THE INS AND OUTS AND ABCS OF ANTIFUNGAL DRUG TRANSPORT: CHARACTERIZING THE ROLE OF MEMBRANE TRANSPORTERS IN PATHOGENIC FUNGI

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THE INS AND OUTS AND ABCS OF ANTIFUNGAL DRUG TRANSPORT: CHARACTERIZING THE ROLE OF MEMBRANE TRANSPORTERS IN PATHOGENIC FUNGI

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ABSTRACT

Pathogenic fungi cause serious disease and even death in humans, animals and plants. In medicine and agriculture alike, fungal infections are widespread and represent a significant threat to global public health. The number and array of fungal species, each exhibiting diverse mechanisms of pathogenesis, makes the challenge of fungal infection prevention and treatment formidable.

The current repertoire of effective antifungal treatment strategies is very limited. As a result of increased use of antifungals to treat and prevent clinical fungal infections in humans, as well as widespread use of fungicides in agriculture, fungal strains that are resistant to each of the classes of antifungals have emerged. A significant rise in the number of fungal infections in recent years, combined with an increasing amount of drug resistant fungal strains is great cause for concern and places urgency on the development of new and more effective fungal infection treatment and prevention strategies.

New fungal drug targets may be discovered with a better understanding of basic fungal biological processes. New or improved fungal infection treatment strategies may stem from a more complete knowledge of fungal response to drug treatment, worldwide trends of

fungal pathogenesis and development of resistance, and even fungal evolutionary relationships.

The goal of this research was to characterize the most basic fungal/drug interactions, which includes the balance of uptake, retention, and efflux of antifungal drugs in the fungal cell. We analyzed a variety of environmental and cellular factors that affect antifungal drug uptake and retention in two medically and agriculturally important pathogenic fungi, *Aspergillus fumigatus* and *Magnaporthe oryzae*. We then identified and characterized a number of *A. fumigatus* plasma membrane ABC transporters that may contribute to antifungal drug resistance due to their role in the efflux of antifungal drugs.

To analyze antifungal drug uptake and retention, we developed an assay to directly measure accumulation of radioactively-labeled azoles in *A. fumigatus* and *M. oryzae*. Our analysis of drug uptake under a variety of cellular and environmental conditions demonstrated that these filamentous fungi import azoles by a facilitated diffusion mechanism. Contrasts between the *M. oryzae* and *A. fumigatus* data revealed interesting differences that suggest variations in expression, induction, or function of efflux transporters in the two organisms.

To analyze antifungal efflux, we cloned and expressed a selection of putative ABC transporter genes from the *A. fumigatus* genome and heterologously expressed each gene in *S. cerevisiae* for direct characterization of drug efflux potential. Our efflux transporter analysis showed differences in substrate specificity, drug susceptibilities, energy-dependent efflux activity, and effect of efflux-inhibitor treatment between the different transporters. These data illustrate the complexity of predicting and counteracting fungal drug treatment response, but also highlight the possibilities for identifying new drug targets.

APPROVAL PAGE

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CHAPTER 1

INTRODUCTION TO AZOLE DRUG RESISTANCE MECHANISMS IN PATHOGENIC FUNGI

Pathogenic Fungi

The incidence of invasive mycoses has increased over the last three decades due to a dramatic rise in the number of immune-compromised individuals. A variety of conditions can lead to patients with severe immune deficiency including AIDS, cystic fibrosis, organ and bone marrow transplants, aggressive cancer chemotherapy, immune suppressant treatments, major surgeries, premature birth and old age (1-4).

While the most common human fungal infections are caused by *Candida albicans* and *Aspergillus fumigatus*, the increased incidence of non-*albicans* and non-*fumigatus* clinical isolates shows an evolving landscape within fungal populations (5-7). Species from a wide variety of fungal phyla are continuously emerging as opportunistic pathogens as they adapt to the changing (host or environmental) selective pressures, and radiate to newly available niches (8, 9). Given the complexity of each individual at-risk patient condition, coupled with the diverse array of potential fungal pathogens, opportunistic mycoses are always a threat. If host conditions are ideal, ANY fungus can potentially cause an infection in a sufficiently immunocompromised individual.

The fungi studied in this dissertation research include *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus fumigatus*, and *Magnaporthe oryzae*.

Saccharomyces cerevisiae

S. cerevisiae has been used as a model organism in research labs around the world for decades. The *S. cerevisiae* genome has been sequenced, its genetics are easily manipulated, molecular technique protocols have been standardized, and the organism is easy to maintain in the lab (10-12). *S. cerevisiae* can exist as a haploid or diploid but most lab strains are maintained in the haploid form. In addition, homologous recombination in *S. cerevisiae* is relatively precise, transformation efficiency is good and *S. cerevisiae* expresses and maintains plasmids (10, 11). The Saccharomyces Genome Database (SGD) is a publicly accessible data repository that has been continually annotated, updated and expanded as new information is submitted by researchers worldwide (11). For all of the above reasons, *S. cerevisiae* has been an invaluable resource in the understanding of fundamental cellular processes of fungi and many other eukaryotes.

Candida albicans

Candida albicans accounts for 9–12 % of all nosocomial bloodstream infections, with a mortality rate of 38 % despite significant advances in diagnosis and increased use of antifungal therapies (8, 9). More than 17 different species of *Candida* have been identified to cause infection. Most *Candida* bloodstream infections are caused by four species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (8, 9).

C. albicans is normally a commensal of the human microflora, but is also a classic opportunistic pathogen causing mucosal thrush, bloodstream and systemic infections, termed candidemia, and invasive candidiasis, respectively. *Candida* infections are normally

associated with individuals who are immunocompromised, traumatized due to major surgery, transplantation recipients or any other invasive medical treatments that disrupt the normal protective microflora (13, 14).

In contrast to most other yeasts, *C. albicans* is an obligate diploid but can exist as a haploid or even polyploid organism (15). For this reason, genetic manipulations such as transformation are more complicated than in haploid yeasts because the gene must be disrupted or altered twice- once for each allele. In addition, protocols that allow for the stable maintenance of a plasmid in *C. albicans* have only recently been developed (12, 15, 16).

Aspergillus fumigatus

Invasive aspergillosis (IA) due to *Aspergillus fumigatus* has a mortality rate of 40–50 %, even with treatment (17, 18). Aspergilli are predominantly saprophytes, growing on dead or decaying matter in the environment. The infectious life cycle of *Aspergillus* and other molds begins with the production of conidia that are easily dispersed into the air. The primary route of human infection is by inhalation of these airborne conidia, followed by conidial deposition in the bronchioles or alveolar spaces (17, 18). In healthy individuals, conidia that are not removed by mucociliary clearance encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung (17, 18). Conidia that evade macrophage killing and germinate become the target of infiltrating neutrophils that are able to destroy hyphae. Developing IA results primarily from a combination of dysfunction of host defenses and characteristics of *A. fumigatus* that permit survival and growth in the lung environment (13, 19).

A. fumigatus is a stable haploid that can be genetically manipulated with a transformation protocol modified specifically for filamentous fungi. However, this modified procedure is much more technically challenging and time consuming, requires a greater quantity of transformable genetic material, and still has poor efficiency with non-homologous recombination likely (12, 20).

Magnaporthe oryzae

Magnaporthe oryzae is a filamentous plant pathogen that causes destructive disease in *Oryza sativa* (rice) crops, known as Rice Blast (21, 22). ‘‘Blast’’ refers to the rapid expansion of the disease within the rice fields. *M. oryzae* infections can cause devastating crop losses and is considered a major threat to worldwide food security (21-24). The plant infection cycle starts when an easily dispersed conidium lands on a rice leaf where physical cues such as hydrophobicity, surface hardness and plant signals, trigger the formation of specialized infection structures called appressoria (21, 22). The appressorium is able to translate high internal turgor into mechanical force to break through the cuticle of the rice leaf, initiating invasive growth and plant lesions (21, 22).

M. oryzae has a stable haploid genome and similarly to other filamentous fungi, is more difficult to genetically manipulate due to frequent and unpredictable non-homologous recombination (12, 20, 25).

Pathogenic fungi, in medicine and agriculture alike, cause devastating disease worldwide representing a significant threat to global public health (2, 23, 24). The sheer number of different fungal species, each adapted to specific hosts and environments and exhibiting diverse mechanisms of pathogenesis, makes the challenge of fungal infection prevention and treatment formidable. However, there are some conserved and unifying

themes in most pathogenic fungal processes that can be taken advantage of to create broad-spectrum antifungals. In addition, species and strain-specific research with epidemiologic analysis can identify unique fungal processes or characteristics that can be targeted for a more precise approach to antifungal therapy.

Antifungal Treatment Options

High mortality rate with invasive mycoses is partly attributed to difficulty in disease diagnoses and delayed treatment caused by the fact that disease symptoms can be ambiguous and the fungal species is often cryptic (3, 4, 21, 22). Since opportunistic fungal infections most commonly occur in a host with poor immune function, there is an additional clinical challenge for selecting the best antifungal course of action and predicting treatment outcome.

Currently available antifungal options for treatment of invasive mycoses are limited and the development of new antifungal agents is more difficult compared to that of antibacterial agents. Since bacteria are prokaryotic, they offer many distinct drug targets and pathways that are not present in the human eukaryotic cell. Conversely, fungi are also eukaryotes and so many fungal drug targets also disrupt human cellular processes leading to toxicity and harmful side effects.

The most widely used classes of antifungals are polyenes, azoles, allylamines, echinocandins and 5-flucytosine. These drug classes and their targets are diagrammed in Figure 1. Drugs with the fewest side effects and least human toxicity, such as the azoles, have narrow modes of action and problems with fungal drug resistance. Conversely, drugs that are more broadly effective with fewer fungal drug resistance concerns, such as the

polyenes, are generally not safe for extended use and can cause life-threatening side effects as the less-selective drug interferes with human cell processes (26-30).

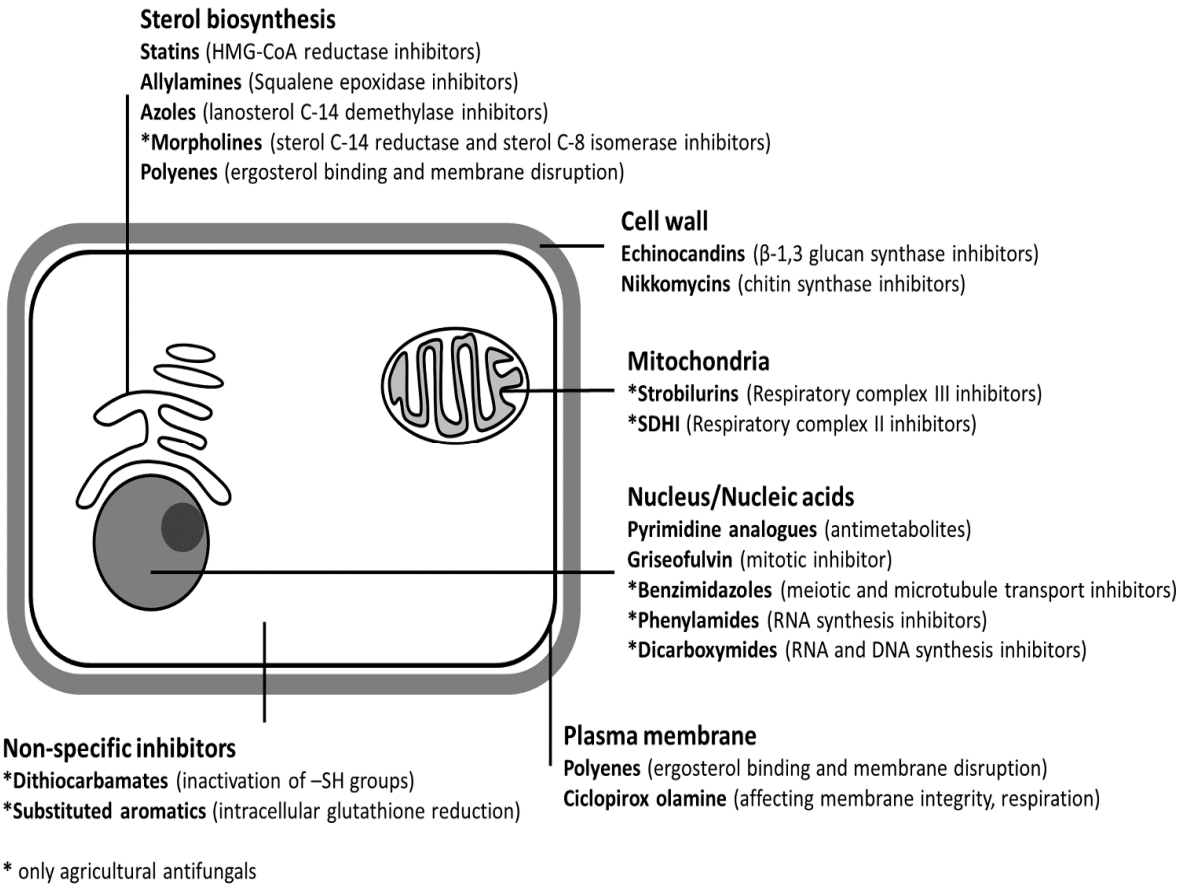

Figure 1.1 Antifungals and Their Targets. Classes of antifungal drugs and their target are diagrammed.

Antifungal drug resistance is difficult to define. The threshold is set individually for each drug, organism and site of infection, respectively. *In vitro* susceptibility testing of filamentous fungi is difficult to standardize. In addition, the *in vitro* test results do not

always agree with the *in vivo* results and so may not be an accurate indication of treatment outcome. Thus, *in vitro* drug susceptibility testing must be used with caution and considered only as a general guideline for treatment prediction purposes (19).

There are two types of antifungal drug resistance. Primary resistance is an intrinsic or inherited characteristic of a species or strain. The fungal species and strain will clearly determine which drugs are effective. Secondary resistance occurs when a previously susceptible isolate develops a resistant phenotype, such as a unique gene mutation, usually as a result of prolonged treatment with antifungals. Both primary and secondary resistance to our current antifungal repertoire is a growing concern.

Selection of an antifungal agent for clinical use is made primarily on the basis of the specific fungal pathogen involved. This approach is useful in avoiding selection of antifungals for species of fungi that are known to have primary resistance to the agent, but less useful in selecting antifungals for species that may develop secondary (drug induced) resistance to a particular agent.

There are several factors that affect the treatment outcome of a fungal infection. A resistant or recalcitrant infection can be the result of a variety of factors associated with the host, the drug, and the fungus. Generally antifungal drugs work alongside the host immune system to control the infection. Infections in immunocompromised patients are generally more recalcitrant to treatment because of the lost additive effect from the host immune system. The infection location (systemic, skin, oral mucosa, vaginal mucosa, eye, brain, etc.) is also an important factor in drug resistance, as some infection sites may be less accessible to drug therapy.

Finally, the cell type or morphological stage of the fungi can alter drug efficacy. Most antifungals are effective only against actively growing fungi, while dormant stages with minimal metabolic activity are usually not responsive to drug. Most fungi exist as various cell types or morphologies, including yeast stages (blastospores), pseudohyphae, hyphae, chlamydospores and conidiospores, each of which can have a specific susceptibility to antifungal drugs.

As a result of increased use of antifungals to treat and prevent fungal infections in a vulnerable human population, as well as widespread use of fungicides in agriculture, fungal strains that are resistant to each of the classes of antifungals have emerged. Some of the newly emerging opportunistic fungal strains have either acquired or intrinsic resistance to the standard antifungal therapy and may require the use of alternative antifungal agents. As those alternative antifungal agents start to be used broadly, strains that are resistant to these agents will most likely emerge as opportunistic pathogenic infections in the coming years.

Azole Drugs

Ergosterol, a sterol homologous to cholesterol and other sterols found in the membranes of other eukaryotic cells, is an essential component of fungal membranes and its metabolism is tightly regulated by the fungal cells. Alterations in ergosterol levels significantly affect the susceptibility of fungal cells to a variety of environmental stresses. Although similar to plant and animal sterol biosynthesis pathways, ergosterol biosynthesis is unique to fungi, and so its metabolism is a prime target of antifungal therapy with minimal negative effects on the animal or plant host. Currently, there are four classes of drugs that target enzymes in the sterol biosynthesis pathway (Figure 1), including statins (HMG-CoA reductase inhibitors; used in mammalian cells), allylamines (squalene epoxidase inhibitors),

azoles (lanosterol 14α-demethylase inhibitors) and morpholines (sterol C-14 reductase and sterol C-8 isomerase inhibitors; only used agriculturally). One additional class of drugs, polyenes, directly targets ergosterol in fungal membranes and negatively affects the native membrane structure.

Azole antifungals, such as fluconazole, are one of the most commonly used drugs to treat fungal infections. Azoles are fungistatic drugs, which do not kill the cells, and are thus more likely to allow cells to develop resistance. As a class of antifungal drugs, azoles are organic compounds consisting of a five-membered nitrogen-containing heterocyclic ring with two nitrogen atoms (imidazole) or three nitrogen atoms (triazole) and a halogenated benzene ring. These two prerequisites are crucial for their antifungal activity, as well as their successful import into the fungal cells (31). The rest of the chemical structure varies among azole drugs, but is crucial for their antifungal activity and selectivity.

Once in the fungal cell, azoles target and inhibit cytochrome P-450-dependent enzyme, lanosterol 14α-demethylase (*ERG11* in *C. albicans* and *S. cerevisiae, CYP51* in *A. fumigatus*), which is located in the endoplasmic reticulum (ER). At the moment there are numerous azoles on the market, both for medical and agricultural use. The medical azoles are highly specific to the fungal enzyme, and so, although they are capable of entering mammalian cells, azoles are generally not effective against the human homolog (21). In contrast, agricultural azoles have poor selectivity for the fungal lanosterol 14α-demethylase over its human homolog, which leads to concerns over agricultural azole side effects on humans and animals (30, 32).

Azole Resistance

Many fungal species display intrinsic resistance to azoles. These fungi include *Candida krusei*, most strains of *Candida glabrata, Fusarium* species and the Zygomycetes. Many filamentous fungi such as *Aspergillus* and *Magnaporthe* are resistant to the azole fluconazole. Long term or prophylactic use of azoles in immune deficient individuals dramatically changes the composition of the individual's mouth, gut and skin microbiome as azole-susceptible commensal organisms are wiped out and are rapidly replaced with the azole-resistant opportunistic organisms. Both *C. krusei* and *C. glabrata* are increasing in frequency in oral and systemic candidiasis in patient populations that use azole drugs for treatment or prophylaxis.

Acquired azole resistance was rare in the 1980s, when azoles were primarily used to treat patients with chronic mucocutaneous candidiasis. However, with the AIDS epidemic in the 1990s, azole resistance in *C. albicans* became a significant problem as oral candidiasis occurred in over 90 % of all HIV positive individuals. In recent years, highly active anti-retroviral therapy, which restores the patient's immune response, is reducing the frequency of most opportunistic fungal infections, and also the need for azole prophylaxis.

In addition to *Candida* species, acquired azole resistance has been detected in isolates of *Aspergillus fumigatus* from patients who have received regular treatment with itraconazole or voriconazole (33). Azoles are also used in surgical wards to prevent systemic candidiasis and as non-prescription drugs to treat fungal skin infections, including athlete's foot. The use of azoles in the environment to treat and prevent fungal pathogens of plant crops is a concern. It has been correlated with an increasing number of agricultural-azole resistant *A. fumigatus* isolates occurring in azole-naïve human patients, and unfortunately these isolates

are also resistant to medical azoles such as itraconazole, posaconazole, and voriconazole (17).

In fungal pathogens naturally susceptible to azoles, there are several mechanisms of azole resistance that can develop as a result of azole treatment (Figure 2). These mechanisms include: (1) reduced azole import, (2) increased azole efflux, (3) import of sterols from host, replacing endogenous ergosterol biosynthesis, (4) increased expression of lanosterol 14α-demethylase, the azole target enzyme, (5) mutation of lanosterol 14α-demethylase, altering its azole affinity, (6) alteration of the ergosterol biosynthetic pathway and (7) additional non-specific metabolic adjustments.

There are additional possible drug resistance mechanisms that have not yet been observed, including intracellular drug sequestration into vesicles/vacuole, or azole degradation. Molecular analyses of antifungal drug resistance have focused mainly on *C. albicans*, which is at the moment the best understood, but have included studies in *C. glabrata*, *C. neoformans, S. cerevisiae* and *A. fumigatus*.

Resistance in *C. albicans* clinical isolates is not usually the result of a single alteration. The resistance is gradually developed through series of independent steps. Each acquired mutation decreases azole susceptibility and increases the fitness of the cell under the drug selective pressure. As a result, this newly acquired allele allows the corresponding clone to outgrow the rest of the population, to become the new major strain. A series of such steps results in development of a highly azole resistant phenotype, where each alteration only partially contributes to the resulting phenotype. This has been well documented in a series of sequential isolates from a single patient during azole treatment (9). All the acquired resistance mechanisms are described in detail in later parts of this chapter, but they include

mutation and overexpression of the azole target enzyme and efflux of the drug from the fungal cells with two types of efflux pumps. The sum of each of these alterations results in the resistant phenotype.

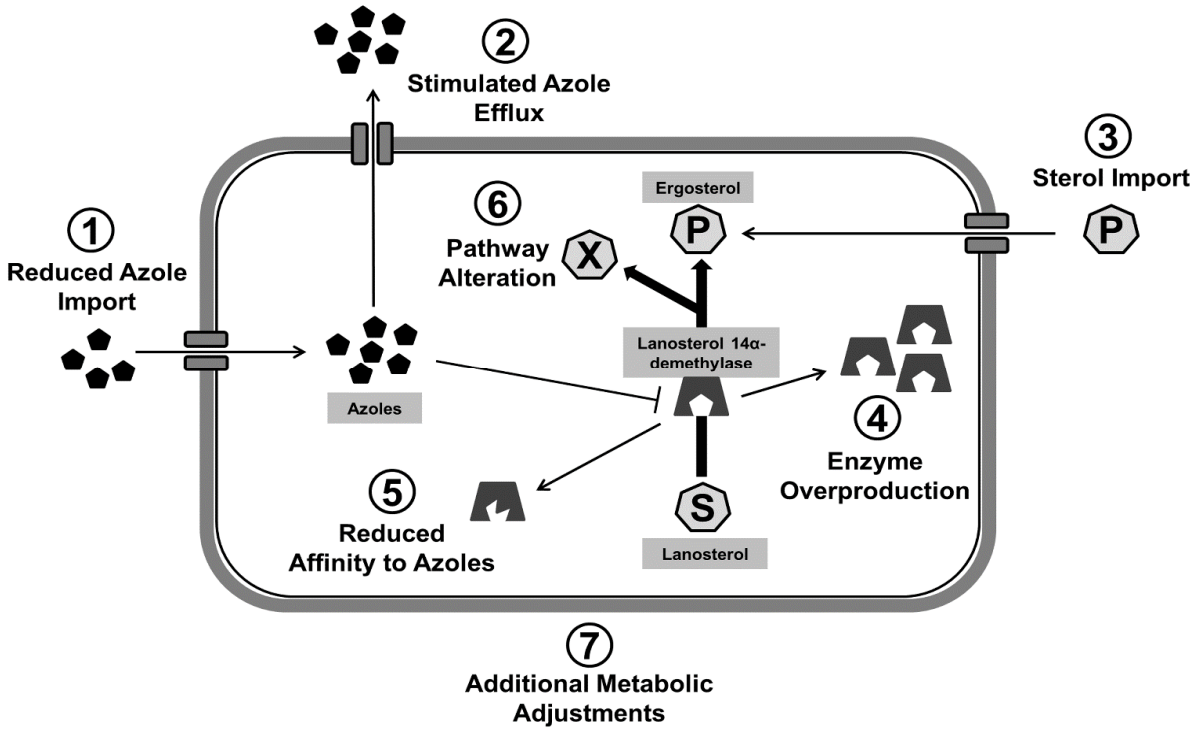

Figure 1.2. Mechanisms of Azole Drug Resistance. Actual mechanisms include 2, 3, 4, 5, 6, and 7. A potential mechanism is represented by 1.

Azole Import

Azole accumulation within a fungal cell is the result of a balance among drug import, retention and efflux. Azole drug accumulation has been studied primarily in *C. albicans*, but has also been described for *S. cerevisiae*, *C. glabrata*, *C. krusei*, *C. neoformans*, *A. fumigatus*, and *M. grisea* (31, 34-37). In general, all fungal species susceptible to azoles are expected to import these drugs.

Based on information obtained from previous studies in our lab on *C. albicans* and our more recent studies on *A. fumigatus* and *M. oryzae* expanded in Chapters 2 and 3, azole import into fungi is independent of ATP or pH. It follows a concentration gradient, is saturable, and proceeds via facilitated diffusion through a yet unknown transporter. All analyzed azoles seem to utilize the same import mechanism. The drug import depends strictly on the chemical structure of azoles, requiring both a halogenated benzene ring and an imidazole or triazole ring in one molecule. Omitting either one results in failure of the molecule to be imported (31, 37). Additional structures, as well as molecule size, seem rather insignificant. There are similarities and differences in azole import between the three organisms. The general mechanism seems to be conserved, including pH, energy-independent, chemical moieties for competition, exponential phase, etc. However differences include the temperature at which optimum uptake occurs, effects of growth media, and time of growth and mycelial development.

In *C. albicans*, conditions that favor the maximum rate of import include an optimal temperature of 30° C, cells harvested from exponential phase of growth and cells grown anaerobically. Hyphae, the tubular, branching structures in filamentous fungi, also display a higher import rate than yeast cells.

So far, no natural environmental compounds have been identified that utilize the same import mechanism and thus compete with azoles for import. Thus the origin and evolutional importance of the import mechanism is unclear. Due to the failure of genetic screens to identify the transporter, it is likely to be either essential for cell viability or part of a multi-member family of transporters with overlapping function. Import is specific for fungi, since

bacteria (*Escherichia coli*) are incapable of fluconazole import (31). Import into mammalian cells has been reported as well, with azoles accumulating in their cellular membranes (21).

