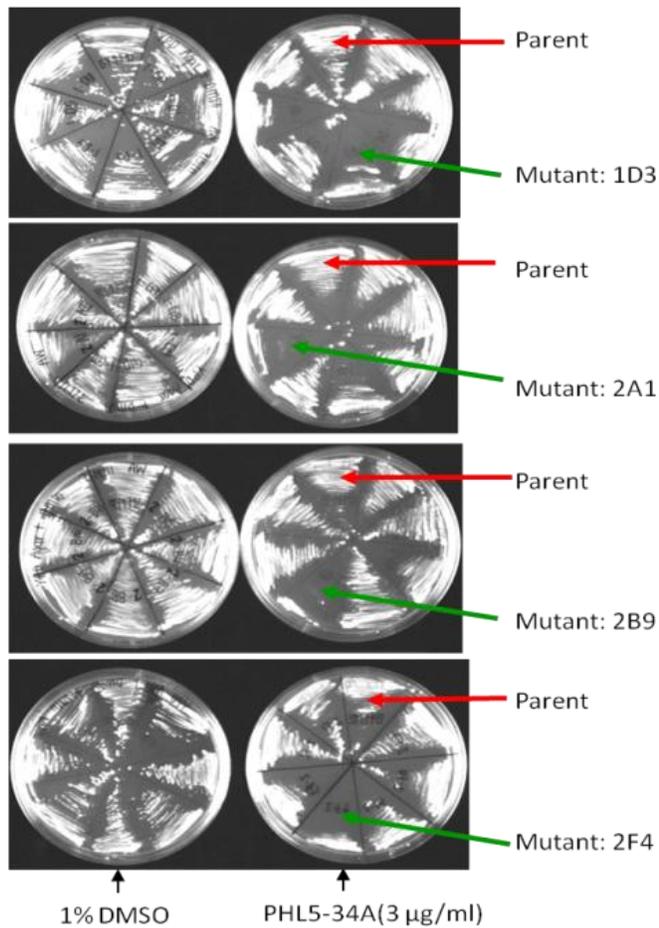


Figure 3. Transcription factor mutant screen. A screen was performed at 3µg/ml concentration of PHL5-34A against 166 yeast mutants. Images taken after 2 days of incubation are shown. The images show four representative mutants that were hypersensitive to PHL5-34A.

Confirming PHL5-34A hypersensitivity of 20 transcription factor mutant strains. After the initial screen, a confirmation test of the twenty hypersensitive mutants was conducted because the initial screen may give false positives. A false positive in the initial screen is a possibility because the cell density was not controlled in the initial screen. The mutant was grown onto a petriplate, and a single colony was inoculated onto the drug-containing petriplate to test for hypersensitivity. Thus, a mutant may

incorrectly appear to be hypersensitive if the colony was small in size or if there were not enough cells present in a colony. On the other hand, in the confirmation test, the density of cells was controlled by taking an OD of the cells in the sample colony, and each culture was diluted to an OD of 3.0. Thus, the same number of cells were present for all mutant strains, including the parent strain.

The parent as well as the twenty hypersensitive mutants were analyzed by drop test assays. This was done by serial dilutions of the cells, and 3 μ L drops of each dilution were placed on the medium. The plates were then incubated at 30°C for two days. Of the twenty mutants that were identified, ten were confirmed through the drop test assay to have hypersensitivity to PHL5-34A (Figure 4). Of the ten confirmed mutants, four mutants have deletions in genes that are required for cell cycle regulation. *ACE2*, *SWI6*, *UME6*, and *MBP1* were the four genes in these mutants that play a role in cell cycle function (based on *Saccharomyces* Genome Database). *ACE2* is a gene that is responsible for the regulation of transcription during the G1 phase of mitotic cell cycle. *SWI6* is responsible for the regulation of transcription of the G1 and S phase of mitotic cell cycle. The gene *UME6* is responsible for reciprocal mitotic recombination, and *MBP1* is a gene that is responsible for the regulation of cell cycle. The remaining six mutants carried deletions in genes that were not of interest because their pathways did not show any specific patterns in their functions.

These results suggest that the cell cycle may be one of the pathways targeted by PHL5-34A. This result is in agreement with a previous mutant assay study conducted on another phloeodictine analog synthesized at the NCNPR. In that study, out of 67 mutants identified from a collection of ~5000 mutants, 9 were involved in cell cycle functions.