Drug import into the fungal cell by facilitated diffusion is a mechanism that might be manipulated by the fungal cell to confer drug resistance and may play a role in azole-resistant isolates. However, direct evidence for this is lacking. Clinical isolates of *C. albicans, A. fumigatus,* and other species show high variability in the rate of azole import, as well as different end points for saturation with azoles. It is difficult to compare data between isolates due to differences in growth rate, auxotrophies, efflux, etc. It is important to note that although reduced azole import can be involved in azole resistance, the reduced import levels cannot always be correlated with the individual strain's azole susceptibilities ((31) and our unpublished results).

Experiments in *A. fumigatus* using itraconazole also showed that azole drug import is saturable and time and concentration dependent (35). The correlation between increased itraconazole resistance and its decreased intracellular accumulation has been reported (34, 35), however, this was most likely due to increased azole efflux, since the cells were not depleted of an energy source.

ERG11/CYP-51

In *C. albicans,* once the azoles have been imported, they target the ER-located enzyme Erg11, which is an essential enzyme in ergosterol biosynthesis. Erg11 is a P450-dependent enzyme containing a heme moiety in its active site. The azoles bind to the heme iron through an un-hindered nitrogen, thus inhibiting the Erg11 catalyzed enzymatic reaction. The second nitrogen of the azoles interacts directly with the apoprotein. The position of the second nitrogen is thought to modulate the affinity of different azoles (38). The inhibited

Erg11 fails to remove methyl groups at the 14α-carbon of ergosterol precursors, resulting in an accumulation of 14α-methyl sterols, which are further processed into toxic intermediates by sterol C5-desaturase (Erg3) (39). These intermediates affect the fluidity and function of the fungal plasma membrane, resulting in higher susceptibility to environmental stress, including host immune system defense mechanisms.

ERG11 Overexpression

One way of gaining resistance through *ERG11* is by its overexpression, which results in an increased production of the encoded enzyme. Increased amounts of enzyme require increased amounts of drug for the same level of inhibition. Normally, as a response to azole treatment (or other ergosterol biosynthesis inhibitors), *ERG11* expression is increased above normal in many fungal species (40). This upregulation often includes other *ERG* genes in the pathway. In certain cases the upregulation of the *ERG11* or the whole pathway can be permanent, leading to the resistant phenotype. The permanent upregulation in *C. albicans* can happen basically by two mechanisms: (1) duplication of chromosome 5, increasing the copy number of the *ERG11* gene located on it, and (2) a gain of function (GOF) mutation in the transcription factor that regulates the ergosterol pathway, Upc2 (Table 2).

GOF mutations in Upc2 lead to overexpression of many ergosterol biosynthesis genes, including the already mentioned *ERG11* (41, 42). Upc2 is a Zn_2-Cys_6 transcriptional factor and central regulator of the ergosterol biosynthesis pathway in *C. albicans* and *S. cerevisiae*. In *S. cerevisiae*, constitutively-active mutants of the two paralogs Upc2 (G888D) and Ecm22 (G790D) induce sterol uptake under aerobic conditions (43-46) but do not alter drug susceptibility phenotype (our data and Chapter 5). However, in *C. albicans*, Upc2

constitutively active mutants in the C-terminus (A643V/T, G648D/S and others) or additional areas increase resistance to the azoles by direct *ERG11* upregulation (47-50). Upc2 forms dimers (our unpublished data) and these GOF mutations can express their full potential in diploid *C. albicans* only when found in the homozygous state (49, 51-54). Homozygous GOF Upc2 (G648D) stimulates *ERG11* promoter expression approximately 4-fold resulting in a 4-fold increase in MIC (54, 55).

Distantly-related fungi (e.g. *Aspergillus* and *Cryptococcus*) are missing Upc2 homologs, but have genes that regulate ergosterol biosynthesis that are functional homologs of the sterol regulatory-element binding protein (SREBP) of higher eukaryotes. The genes are *srbA* in *Aspergillus* and *SRE1* in *Cryptococcus*. These proteins belong to helix-loop-helix transcriptional factors, are structurally unrelated to Upc2, and also have a different mechanism of activation. No GOF mutations have been identified in these proteins to date. However, the *A. fumigatus srbA* deletion results in azole hyper-susceptibility similar to *upc2* mutants in *Candida* (56). Recently, increased expression of *Aspergillus cyp51A* has been linked to a P88L mutation in the unrelated transcription factor HapE (57), which may interact with SrbA.

Pressure for increased CYP51A expression by use of agricultural triazoles has led to selection of *A. fumigatus* strains with promoter mutations, allowing overexpression of the azole target enzyme (30, 58). This mutant strain has now been found to have spread to many areas of the world rapidly, leading to concerns about agricultural azole use interfering with medical azole efficiency in human fungal infections.

Azole Efflux

In *C. albicans*, there are several transporters involved in azole efflux, the major ones are Cdr1, Cdr2, Mdr1 and Flu1. Upregulation of *CDR1* and *CDR2* (*Candida* drug resistance) is the most frequent azole resistance mechanism in *C. albicans* (59). While Cdr1 and Cdr2 belong to the class of ABC (ATP binding cassette) transporters using ATP for transport, Mdr1 (multidrug resistance) and Flu1 (fluconazole resistance) are representatives of the major facilitator superfamily (MFS), which use a membrane proton (H^+) gradient for drug/H^+ antiport.

Mrr1 and Tac1 are two transcriptional activators in *C. albicans* whose GOF mutations affect azole resistance through upregulation of the above mentioned efflux pumps. Mrr1 and Tac1 both belong to Zn_2-Cys_6 type of zinc cluster transcription factors, similar to Upc2. In clinically resistant *C. albicans* isolates, the mutations in Mrr1 and Tac1 are often combined with each other and with the GOF Upc2 version. The azole resistance in these strains is significantly higher than it is in strains containing a single GOF mutation in one of these three transcriptional factors (28).

Tac1 is perhaps the most important transcription factor involved in azole resistance of clinical isolates. It is the transcriptional activator of *CDR1*, *CDR2* and *PDR16*, which binds to the drug response element (DRE) sequence in the promoters of these genes (52, 60). The ABC transporters Cdr1 and Cdr2, are more efficient fluconazole efflux pumps than the major facilitator Mdr1 (61).

Like GOF Upc2, both GOF Mrr1 and GOF Tac1 increase their effect with homozygosity, selecting for the loss of heterozygosity under azole selective pressure (53, 55, 62). In the presence of homozygous GOF Mrr1 (P683S), the *MDR1* promoter displays

approximately 50-fold induction. Homozygous GOF Tac1 (G980E) stimulates the *CDR1* promoter approximately 8-fold and *CDR2* promoter 10-fold. The increase in MICs for homozygous GOF alleles are 8-fold for *TAC1* and 16-fold for *MRR1* (54, 55).

Compared to the other studied species, the fungal pathogen *A. fumigatus* contains an unusually high number of genes encoding ABC (close to 50) and MFS (close to 300) transporters, which are mostly uncharacterized to date (63). Although several ABC and MFS transporters have been proposed to be azole transporters (64), only *abcC/cdr1B* and *mdr1* have been shown to play a direct role in azole resistance (65). In contrast to *C. albicans*, and similar to *C. glabrata*, some ABC and MFS transporters are induced in the presence of azoles (65, 66). Many transporters are also induced in biofilms (67), possibly playing a similar role to the transporters in *C. albicans*.

Sterol Import

Sterol import is a potential azole resistance mechanism identified relatively recently. To date, this phenomenon is related only to two fungal species, the model organism *S. cerevisiae* (68) and the closely-related related pathogen *C. glabrata* (69-72). Although *C. albicans* is also capable of sterol import, the rate is insufficient to replace the endogenous ergosterol biosynthesis (69). *S. cerevisiae* and *C. glabrata* are the only two described species able to import extracellular sterols in quantities sufficient to replace endogenous ergosterol biosynthesis in the presence of azoles. The difference in *S. cerevisiae* and *C. glabrata* from other fungi is the presence of *AUS1* and *PDR11*, ABC types of sterol importers (71, 73). These importer have not been identified in any other fungal species.

In *S. cerevisiae* sterols are exclusively imported anaerobically (68), or in strains that mimic anaerobic conditions caused by heme biosynthesis defects (*HEM1* mutations) or a GOF Upc2/Ecm22 allele (43, 74). The GOF Upc2 G/D 888 mutant takes up exogenous ergosterol in aerobic conditions, although this does not change the drug susceptibility profile from wild-type UPC2. However, it may complement an *ERG2* deletion mutant, which is susceptible to the polyene antifungal amphotericin B (AMB) and is slow growing due to membrane disruption caused by a faulty ergosterol pathway (work in progress).

Like *S. cerevisiae*, *C. glabrata* also imports sterols anaerobically and in mutants with defective *HEM1* (70). Additionally, sterol import is observed in mutant strains with defects in the early steps of ergosterol biosynthesis (*ERG1* - squalene epoxidase, or *ERG7* - lanosterol synthase) (70). *C. glabrata* also imports small amounts of sterols aerobically, and this can be greatly stimulated in the presence of blood serum together with a block in ergosterol biosynthesis caused by azoles (69, 72). Thus, an azole-induced block in ergosterol biosynthesis can be compensated *in vivo* by sterol import from the host. Similarly, a defect in the ergosterol biosynthesis pathway can also trigger sterol import from the host.

Finally, sterol import has also been reported for *A. fumigatus* (75). The presence of cholesterol or blood serum in the growth medium, and the subsequent cholesterol uptake, reduces the susceptibility to itraconazole and voriconazole. However, compared to *C. glabrata*, the rescue is only partial, suggesting that the rate of sterol import is insufficient for complete replacement of endogenous ergosterol biosynthesis. The sterol transporter in *A. fumigatus* has not yet been identified, but there is no direct homolog of the *AUS1*/*PDR11* type of sterol transporter in its genome.

Drug Combinations

In recent years a variety of drugs have been reported to have a synergistic effect when combined with azoles, which provides new hope for combined therapy that will be less likely to result in the rapid development of drug resistance. One treatment combination would be to use antifungal drugs from different drug classes/families. For example, the composition and genetic regulation of *Candida* biofilm matrix is a promising target for the development of treatments for medical device-associated infections. The synergistic effect of azoles and echinocandins would be expected to negatively affect biofilms that were previously unresponsive to either single drug treatment alone (76). Echinocandins exhibit their own direct antifungal activity (77), and they also significantly increase *in vitro* susceptibility of *Candida* and *Aspergillus* species to azoles and other antifungals.

Another group of drugs that might be considered for combined therapy are efflux pump inhibitors. Several have been identified to date. Two inhibitors clorgyline (monoamine oxidase A inhibitor) and ebselen (antioxidant under investigation for several medical uses) were described to have multi-purpose functions, including potentially inhibiting several efflux pumps in *C. albicans, C. glabrata* and *C. krusei* (78). Clorgyline is also active against, and shows better potency with the *C. albicans* MFS pump Mdr1.

Our ^{3}H-FLC efflux data in both *A. fumigatus* (Chapter 2) and *M. oryzae* (Chapter 3), and efflux data from the heterologous expression of *A. fumigatus* putative transport protein AF14 in *S. cerevisiae* (Chapter 4), show the potential for clorgyline or similar compounds to act synergistically with azoles to inhibit azole efflux by energy-dependent transporters.

Clorgyline and other efflux inhibitors may not exhibit their own direct antifungal effect, but work by chemosensitizing the cell to the other combination drug. These drug

combinations induce a better response in drug resistant pathogens, turning fungistatic drugs to fungicidal, and delaying or disrupting the acquisition and spread of new single-drug resistance fungal strains.

Several of the above drugs are already marketed for medical use. Therefore clinical trials are needed to show efficacy, but not to ascertain their safety in humans. Synergy *in vivo* is not guaranteed due to the limits in physiological concentrations and possible alterations in metabolism. Numerous efflux pump inhibitors are used in traditional medicine and clearly warrant clinical trials for safety and efficacy. It is clear that as we learn about mechanisms of resistance in the fungi, we are also identifying strategies to interfere with these mechanisms.

Conclusions

Pathogenic fungi are numerous and diverse, causing a wide range of diseases in plants and animals worldwide. *C. albicans* and *A. fumigatus* are the most common cause of fungal infections in humans although many other fungal species are known to cause disease. The most commonly used antifungal treatment for many infections is the class of azole drugs.

Prolonged clinical or environmental exposure of fungal pathogens to azoles and other classes of antifungals often results in development of resistance to these drugs. Azole susceptibility or potential for resistance is determined by strain-specific characteristics that affect drug import, drug efflux, sterol import, drug target mutations or drug target changes in expression to name a few. There are many other poorly characterized and yet-unidentified antifungal resistance mechanisms in fungi.

The increasing numbers of antifungal-resistant *C. albicans* and *A. fumigatus* isolates, as well as a continual emergence of drug-resistant populations of less common fungal

species, are very concerning for the future of pathogenic fungi management and demonstrates the need for expanded development of our antifungal repertoire.

While the threat posed by medical and agricultural antifungal resistance has increased in recent years, constant advances in understanding the mechanisms underlying the development of resistance will continue to lead to improved treatment, diagnostics and prevention of fungal infections.

Table 1.1 Genes Encoding Proteins Involved in Azole Resistance

Organism	***Ca***	***Cg***	***Sc***	***Af***	***Cn***
Lanosterol 14α-demethylase	*ERG11*	*ERG11*	*ERG11*	*cyp51A* *cyp51B*	*ERG11*
Sterol metabolism regulators	*UPC2*	*UPC2A* *UPC2B*	*UPC2* *ECM22*	*srbA*	*SRE1*
MFS transporters	*MDR1* *FLU1*	*FLR1* *QDR2*	*QDR2* *FLR1 (?)*	*mdr3 (?)*	
Regulators of MFS transporters	*MRR1*	*YAP1* *PDR1*	*GCN4* *YAP1 (?)*		
ABC transporters	*CDR1* *CDR2*	*CDR1* *PDH1* *SNQ2*	*PDR5*	*abcC* *mdr1* *atrF (?)* *mdr4 (?)*	*AFR1*
Regulators of ABC transporters	*TAC1*	*PDR1*	*PDR1* *PDR3*		

Ca, *Candida albicans*; *Cg*, *Candida glabrata*; *Sc*, *Saccharomyces cerevisiae*; *Af*, *Aspergillus fumigatus*; *Cn*, *Cryptococcus neoformans*.

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CHAPTER 2

AZOLE DRUG IMPORT INTO THE PATHOGENIC FUNGUS *ASPERGILLUS FUMIGATUS*

Chapter Summary

The fungal pathogen *Aspergillus fumigatus* causes serious illness and often death when it invades tissues, especially in immunocompromised individuals. The azole class of drugs is the most commonly prescribed treatment for many fungal infections and acts on the ergosterol biosynthesis pathway. One common mechanism of acquired azole drug resistance in fungi is prevention of drug accumulation to toxic levels in the cell. While drug efflux is a well-known resistance strategy, reduced azole import would be another strategy to maintain low intracellular azole levels. Recently, azole uptake in *Candida albicans* and other yeasts was analyzed using ^{3}H-fluconazole. Defective drug import was suggested to be a potential mechanism of drug resistance in several pathogenic fungi including *C. neoformans*, *C. krusei* and *S. cerevisiae*. We have adapted and developed an assay to measure azole accumulation in *A. fumigatus* using radioactively labeled azole drugs, based on previous work done with *C. albicans*. We used this assay to study differences in azole uptake in *A. fumigatus* under a variety of drug treatment conditions, with different morphologies, and with a select mutant strain with deficiencies in the sterol uptake and biosynthesis pathway. We conclude that azole drugs are specifically selected and imported into the fungal cell by a pH and ATP-independent facilitated diffusion mechanism, not by passive diffusion. This method of drug transport is likely to be conserved across most fungal species.

Introduction

The fungal pathogen *Aspergillus fumigatus* is one of the most common and ubiquitous environmental molds. *Aspergillus* infections represent a significant human health burden. It causes serious illnesses, ranging from sinus infections to invasive or chronic aspergillosis in immunocompromised individuals (1-3). Infection can be fatal when *Aspergillus* colonizes or invades tissues, such as the lungs and blood vessels (2). The patients most at-risk for these infections are those with prior lung conditions such as asthma, tuberculosis, COPD (chronic obstructive pulmonary disease), or cystic fibrosis, as well as bone marrow transplant patients and people living with HIV, AIDS or other immune deficiencies (1-4). *Aspergillus* infections greatly affect the quality of life, cause dramatically lengthened hospital stays, and cost the US over a billion dollars each year (3, 5). In recent years, there has been a significant rise in the number of fungal infections due to the growing subpopulation of individuals with weakened immune health (3).

If not swiftly and appropriately treated, these fungal infections can progress to serious illness and rapidly become fatal (2, 6). The azole class of drugs is still the most commonly prescribed treatment for many fungal infections because the drugs are relatively cheap to produce, are generally non-toxic to humans, and are usually more effective at controlling an infection than other classes of antifungals (5, 7). Although *A. fumigatus* has an intrinsic reduced susceptibility to the most common azole drug, fluconazole (FLC), other azoles such as voriconazole, itraconazole, and posaconazole are common drugs of choice for prevention and treatment of aspergillosis (5, 7).

In a susceptible cell, azole drugs target the ergosterol biosynthesis pathway, which is unique to fungi but similar to the human cholesterol biosynthesis pathway (7). Specifically,

azoles enter the fungal cell and inhibit the fungal cytochrome P450-dependent enzyme, lanosterol 14-alpha demethylase, encoded by the *cyp51A* and *cyp51B* genes in *A. fumigatus* (7, 8). Disruption of the ergosterol biosynthesis pathway leads to increased membrane permeability and instability in the fungal cell, which is deleterious to cell growth and replication (6, 9). *A. fumigatus'* intrinsic resistance to FLC is possibly due to the differential FLC binding affinity to the Cyp51A and Cyp51B proteins (8, 10).

In response to reduced sterol availability such as the result of azole treatment, the fungal cell may activate transcription factors such as SrbA in *A. fumigatus* or Upc2 in *C. albicans* that regulate the ergosterol biosynthesis and uptake pathways in an attempt to maintain sterol homeostasis (11, 12). In *A. fumigatus*, a deletion of the SrbA transcription factor leads to a mutant strain with FLC susceptibility, showing critical roles for sterol biosynthesis regulators such as SrbA in response to azole treatment (11, 12). Further, given the presumed intracellular localization of the azole target enzyme, the FLC susceptible phenotype is highly indicative of cellular entry of FLC into *A. fumigatus*.

Azole drugs are frequently used prophylactically to prevent *Aspergillus* infections, as well as to treat these infections, sometimes for long periods of time (1, 13). In addition, agricultural azoles are routinely used to control plant fungal pathogens (5). All of these factors have led to an emergence of fungal strains that have acquired molecular mechanisms of azole drug resistance (5, 14-16). Common mechanisms of acquired drug resistance in fungi include mutations in, or overexpression of, the azole drug target enzyme lanosterol 14α-demethylase; other mutations in genes encoding enzymes in the sterol biosynthesis pathway; and prevention of drug accumulation to toxic levels in the cell (9, 17, 18).

The prevention of toxic levels of drug accumulation in the cell frequently involves the action of membrane efflux pumps. Increased expression and activity of cell membrane transporters that export antifungal drugs, such as ATP binding cassette (ABC) and Major Facilitator (MFS) efflux pumps, are well-known mechanisms of drug resistance and have been extensively characterized in many fungal species. However, reduced azole import into the cell may be another mechanism to prevent drug accumulation (5, 19, 20). Mechanisms by which the drug enters the fungal cell, or mechanisms that prevent the drug from entering, have not been well characterized. Mansfield et al. (19) have analyzed azole uptake extensively in *Candida albicans* using radioactively labeled azoles to measure how drug accumulation in the fungal cell changes under a variety of *in vitro* conditions. Some of the conditions examined included changes in temperature, pH, and oxygen availability, as well as different *C. albicans* cellular morphologies and *C. albicans* clinical isolates (19). The results of the *C. albicans* experiments using ^{3}H-FLC show that azoles are not taken up solely by passive diffusion in *C. albicans* and suggest that deficient drug import could be a potential mechanism of drug resistance in *C. albicans*.

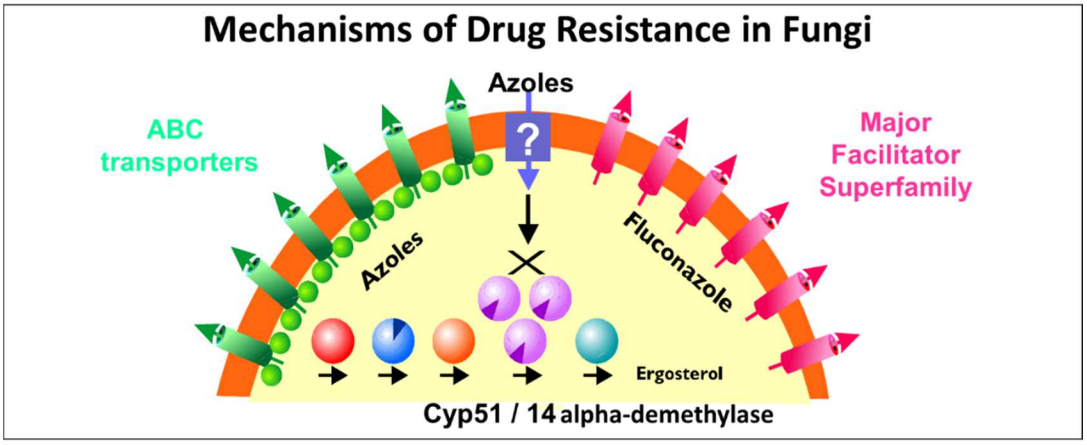


Figure 2.1. Mechanisms of Drug Resistance in Fungi.

In *Aspergillus*, there has been an increase in azole resistant *A. fumigatus* isolates (17, 21, 22). While many of these azole resistant isolates have mutations in *cyp51A* or increased efflux, a significant fraction of clinical isolates have unknown resistance mechanisms that are independent of Cyp51A and efflux pumps (15, 23). Some specific isolates have been characterized to have an efflux-independent reduction in cellular drug accumulation (24, 25). Thus, in the interest of public health, there is an urgent need to better characterize biological processes in fungi, especially those that can lead to drug resistance, as well as to continue tracking and predicting future azole resistance mechanisms (22). This knowledge will be used to improve existing therapeutic strategies or facilitate the development of new approaches for more effective treatments and preventions of fungal infections (5).

In this study, we developed an assay to directly measure azole drug accumulation in *A. fumigatus* under a variety of conditions that were previously examined in *C. albicans*, and in a variety of *A. fumigatus* morphologies and strains. Consistent with the *Candida* work, our import experiments with *A. fumigatus* have shown that azole drugs do not accumulate in the fungal cell solely by passive diffusion. Evidence for azole import other than passive diffusion includes: **(1)** dramatically reduced azole accumulation in heat inactivated cells compared with living cells, **(2)** decreased drug accumulation at higher temperatures, **(3)** competition for import by other compounds, **(4)** import specificity for certain chemical moieties present only on azoles and azole-like compounds, **(5)** differences in drug accumulation between different morphologies, including conidia, germlings and mycelia, and **(6)** differences in drug accumulation in exponentially growing and stationary cells. We also found a significant decrease in drug accumulation in the *A. fumigatus srbA* deletion mutant compared to the wild-type. We conclude that azole drugs are specifically selected and

imported into the fungal cell by a pH and ATP-independent facilitated diffusion mechanism. This method of drug transport is likely to be conserved across most fungal species.

Materials and Methods

Strains, Media, Materials, and Strain Preparation

The *A. fumigatus* wild-type, sequenced strain CEA10 (CBS 144.89) was used for all azole import experiments unless otherwise noted. The *srbA* null mutant strain SDW1 [*ΔsrbA*::*A. parasiticus pyrG pyrG1*] and the complement strain SDW2 [*ΔsrbA*::*A. parasiticus pyrG1 + srbA*] were used in the mutant strain experiment. SDW1 and SDW2 were created in a CEA17 (*pyrG1*) background strain, a uracil auxotroph of wild-type strain CEA10. All strains were provided by Dr. Robb Cramer (Dartmouth U.). Strains can be found in Table 2.1.

Table 2.1. Strains Used in This Study

Strain name	Description	Genotype	Alternative names	Reference
CEA10	Wild-type, sequenced strain	Wild-type	CBS 144.89	12
SDW1	*srbA* null mutant	*ΔsrbA*::*A. parasiticus pyrG pyrG1*	Δ*srbA*	12
SDW2	*srbA* null complement	*ΔsrbA*::*A. parasiticus pyrG + srbA*	Re-*srbA*	12

The strains were grown either in liquid or agar CSM complete medium (0.75 g CSM [Bio 101; Vista, CA], 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate, 20 g glucose per liter) at 37° C. Conidia were harvested from 5-7 day old agar plates by pipetting 7.0 mL 0.01 % Tween 20/water directly onto sporulating plates

and harvesting conidia using a sterilized glass spreader. The dark green suspension was allowed to settle in a 14 mL round bottom tube for 10-15 minutes and then 500 µL of supernatant was aliquoted into cryotubes containing 500 µL of 60 % glycerol and vortexed for a final glycerol concentration of 30 %. The conidia concentration was determined by hemocytometer, containing approximately 1-5 X 10^8 conidia/mL. Stock solutions were stored at -80° C.

Liquid cultures were started from 20 µL of the glycerol conidia stock solution, inoculated into 5 ml and grown in 50 mL conical tubes in a 37° C shaking incubator at 180 rpm for 24 hr unless noted otherwise, in which time they formed mycelial masses, or fungal balls, approximately 3 mm in diameter.

Medium components and plastic ware were obtained from Fischer Scientific (Pittsburgh, PA) or Bio 101 (Vista, CA). General chemicals and unlabeled drugs used for competition were obtained from Fisher Scientific, or Sigma-Aldrich (St. Louis, MO).

Radioactively Labeled Azole Import by *A. fumigatus*

Radioactive drugs included ^{3}H-FLC (481 GBa/mmol, 13 Ci/mmol, 1 µCi/µL; 77 µM FLC; custom synthesis by Amersham Biosciences), UK; ^{3}H-KTC (370 GBa/mmol, 10 Ci/mmol, American Radiolabeled Chemicals), or ^{3}H-CLT (740 GBa/mmol, 20 Ci/mmol, American Radiolabeled Chemicals). Drug concentrations used during the import assay were well below (10 fold below) the Minimum Inhibitory Concentration (MIC) for the strain.

All experiments were performed as biological triplicates unless noted otherwise. To determine azole import in *A. fumigatus*, we used ^{3}H-FLC, ^{3}H-KTC or ^{3}H-CLT in our drug uptake assay. Conidia were grown overnight in liquid CSM complete medium with 2 %

glucose at 37° C and 180 rpm shaking for 20-24 hr at which point they were mycelial masses or fungal balls, unless otherwise noted. Every triplicate biological sample was tested separately. The fungal balls were harvested from each 50 mL tube and transferred to a 2 mL microcentrifuge tube and washed by centrifugation and resuspension three times with YNB complete (1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate per liter, pH 5.0) without glucose, unless otherwise noted. After the washing, the fungal ball pellet was transferred to 14 mL round bottom tubes containing 1 mL of YNB for a 2 hr glucose-deprived (starvation) de-energizing period. The glucose starvation was done to keep the cells in a de-energized state. The de-energized cells showed no further growth for the extent of the import assay, as determined by dry weight at the conclusion of the assay. The cells were still viable after the assay, as shown by agar plating (with the exception of the heat killed samples). After the glucose starvation period, reaction mixes were made consisting of 1 mL of YNB containing no glucose, with fungal balls and 25 µL of diluted ^{3}H-FLC (freshly diluted 1/100X from stock). The resulting final ^{3}H-FLC concentration is 19.25 nM (5.89 ng/ml), which is > 50 fold less than the MIC value of the ΔsrbA mutant (MIC = 1 ug.ml) that is susceptible to FLC, and the wild-type strain (CEA10), which has an MIC of ≥50 µg/ml to FLC. Thus, the azole concentration used for the import assay is not expected to have any effect on cell viability.

After 24 hr incubation, or at other specified times, a 5 ml stop solution (YNB +20 mM [6 mg/L] unlabeled FLC) was added to each 14 mL round bottom tube sample. The tube was filtered by vacuum over pre-weighed and wetted glass fiber filters (24 mm GF/C; Whatman; Kent, UK). After filtering, another 5 ml stop solution was used to wash each sample again. The filters with fungal balls were either allowed to dry for 24-48 hr or were

baked in a drying oven for 15 minutes at 95° C. Filters with attached fungal balls were then weighed to obtain the dry mass of each fungal sample. The weighed filters were transferred to 5 mL scintillation vials. A 3 ml volume of scintillation cocktail (Ecoscint XR, National Diagnostics, Atlanta GA) was added and the radioactivity associated with the filter was measured in a liquid scintillation analyzer (Beckman Coulter, LS 6500 multipurpose scintillation counter). Results were calculated as CPM/mg of mycelial mass. While absolute CPM values varied between experiments, relative import differences between samples remained consistent.

Characterization of Conditions that Affect FLC Import

Further studies were done to determine the effect of changes in the growth or incubation conditions on FLC import.

Heat killed cells. Uptake of ^{3}H-FLC was measured in cells inactivated (killed) by heat (95° C for 30-40 min). This killing method decreased colony forming units greater than 100 fold compared to the non-heat treated culture. Samples were processed according to the above protocol with the exception that the heat killing step was performed during the final 45 minutes of the glucose starvation period. Heat killed samples were treated with ^{3}H-FLC and analyzed identically to the live cell samples. The results were compared with live *A. fumigatus* data. Heat killed samples were used as a control for baseline drug accumulation and non-specific cell surface binding in all testing conditions unless otherwise noted.

Other methods of cell inactivation, including UV treatment (data not shown), amphotericin B treatment, and sodium azide treatment also each reduced azole import significantly (Fig. 2.2). Heat killing was found to be the most reliable at reducing viable cell counts to less than 1 %. The reduced import due to amphotericin B treatment suggests that

membrane permeability allows drug to pass through the membrane in both directions. Caspofungin treatment at concentrations 4 fold above MIC did not affect drug import, suggesting that cell wall disruption does not influence azole import.

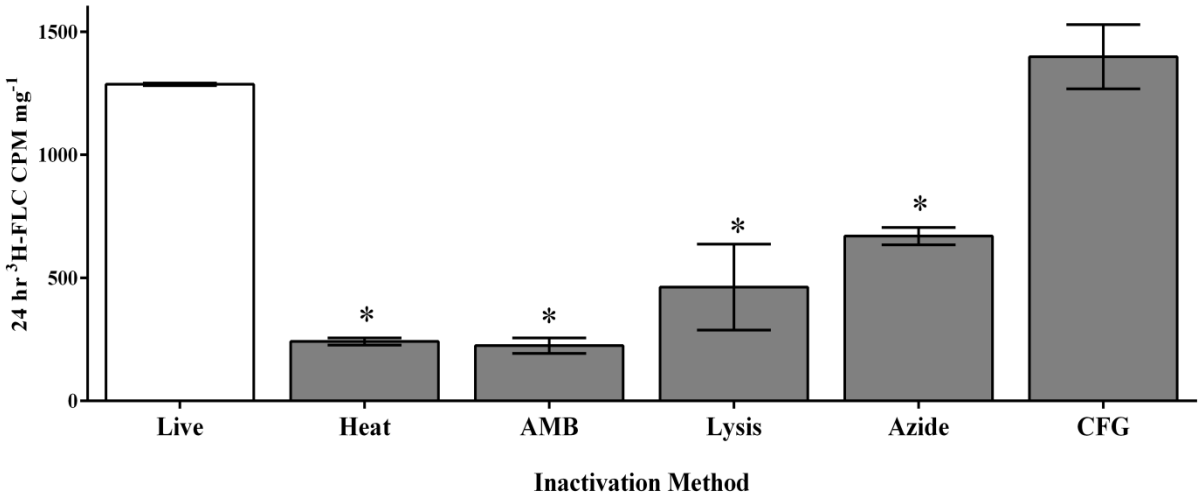


Figure 2.2. *A. fumigatus* Inactivation Methods before ^{3}H-FLC Treatment. ^{3}H-FLC accumulation was measured after 24 hr in samples that were exposed to various inactivation methods. Live: no inactivation, Heat: 95° C for 30 min, AMB: 8 µg/ml amphotericin B, Lysis: 600 µL Qiagen RLT buffer, Azide: 20 % NaN_3, CFG: 16 µg/ml caspofungin. Error bars represent standard deviation of biological triplicates for each condition. Asterisk indicates a statistical significance of $P < 0.05$.

Energy depletion. To determine whether ^{3}H-FLC import was energy-dependent, cells were de-energized either by glucose starvation for 2 hr in glucose-depleted media or by treatment with the glycolysis inhibitor 2-deoxy-glucose (5mM) for 2 hr. Following the de-energization period, the cells were then treated with ^{3}H-FLC for 24 hr in glucose-depleted media and processed as described above.

Temperature. Cells were grown overnight at 37° C as previously discussed, then incubated with ^{3}H-FLC at either 4° C, room temperature (20° C), 30° C, 37° C, or 42° C for 24 hr. Samples were then processed as described above. As an alternative temperature characterization, cells were also grown overnight at different temperatures (20° C, 30° C, 37° C, 42° C), and then incubated with ^{3}H-FLC at room temperature (20° C) for 24 hr per standard treatment and processing.

pH. Cells were incubated with ^{3}H-FLC in YNB without supplementation, adjusted to pH 5, 7 or 9 with potassium hydroxide (to increase pH) or hydrochloric acid (to decrease pH) and buffered with 100 mM MOPS, TRIS, or HEPES respectively. All samples were processed after 24 hr as described above.

Exponential vs stationary phase uptake of ^{3}H-FLC. Conidia were grown at 37° C in a shaking incubator at 180 rpm in CSM complete media with glucose for either the standard 24 hr (exponentially growing) or for 48 hr or 72 hr (stationary phase). These 1, 2 or 3 day old fungal balls were then treated with ^{3}H-FLC as described previously.

Competition for azole import. To determine if compounds compete for azole uptake in *A. fumigatus*, we processed samples as described above by our ^{3}H-FLC assay, but in addition, treated the samples simultaneously with potential competitive inhibitors at 1.95 µM (100X molar excess of the labeled FLC). ^{3}H-FLC uptake was measured as usual after 24 hr incubation with ^{3}H-FLC and competitor. Decreased ^{3}H-FLC uptake in the presence of an unlabeled competitor suggests that both drugs use the same transporter.

^{3}H-FLC uptake in distinct *A. fumigatus* morphologies. To compare ^{3}H-FLC uptake between the different morphological stages of *A. fumigatus*, conidia were harvested from agar plates as described previously. For conidia samples, conidia were washed, starved and

treated immediately. For germling and mycelial samples, the harvested conidia were allowed to germinate in CSM complete liquid media with glucose, shaking at 180 rpm at 37° C for 4.5 hr (clumping of conidia and apical extension visible), 7.5 hr (germ tubes are long and distinct but not branching), or 24 hr (balls of mycelial mass). Once the cells reached the desired time point, they were washed and starved in YNB with no glucose and incubated with ^{3}H-FLC for 24 hr as described previously. Because there was no carbon source during the ^{3}H-FLC treatment, the morphologies were maintained and there was no further growth of the *A. fumigatus*. ^{3}H-FLC uptake was calculated as CPM/mg dry mass for each sample.

^{3}H-FLC import in *A. fumigatus srbA* deletion strain. ^{3}H-FLC accumulation in *A. fumigatus* wild-type strain CEA10 was compared with the SDW1 and SDW2 strains. Heat killed controls for each strain was included. Overnight growth, ^{3}H-FLC treatment and processing was done as described previously. The mutant strains grew similarly to the wild-type in shaking liquid cultures.

Efflux kinetics. Fungal balls were preloaded with ^{3}H-FLC by treating them at 19.5 nM for 24 hr per the standard assay. The cells were then washed and diluted into YNB media, and the amount of labeled drug associated with the cells was determined as a function of time at 4, 8 and 24 hr. Efflux was evaluated in both glucose-energized (2 % glucose) and de-energized (glucose-starved) cells (see above).

Statistical Analysis

Differences between sets of samples were evaluated by an unpaired two-tailed Student's *t* test. A P value of < 0.05 was considered significant.

Results

Radioactively-labeled azole drugs have been used to analyze ^{3}H-FLC uptake in *C. albicans* (19). We have adapted the assay used in the previous study to characterize azole uptake in *A. fumigatus*. The basic protocol is described in the Materials and Methods and outlined in Fig. 3A. As cell numbers are difficult to calculate with *A. fumigatus*, all values are expressed as drug accumulation in counts normalized to dry weight of the sample. For our analysis, *A. fumigatus* was treated with ^{3}H-labeled azoles, including FLC, KTC and CLT (Fig. 3B) under a variety of conditions, and drug accumulation in the fungal cell was measured.

Azole Uptake in *A. fumigatus*

Figure 2.3B shows that all three radioactively labeled azoles (FLC, KTC and CLT) are taken up by living *A. fumigatus* cells, while the heat killed control cells show significantly reduced drug accumulation. Heat inactivated cells were subsequently used in all experiments as a baseline/background drug uptake control. The heat killed cells treated with labeled KTC and CLT showed higher baseline counts when compared to labeled FLC.

A. fumigatus and many other filamentous fungal species and molds are intrinsically resistant to FLC (26). However, due to the limited supply and expense of radioactively labeled KTC and CLT; and the evidence that all three azoles are indeed taken up by *A. fumigatus* (Fig 2.3B), subsequent experiments were performed using readily available ^{3}H-FLC. Figure 2.3C shows that ^{3}H-FLC accumulation over a period of 72 hr steadily increased up to 24 hr where a maximum accumulation was reached. For this reason, unless stated otherwise, treatment assays were stopped at 24 hr and azole accumulation measured at this time.

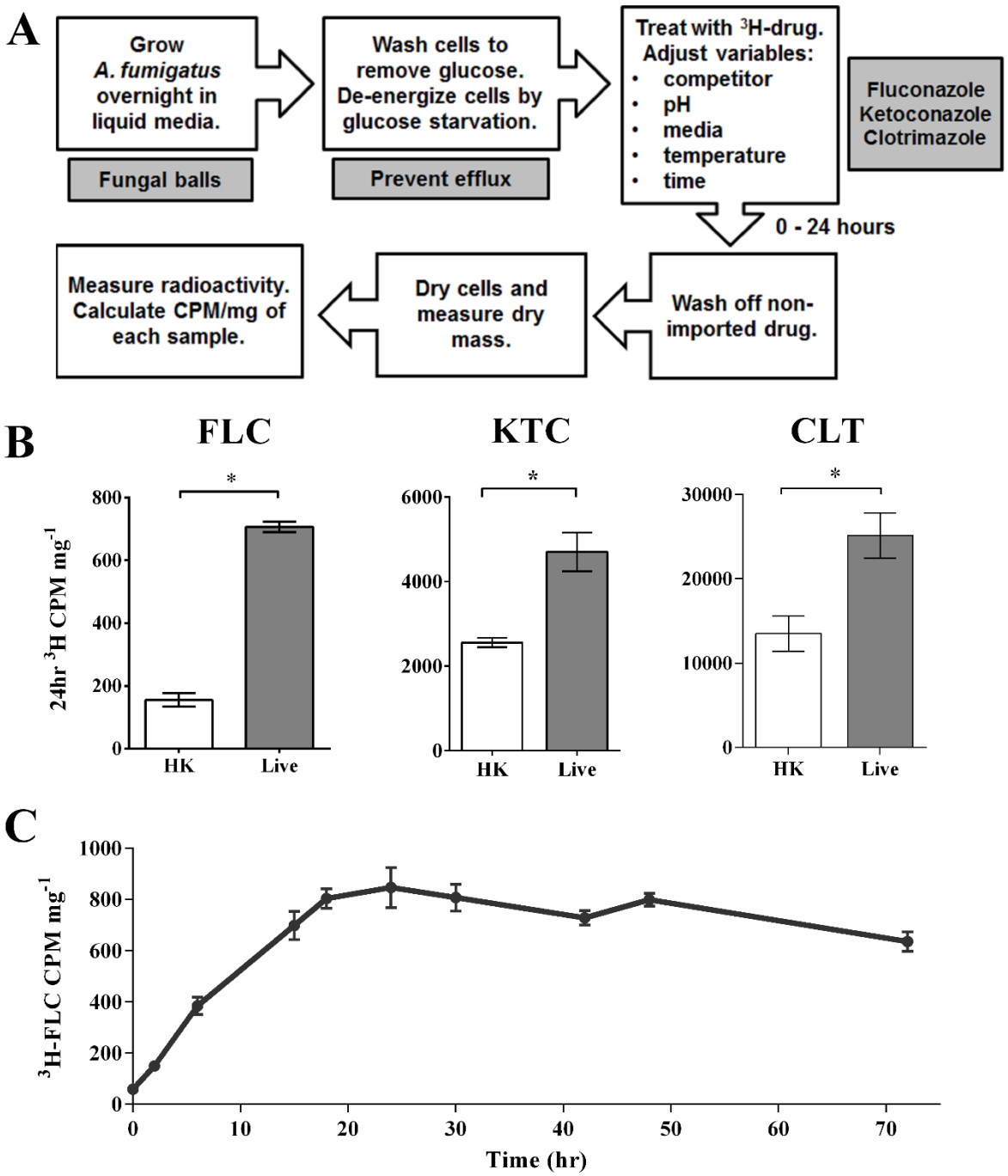


Figure 2.3. FLC Import in *A. fumigatus*. A. Schematic of the import assay using radioactively labeled azoles to directly measure drug accumulation in *A. fumigatus* cells under a variety of conditions. **B.** Comparison of ^{3}H-labeled azole uptake of FLC, KTC and CLT (dark grey bars). Heat inactivated (killed) cells were used as a control (white bars). Error bars represent standard deviation of biological triplicates for each condition. Asterisk indicates a statistical significance of $P < 0.05$. **C.** FLC accumulation in *A. fumigatus* wild-type strain CEA10 over a 72 hr time course.

Import of ^{3}H-FLC with Varied *In Vitro* Treatment Conditions

Availability of energy: To examine the effect of energy-requiring efflux mechanisms and to determine whether the accumulation of ^{3}H-FLC in *A. fumigatus* requires energy, the effect of glucose was examined in the assay. Cells were de-energized by glucose starvation in glucose-depleted media or by treatment with the glycolysis inhibitor 2-deoxy-glucose. ^{3}H-FLC accumulation of the de-energized samples were compared with ^{3}H-FLC accumulation of energized samples grown in 2 % glucose replete media (Fig 2.4A). ^{3}H-FLC uptake was observed in both de-energized and energized cells. However, the energized cells showed significantly reduced intracellular ^{3}H-FLC concentration compared to de-energized cells. The reduced azole accumulation in the energized cells is most likely the result of glucose activation of the efflux pumps. For subsequent experiments, we used de-energized (glucose starved) cells to focus solely on drug uptake and eliminate efflux mechanisms.

pH: To determine if ^{3}H-FLC import is pH dependent or affected by a proton gradient (Fig. 2.4B), we measured drug accumulation after ^{3}H-FLC treatment at pH 5, 7 and 9 using the buffers MOPS, Tris and HEPES respectively. There was ^{3}H-FLC accumulation at all pHs tested, but there was no statistically significant difference *between* the different pHs, indicating ^{3}H-FLC import is not pH dependent. There was a trend towards alkaline sensitivity for drug uptake as seen by a decrease in ^{3}H-FLC uptake in samples at pH 9 media. However, a deficiency in cell growth and robustness is observed in *A. fumigatus* cells at pH 9, so import at this pH may be affected by other cellular factors directly or indirectly related to pH and proton gradients.

Temperature: Figure 2.4C shows that ^{3}H-FLC accumulation is affected by incubation temperature during treatment. Samples treated at 4° C and 42° C showed baseline drug

accumulation. Samples treated at room temperature (20° C) show significantly higher ^{3}H-FLC accumulation than the other temperatures while 30° C and 37° C showed an intermediate amount of accumulation. Cells were viable at all temperatures (data not shown). The decrease in drug accumulation at the higher temperatures argues against passive diffusion of ^{3}H-FLC in *A. fumigatus.*

Stationary vs exponential cells: Figure 2.4D shows that ^{3}H-FLC accumulation is affected by the stage of hyphal growth of the *A. fumigatus* cells before treatment. Samples grown for the standard 24 hr before drug treatment were compared to samples grown for 48 or 72 hr before drug treatment. After ^{3}H-FLC treatment, the exponentially growing (24 hr) samples accumulated significantly more ^{3}H-FLC than the stationary phase (48 and 72 hr) samples.

Competition for ^{3}H-FLC Import in *A. fumigatus* with Azoles and Other Compounds

To determine whether all azoles use the same transporter or family of transporters in *A. fumigatus*, non-radioactively labeled azoles were tested for competition against labeled FLC (Fig. 2.5, Tables 2.2 and 2.3). The concentration of all competitors was 1.95 μM (100x molar excess to ^{3}H-FLC). All non-radiolabeled azoles that were tested [fluconazole (FLC), clotrimazole (CLT), itraconazole (ITC), ketoconazole (KTC), metconazole (MET), miconazole (MCZ), posaconazole (POS), prochloraz (PCZ), propiconazole (PROP), prothioconazole (PROT) and tebuconazole (TBZ)] competed for import with ^{3}H-FLC, as indicated by a reduction of the ^{3}H-FLC accumulation to baseline levels. These azoles include medically important azoles (FLC, CLT, ITC, KTC, MCZ, POS) as well as agriculturally important azoles (MET, PCZ, PROP, PROT, TEB). 1-(triphenylmethyl)-imidazole (1-TRI), a CLT analog, also competed with FLC for import.

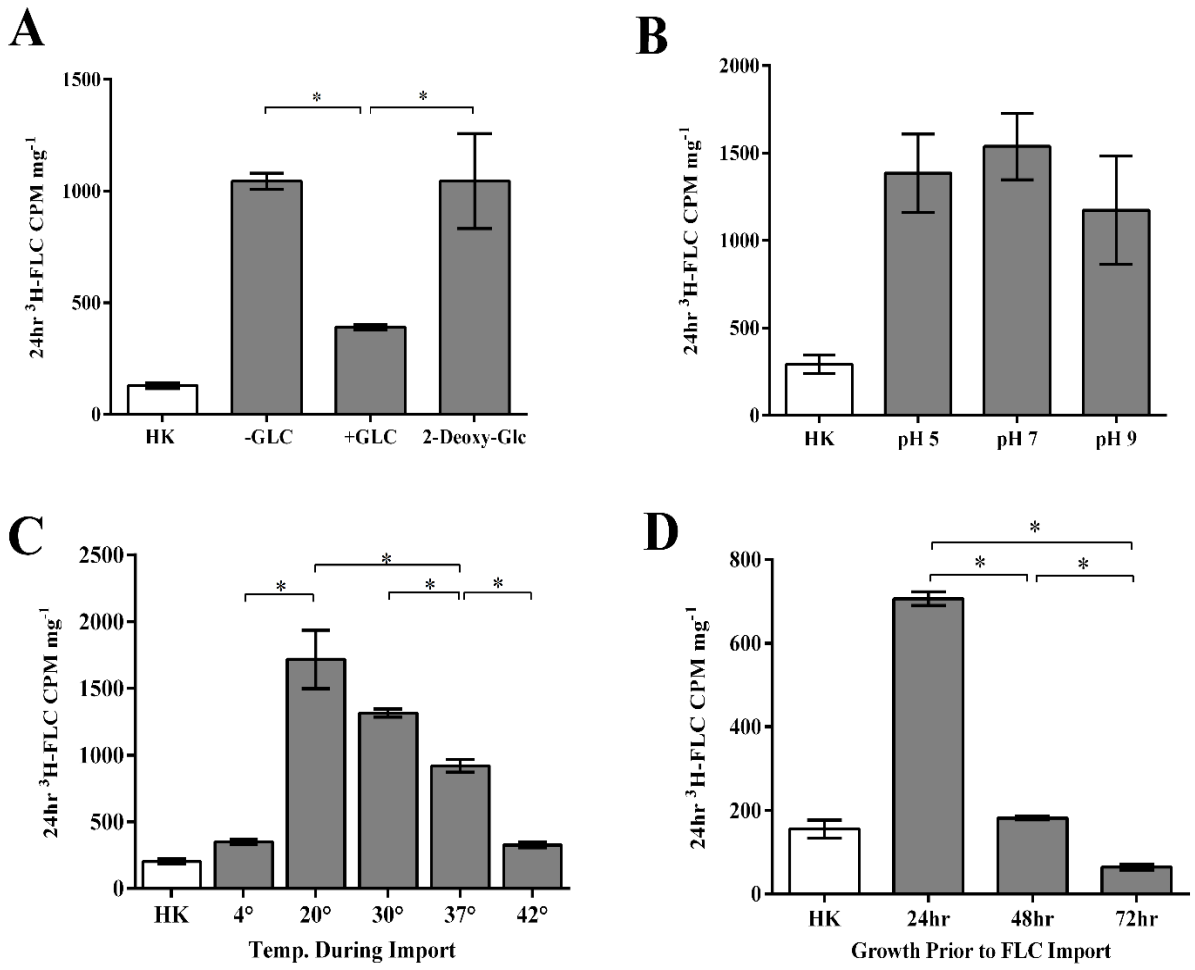


Figure 2.4. Effects of Glucose, pH, Temperature, and Cell Phase on FLC Import. **A. Glucose:** Cells were de-energized either by glucose-depleted media (-GLC) or by 5 mM treatment with the glycolysis inhibitor 2-deoxy-glucose (2-Deoxy-Glc), compared to cells in the presence of glucose (+GLC) and measured for FLC accumulation. **B. pH:** ^{3}H-FLC was imported into *A. fumigatus* at pH 5, 7 and 9. **C. Temperature:** Cells grown overnight at the standard 37° C were treated with ^{3}H-FLC and then incubated at 4° C, room temperature (20° C), 30° C, 37° C, or 42° C. The difference in import between 20 and 30° C treated cells is not statistically significant. All other temperatures showed statistically significant import differences compared to 20° C. **D. Phase:** Cells were grown in shaking liquid media for 24 hr, 48 hr or 72 hr and then treated with ^{3}H-FLC for 24 hr. For each panel: Error bars represent standard deviation of biological triplicates for each condition. Asterisks indicate a statistical significance of $P < 0.05$ between two conditions. HK = heat killed control. Statistical differences to HK are not shown.

The results of these competition experiments are consistent with azole import being mediated by facilitated-diffusion carrier(s), and suggests that these azoles use the same transport system to enter *A. fumigatus.*

Other compounds were also tested for competition against ^{3}H-FLC (Fig. 2.5, Tables 2.2 and 2.3), including azole-like compounds as well as other common antifungals [azoxystrobin (AZO), 1-benzylimidazole (1-BENZ), caspofungin (CFG), 5-flucytosine (5-FC), hematin (HEM), 4-(imidazole-1-yl) phenol (4-IMID), nicotinamide (NIC), rhodamine 6-G (R-6G), rufinamide (RUF), terbinafine (TRB), and vinclozolin (VIN)]. Most of the non-azole compounds did not compete with ^{3}H-FLC for import into *A. fumigatus*. These include medically important antifungals (CFG, 5-FC, TRB), as well as agricultural antifungals (AZO, VIN*)* and molecules with some similarity to the medical azoles (1-BENZ, NIC, RUF). The results of these competition experiments indicate transport specificity to certain chemical structures, and suggesting that they do not use the same transporter(s) to enter *A. fumigatus.*

The structure of FLC and the compounds tested in this assay are shown in Tables 2.2 and 2.3. FLC has two 5-membered triazole rings containing 2 nitrogen atoms, and a 6-member halogenated benzene ring. Previous screens of moieties important for import in *C. albicans* are consistent with this result. Analyses in both *C. albicans* and *A. fumigatus* suggest that to compete for FLC import, a compound requires a 5-membered ring with two (imidazole) or three (triazole) nitrogen atoms, in addition to a halogenated 6-membered ring with the halogen in position 1 or 3, but not necessarily both (19). The only exception to this has been the competition of 1-TRI, which has a five membered imidazole ring as well as three 6 membered rings, but is not halogenated.