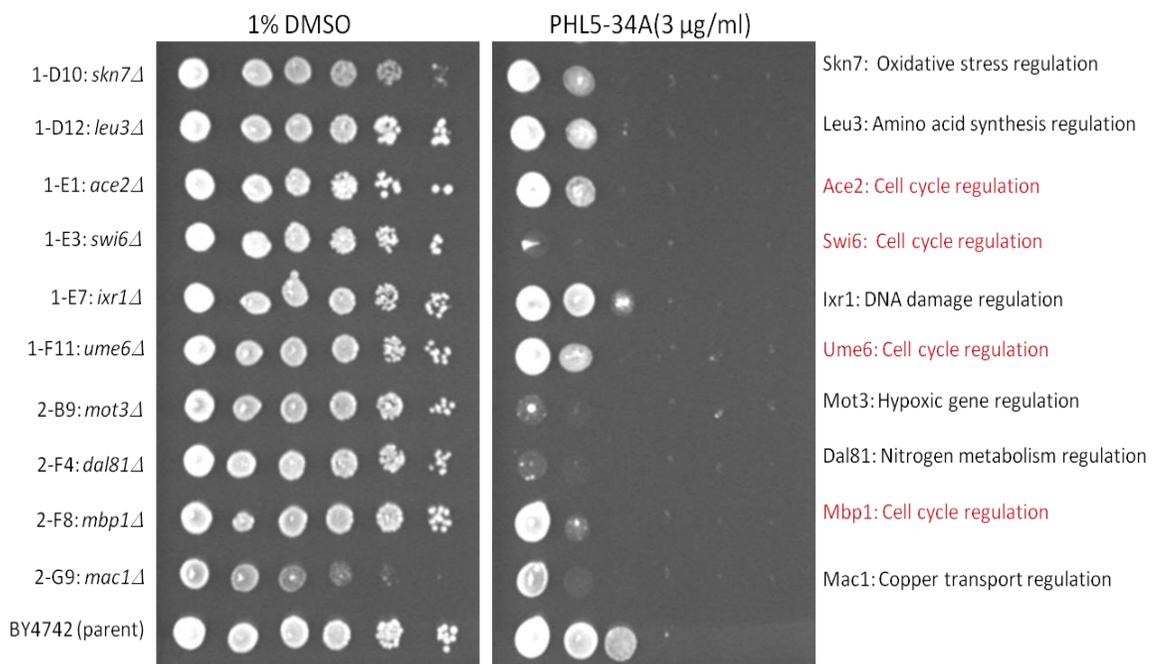


Figure 4. Confirming PHL5-34A hypersensitivity of 20 transcription factor mutant strains. A screen was performed to confirm the twenty hypersensitive mutants from the initial screen. Ten of the twenty hypersensitive mutants from the initial screen confirmed. Image taken after 2 days of incubation is shown. Mutants with deletions in cell cycle genes are shown in red.

PCR confirmation of 4 transcription factor mutant strains. A polymerase chain reaction (PCR) was performed on the four transcription factor mutant strains that contained deletions in cell cycle genes. The purpose of the PCR analysis was to confirm that the

mutant strains carried deletions in the correct genes. The deletion mutant strains that were used in this project were purchased from a commercial vendor, so there is possibility that errors may occur in the designation of the mutants.

The four strains under study were streaked onto agar plates to get single colonies. The PCR reactions were performed on a single colony for each strain using the appropriate primer conditions. The PCR reactions were then analyzed by agarose gel electrophoresis. As shown in Figure 5, in the normal *ACE2* gene, the ORF or Open Reading Frame is in the middle, and the 5' and 3' regions are at the ends. In the deleted *ACE2* gene, the *ACE2* ORF is replaced with the KanMX ORF. Primers are designed to correspond to the appropriate regions in the gene. For example, in the *ACE2* deletion mutant, with the ACE2-A and ACE2-B primers, we should get no PCR product. Similarly, ACE2-C and ACE2-D primers give no PCR product. But ACE2-A and KanB primers will give a PCR product, and also ACE2-D and KanC will give a PCR product.

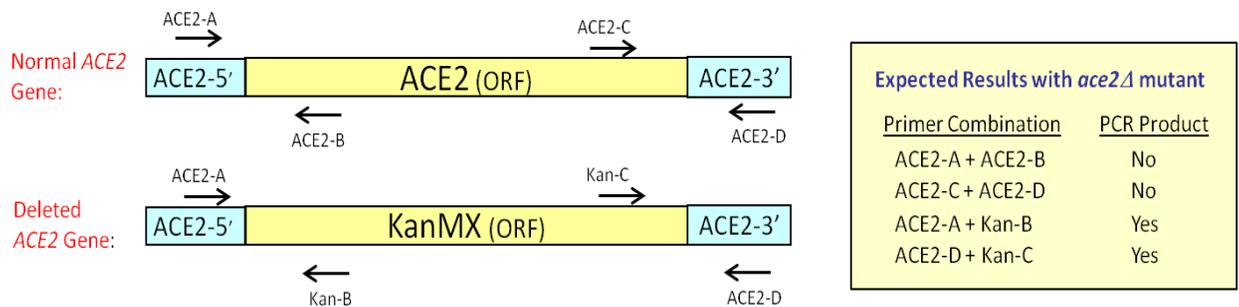


Figure 5. Expected PCR results for *ace2Δ* mutant. The diagram on the left shows the positions of the primers in the normal and deleted *ACE2* gene. The table on the right shows the expected PCR results.