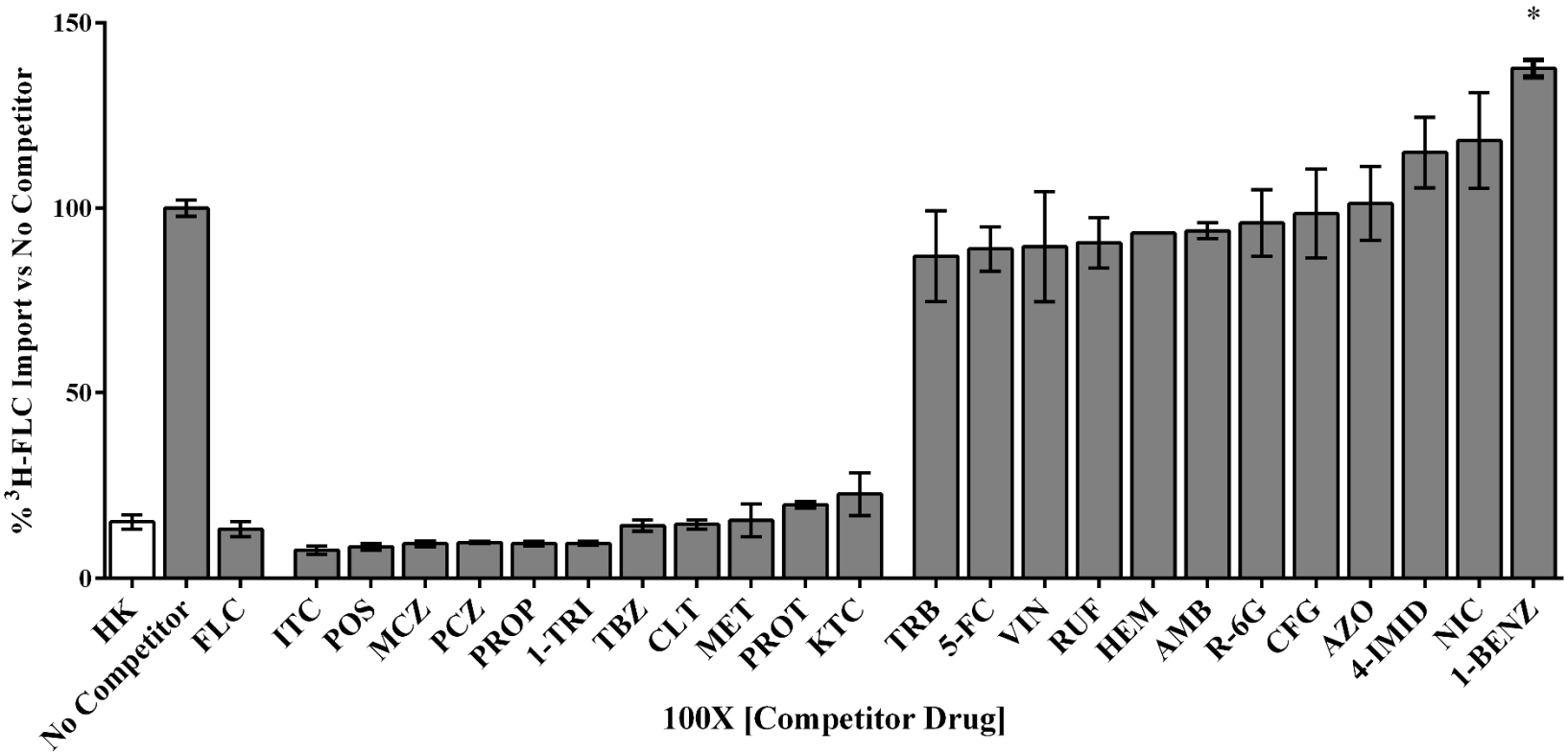


Figure 2.5. Competition for ^{3}H-FLC Import in *A. fumigatus*. Compounds were tested for competition at 1.95 μM (100x molar excess to ^{3}H-FLC) during simultaneous treatment with ^{3}H-FLC. ^{3}H-FLC accumulation was measured after 24 hr incubation with competitors. Error bars represent standard deviation of biological triplicates for each condition. Drug abbreviations are described in the Results section. Compounds clustered to the left side of the figure compete with ^{3}H-FLC for import and are statistically difference from the no competitor sample. Compounds clustered to the right side of the figure do not compete with ^{3}H-FLC and are not statistically different from the no competitor sample with the exception of 1-benzylimidazole, which had statistically higher FLC import.

Table 2.2. Compounds That Compete at 100X Molar Excess with FLC for Import in *A. fumigatus* CEA10

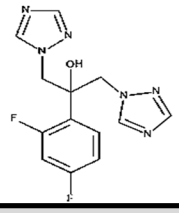
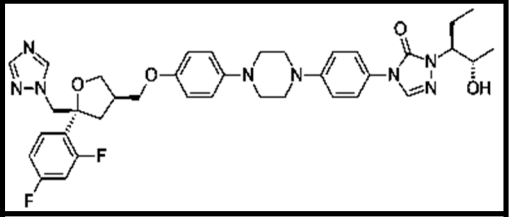
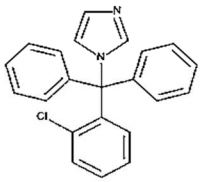
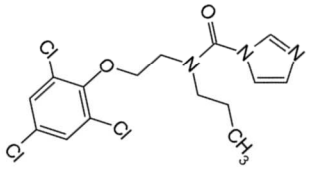
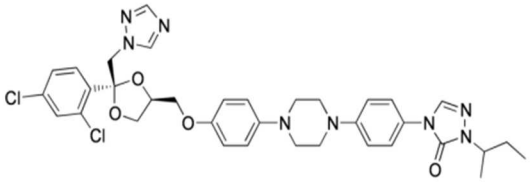
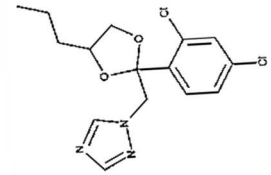
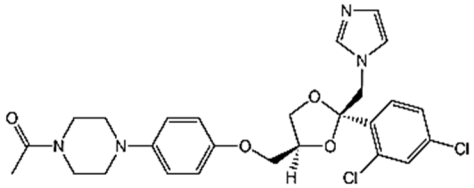
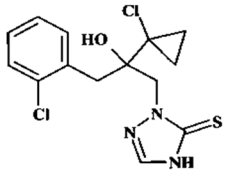
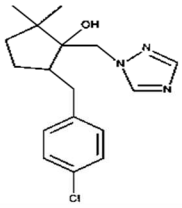
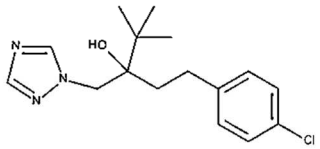
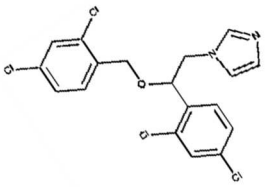
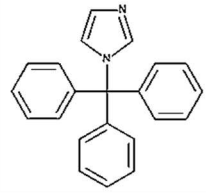
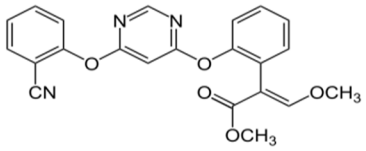
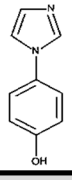
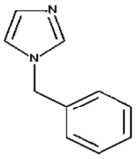
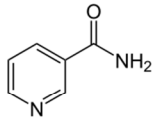
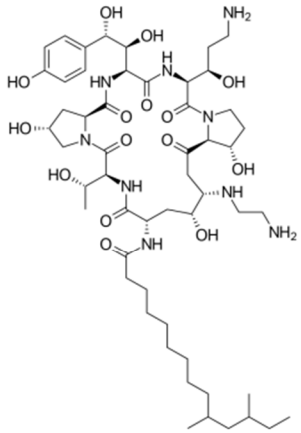
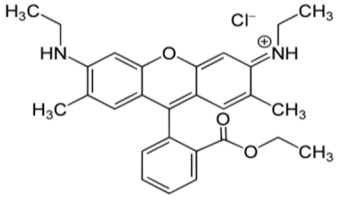
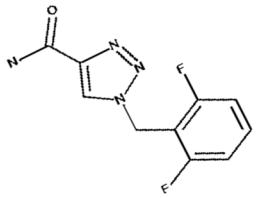
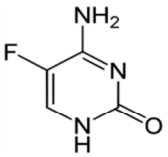
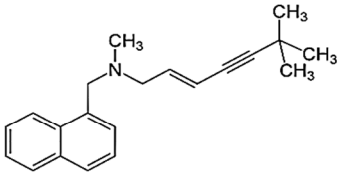
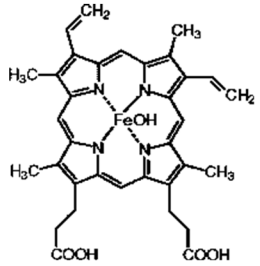
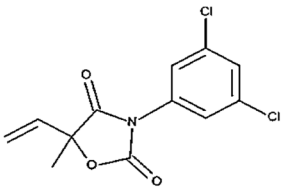
Fluconazole	Posaconazole
	
Clotrimazole	**Prochloraz**
	
Itraconazole	**Propiconazole**
	
Ketoconazole	**Prothioconazole**
	
Metconazole	**Tebuconazole**
	
Miconazole	**1-(triphenylmethyl) imidazole**
	

Table 2.3. Compounds That Do Not Compete at 100X Molar Excess with FLC for Import in *A. fumigatus* CEA10

Azoxystrobin	4-(imidazole-1-yl) phenol
	
1-Benzyl-imidazole	**Nicotinimide**
	
Caspofungin	**Rhodamine 6G**
	
	Rufinimide
	
5-Flucytosine	**Terbinafine**
	
Hematin	**Vinclozoline**
	

Import of ^{3}H-FLC in Different *A. fumigatus* Morphological Forms

A. fumigatus undergoes several stages of growth from conidia through germlings to mature hyphae. Each is important for different aspects of survival and pathogenesis. The different stages analyzed are pictured in Figure 6A-C.

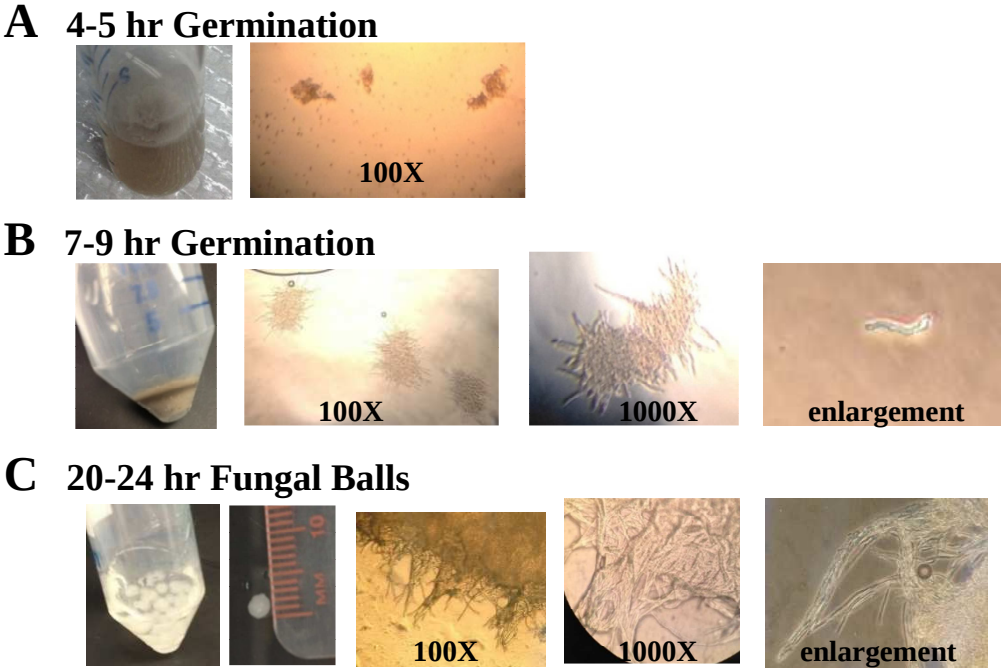


Figure 2.6. Images of *A. fumigatus* CEA10 Different Morphologies. Conidia were harvested from agar plates and grown in CSM complete with glucose for the following times. **A.** 4-5 hr, conidia began to cluster and elongation was slightly visible as germ tubes started to form; **B.** 7-9 hr, distinctive germ tubes were present but no hyphal branching had occurred; **C.** 20-24 hr, the mycelia form small (3 mm) fungal balls.

We determined azole accumulation during several stages of growth (Figure 2.7). The amount of radioactivity inside the cells was measured per sample biomass and compared. Conidia showed baseline ^{3}H-FLC accumulation, while the young germlings (4.5 hr of germination), older germlings (7.5 hr of germination), and the standard mature mycelia (24

hr fungal balls) accumulated significantly more ^{3}H-FLC at each stage with 24 hr being maximal. The mycelial fungal balls accumulated the greatest amount of ^{3}H-FLC compared to the other morphological forms. Thus, the 24 hr mycelial form was used for other characterizations. The difference in import between the 4.5 and 7.5 hr germlings was not statistically significant. Peculiarly, the HK 7.5 hr germlings showed reduced baseline counts.

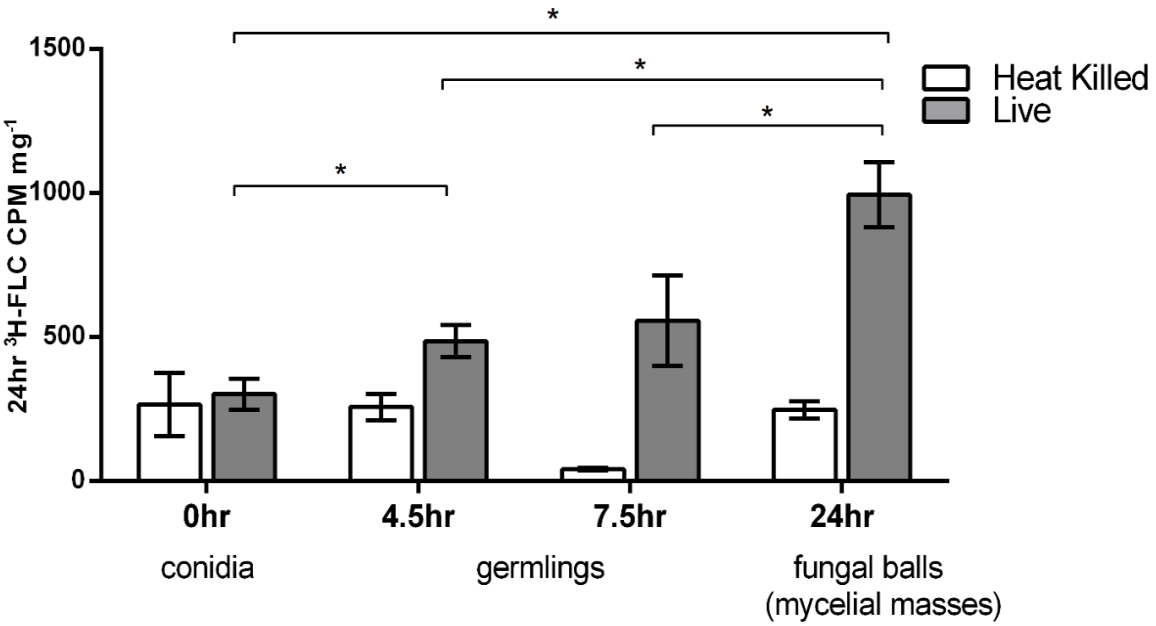


Figure 2.7. *A. fumigatus* Fungal Balls Take Up More ^{3}H-FLC than Conidia or Germlings. The different growth stages of *A. fumigatus* were incubated with 19.25 nM ^{3}H-FLC for 24 hr and drug uptake was measured. Error bars represent standard deviation of biological triplicates for each condition. Asterisks indicate a statistical significance of $P < 0.05$ between two conditions.

Import of ^{3}H-FLC in Mutant *A. fumigatus* Strains

SrbA is a sterol regulatory element binding protein in *Aspergillus* that primarily regulates ergosterol biosynthesis, but also regulates many genes important for basal, as well as stressed, cell activity. This includes transcriptional activation of a variety of transporter or

putative transporter genes, as well as genes important for membrane stability and function (11, 12). Import analyses on mutant *Aspergillus* strains showed differences in drug accumulation when a gene for the major transcription factor SrbA is deleted, SDW1, compared to the wild-type strain and the complemented strain SDW2 (Fig. 2.8).

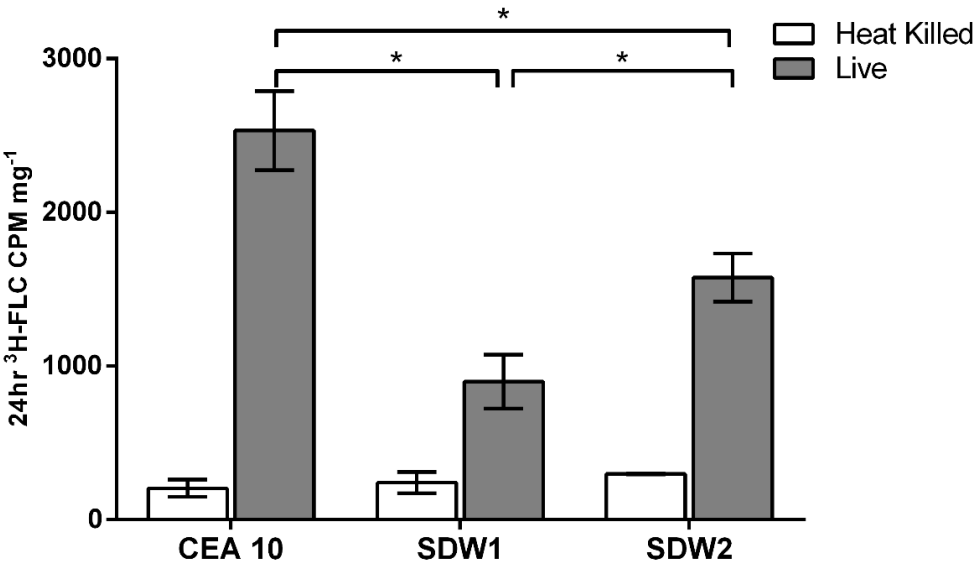


Figure 2.8. *srbA* Deletion Affects ^{3}H-FLC Accumulation. ^{3}H-FLC uptake in the *A. fumigatus* wild-type strain CEA10 was compared to the SDW1 *srbA* mutant and SDW2 +*srbA* complemented strains. SDW1 accumulated significantly less ^{3}H-FLC than the wild-type CEA10 and SDW2 strains, while the SDW2 strain showed intermediate drug uptake. Error bars represent standard deviation of biological triplicates for each condition. Asterisk indicates a statistical significance of $P < 0.05$ between two conditions.

The *srbA* mutant strain SDW1 shows significantly reduced ^{3}H-FLC accumulation compared to the wild-type strain and complementation strain SDW2. This suggests that the SrbA transcription factor is important for azole import, whether by direct or indirect effects. SDW2 recovered the wild-type drug susceptibility and morphological phenotype (11, 12). However, the drug import was not restored to CEA10 wild-type levels, instead there was an

intermediate level of drug accumulation in this strain; lower than wild-type and higher than the deletion mutant (Fig. 2.8). This could be due to differences in transcriptional activity in the ectopic reinsertion of the *srbA* gene, additional point mutations in this strain, or the CEA17 mutant background strain.

Efflux of ^{3}H-FLC from Preloaded Cells

Given the import of radiolabeled azoles over 24 hr, it was important to study how the drug is exported after import. Figure 2.9 shows cells that were preloaded with ^{3}H-FLC at 19.5 nM for 24 hr. The cells were diluted into a 50-fold excess volume of YNB media, and the amount of labeled drug associated with the cells was determined as a function of time. Efflux was evaluated in both glucose-energized (gray line with squares) and de-energized (glucose starved) (black line with circles) cells.

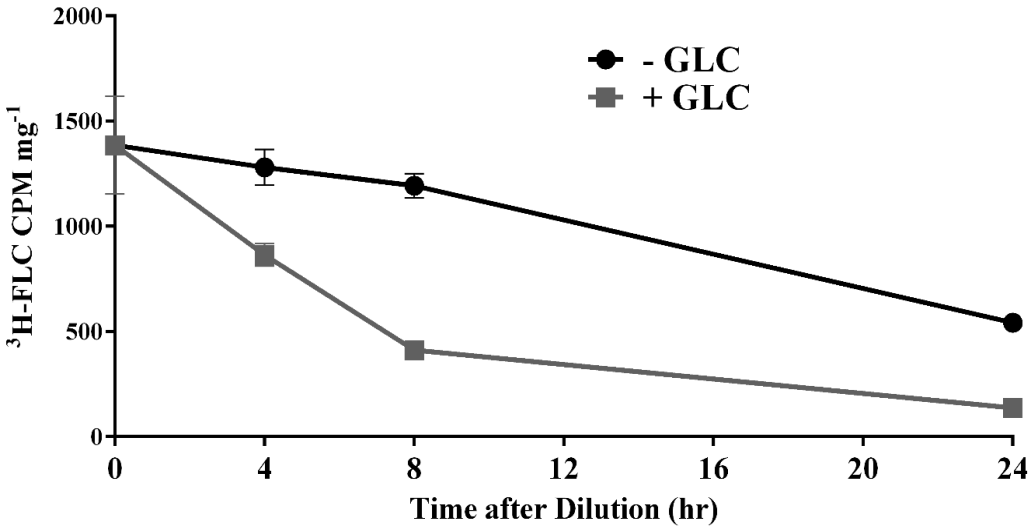


Figure 2.9. Efflux of ^{3}H-FLC from Preloaded Cells. Cells were preloaded with 19.25 nM ^{3}H-FLC for 24 hr. The cells were washed with YNB and placed in either de-energized (glucose free) (solid black line with circles) or a 2 % glucose-energized (gray line with squares) media for 4, 8 and 24 hr to measure the efflux of ^{3}H-FLC in *A. fumigatus*. Error bars represent standard deviation of biological triplicates for each condition. Some error bars are hidden by the symbols.

^{3}H-FLC efflux was slow and dependent on energy, consistent with the activation of membrane efflux transporters to pump the azole out of the cell. By 24 hr, most of the ^{3}H-FLC was exported from the cells in both energized and de-energized conditions. Those samples incubated in the presence of glucose show faster export of labeled drug, consistent with the idea that membrane efflux pumps require energy and that glucose starvation limited the energy and thus the efflux pump activity.

Discussion

In this work, we have analyzed a potential molecular mechanism of azole drug resistance in *A. fumigatus,* by characterizing azole import into the fungal cell under a variety of environmental conditions. Azole drug uptake is required so that the drug can inhibit the intracellular Cyp51 target enzyme. Therefore reduced or modified drug import may help to explain why some pathogenic fungi are more resistant to azoles than others. In *A. fumigatus,* there is an increasing number of cases of azole-resistant isolates in which the resistance mechanism is unknown (15). Many of these isolates do not have a mutation in the azole target enzyme Cyp51, nor do they show active efflux of the drug from the cells. Thus, we hypothesize that these *A. fumigatus* isolates may well have alterations in drug import. Our assay will further be used to compare drug import in clinical isolates and resistant strains of *A. fumigatus* and other filamentous fungi.

The study of drug import in *A. fumigatus* using radioactively labeled azoles has not been done previously. This approach includes novel experiments that may identify differences in substrate specificities of medically relevant compounds. Our experiments thus far, in agreement with Mansfield et al's work in *C. albicans* (19), have demonstrated that

azoles are not passively diffused into the *A. fumigatus* cell, nor are they actively transported in an energy-dependent fashion. Instead, azoles most likely enter the cell by facilitated diffusion via a membrane protein carrier that recognizes a specific moiety found in azole drugs.

The labeled azoles do not simply bind to the cell surface as demonstrated by baseline drug accumulation in heat-inactivated cells compared to living cells. Drug uptake can be measured over time in live cells reaching a maximum accumulation after 24 hr of treatment (Fig. 2.3C), while heat inactivated cells maintain a constant drug concentration.

Import is not diffusion limited, since the internal drug concentration is much lower than that of the external medium (data not shown). The drug concentrations used in our import analysis was in the nanomolar range. It cannot be ruled out that normal diffusion or non-specific-carrier transport occurs at higher drug concentrations. It is also not known whether the import transporters also act as efflux transporters for azoles or other molecules.

Drug uptake by this carrier is energy independent, as seen by drug accumulation measured in de-energized cells. Azoles were imported in both energy replete and glucose-containing media. Even cells treated with 2-deoxyglucose, a glycolytic inhibitor, showed drug uptake (Fig. 2.4A). However, media that contained glucose showed reduced final drug accumulation levels, presumably due to activation of glucose-dependent efflux pumps. It is possible that cells are better able to metabolized and degrade FLC in energized conditions. However, the radioactive label would still be detected regardless of cleavage or FLC degradation. There has been no evidence to date that FLC is degraded by fungal cells. Our evidence indicates that efflux of azoles is dependent on energy (Fig. 2.9), suggesting distinct

transporters for influx and efflux of azoles. Import of azoles also did not require a proton gradient as shown by no change in uptake over a range of buffered pHs (Fig. 2.4B).

Changes in treatment temperatures had a significant effect on azole drug uptake in *A. fumigatus* (Fig. 2.4C). *A. fumigatus* has a remarkable ability to thrive at a range of environmental temperatures including the 4° C to 42° C. However, drug accumulation was near baseline levels for both 4° C and 42° C, with the highest accumulation at 20-22° C (Fig. 2.4C). These dramatic differences in temperature-dependent accumulation are being further analyzed but could be due to changes in gene expression, membrane fluidity, drug stability, or protein folding among other things.

A comparison of drug import in exponential vs post-exponentially growing cells (Fig. 2.4D) shows a dramatic decrease in accumulation in the older cells. This is in agreement with post-exponential cells being less metabolically active than exponentially growing cells. A cessation of cell division, decrease in macromolecular production, and other changes in cellular regulation could be responsible for this decrease in azole uptake in post-exponential cell growth.