PCR reactions on all four mutants were conducted, and in each case, PCR products were not visible with A+B and C+D primer. But PCR products were visible with A+KanB and D+KanC primers. All of the mutants gave the expected PCR products (Figure 6), and the mutants were confirmed to carry deletions in the correct genes.

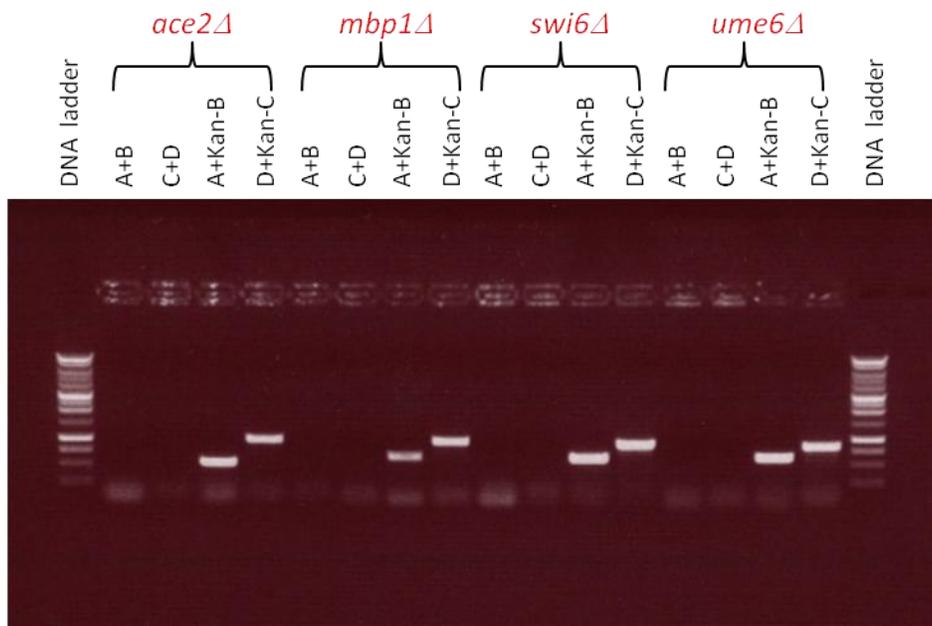


Figure 6. PCR confirmation of 4 transcription factor mutant strains. PCR analysis was performed to confirm that the four hypersensitive mutants carried deletions in the correct genes. Image taken after the agarose gel electrophoresis was completed is shown.

CONCLUSIONS

In this project, a MOA study was conducted on the novel antifungal compound, PHL5-34A, using a collection of yeast mutants that contained deletions in transcription factors. A screen of this type identifies mutants that are hypersensitive to a novel compound, and the genes deleted in those mutants provide clues about the pathways targeted by the compound. Of the 166 mutants that were screened in this study, ten confirmed mutants showed hypersensitivity to PHL5-34A. Interestingly, four of these ten mutants carried deletions in cell cycle genes. This suggests that cell cycle is one of the pathways targeted by PHL5-34A. While there are several anticancer drugs such as Taxol that target the cell cycle (Gascoigne and Taylor, 2009), the current antifungal drugs don't inhibit cell cycle. Thus, this a potentially novel antifungal target for new drug discovery. Given the central role played by the cell cycle in cell division and growth, inhibiting the cell cycle will have a dramatic effect on cell viability. On the other hand, since the cell cycle is an important process in all eukaryotic cells, it is possible that PHL5-34A may cause an inhibitory effect on not only fungal cells but also on the human host cells. However, the in vitro cytotoxicity studies on PHL5-34A indicate that it has low inhibitory activity against mammalian cells (see Table 2) – thus, it may target specific aspects of the fungal cell cycle. In addition, PHL5-34A may serve as an important tool in further exploring how the fungal cell cycle is regulated. In conclusion, this study

indicates that PHL5-34A may target the cell cycle, and it provides a useful starting point for further studies to determine the precise MOA of this class of compounds.

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