Evidence for a saturable protein carrier is shown with competitive inhibition of ^{3}H-FLC uptake by other azoles (Fig. 2.5). ^{3}H-FLC import was significantly inhibited by simultaneous treatment with an excess of unlabeled azoles. However, other antifungal drugs did not compete for import into the cell. This indicates substrate specificity for moieties found in the azole structure. Tables depicting the structure of competitive inhibitors and drugs that did not compete for import are shown in Tables 2.2 and 2.3 respectively.

Differences in import between the different *Aspergillus* morphologies (Fig. 2.7) is interesting but expected since there are many differentially expressed genes and cellular changes during the different growth phases of this organism. The inactive conidia accumulated only baseline levels of azole per weight, while the germlings had an increased azole accumulation. Mature hyphae forming mycelial masses imported the highest amount of drug per mass. Images of the cellular morphologies used can be seen in Figure 2.6A-C. A major factor to consider is whether comparing drug import per mass is appropriate for each morphology. The weight per volume ratio is very likely different between the cellular morphologies. Conidia and germlings are perhaps more dense than actively growing cells due to nutrient and protein reserves. In addition to these considerations, many cell wall changes take place during germination and hyphal growth, including variations in surface expressed proteins.

Import analyses on mutant *Aspergillus* strains showed differences in drug accumulation when the gene for a major transcription factor SrbA, is deleted (Fig. 2.8). The deletion strain showed significantly reduced azole import compared with the wild-type CEA10 strain. It is possible that this mutant has a disrupted cell membrane, altering membrane transport. Many genes regulated by the SrbA transcription factor, such as the azole drug targets *cyp51A/B*, have altered expression in this strain and may be responsible for differences in drug accumulation data observed in the mutant. It is also possible that SrbA regulates a drug transporter involved in bringing azoles into the cell so that when this transcription factor is deleted, the transporter is downregulated. Future work should be done to determine if the azole importer protein is regulated by the SrbA transcription factor. It is

curious that reinsertion of the *srbA* gene in SDW2 did not fully restore azole import to wild-type levels. However, this could be the result of the ectopic reintroduction of the gene or additional point mutations in the mutant background strain.

While we have performed a preliminary investigation of drug transport across the cell membrane, the next steps are to identify the proteins that are involved in drug import. Identifying the specific channel or transport protein by which this occurs is still in progress and is a major goal of our research. Determining the proteins involved in transporting azoles into the fungal cell would provide insight into how our current drug treatments are working. Novel import proteins could potentially provide new targets for drug treatment. The long-term goal of this research is to discover and to better characterize the cellular mechanisms of drug resistance in *A. fumigatus* that can be targeted by new and better-designed drugs for more effective treatments of fungal infections.

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CHAPTER 3

AZOLE DRUG IMPORT INTO THE FUNGAL PLANT PATHOGEN *MAGNAPORTHE ORYZAE*

Chapter Summary

The fungal plant pathogen *Magnaporthe oryzae* is a destructive agricultural mold that causes disease in rice, resulting in devastating crop losses. Since rice is a world-wide staple food crop, *M. oryzae* poses a serious global food security threat.

M. oryzae shares many characteristics associated with other cereal pathogens and so elucidating the mechanisms of drug resistance and susceptibility in this species will help advance our understanding of other related fungal diseases. Anticipating future drug resistance evolution, identifying new drug targets, and finding treatment strategies using combinations of existing drugs, are of great importance for efficient crop protection.

Fungicides, including agricultural azoles, are used to combat *M. oryzae*. The target of azole antifungals is CYP51, an enzyme required for ergosterol biosynthesis. In this study, we extended our azole import assay from Chapter 2, to examine azole uptake in *M. oryzae* under a variety of conditions. Comparisons and contrasts between the results of these experiments in *M. oryzae* and *A. fumigatus* have revealed interesting similarities and variations. Consistent with our lab's import experiments on *C. albicans* and *A. fumigatus*, we conclude that azole drugs are specifically selected and imported into the *M. oryzae* cell by a pH-independent and ATP-independent facilitated diffusion mechanism. This method of drug transport into the cell is likely to be conserved across most fungal species.

Introduction

The fungal plant pathogen *Magnaporthe oryzae* is considered to be one of the most dangerous and destructive agricultural molds. Named after the host it infects, *M. oryzae* causes disease in rice (*Oryza sativa*) called rice blast, which can infect all parts of the rice plant causing lesions on leaves, stems, seeds, and even roots (1, 2). Hundreds of millions of people world-wide depend on rice as a staple food crop making rice the most important grain with regard to human diet and nutrition (3, 4). Consequently, rice blast poses a serious global food security threat. So great is the potential risk for crop failure or severely reduced crop yield due to rice blast, that *M. oryzae* has been ranked among the most significant plant pathogens (3, 5).

M. oryzae growth is favored in environments of moderate temperatures and high moisture; conditions that describe most rice fields. This pathogen produces abundant spores, which spread easily between and within crops by wind, rain, and harvest (6-9). Managing rice blast requires vigilance and careful integration of many strategies and techniques. A single mistake or change in the way in which rice is grown, or infection is treated, can result in significant crop losses (5, 10-16).

Fungicides, including agricultural azoles such as propiconazole, prothioconazole, tebuconazole and prochloraz are used to combat rice blast (5, 10-16). Fungicides are utilized as both seed treatment to prevent infection of the seedlings after germination, and also as foliar sprays on mature leaves to prevent infection and spread during the growing season (7, 11-16). Even when both treatment strategies have been employed, fungicides alone are not considered to be highly successful (5, 10-16).

The target of azole antifungals is the fungal CYP51, or sterol 14α-demethylase. This enzyme is a member of the cytochrome P450 superfamily of enzymes required for ergosterol biosynthesis in fungi. The fungal ergosterol biosynthesis pathway is unique to fungi but closely related to the mammalian cholesterol biosynthesis pathway as well as the plant sterol biosynthesis pathways, and so drug design and target specificity is critically important to prevent disruption of host organism processes.

Medical azoles are used in the treatment of fungal infections in humans and animals. These azoles are designed to be selective for inhibiting the fungal CYP51 enzyme over the ***mammalian*** homolog, and so there are generally few side effects from incorrect inhibition of the mammalian enzyme (17-19). Agricultural azoles used to treat fungal infections in plants, however, were developed primarily for selectivity against the fungal CYP51 over the ***plant*** homolog, with less regard for the possibility of any effects on the human enzyme (18-20). This has raised some concerns about human and animal safety of direct azole exposure from widespread use of agricultural azoles in the environment as well as the dangers of consuming azole-treated food products (21-24). More alarming is the possibility that extensive use of agricultural azoles may exert an azole resistance selective pressure on environmentally-dwelling, opportunistic human fungal pathogens, so that human fungal infections are no longer treatable with traditional medical azoles (25-29).

In addition to concerns about the direct effect on *humans* and *human* fungal pathogens, evolution of plant pathogen drug resistance has mirrored the pattern of increase fungicide use to a troubling degree (3, 6-9, 28, 30). Some of the fungicide resistance mechanisms that have arisen from environmental drug use include mutations in fungal gene

promoter elements that have been shown to allow upregulation or inducibility of fungicide target genes (28, 31-33). And mutations in the target gene active site may cause weakened antifungal drug binding or even change the protein folding to allow constitutive activation of the target enzyme (17, 34).

Additionally, target gene duplication events have led to intrinsic resistance to many fungicides. For example, filamentous fungi often possess multiple CYP51 paralogs: two in *M. oryzae, A. fumigatus* and *A. nidulans*, and three in *A. flavus* and species of *Fusarium* (17, 29, 35). These filamentous fungal species and molds are intrinsically resistant to fluconazole (FLC) partly due to weak binding and thus incomplete inhibition of the Cyp51 target enzyme compared to other azoles with stronger Cyp51 binding affinity (29, 30, 36). In organisms that have a single copy of Cyp51, even a transitory inhibition of the enzyme's activity by FLC is enough to cause a disruption in the ergosterol biosynthesis pathway and an inhibition of fungal cell growth. Conversely, in organisms that have multiple Cyp51 enzymes, the transitory binding of FLC to Cyp51 is not enough to overcome the activity of the additional paralogs and ergosterol biosynthesis remains sufficient for continued growth and replication (29).

Importantly to our research, *M. oryzae* shares many characteristics associated with other notable cereal pathogens (5, 10). Advances in understanding the genes and mechanisms that govern the resistance and susceptibility in this species, will help advance our understanding of other related fungal diseases. Identifying new fungal-specific drug targets, or finding treatment strategies using combinations of existing drugs, is of great importance to control the spread of resistant fungi and increase the efficiency of crop

protection. Continued analysis of even the most generic processes in the *Magnaporthe* cell can be helpful for targeted or broad-spectrum crop disease intervention, management, and diagnostics.

In this study, we extended our azole drug accumulation assay from Chapter 2, to examine azole uptake in the filamentous fungal plant pathogen *M. oryzae* under a variety of conditions. Comparisons and contrasts between the results of these experiments in *M. oryzae* and *A. fumigatus* have revealed interesting similarities and variations.

Consistent with our import experiments on the filamentous human fungal pathogen *A. fumigatus*, our import experiments with *M. oryzae* have shown that azole drugs do not accumulate in the fungal cell solely by passive diffusion. Instead there is evidence for azole import by a facilitated diffusion mechanism including **(1)** dramatically reduced azole accumulation in heat inactivated cells as well as chemically inactivated cells compared with living cells, **(2)** competition for import by other compounds, **(3)** differences in drug accumulation in exponentially growing and stationary cells.

We also found significant differences in drug accumulation in *M. oryzae* depending on the growth media used, which was not observed in *A. fumigatus*. We conclude that azole drugs are specifically selected and imported into the fungal cell by a pH-independent and ATP-independent facilitated diffusion mechanism. This method of drug transport into the cell is likely to be conserved across most fungal species.

Finally, we used ^{3}H-FLC to explore azole efflux in *M. oryzae*. Some of the variations in ^{3}H-FLC accumulation between *M. oryzae* and *A. fumigatus* suggest differential expression, induction, or function of efflux transporters in the two organisms. These distinctions between

organisms should be taken into consideration for future broad-spectrum drug design as well as organism-specific treatment strategies. We demonstrated the potential usefulness of combination drug therapies and the idea of repurposing archived compounds for alternative uses as the threat of resistance to current fungal treatments emerges.

Materials and Methods

Strains, Media, Materials, and Strain Preparation

The *M. oryzae* wild-type, sequenced strain 8958 (70-15), was used for all azole import experiments. *M. oryzae* cultures were routinely grown and maintained in either liquid YAD medium (1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate and 20 g glucose per liter) or on oatmeal agar plates with 20 g glucose per liter, at room temperature (27° C). Conidia were harvested from 7-day old agar plate cultures by pipetting 7.0 mL 0.01 % Tween 20/water directly onto sporulating plates and loosening conidia using a sterilized glass spreader. The conidial suspension was allowed to settle in a 14 mL round bottom tube for 10-15 minutes to roughly separate the conidia (which stay suspended) from mycelia (which settle).

To propagate *M. oryzae*, 200 µL of the supernatant from the harvested conidial suspension was either directly inoculated into 5 ml liquid YAD media with glucose and grown in 50 mL conical tubes in a room temperature shaking at 180 rpm, or plated on oatmeal agar plates. For storage, oatmeal agar plates were overlaid with 3-5 Whatman filter paper disks before inoculating with conidial suspension. After 7-10 days of growth, filter paper disks covered with a lawn of *M. oryzae* were collected, desiccated in a Tupperware

container with Drierite desiccant stones (W.A. Hammond Drierite Co. LTD. Xenia, OH), and stored at -20° C.

Medium components, plastic ware, general chemicals, and unlabeled drugs used for competition were obtained from Fisher Scientific (Pittsburg, PA), or Sigma-Aldrich (St. Louis, MO).

M. oryzae E-test Susceptibility Testing to Common Antifungals

E-test strips (bioMérieux, United States) were used to test *M. oryzae* susceptibility and determine the MIC to common antifungals belonging to different classes of drugs, including the medical azoles fluconazole (FLC), ketoconazole (KTC), itraconazole (ITC), posaconazole (POS) and voriconazole (VRC), an echinocandin caspofungin (CFG), a polyene amphotericin B (AMB), and a DNA and RNA synthesis inhibitor flucytosine (5FC). One side of the plastic E-test strip is calibrated with MIC values of the drug in µg/ml. The drug-gradient on the strip covers 15, two-fold dilutions.

Yeast extract Peptone Dextrose, or YEPD or YPD (10 g yeast extract, 20 g peptone and 20 g glucose) plates were inoculated with a lawn of *M. oryzae* conidia and allowed to dry. A single E-test strip was placed on each inoculated plate and kept at 27° C for 96 hours with daily monitoring. The MIC was determined based on the drug concentration on the E-test strip in which the zone of inhibition, or ellipse of non-growth occurred.

Radioactively Labeled Azole Import by *M. oryzae*

Radioactively labeled FLC (^{3}H-FLC), (481 GBa/mmol, 13 Ci/mmol, 1 µCi/µL; 77 µM FLC) was custom synthesized by Amersham Biosciences. The drug concentration used

during the import assay was well below the Minimum Inhibitory Concentration (MIC) for the strain (*M. oryzae* FLC MIC >32 µg/ml).

To directly measure azole import in *M. oryzae,* we used ^{3}H-FLC in our drug uptake assay, as described in Chapter 2 and below, as modified for *M. oryzae*. Unless specifically noted, 5 ml YAD medium with 2 % glucose in 50 mL conical tubes was inoculated with *M. oryzae* conidia and grown at 27° C, 180 rpm shaking, for 48 hr at which point they were mycelial masses or fungal balls, approximately 3 mm in diameter. The fungal balls were transferred to 2 mL microcentrifuge tubes and washed by centrifugation and resuspension three times with YNB complete (1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate per liter, pH 5.0) without glucose unless specifically noted.

After the washing, the fungal ball pellet was transferred to 14 mL round bottom tubes containing 1 mL of YNB for a 2 hr glucose-deprived (starvation) de-energizing period. The glucose starvation was done to keep the cells in a de-energized state. The de-energized cells showed no further growth for the extent of the import assay, as determined by dry weight at the conclusion of the assay. After the glucose starvation period, reaction mixes were made consisting of 1 mL of YNB containing no glucose, with fungal balls and 25 µL of diluted ^{3}H-FLC (freshly diluted 1/100X from stock). The resulting final ^{3}H-FLC concentration is 19.25 nM (5.89 ng/ml). The *M. oryzae* strain (70-15) has an MIC of > 32 µg/ml to FLC. Thus, the azole concentration used for the import assay is not expected to have any effect on cell viability.

After 24 hr incubation with ^{3}H-FLC, a 5 ml stop solution (YNB +20 mM [6 mg/L] unlabeled FLC) was added to each 14 mL round bottom tube sample. The tube was filtered

by vacuum over pre-weighed and wetted glass fiber filters (24 mm GF/C; Whatman; Kent, UK). After filtering, another 5 ml stop solution was used to wash each sample again to remove non-specific, cell surface binding. The filters with fungal balls were either allowed to dry for 24-48 hr or were baked in a drying oven for 15 minutes at 95° C. Filters with fungal balls were then weighed to obtain the dry mass of each fungal sample. The weighed filters were transferred to 5 mL scintillation vials. 3 ml of scintillation cocktail (Ecoscint XR, National Diagnostics, Atlanta GA) was added and the radioactivity associated with the filter was measured in a liquid scintillation analyzer (Beckman Coulter, LS 6500 multipurpose scintillation counter).

As cell numbers are difficult to calculate with filamentous fungi such as *M. oryzae,* all values are expressed as drug accumulation in counts normalized to dry weight of the sample. Results were calculated as CPM/mg of mycelial mass. While absolute CPM values varied between experiments, relative import differences between samples remained consistent.

Characterization of Conditions That Affect FLC Import

Further studies were done to determine the effect of changes in the growth or incubation conditions on FLC import.

Heat killed cells. Uptake of ^{3}H-FLC was measured in cells inactivated (killed) by heat (95° C for 30-40 min). This killing method decreased colony-forming units greater than 100 fold compared to the non-heat treated culture. Samples were processed according to the above protocol with the exception that the heat-killing step was performed during the final 45 minutes of the glucose starvation period. Heat killed samples were treated with ^{3}H-FLC and

analyzed identically to the live cell samples. The results were compared with live *M. oryzae* data. Heat killed samples were used as a control for baseline drug accumulation and non-specific cell surface binding in all testing conditions unless otherwise noted.

Other methods of cell inactivation. ^{3}H-FLC accumulation was measured after 24 hr in samples that were exposed to various inactivation methods. Methods of cell inactivation included treatment with high concentrations of AMB (8 µg/ml), Qiagen RLT Lysis solution (600 µL), and CFG (16 µg/ml). Heat killing as described above was found to be the most reliable at reducing viable cell counts to less than 1 %. And so this method of inactivation was used as a no-import, baseline control, although the other methods of inactivation were useful to confirm that import is reduced in inactivated cells as opposed to being an artifact of heat treatment.

Energy depletion. To determine whether ^{3}H-FLC import was energy-dependent, mycelial balls were washed 3 times and resuspended in YNB media deplete of glucose. Samples were considered to be de-energized following 2 hr in this glucose starvation media. Following the de-energization period, the cells were then treated with ^{3}H-FLC for 24 hr in glucose-depleted media and their ^{3}H-FLC accumulation was compared to cells that were treated with ^{3}H-FLC for 24 hr in media containing 2 % glucose.

pH. Cells were incubated with ^{3}H-FLC in YNB without supplementation (pH 5), with no pH adjustment, or with YNB medium adjusted to pH 4 or 7 with a 1M citric acid and 2M Na_2HPO_4 mixed buffer. Increasing the ratio of the citric acid to Na_2HPO_4 to lower pH and increasing the ratio of Na_2HPO_4 to citric acid solution to increase pH. All samples were processed after 24 hr as described above.

Growth media: *M. oryzae* liquid cultures were grown from conidia to mature mycelial masses (48 hr) in Complete Supplemental Medium or CSM (0.75 g CSM, 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate, 20 g glucose per liter); YAD Medium (1.7 g yeast nitrogen base without amino acids or ammonium sulfate, 5 g ammonium sulfate and 20 g glucose per liter); and YPD as described above. The mycelial masses were then washed and treated with ^{3}H-FLC in YNB for 24 hr as described for the standard import assay.

Exponential vs stationary phase uptake of ^{3}H-FLC. Conidia were grown at 27° C in a shaking incubator at 180 rpm in YAD complete media with glucose for either the standard 48 hr (exponentially growing) or for 72 hr or 96 hr (stationary phase). These 2, 3 or 4 day old fungal balls were then treated with ^{3}H-FLC for 24 hr as described previously.

Competition for azole import. To determine if compounds compete for azole uptake in *M. oryzae*, we processed samples as described above by our ^{3}H-FLC assay, but in addition, treated the samples simultaneously with potential competitive inhibitors at 1.95 µM (100X molar excess of the labeled FLC). ^{3}H-FLC uptake was measured as usual after 24 hr incubation with ^{3}H-FLC and competitor. Decreased ^{3}H-FLC uptake in the presence of an unlabeled competitor suggests that both drugs use the same transporter. Non-radiolabeled drugs used for import competition experiments included: amphotericin B (AMB), caspofungin (CFG), fenpropimorph (FENP), fluconazole (FLC), itraconazole (ITC), ketoconazole (KTC), metconazole (MET), tebuconazole (TBZ), terbinafine (TRB), and 1-triphenylmethyl imidazole (1-TRI).

Azole Efflux in *M. oryzae*

Efflux kinetics. Fungal balls were preloaded with ^{3}H-FLC by treating them at 19.5 nM for 24 hr per the standard assay. The cells were then washed and diluted into YNB media, and the amount of labeled drug associated with the cells was determined as a function of time at 4, 8 and 16 hr. Efflux was evaluated in both glucose-energized (2 % glucose) and de-energized (glucose-starved) cells.

Efflux inhibition by Clorgyline. To test the effect of Clorgyline as a possible inhibitor of energy-dependent efflux in *M. oryzae,* we treated energized cells (2 % glucose, efflux active) with either ^{3}H-FLC alone (19.25 nM) or ^{3}H-FLC with clorgyline [N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy)propylamine hydrochloride] (233 µM) and compared the results to ^{3}H-FLC treatment in de-energized (glucose-starved, efflux inactive) cells. ^{3}H-FLC accumulation was measured in all conditions after 20 hr.

Statistical Analysis

Differences between sets of samples were evaluated by an unpaired two-tailed Student's t test. A P value of < 0.05 was considered significant.

Results

Recently, radioactively-labeled drugs were used to analyze azole uptake in the human pathogenic yeast *C. albicans* and *Cryptococcus neoformans*, the model yeast *Saccharomyces cerevisiae*, and the human pathogenic mold *A. fumigatus* (37, 38). In those studies, FLC was shown to be taken up by a facilitated diffusion mechanism and this import was affected by several in-vitro environmental conditions (37, 38). Here we have adapted the assay used in

the previous studies for an initial characterization of azole uptake and efflux in the plant pathogenic mold *M. oryzae*.

M. oryzae MICs to Common Antifungals

E-test strips were used to establish *M. oryzae* susceptibility to common medical antifungals belonging to different classes of drugs and determine if a study using medical azoles was relevant in *M. oryzae*. The MIC was reported as the drug concentration at which a clearing of cells intersected with a specific concentration of drug on the test strip as seen in Figure 3.1 and Table 3.1. The drugs tested included the medical azoles FLC, ITC, KTC, POS, and VRC, the echinocandin CFG, the polyene AMB, and the RNA and DNA synthesis inhibitor 5FC.

M. oryzae did not show susceptibility to 5FC or FLC at the drug concentrations tested, and although there was a 'phantom' zone of clearing around the CFG E-test, there were enough CFG-resistant colonies in the clearing for this strain to be interpreted as resistant at the concentrations tested. There was measurable susceptibility of *M. oryzae* to the other drugs tested (Figure 3.1 and Table 3.1).

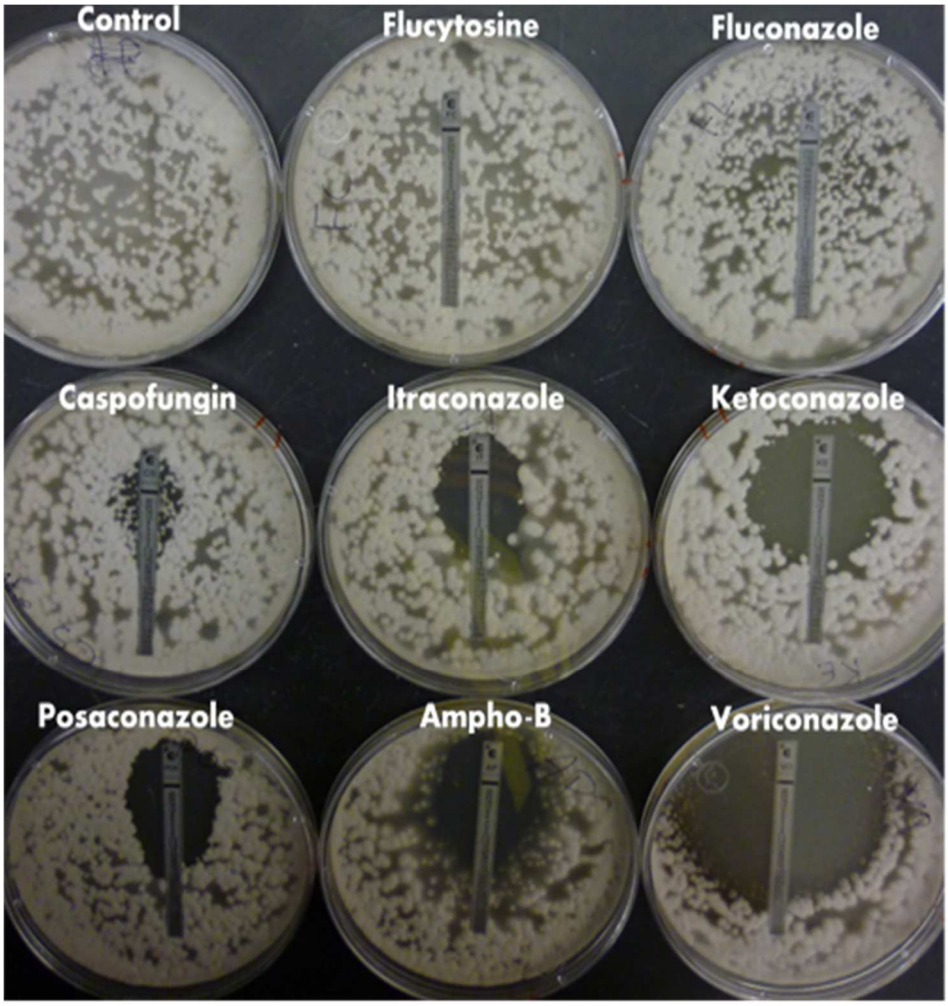


Table 3.1.

MICs by E-test

Drug	µg/ml
5FC	>32
FLC	>32
CFG	>32
ITC	0.5
KTC	0.5
POS	0.38
AMB	0.25
VRC	0.06

Figure 3.1. *M. oryzae* Drug Susceptibility to Common Classes of Medical Antifungals. E-test strips were used to test *M. oryzae* susceptibility and determine the MIC to common antifungals belonging to different classes of drug. Plates were allowed to grow at 27° C for 96 hours. The MIC was determined based on the ellipse of non-growth intersecting with the drug gradient marked on the test strip.

^{3}H-FLC Import in Inactivated Cells

Cell inactivation by heat, AMB treatment and lysis solution each reduced azole import significantly (Fig. 3.2). Heat killing was found to be the most reliable at reducing viable cell counts to less than 1 %. The reduced import due to AMB treatment suggests that membrane permeability allows drug to pass through the membrane in both directions. CFG treatment at 16 µg/ml did not significantly affect drug import, suggesting that cell wall disruption does not influence azole import. However, the CFG-treated cells showed high import variability and a trend towards reduced azole import.

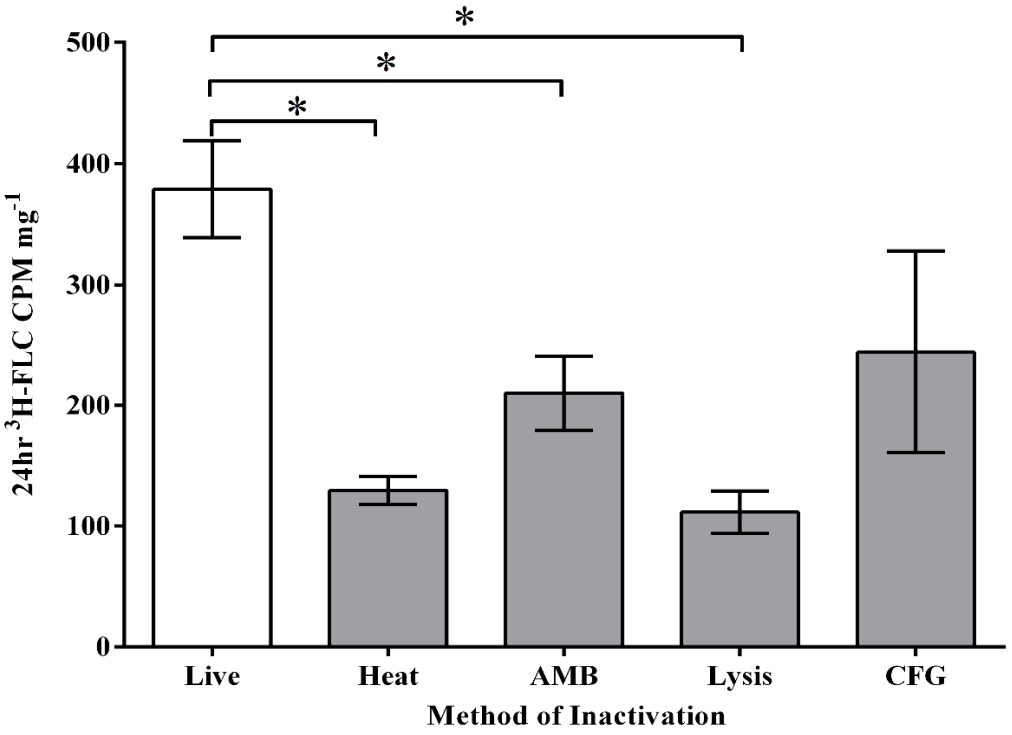


Figure 3.2. *M. oryzae* Inactivation Methods before ^{3}H-FLC Treatment. ^{3}H-FLC accumulation was measured after 24 hr in samples that were exposed to various inactivation methods. Live: no inactivation, HK: 95° C for 30 min, AMB: 8 µg/ml, Lysis: 600 µL Qiagen RLT buffer, CFG: 16 µg/ml. Error bars represent standard deviation for each condition. Asterisk indicates a statistical significance of $P < 0.05$.

Import of ^{3}H-FLC with Varied *In Vitro* Treatment Conditions

Availability of energy: To examine the effect of energy-requiring efflux mechanisms and to determine whether the accumulation of ^{3}H-FLC in *M. oryzae* requires energy, the effect of glucose was examined in the assay. Cells were de-energized by glucose starvation in glucose-depleted media. ^{3}H-FLC accumulation of the de-energized samples were compared with ^{3}H-FLC accumulation of energized samples grown in 2 % glucose replete media (Fig 3.3A).

^{3}H-FLC uptake was observed in both de-energized and energized cells. However, the energized cells showed significantly reduced intracellular ^{3}H-FLC concentration compared to de-energized cells. The reduced azole accumulation in the energized cells is most likely the result of glucose activation of the efflux pumps. For subsequent experiments, we used de-energized (glucose starved) cells to focus solely on drug uptake and eliminate efflux mechanisms.

Growth media: Differences in ^{3}H-FLC accumulation was compared between *M. oryzae* that was grown from conidia to mature mycelial masses in CSM, YAD or YPD media (Fig. 3.3B). CSM is a Complete Supplemental Mixture that is considered synthetic defined, complete medium, containing chemically defined components Yeast Nitrogen Base (YNB), Ammonium Sulfate (AS) and a carbon source, glucose. It also contains a complete amino acid supplement mixture and so is non-selective.

YAD is considered a synthetic defined, minimal medium, containing chemically defined components YNB, AS and glucose, but without any supplemented amino acids so only prototroph strains can grow in this media.

YPD, also called YEPD, is considered an undefined, non-selective, rich medium. YPD contains Yeast extract, a complex nutrient base derived from killed yeast cells; Peptone, an enzymatic digest of animal protein that contains nitrogen and a high peptone and amino acid content, and glucose. This medium provides an excess of amino acids, nucleotide precursors, vitamins, and essential metabolites (Fig. 3.3B).

Cells that were grown from conidia to mycelial balls in CSM took up the least amount of ^{3}H-FLC after 24 hr treatment. Cells grown in YPD media took up the most ^{3}H-FLC, however the baseline control was also slightly higher in the YPD-grown cells as well. Cells grown in YAD took up an intermediate level of ^{3}H-FLC, but surprisingly, uptake was more similar to cells from YPD media compared to the CSM. Both YAD and YPD-grown samples had significantly higher import compared to CSM-grown cells, which only had near baseline levels of import.

pH: To determine if ^{3}H-FLC import is pH dependent or affected by a proton gradient, we measured drug accumulation after ^{3}H-FLC treatment in unbuffered YNB media or at pH 4 or 7 using a 1M citric acid and 2M Na_2HPO_4 mixed buffer (Fig. 3.3C). There was significant ^{3}H-FLC accumulation at all pHs tested, but there was no statistically significant difference between the different pHs, indicating ^{3}H-FLC import is not pH dependent.

Stationary vs exponential cells: Figure 3.3D shows that ^{3}H-FLC accumulation is affected by the stage of hyphal growth of the *M. oryzae* cells before treatment. Samples grown for the standard 48 hr before drug treatment were compared to samples grown for 72 or 96 hr before drug treatment.

After ^{3}H-FLC treatment, the exponentially growing (48 hr) samples accumulated significantly more ^{3}H-FLC than the stationary phase (72 and 96 hr) samples.

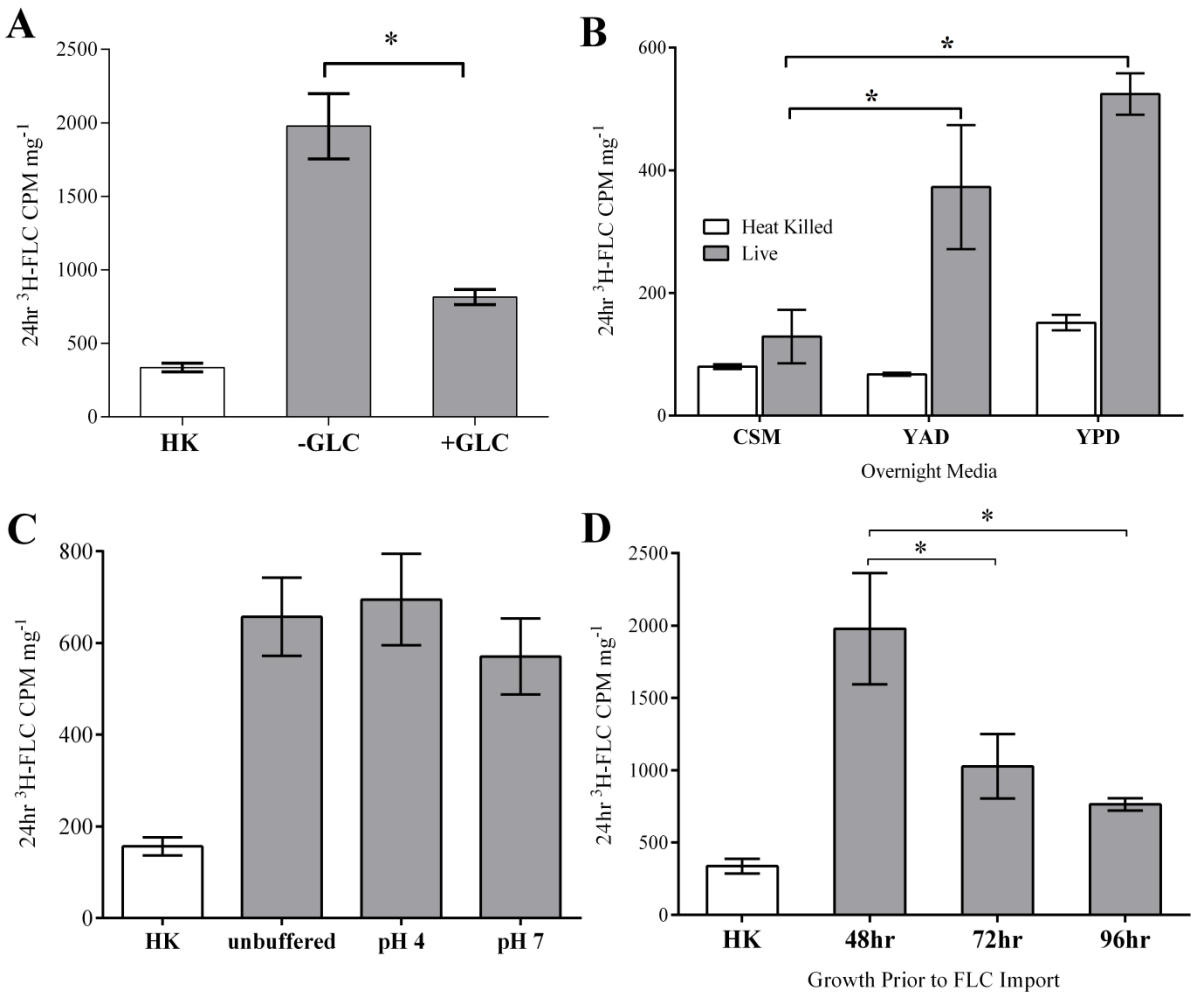


Figure 3.3. Effects of Glucose, Media, pH and Cell Phase on FLC Import. A. Glucose: Cells were de-energized by glucose-depleted media (-GLC) compared to cells in the presence of glucose (+GLC) and measured for FLC accumulation. **B. Growth Media:** Cells were grown from conidia to mycelial masses for 48 hours in either CSM complete, YAD, or YPD media. **C. pH**: ^{3}H-FLC was imported into *M. oryzae* in unbuffered media or at pH 4 and 7 in 100 mM citric acid buffers. There were no statistically significant differences between import at the three conditions. **D. Phase:** Cells were grown in shaking liquid media for 48 hr, 72 hr or 96 hr and then treated with ^{3}H-FLC for 24 hr. For each panel: Error bars represent standard deviation for each condition. Asterisks indicate a statistical significance of $P < 0.05$ between two conditions. HK = heat killed control. Statistical differences to HK are not shown.

Competition for ^{3}H-FLC Import in *M. oryzae* with Azoles and Other Compounds

To determine whether all azoles use the same transporter or family of transporters in *M. oryzae,* non-radioactively labeled azoles were tested for competition against labeled FLC (Fig. 3.4, Tables 3.2 and 3.3). The concentration of all competitors was 1.95 μM (100x molar excess to ^{3}H-FLC). All non-radiolabeled azoles that were tested [FLC, ITC, MET, and TEB] competed for import with ^{3}H-FLC, as indicated by a reduction of the ^{3}H-FLC accumulation to baseline levels. These azoles include medically important azoles (FLC, ITC) as well as agriculturally important azoles (MET, TEB). 1-TRI, a CLT analog, also competed with FLC for import. The results of these competition experiments are consistent with azole import being mediated by facilitated diffusion carrier(s), and suggests that these azoles use the same transport system to enter *M. oryzae.*

Other compounds were also tested for competition against ^{3}H-FLC including azole-like compounds as well as other common antifungals (Fig. 3.4 and Tables 3.2 and 3.3). The non-azole compounds did not compete with ^{3}H-FLC for import into *M. oryzae.* These include medically important antifungals TRB, CFG, AMP, and FENP. The results of these competition experiments indicate transport specificity to certain chemical structures, and suggesting that they do not use the same transporter(s) to enter *M. oryzae.*

The structure of FLC and the compounds tested in this assay are shown in Tables 3.2 and 3.3. FLC has two 5-membered triazole rings containing 3 nitrogen atoms, and a 6-member halogenated benzene ring. Previous screens of moieties important for import in *C. albicans* are consistent with this result. Analyses in *C. albicans, A. fumigatus* and *M. oryzae* suggests that to compete for FLC import, a compound requires a 5-membered ring with two

(imidazole) or three (triazole) nitrogen atoms, in addition to a halogenated 6-membered ring with the halogen in position 1 or 3, but not necessarily both (37, 38). The only exception to this has been the competition of 1-TRI, which has a five membered imidazole ring as well as three 6 membered rings, but is not halogenated.

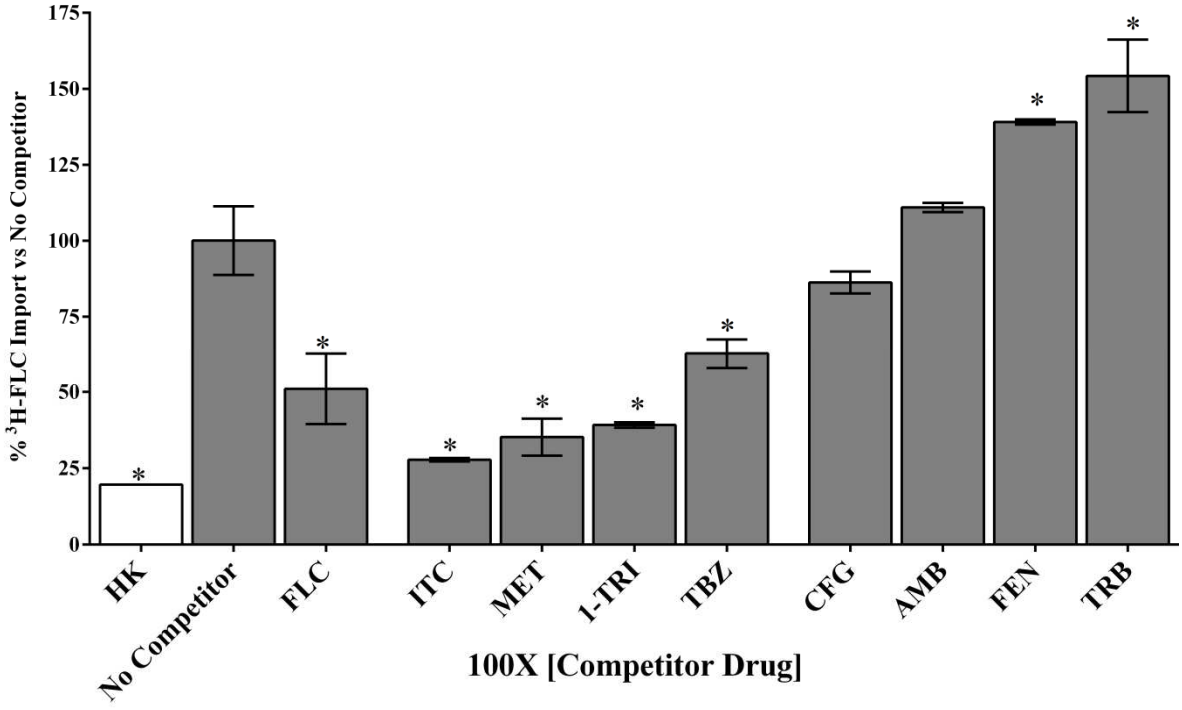


Figure 3.4. Competition for ^{3}H-FLC Import in *M. oryzae.* Compounds were tested for competition at 1.95 μM (100x molar excess to ^{3}H-FLC) during simultaneous treatment with ^{3}H-FLC. ^{3}H-FLC accumulation was measured after 24 hr incubation with competitors. Error bars represent standard deviation of biological triplicates for each condition. Drug abbreviations described in Materials and Methods.

Table 3.2. Compounds That Compete at 100X Molar Excess with FLC for Import in *M. oryzae*

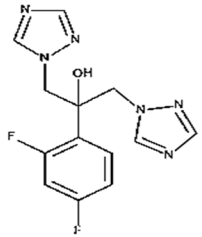
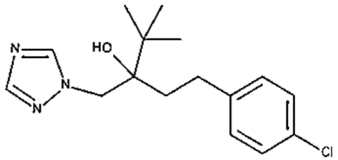
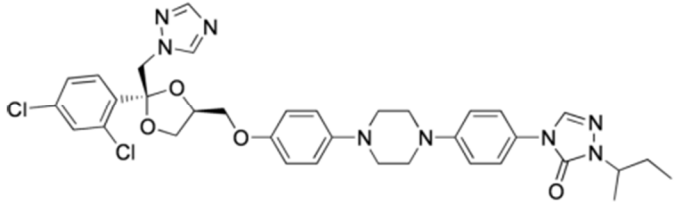
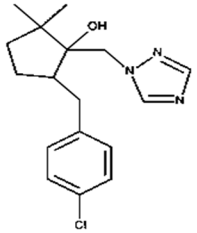
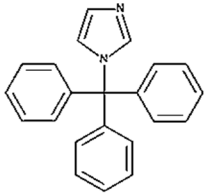
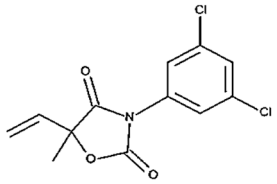
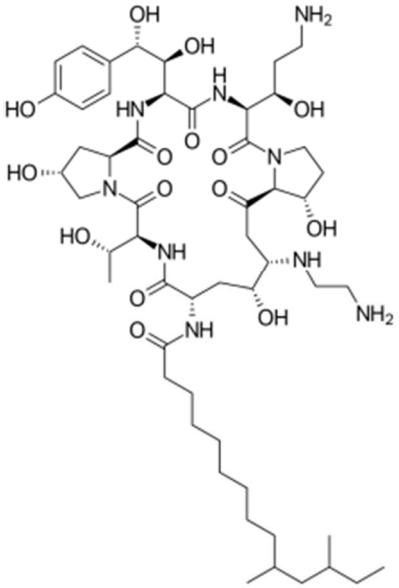
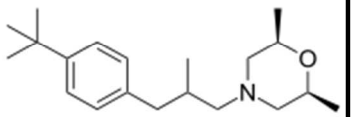
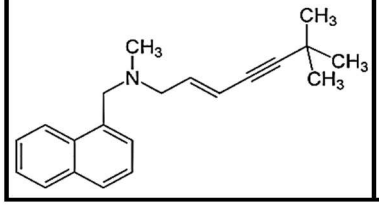
Fluconazole	Tebuconazole
	
Itraconazole	
	
Metconazole	**1-(triphenyl-methyl) imidazole**
	

Table 3.3. Compounds That Do Not Compete at 100X Molar Excess with FLC for Import in *M. oryzae*

Amphotericin B	Caspofungin
	
Fenpropimorph	
	
Terbinafine	
	

Efflux of ^{3}H-FLC from Preloaded Cells

Drug accumulation in a fungal cell can be thought of as the net effect of uptake and efflux mechanisms. The conditions of our ^{3}H-FLC import assay remove the efflux component so that we can focus on drug entry into the cell. Contrarily, Figure 3.5 demonstrates efflux of FLC from *M. oryzae* cells. The cells were preloaded with ^{3}H-FLC at 19.5 nM for 24 hr following the standard import assay protocol and then were washed and resuspended in fresh media with and without glucose so as to see the effect of energy-dependent efflux on intracellular ^{3}H-FLC concentration. The amount of labeled drug associated with the cells was determined at 4 hr, 8 hr and 16 hr after being transferred to fresh media. Efflux was evaluated in both glucose-energized (gray line with squares) and de-energized (glucose starved) (black line with circles) cells.

By 16 hr, most of the ^{3}H-FLC was exported from the cells in both energized and de-energized conditions. The samples incubated in the presence of glucose show faster export of labeled drug, consistent with the idea that membrane efflux pumps require energy and that glucose starvation limited the energy and thus the efflux pump activity. The difference in rate of efflux between energized and de-energized cells was relatively modest, at least when compared to the results of a similar assay performed with *A. fumigatus* (Fig. 3.5A and B).

Clorgyline as a Potential Efflux Inhibitor

Clorgyline is a monoamine oxidase-A inhibitor (MAOI) that has been used for decades as a clinical antidepressant (39, 40). As with many MAOIs, side effects include a hypersensitivity to tyramine-containing foods (especially cheese), and this negative dietary interaction has led to improvements and drug alternatives so that clorgyline use has markedly declined for treatment of depressive illnesses (39, 40).

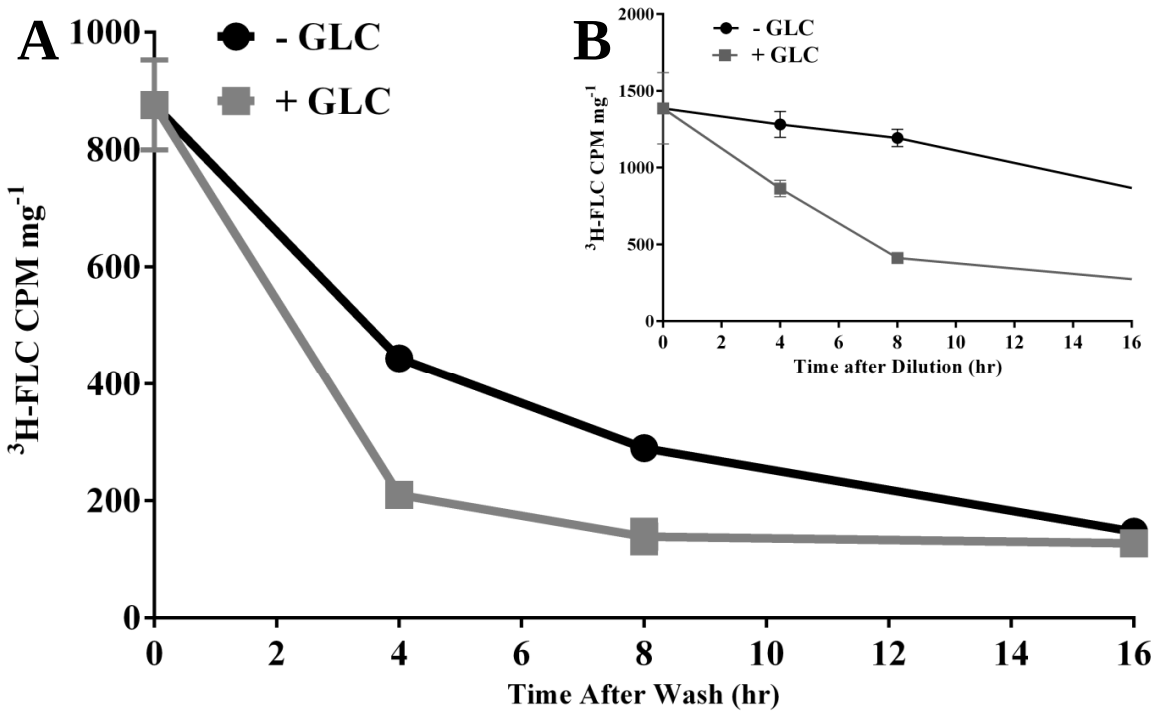


Figure 3.5. Efflux of ^{3}H-FLC from Preloaded Cells. **A.** *M. oryzae* and **B.** *A. fumigatus* cells were preloaded with 19.25 nM ^{3}H-FLC for 24 hr. The cells were washed with YNB and placed in either de-energized (glucose free) (solid black line with circles) or a 2 % glucose-energized (gray line with squares) media for 4, 8 and 16 hr to measure the efflux of ^{3}H-FLC. Error bars represent standard deviation for each condition. Some error bars are hidden by the symbols

However, clorgyline remains in the Library of Pharmacologically Active Compounds (LOPAC), which is a collection of compounds, marketed drugs and pharmaceutically relevant structures annotated with biological activities. These compounds can be purchased pre-solubilized, normalized, easily resupplied, and ready-to-use for studies on repurposing applications (Sigma-Aldrich).

Clorgyline has recently been identified in a screen as an inhibitor of two *C. albicans* ABC efflux pumps, CaCdr1p and CaCdr2p, as well as reversing FLC resistance in *S. cerevisiae* strains expressing ABC transporters (Cannon, Monk). To examine the effect of clorgyline as a possible inhibitor of energy-dependent efflux in *M. oryzae*, we treated

energized (efflux active) cells with either ^{3}H-FLC alone or ^{3}H-FLC with clorgyline and compared the results to ^{3}H-FLC treatment in de-energized (efflux inactive) cells (Fig. 3.6).

Consistent with Figure 3.3A, ^{3}H-FLC uptake was observed in both de-energized and energized cells, with the energized cells showing significantly reduced intracellular ^{3}H-FLC concentration compared to de-energized cells (Fig. 3.6). The reduced azole accumulation in the energized cells is most likely the result of activation of energy-dependent efflux pumps transporting the ^{3}H-FLC out of the cell. However, when the energized, efflux active cells were treated with clorgyline, there was a significantly increased intracellular ^{3}H-FLC accumulation, indicating energy-dependent efflux was at least partially prevented by the addition of clorgyline.

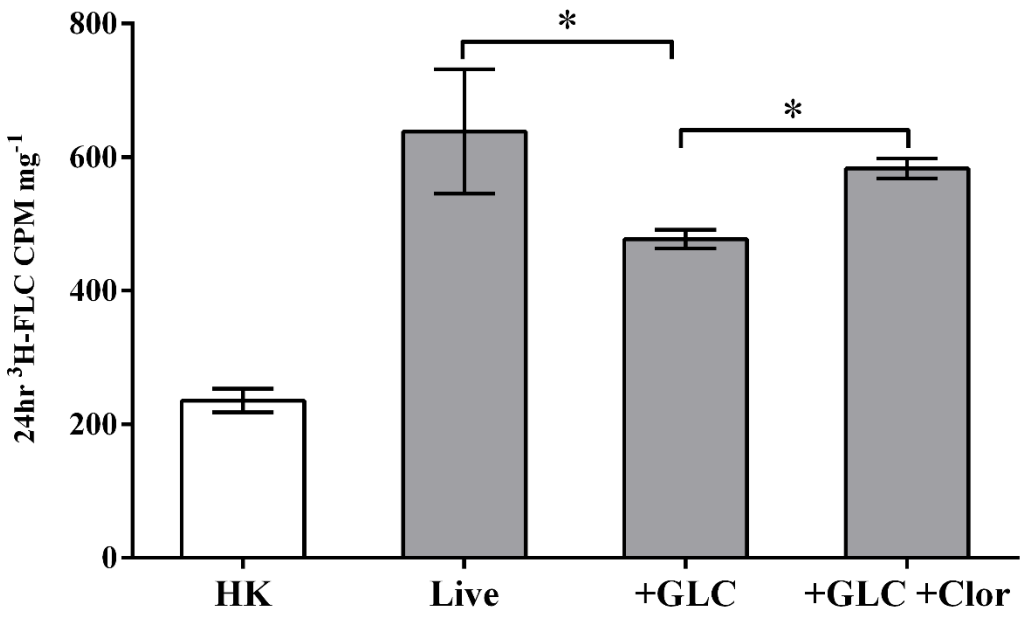


Figure 3.6. Clorgyline as a Potential Efflux Inhibitor. Energized cells (2 % glucose, efflux active) were treated with either ^{3}H-FLC alone (19.25 nM) or ^{3}H-FLC with clorgyline (233 µM) and the results were compared to ^{3}H-FLC treatment in de-energized (glucose-starved, efflux inactive) cells. ^{3}H-FLC accumulation was measured after 20 hr in all conditions.

Discussion

There is a continual emergence of fungicide-resistant pathogens in clinical and agricultural isolates (36). Understanding even the most basic cellular processes and epidemiology of these isolates is imperative to prevent their spread, determine treatment and prevention strategies, optimize future drug design, and to predict future evolution of resistance (8).

Many fungi with intrinsic resistance to antifungal agents already exist in our environment (36). We illustrated this with E test susceptibility testing of *M. oryzae,* which displayed FLC and CFG resistance (Fig. 3.1). The E-tests confirmed that even azole drugs used to treat human fungal pathogens are taken up by the plant pathogen *M. oryzae* as evidence by growth inhibition seen with the medical azoles ITC, KTC, POS and VRC. Resistance to FLC was expected because *M. oryzae* has 2 copies of the azole target CYP51 as discussed previously. However, this may be the first time reporting MICs of medical antifungals on a distinctly plant pathogen. The susceptibility and resistance patterns suggest a common mechanism of action of azoles on all fungal species, including the requirement for entry into the cell.

The resistant fungi have and will continue to emerge opportunistically when antifungal agents with similar fungal drug targets or mechanisms of action are used broadly, whether in agriculture or in human and animal patients. In order to continue with optimal fungal diagnosis, treatment and prevention strategies, it is important that researchers communicate and collaborate across fields of study (clinicians, microbiologists, agricultural and food scientists) to be aware of the epidemiology of wide variety of fungal species. This includes monitoring and sharing patterns of antifungal resistance and becoming familiar with

the current repertoire of antifungals and changing treatment strategies in agriculture and medicine.

In this work, we have begun to analyze potential molecular mechanisms of azole drug resistance in *M. oryzae*, by characterizing azole import into the fungal cell under a variety of environmental conditions as well as azole efflux from the cell using radioactively labeled FLC. Azoles must enter the fungal cell in order to inhibit the intracellular Cyp51 target enzyme. Therefore reduced or modified drug import may help to explain why some pathogenic fungi are more resistant to azoles than others. Our assay can be used to compare drug import in agricultural, medical and other pathogenic fungi

Our experiments thus far have demonstrated that azoles are not passively diffused into the *M. oryzae* cell, nor are they actively transported in an energy-dependent fashion. Instead, azoles most likely enter the cell by facilitated diffusion via a membrane protein carrier that recognizes a specific moiety found in azole drugs. This finding is in agreement with studies on the human pathogenic fungi *C. albicans*, *C. neoformans* and *A. fumigatus* as well as the model yeast *S. cerevisiae* (37, 41).

The labeled FLC does not simply bind to the cell surface as demonstrated by baseline drug accumulation in heat and chemical inactivated cells compared to living cells (Fig 3.2).

Import is not diffusion limited, since the internal drug concentration is much lower than that of the external medium (data not shown). The drug concentrations used in our import analysis was in the nanomolar range. It cannot be ruled out that normal diffusion or non-specific-carrier transport occurs at higher drug concentrations. It is also not known whether the import transporters also act as efflux transporters for azoles or other molecules.

Import of azoles also did not require a proton gradient as no change was observed in uptake over a range of buffered pHs (Fig. 3.3C). There was a trend towards alkaline sensitivity for drug uptake as seen by a decrease in ^{3}H-FLC uptake in samples at pH 7 media. However, a deficiency in cell growth and robustness is observed in *M. oryzae* cells at pH 7, so import at this pH may be affected by other cellular factors directly or indirectly related to pH and proton gradients.

We did however find significant differences in drug accumulation in *M. oryzae* depending on the growth media used as shown in Figure 3.3B. This effect of growth media was not observed in *A. fumigatus*. Based on these import results and a comparison of the components of these three medias, it is difficult to identify a single factor that would cause such a dramatic difference in uptake between CSM and the other two medias. CSM and YNB both contain YNB and AS. CSM and YPD both contain amino acids. The CSM supplemental amino acids or the quantities of amino acids in the CSM supplement possibly account for the differences in uptake. Regardless, it appears that choice of growth media should be tested and strongly taken into consideration with experiments performed with *M. oryzae*.

A comparison of drug import in exponential vs post-exponentially growing cells (Fig. 3.3D) shows a dramatic decrease in drug accumulation in the older cells. This is consistent with major differences in cell activity when comparing exponentially growing cells and stationary phase cells, including changes to transcription, protein translation, modifications, and secretion, membrane maintenance, and other vital cell processes. Exponentially growing cells are considered to be more active and responsive, while stationary phase cells shift to a

period of maintenance and conservation (42). So this could be responsible for the decrease in azole uptake in post-exponential cell growth.

Evidence for a saturable protein carrier is shown with competitive inhibition of ^{3}H-FLC uptake by other azoles (Fig. 3.4). ^{3}H-FLC import was significantly inhibited by simultaneous treatment with an excess of unlabeled azoles. However, other antifungal drugs did not compete for import into the cell. This indicates substrate specificity for moieties found in the azole structure. The structure of competitive inhibitors and drugs that did not compete for import are shown in Tables 3.2 and 3.3 respectively.

Drug uptake by this carrier is energy independent, as seen by drug accumulation measured in de-energized cells. Azoles were imported in both energy replete and glucose-containing media (Fig. 3.3A). However, media that contained glucose showed reduced final drug accumulation levels, presumably due to activation of glucose-dependent efflux pumps.

Our evidence indicates that the efflux of azoles is partly dependent on energy (Fig. 3.5), suggesting distinct transporters for influx and efflux of azoles. The efflux of ^{3}H-FLC occurred much faster in *M. oryzae* compared to *A. fumigatus* in non-energized conditions and so earlier time points are needed for more accurate efflux kinetics in *M. oryzae* (Fig. 3.5A and B). The difference in azole efflux could indicate important differences in either transporter expression or function between the two organisms. Intracellular ^{3}H-FLC measurements at 1 hr and 2 hr time points might have been more appropriate for *M. oryzae*.

Addition of clorgyline, a compound in the LOPAC collection and potential inhibitor of energy-dependent efflux, showed reduced efflux of ^{3}H-FLC from energized *M. oryzae* cells (Fig. 3.6). A more thorough examination of clorgyline as an efflux inhibitor is needed, but our and others' preliminary data with clorgyline suggest it might warrant investigation

into whether clorgyline or similar compounds could be used as combination therapy in either agriculture or clinical practice, to act in synergy with azoles or other drug that might be exported from the cell using energy-dependent efflux transporters.

The ability to increase antifungal drug accumulation in the cell or prevent the rapid efflux of drug makes clorgyline and similar compounds an exciting candidate to be considered for combination therapy to act in synergy with azole treatment of fungi. The notion of considering medically now-outdated drugs for use as agricultural treatments unlocks many new possibilities. Repurposing compounds from the LOPAC collection would shave years off the drug development process as these compounds have previously been well characterized, synthesized, stabilized, near optimized, etc.

In conclusion, a broad-spectrum analysis of drug uptake in the human and animal pathogenic yeasts has included *C. albicans*, *C. neoformans*, *S. cerevisiae*, the plant and animal pathogenic mold *A. fumigatus*, and now the plant pathogenic mold *M. oryzae* (37, 41). While each organism is extremely specialized for their niche environment and has specialized infection mechanisms, we have shown common factors between the organisms such as azole drug uptake by a similar mechanism of facilitated diffusion.

While an antifungal mechanism of action may be very well conserved across a broad-spectrum of pathogens, the drugs are often only tested and considered for a small subset of organisms or diseases. Commonly the reason for drug trial failures or retirement of drugs from treatment circulation is more dependent on the host response rather than the effectiveness of the drug at clearing the pathogen. Our work challenges this short-sighted approach and suggests keeping a broader picture in mind. It might be more practical to reconsider ‘failed’ human treatment strategies, whether due to pharmacologic properties of

absorption, distribution, adherence, etc. in humans, for use in plant fungal pathogens. Likewise, agricultural drugs discarded from production and use because of expense of application etc. could possibly be used in smaller, more affordable dosages in humans and animals.

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CHAPTER 4

FUNCTIONAL AND INDUCIBLE EXPRESSION OF *A. FUMIGATUS* PUTATIVE EFFLUX TRANSPORTERS IN *S. CEREVISIAE*

Chapter Summary

Resistance to commonly used antifungals can develop due to overexpression or increased activity of fungal plasma membrane transporters. The ATP-binding cassette (ABC) superfamily of efflux pumps are well characterized and widely recognized as a cause of antifungal drug treatment failure in many fungal pathogens. Bioinformatics evidence from sequence orthology infers that there is a relatively large number of genes in *A. fumigatus* that encode ABC efflux transporters. However, very few of these transporters have been directly characterized and analyzed for their potential role in drug resistance.

Considering the importance of fungal efflux transporters in the process of drug development, treatment and resistance, the focus of this research has been to analyze *A. fumigatus* genes that may encode ABC efflux proteins and better characterize their role as transporters. We have created recombinant *Saccharomyces cerevisiae* strains with inducible overexpression of individual transporters from *A. fumigatus*. We used a number of techniques to directly measure expression, localization, efflux activity, substrate specificity, and potential role in drug resistance of the *A. fumigatus* heterologously-expressed proteins.

Introduction

Drug Efflux is a Common Resistance Mechanism in Fungi

The filamentous fungal pathogen *Aspergillus fumigatus* is the most common cause of invasive mold infection in humans and is associated with an alarmingly high mortality rate (1). Currently available antifungal drugs to treat invasive aspergillosis are very limited, either due to issues with safety and toxicity to the host, or because they have narrow modes of action leading to the potential for the development of drug resistance (2-4). In addition, filamentous fungi are intrinsically resistant to some antifungals that are commonly used to treat other types of fungal infections (5, 6). In some cases the intrinsic mechanism of resistance can be explained based on our biological understanding of the organism. For many cases the basis of intrinsic resistance is unknown. Both intrinsic and acquired resistance in *A. fumigatus* to the current classes of antifungal chemotherapeutics is a growing concern (5-8).

In many well-studied fungal pathogens, multidrug resistance is thought to be caused by overexpression or increased activity of fungal plasma membrane transporters (9-12). Commonly, the transporters belong to the ATP Binding Cassette (ABC) superfamily of proteins that carry a broad range of substrates including, but not limited to, antifungal drugs across biological membranes (12-17). The best characterized fungal ABC transporter is that encoded by the *PDR5* gene in *S. cerevisiae* (18). Overexpression of *PDR5* leads to resistance to a number of structurally unrelated drugs, while deletion of *PDR*5 leads to hypersensitivity to these drugs (19). Our analysis of radioactively labeled azole transport in Chapters 1 and 2 revealed a measurable, energy-dependent efflux of fluconazole in the filamentous fungal

pathogens *A. fumigatus* and *M. oryzae*. Compared to yeast species such as *Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans, Candida glabrata,* and *Candida krusei*, filamentous fungi contain an unusually high number of genes encoding predicted ABC transporters (14, 15, 20). However, very few of these genes have been directly demonstrated to encode functional transporters and analyzed for their potential role in drug resistance (14, 20).

While the number of transporter genes within genomes is variable and the gene sequences between species can be extremely diverse, there are several characteristic ABC transporter motifs that are conserved across organisms. The hallmark structures of ABC transporters include nucleotide-binding domains (NBD) that bind ATP, and transmembrane domains (TMD) that are thought to play a role in substrate recognition and specificity (14, 20). The number, arrangement and topology of these domains can vary within and between organisms. The extreme multiplicity of fungal ABC transporters allows for a diversity of physiological functions that go beyond membrane transport and drug resistance (14, 19-23).

The focus of this research is to directly characterize *A. fumigatus* genes that may encode ABC efflux transport proteins that could be involved in antifungal drug transport and determine their functionality, substrate specificity and potential role in drug resistance. Identifying *A. fumigatus* transporters that have substrate specificity for common antifungals could potentially lead to the development of compounds to be used as efflux inhibitors. The inhibition of drug efflux pumps is a potential strategy to overcome antifungal resistance, especially when given as co-therapy with fungal-specific drug treatments (13, 24-28).

In addition, determining which *A. fumigatus* putative ABC transporter genes are transcribed and translated into functional efflux pumps will improve gene annotation databases and will help researchers gain more insight into fungal evolutionary relationships as well as provide a better understanding of intrinsic resistance mechanisms.

There are multiple challenges to a study of this nature including the presence of a relatively high number of encoded transporters in the *A. fumigatus* genome and the fact that *A. fumigatus* is not typically a model organism. The presence of multiple introns and large transporter gene size (many ~4500bp) in *A. fumigatus* adds further complications to molecular-based experiments (29, 30). In addition, the filamentous and non-homogenous growth of molds makes drug susceptibility testing hard to standardize and the analysis of growth characteristics difficult (31).

For a more straightforward approach to studying the function of individual *A. fumigatus* genes, we have created recombinant *Saccharomyces cerevisiae* strains with inducible overexpression of individual transporters from *A. fumigatus*. The individual *A. fumigatus* transporter genes were cloned and expressed individually in several *S. cerevisiae* host strains (Table 4.1) including lab-created strains in which the highly active endogenous ABC transporter (Sc*PDR5*) was deleted. To screen for additional phenotypes, we expressed some *A. fumigatus* genes in the *S. cerevisiae* strain ScADKan, which has seven ABC transporter genes deleted. The use of these particular recipient strains allowed the heterologously-expressed pump activity to be assayed in a background of depleted endogenous pumps.

We used a number of techniques to directly measure expression and localization, efflux activity, substrate specificity, and effect on drug resistance of the *A. fumigatus* heterologously-expressed proteins. These techniques included fluorescent substrate efflux assays, E-test drug susceptibility testing and broth microdilution drug susceptibility testing. We also used GFP tagging and fluorescent microscopy of one specific transporter that showed a particularly strong efflux-active phenotype in order to visualize transporter expression and localization.

The controlled over-expression of *A. fumigatus* genes in an *S. cerevisiae* background deficient for its major endogenous transporter allows the functional analysis of transport activity of Pdr5-like putative ABC transporters from an important human pathogen. Drug resistance was correlated with energy-dependent drug efflux in *S. cerevisiae* strains expressing a number of *A. fumigatus* transporters. Strains expressing the *A. fumigatus* transporters *AF3-10, 14, 15* and *17* demonstrated varying degrees of resistance to an assortment of drugs that are thought to be efflux pump substrates. Complementarily, these strains also showed a variable range of inducible, energy-dependent transporter activity as seen by the direct measurement of efflux of the fluorescent dye rhodamine 6G from the cells. Some of the AF transporters showed an impairment in activity in response to treatment with the efflux inhibitors FK506, farnesol and clorgyline.

Materials and Methods

A. fumigatus Efflux of ^{3}H-FLC

Mycelial masses of *A. fumigatus* strain AF293 were preloaded with ^{3}H-FLC by treating them at 19.5 nM for 24 hr per the standard radioactive import assay as described in

Chapter 2. The cells were then washed and resuspended in a 10 mL of YNB media, and intracellular ^{3}H-FLC was evaluated over time in both glucose-energized (YNB media with 2 % glucose) and de-energized (glucose starved) cells. Radioactivity was quantified by liquid scintillation counting and calculated as CPM per mg of *A. fumigatus* biomass as a function of time at 4, 8 and 24 hr time points.

Efflux Inhibition by Clorgyline

To test the effect of clorgyline as a possible inhibitor of energy-dependent efflux in *A. fumigatus*, we treated energized (2 % glucose), efflux active cells with either ^{3}H-FLC alone (19.25 nM) or ^{3}H-FLC with clorgyline [N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride] (233 µM) and compared the results to ^{3}H-FLC treatment in de-energized (glucose-starved), efflux inactive cells. Radioactivity was quantified by liquid scintillation counting after 20 hr of treatment. Intracellular ^{3}H-FLC accumulation in *A. fumigatus* was calculated as CPM per mg fungal biomass.

Strains and Media for Heterologous Gene Expression

The strains used in this study, and their genotypes, are listed in Table 4.1. Homologous recombination was performed in the wild-type *S. cerevisiae* strain W303 (29, 32). Subsequent plasmid recipient strains used for functional analysis were ABC transporter mutants with either *PDR5* deleted or with seven ABC transporters deleted.

The two recipient strains with *PDR5* deleted were derived from wild-type *S. cerevisiae* strains BY4741 or W303. We deleted the *S. cerevisiae* Pdr5p plasma membrane ABC transporter using a Kanamycin deletion cassette providing selectable Kanamycin resistance in these strains (33, 34). Oligos for the deletion cassette are listed in Table 4.2.

Both the BY4741 *PDR5* deletion strain (BΔ*pdr5)* and the W303 *PDR5* deletion strain (WΔ*pdr5)* were confirmed to have the kanamycin resistance gene inserted into the *PDR5* genomic locus. Both strains also showed a multi-drug susceptibility after deletion of the major efflux transporter (Fig. 4.1). (WΔ*pdr5)* grows as red colonies due to an *ade2*-mutation.

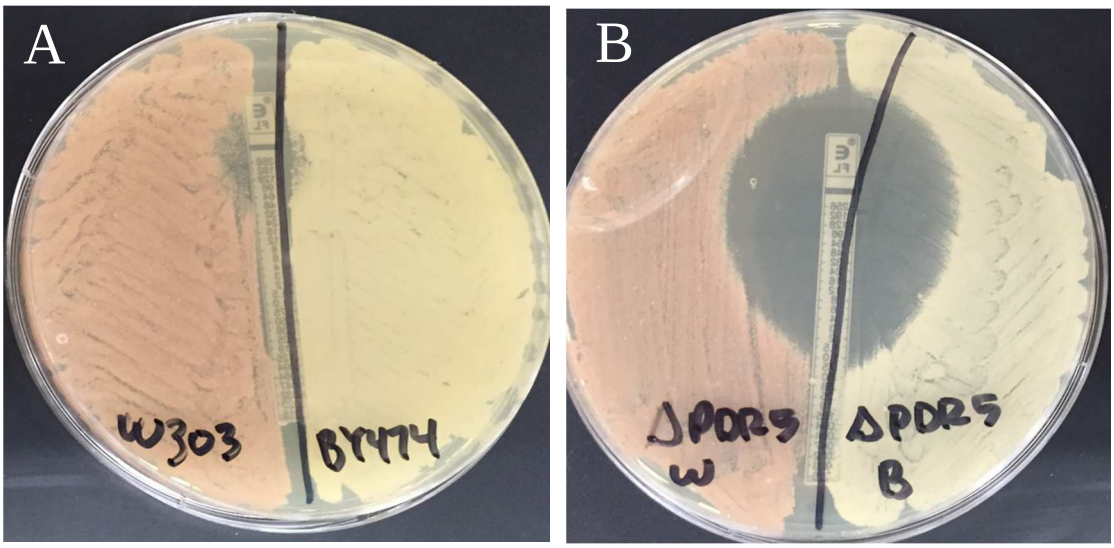

Figure 4.1. *PDR5* Deletion in Recipient *S. cerevisiae* Strains Causes Drug Susceptibility. **A)** Parent *S. cerevisiae* strains W303 and BY4741 were compared to their *PDR5* deletion derivative strains **B)** WΔ*pdr5* and BΔ*pdr5* for fluconazole susceptibility. Deletion of *PDR5* causes drug susceptibility.

The *S. cerevisiae* strain ScADKan, which has seven ABC transporter genes deleted (*YOR1, SNQ2, PDR10, PDR11, YCF1, PDR5,* and *PDR15*) was used with the idea that a strain with the fewest possible endogenous pumps would better elucidate efflux phenotypes of the *A. fumigatus* transporters that may have been obscured by endogenous pump activity. However, the ScADKan strain was only used for a small number of drug susceptibility experiments because of its extreme slow growth.

Table 4.1. Strains Used in This Study

Strain name	Description	Genotype
AF293	Wild-type, sequenced *A. fumigatus* strain from which RNA was isolated.	Wild-type
B +p0	Wild-type, sequenced *S. cerevisiae* strain used as wild-type control for BY4741-dervied strains. Contains empty pYES plasmid for uracil selection.	Wild-type *ura3-* + p (empty) with Ura^+
B∆*pdr5* +p0	BY4741 strain with *PDR5* replaced by kanamycin deletion cassette. Contains empty pYES plasmid for uracil selection	*ura3-*, *pdr5::Kan*^{r} + p (empty) with Ura^+
B∆*pdr5* +p*AFX*	B∆*pdr*5 background expressing a single AF gene *AF3-10*, *AF14*, *AF15* or *AF17* in pYES GAL-inducible plasmid	*ura3-*, *pdr5::Kan*^{r} *+ p (AF3-10, 14, 15, 17)* with Ura^+
B∆*pdr5* +p*14*GFP	B∆*pdr*5 background expressing *AF14* with GFP tag in pYES GAL-inducible plasmid	*ura3-*, *pdr5::Kan*^{r} + p (*14*GFP) with Ura^+
W303	Wild-type, sequenced strain used for homologous recombination	Wild-type *ade2- ura3-*
W +p0	Wild-type with empty pYES plasmid, used as wild-type control for W303 derived strains. Contains empty pYES plasmid for uracil selection.	Wild-type *ade2- ura3-*, + p (empty) with Ura^+

Cont. Strain name	Description	Genotype
W∆*pdr5*	W303 strain with *PDR5* replaced with kanamycin deletion cassette. Contains empty pYES plasmid for uracil selection.	*ade2-*, *ura3-*, *pdr5::Kan*^{r} + p (empty) with Ura^{+}
W∆*pdr5* +p*AFX*	W∆*pdr*5 expressing a single AF gene *AF14* or *AF17*in pYES GAL-inducible plasmid	*ade2-*, *ura3-*, *pdr5::Kan*^{r} + p (*AF14* or *AF17)* with Ura^{+}
W∆*pdr5* +p*14*GFP	Expressing *AF14* with GFP tag in pYES GAL-inducible plasmid	*ade2- ura3-*, *pdr5::Kan*^{r} + p (*14*GFP) with Ura^{+}
ScADKan +p0	AD1-8u derivative (S288C derived mutant strain with 7 transporters and their regulators deleted) and leu^{-}. Contains empty pYES plasmid for uracil selection.	*leu2::Kan*^{r} *MATα, pdr1-3, ΔURA3, yor1Δ::hisG, snq2Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG, pdr5Δ::hisG* + p (empty) with Ura^{+}
ScADKan +p*AFX*	AD1-8u derivative (S288C derived mutant strain with transporters and their regulators deleted) and leu^{-} Expressing a single AF gene *AF14* or *AF17* in pYES GAL- inducible plasmid	*leu2::Kan*^{r} *MATα, pdr1-3, ΔURA3, yor1Δ::hisG, snq2Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG, pdr5Δ::hisG* + p (*AF14* or *AF17*) with Ura^{+}

Table 4.2. Oligos Used for *PDR5* Deletion with Kanamycin Deletion Cassette

ScPDR5::KanX F	GTCTCCGCGGAACTCTTCTACGCCGTGGTACGATATCTGTCG GATCCCCGGGTTAATTAA
ScPDR5::KanX R	CCTAGGAATAAAATTCTCGGAATTCTTTCGGACATTGAACGA ATTCGAGCTCGTTTAAAC

A. fumigatus RNA Isolation and cDNA Synthesis

A. fumigatus hyphae were harvested as mycelial masses from overnight liquid cultures of *A. fumigatus* strain AF293 grown at 37° C in CSM complete media with 2 % glucose. Mycelial masses were loaded onto a Whatman paper filter attached to a vacuum and vacuumed to dryness. The mycelial mass was divided into approximately 60 mg portions and then each portion was transferred to a 2 mL microcentrifuge tube and stored at -80° C for later use. RNA was extracted from the frozen fungal masses using the Qiagen RNeasy Mini Kit for plant, tissues and filamentous fungi. The isolated RNA was quantitated by NanoDrop and used for cDNA construction using Thermo Scientific Superscript III cDNA synthesis kit. The cDNA was synthesized using a combination of random hexamer and polyDT oligos.

PYES Plasmid Construction

pYES2 is a 5.9 kb vector designed for inducible expression of recombinant proteins in *S. cerevisiae*. Transformants are selected by uracil prototrophy. The vector contains the yeast *GAL1* promoter for high level, inducible protein expression in yeast by galactose and a *CYC1* transcriptional terminator. The plasmid also contains an Ampicillin resistance gene for selection in *E. coli*.

A. fumigatus putative transporter genes of interest are listed in Table 4.3 and are designated in this paper as *AF3* through *AF10, AF14, AF15* and *AF17* (See Table 4.3 for name designations). The genes were PCR amplified from the cDNA using Phusion polymerase using oligos shown in Table 4.3.

Table 4.3. *A. fumigatus* Gene Names and Oligos Used for cDNA Amplification

Shorthand Name	Systematic Name	Forward	Reverse
AF3	Afu2g14020	AAGCGGCCGCTGACTCAGTTGGTGTTCACC	AATCTAGACCCAGTACAATCGCAACAAC
AF4	Afu6g05080	AAGCGGCCGCTCAACAAGCCAAATAGTCTT	AATCTAGAAATGAAAGGAGAGAATTTGA
AF5	Afu5g00790	AAGCGGCCGCCACCTCGGTTGATCGACCCT	AATCTAGAATTCTATCCATGAGTGCGAA
AF6	Afu2g15130	AAGCGGCCGCTTGGTGTGAGTCGTGCAGCG	AATCTAGAATAAGTCAGGTCCGTTCCCC
AF7	Afu6g08020	AAGCGGCCGCCTGATCTGCAGCCTGCACCA	AATCTAGATTATGGTTCCGGTAGCACTA
AF8	Afu4g01050	AAGCGGCCGCGGTTAAGCTGATCGTGCAGA	AATCTAGATCTTGTCTATATAAGGTTGT
AF9	Afu3g01400	AAGCGGCCGCAGGTTGGTGTCTGCTGAGCC	AATCTAGAAGCGAGGAACCCAGAGAATA
AF10	Afu5g09460	AAGCGGCCGCCCTTGGTGTCGACTGCATCC	AATCTAGAGTAGACGCCATATTAAAACG
AF14	Afu1g14330	AAGCGGCCGCGTCTCACTCAATCGGCCATC	AATCTAGAAAGGGAAGGAGGTCAGGCTG
AF15	Afu6g07280	AAGCGGCCGCTCTTCGCGTACAAATAAGTC	AATCTAGAGGGTAAAGTACCCGAAACAT
AF17	Afu1g17440	AAGCGGCCGCAAGCCAGAACCCACGCAAAC	AATCTAGAGTCCCAATGAAAAACAACGC

After successful cDNA amplification, genes were again amplified with oligos designed for homologous recombination into the pYES2 plasmid (oligos are listed in Table 4.4). The forward oligos contained 40 bp homology with the *GAL1* promoter on the pYES2 plasmid and the reverse oligos contained 40 bp homology to the *CYC1* terminator region on the pYES2 plasmid. All genes amplified as expected from *A. fumigatus* genomic DNA. However we were unable to amplify some genes from cDNA.

Table 4.4. Oligos Used for Homologous Recombination Into pYES Plasmid

Gene	Forward	Reverse
AF3	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCTGACTCAGTTGG TGTTCACC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATCCCAGTACAATCGCAACAAC
AF4	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCTCAACAAGCCA AATAGTCTT	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATAATGAAAGGAGAGAATTTGA
AF5	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCCACCTCGGTTGA TCGACCCT	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATATTCTATCCATGAGTGCGAA
AF6	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCTTGGTGTGAGTC GTGCAGCG	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATATAAGTCAGGTCCGTTCCCC
AF7	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCCTGATCTGCAGC CTGCACCA	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATTTATGGTTCCGGTAGCACTA
AF8	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCGGTTAAGCTGAT CGTGCAGA	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATTCTTGTCTATATAAGGTTGT
AF9	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCAGGTTGGTGTCT GCTGAGCC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATAGCGAGGAACCCAGAGAATA
AF10	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCCCTTGGTGTCGA CTGCATCC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATGTAGACGCCATATTAAAACG
AF14	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCGTCTCACTCAAT CGGCCATC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATAAGGGAAGGAGGTCAGGCTG
AF15	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCTCTTCGCGTACA AATAAGTC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATGGGTAAAGTACCCGAAACAT
AF17	GTTAATATACCTCTATACTTTAACGTC AAGGAGAAAAAACCAAGCCAGAACC CACGCAAAC	GGGCGTGAATGTAAGCGTGACATAACTAA TTACATGATGTCCCAATGAAAAACAACGC

The pYES2 plasmid was cut with *Hin*dIII and *Pvu*II and dephosphorylated with alkaline phosphatase (New England Biolabs, Beverly, Mass.). Homologous recombination

of the PCR products and the linearized plasmid was done in *S. cerevisiae* wild-type strain W303 using the standard Saccharomyces LiAc Transformation Protocol (32).

The pYES2 + *AF(*transporter) plasmids were isolated from ura^+ colonies and transformed into *E. coli* using the DH5α heat shock protocol. *E. coli* transformants were selected on LB plates containing 50 to 100 μg/ml ampicillin. Plasmids were isolated from amp^+ selected colonies and checked for plasmids carrying the correct size insert as determined by restriction digestion and agarose gel electrophoresis. The correct orientation and insertion of the *A. fumigatus* genes in the plasmid was confirmed by sequencing.

The pYES2 + *AF(*transporter) plasmids were transformed into three different *S. cerevisiae* ABC transporter mutant strains (described in Table 4.1) for analysis. Colonies were selected from CSM–ura plates. All *S. cerevisiae* strains used in functional analysis assays contained the pYES2 plasmid (+/- gene insert) conferring ura^+ prototrophy and so all strains were grown on CSM –ura with 2 % glucose or 2 % galactose on agar plates or in liquid culture at 30° C.

Functional Expression Analysis of the Recombinant *A. fumigatus* Genes

Functional expression of the recombinant genes was analyzed by E-tests, MICs, and rhodamine 6G efflux assay. *A. fumigatus* genes were induced and overexpressed under the control of the *GAL1* promoter in media containing 2 % galactose.

Minimum Inhibitory Concentration Drug Susceptibility Test

The MICs of compounds for recombinant *S. cerevisiae* strains were determined in accordance with the M27-A3 broth microdilution method, except the method was modified by using a CSM-ura based medium because *S. cerevisiae* strains do not grow in the RPMI

medium used in the CLSI method. Briefly, *S. cerevisiae* strains were inoculated in microtiter plates in the presence of a series of 2-fold dilutions of a variety of compounds and antifungal drugs. The plates were incubated in CSM-ura + 2 % glucose (plasmid expression repressed) at 30° C for 48 hours or CSM-ura + 2 % galactose (plasmid expression induced) at 30° C for 72 hours to account for the slower growth in galactose. The MIC values between strains were compared in each media. The cell growth at each drug concentration was read at OD_{600} with a microplate reader (Bio-Tek). The MIC_{80} was determined to be the drug concentration at which 80 % of cells were inhibited compared to the growth found for a no-drug control.

Drugs used for MIC testing include amphotericin B (AMB), benomyl (BEN), chloramphenicol (CHL), clotrimazole (CLT), cycloheximide (CH), doxycycline (DOX), fenpropimorph (FEN), fluconazole (FLC), hygromycin B (HYB), itraconazole (ITC), ketoconazole (KTC), metconazole (MET), miconazole (MIC), nystatin (NYT), posaconazole (POS), prothioconazole (PRO), reserpine (RES), rhodamine 6G (R-6G), tebuconazole (TBZ), terbinafine (TRB), voriconazole (VRC) and 4-nitroquinolone (4-NQ),

Rhodamine 6G Efflux Assay

A. fumigatus ABC efflux transporters were analyzed for potential functionality using a microtiter assay of the fluorescent dye rhodamine 6G (R-6G), which measured R-6G efflux into the supernatant over time.

Yeast cells of *AF* gene-expressing strains were each grown in 50 mL CSM-ura with galactose to an OD_{600} 0.6. The cells were harvested by centrifugation at 5000 g for 10 min and the pellets were washed twice with 5 mL of phosphate buffered saline (PBS) and then resuspended in 5 mL of glucose-free PBS buffer.

A stock solution of R-6G was prepared by dissolving the dye in 100 % ethanol at a concentration of 10 mM. To load the cells with dye for the efflux assay, a final concentration of 10 µM of R-6G (5 µl in 5 ml) was added to the cell suspension and incubated at 35° C in a reciprocating shaker for 1 hr. After the 1 hr pre-loading, excess dye was removed by washing in PBS and resuspended in 5 ml PBS with 2 % glucose to activate ATP-driven efflux or without glucose to use as a baseline comparison. At each time point, 400 uL samples were withdrawn and centrifuged at 9000 g for 1 min to pellet the cells. The supernatants (200 µL) were collected and pipetted into a black microtiter plate. The fluorescence was measured at 485/520 nm excite/emission in the Synergy2, Biotek microplate reader. The time points included time 0 immediately after the dye-loaded cells were resuspended in media, as well as after 10, 20, 40, and 60 min of incubation.

For R-6G efflux assays with a competitor or inhibitor, the samples were treated with R-6G as per above and allowed to incubate for 30 minutes. The competitor or inhibitor was then added to the sample and allowed to co-incubate with the R-6G for another 30 minutes. After the pre-loading and co-incubation, the samples were washed as above and resuspended in media with the competitor or inhibitor. Samples were treated with FK506 at an approximate 1:2 ratio of drug to R-6G at 5 µM FK506. Competition with FLC, as well as inhibition with farnesol and clorgyline was performed at a 50:1 ratio of drug to R-6G at 500 µM FLC, FAR, or CLOR.

Statistical Analysis

Error bars represent standard deviation of the means of biological triplicates for each condition. Differences between sets of samples were evaluated by an unpaired two tailed

Student's t test. A P value of < 0.05 was considered significant.

GFP Tagging

The *AF14* gene was tagged by homologous recombination of the *AF14* gene insert into a pYES2 plasmid containing a green fluorescent protein (GFP). The GFP-containing plasmid was provided by Dr. Alex Idnurm (formerly UMKC, Missouri). The *AF14* gene was amplified by a forward primer that had a 40-bp overlap with the *GAL1* promoter and 20 nucleotides of the *AF14* gene *and* a reverse primer with 40 nucleotides of the GFP gene sequence and 20 nucleotides of the *AF14* gene with the stop codon removed. The GFP-containing plasmid was digested by *Xba*I to linearize the plasmid at the MCS and treated with alkaline phosphatase. The amplified *A. fumigatus* gene insert was cloned into the pYES plasmid by homologous recombination in *S. cerevisiae* to generate pYES-*AF14*-GFP.

Transformant colonies were selected from –ura media and correct insertion of the *AF* gene insert was confirmed by restriction enzyme digestion and agarose gel electrophoresis to check its size as well as PCR amplification of the region between the *GAL* promoter and the *CYC1* terminator. Once the plasmid and insert size were confirmed to be correct, cells expressing the GFP plasmid were analyzed by fluorescent microscopy.

Microscopy

To test the expression and surface localization of the heterologous proteins, yeast cells were grown in CSM–ura with 2 % galactose. After a brief wash with water, yeast cells were heat fixed to a microscope slide and the GFP-tagged AF14 fusion protein was analyzed using fluorescent microscopy by the University of Missouri, Kansas City School of Dentistry Confocal Core.

E-test Drug Susceptibility Testing

E-test strips (bioMérieux, United States) were used to determine the strain MIC to FLC and confirm that the functionality of the transporter had not been disrupted by the addition of the GFP fusion. MICs of W-Δ*pdr*5 and BY-Δ*pdr*5 strains carrying an empty pYES plasmid were compared to W-Δ*pdr*5 and BY-Δ*pdr*5 strains expressing the pYES plasmid containing the *AF14*+GFP fusion. One side of the plastic E-test strip is calibrated with MIC values of the drug in µg/ml. The drug gradient on the strip covers 15 two-fold dilutions.

Overnight cultures of the strains were grown in CSM-ura + 2 % galactose at 30° C shaking and diluted to an OD_{600} of 0.1. CSM-ura agar plates with 2 % galactose were inoculated with the diluted cultures using a cotton swab spread 3 ways over the plate and allowed to dry. A single E-test strip was placed on each inoculated plate and kept at 30° C for 48 hours with daily monitoring. The MIC was determined based on the drug concentration on the E-test strip in which the zone of inhibition, or ellipse of non-growth, occurred.

Results

Azole Efflux from *A. fumigatus*

^{3}H-FLC efflux is energy-dependent. Figure 4.2A shows efflux of ^{3}H-FLC from *A. fumigatus* cells that were preloaded with ^{3}H-FLC at 19.5 nM for 24 hr as previously shown in Chapter 2. Efflux was measured over time in both glucose-energized and de-energized (glucose starved) cells. The samples incubated in the presence of glucose [gray line with squares] show dramatically faster export of labeled drug compared to the glucose starved

[black line with circles] cells (Fig. 4.2A), suggesting an energy-dependent efflux mechanism. This is consistent with the action of ABC transporters that have been characterized in many fungal species to actively transport azoles out of the fungal cell and thus may be a mechanism of drug resistance. Since ABC transporters require energy, the glucose-depleted conditions limit the efflux pump activity as evident by the dramatically slower efflux rate. By 24 hr, most of the ^{3}H-FLC was exported from the energized samples, while a measureable amount ^{3}H-FLC remained the de-energized samples (Fig 4.2A).

Clorgyline inhibits ^{3}H-FLC efflux. Clorgyline is a monoamine oxidase-A inhibitor (MAOI) that has been used for decades as a clinical antidepressant in humans (35, 36). Clorgyline has recently been identified in a screen as an inhibitor of two *C. albicans* ABC efflux pumps, CaCdr1p and CaCdr2p, as well as reversing FLC resistance in *S. cerevisiae* strains expressing ABC transporters (27). We showed in Chapter 3 that clorgyline acts to inhibit energy-dependent efflux of FLC in *M. oryzae* as well, indicating that the mechanism of clorgyline transport inhibition is broadly conserved. To examine the effect of clorgyline as a possible inhibitor of energy-dependent efflux in *A. fumigatus*, we treated energized, efflux-active cells with either ^{3}H-FLC alone [+^{3}H-FLC +GLC] or ^{3}H-FLC with clorgyline [^{3}H-FLC +GLC +CLOR]. We compared the results to ^{3}H-FLC treatment in de-energized, efflux-inactive cells with and without clorgyline [+^{3}H-FLC -GLC] and [+^{3}H-FLC –GLC +CLOR], as well as heat inactivated background control cells, [HK] (Fig. 4.2B).

After 20 hours of incubation, ^{3}H-FLC uptake was observed in both de-energized and energized cells, with the energized cells showing significantly reduced intracellular ^{3}H-FLC concentration compared to de-energized cells (Fig 4.2B -GLC vs +GLC samples). The

reduced azole accumulation in the energized cells is most likely the result of activation of energy-dependent efflux pumps transporting the ^{3}H-FLC out of the cell. However, when the energized, efflux-active cells were treated with clorgyline [+^{3}H-FLC +GLC +CLOR], there was a significantly increased intracellular ^{3}H-FLC accumulation, indicating energy-dependent efflux was at least partially prevented by the addition of clorgyline. Clorgyline had no effect on ^{3}H-FLC accumulation in efflux-inactive cells [+^{3}H-FLC –GLC +CLOR]. This effect in *A. fumigatus* contributes to the idea that clorgyline may inhibit energy-dependent ABC transporters in a diversity of fungi and is an exciting prospect for use in drug combination therapy with other antifungals that may normally be transported out of the fungal cell by ABC transporters.

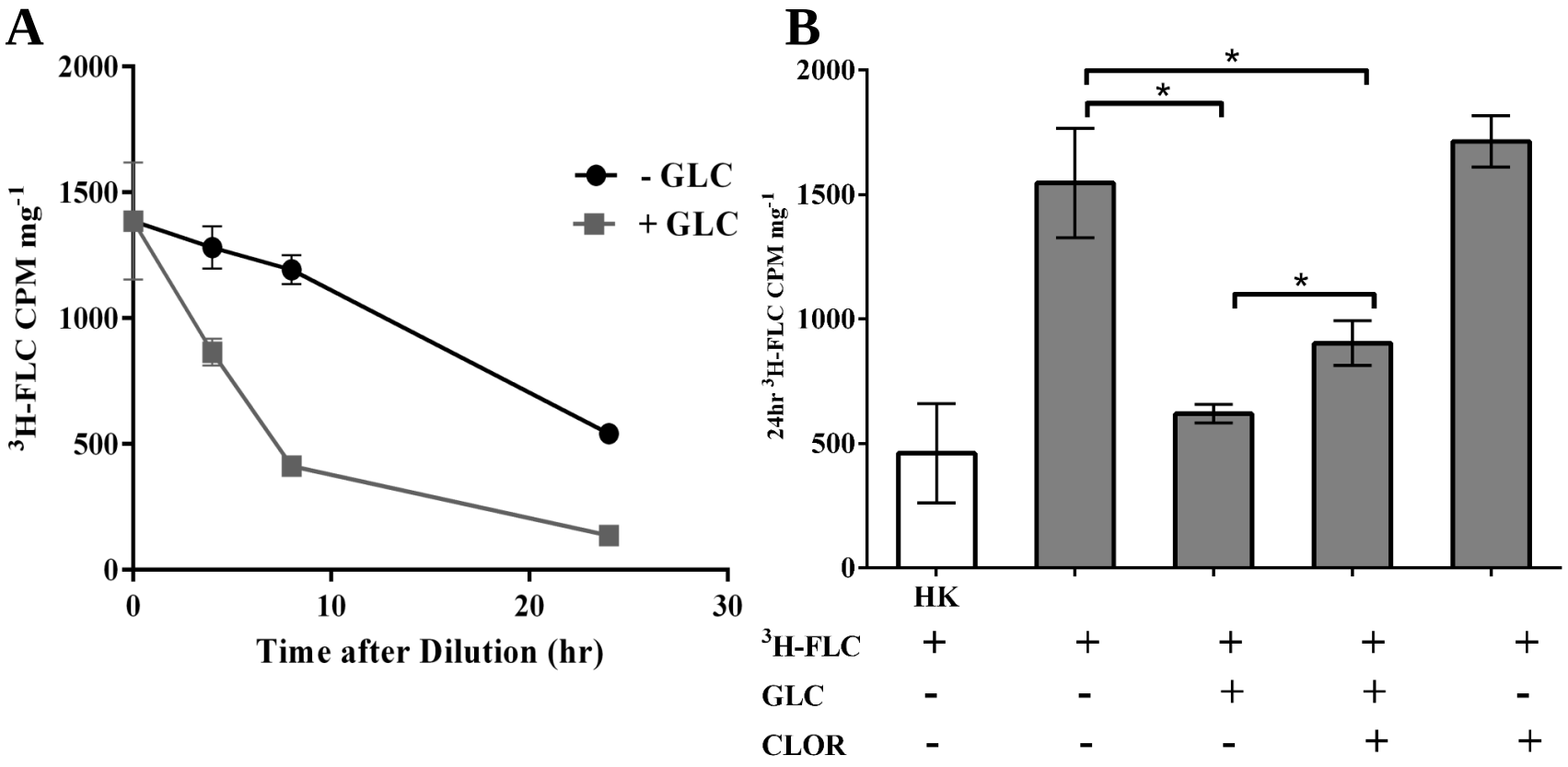

Figure 4.2. Energy Dependent Azole Efflux and Potential for Efflux Inhibition with Clorgyline. A) *A. fumigatus* showed energy-dependent azole efflux as intracellular ^{3}H-FLC concentration was measured over time in energized [gray squares] and de-energized [black circles] samples. **B)** Intracellular ^{3}H-FLC concentration was measured in cells that were treated with; ^{3}H-FLC alone (19.25 nM), ^{3}H-FLC with 2 % glucose (efflux active), ^{3}H-FLC with 2 % glucose and clorgyline (233 µM), and with ^{3}H-FLC with clorgyline. Heat killed [HK] represents the non-specific, background level ^{3}H-FLC accumulation. ^{3}H-FLC accumulation was measured after 20 hr in all conditions. Error bars represent standard deviation of biological triplicates for each condition.

Expression of *A. fumigatus* Putative Transporter Genes in *S. cerevisiae*

S. cerevisiae is unable to splice introns from *A. fumigatus* genomic DNA. The *A. fumigatus* genome contains multiple introns throughout many ORFs, which is in contrast to the *S. cerevisiae* genome that has relatively few introns (37-40). We first attempted to amplify genes from *A. fumigatus* genomic DNA to be used for plasmid expression in *S. cerevisiae* in the hopes that *S. cerevisiae* splicing machinery could remove the introns from the *A. fumigatus* genes. However, after isolating RNA from the *S. cerevisiae* cells and synthesizing cDNA, it was clear that the introns were still present in the mRNA for the *A. fumigatus* genes as confirmed by PCR amplification using intron-specific oligos (Table 4.5 and Figure 4.3). Since the *S. cerevisiae* splicing machinery was not capable of removing the multiple introns from *A. fumigatus* genes, cDNA was synthesized from RNA isolated from the wild-type, sequenced *A. fumigatus* strain AF293. Since high basal efflux pump expression is more often the cause of resistance as compared to the pumps that are induced (41-43), RNA was isolated from *A. fumigatus* under normal, non-drug treatment conditions. Some genes did not successfully amplify from cDNA and so were left out of this study.

Table 4.5. Oligos Used to Check for Introns in cDNA

Systematic Name	Forward	Reverse
Afu5g00790	CCAAGGCGATCCTGACCGTCGGCTC	GGCCGACGGGGTAGAACCAGGCCAGG
Afu1g14330	GGTGGCGAGTCGCTCAACCAG	GGGACGTTGAGCATAGAGTGTC
Afu1g17440	GAGCTTGCAGCCAGACCCCAAC	GCGCTGAAACAGCATGGCAG

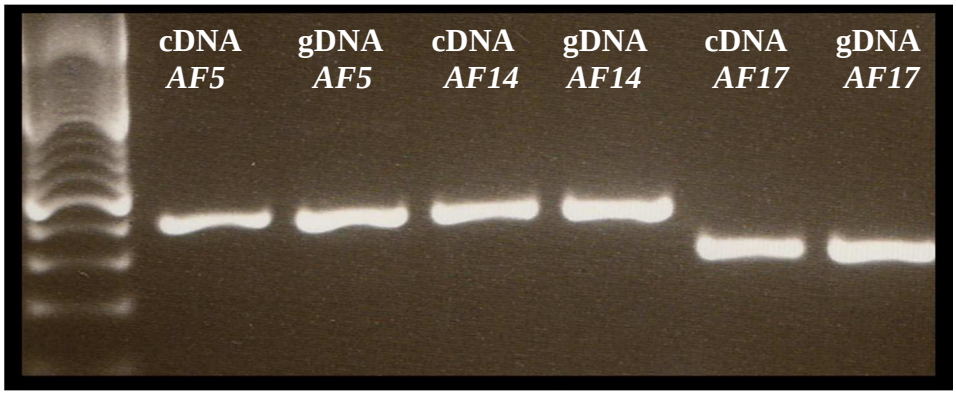

Figure 4.3. ***S. cerevisiae*** **was Not Able to Splice Introns from Heterologously Expressed** ***A. fumigatus*** **Transporter Genes.** Oligos designed for either side of introns were used to amplify cDNA of yeast that heterologously expressed AF transport genes *AF5*, *AF14* and *AF17*. The PCR products were compared to genomic DNA of AF transport genes known to contain introns.

Deletion of endogenous *PDR*5 in *S. cerevisiae* recipient strain allows analysis of heterologous gene phenotype. To characterize *A. fumigatus* putative transport genes for cellular drug efflux, to distinguish potential substrate specificities, and to analyze their contribution to antifungal drug resistance, we constructed *S. cerevisiae* recipient strains suitable for the functional expression of *A. fumigatus* putative transporter genes in efflux-deficient *S. cerevisiae* recipient strains. The gene encoding the *A. fumigatus* transporter of interest is integrated downstream of *GAL1*, a galactose-induced promoter for highly inducible gene overexpression. The B-Δ*pdr*5 and W-Δ*pdr*5 recipient strains exhibited no obvious changes in growth phenotypes compared to the parent strains on complete media, but were severely hypersusceptible to multiple drugs due to the lack of endogenous Pdr5 pump and showed deficient efflux activity. Deletion of this major *Saccharomyces* transporter allowed activity of the heterologous *A. fumigatus* genes to be assayed.

An additional *S. cerevisiae* recipient strain with deletions of 7 major transport genes and 2 transport regulators was used to confirm that no drug resistance or efflux phenotype of the *A. fumigatus* genes was masked by endogenous *S. cerevisiae* efflux activity. The ScADKan strain had a reduced growth rate phenotype, poor transformation efficiency and is hyper-susceptible to a variety of drugs (44).

The controlled overexpression of *A. fumigatus* transporter genes in an *S. cerevisiae* background deficient for its major endogenous transporter allows the functional analysis of potentially significant Pdr5-like ABC transporters involved in drug efflux from an important human pathogen.

Drug Susceptibility Testing

Broth microdilution drug susceptibility assay was performed on all three background strains (BY4741 Δ*pdr5*, W303 Δ*pdr5* and ScADKan) carrying either an empty plasmid or a plasmid expressing an *A. fumigatus* transporter gene. The Minimum Inhibitory Concentration (MIC_{80}), which calculates the minimum concentration of drug required to inhibit fungal growth by 80 % when compared to a non-treated sample. Various drugs on the *S. cerevisiae* strains were compared and are listed in Materials and Methods. The assay was performed in both glucose (plasmid-expression repressed) and galactose (plasmid expression induced) conditions. MIC_{80} results for the galactose-induced plasmid expression are shown in Table 4.6. Four fold changes in drug susceptibility of the AF-expressing strains compared to the *PDR5*-deletion strains are highlighted in red.

The transporters *AF5*, *6*, *9*, *10*, and *14* show substrate specificity for different azoles and R-6G. Table 4.6 shows the MIC_{80} results comparing the efflux-deficient strains B-

Δ*pdr5*, W-Δ*pdr5*, and ScADKan, carrying either an empty plasmid (p0), or a plasmid with the *A. fumigatus* gene of interest [*AF3-10, 14, 15* or*17*]. Samples that had been grown overnight in repressing (glucose) media showed no difference in MIC_{80}s between the AF-transporter-carrying strains, which was used as a representative, non-induced control. The wild-type strain showed a higher MIC_{80} to many of the drugs tested compared to the AF-transporter-carrying strains under glucose (repressing) conditions (data not shown). This was expected since, even in non-inducing conditions, the wild-type strain is still expressing the endogenous SC efflux transporters.

Even under galactose induction, some of the heterologously expressed *A. fumigatus* genes provided no measurable phenotype to the drugs tested. The genes that showed neither a drug resistance nor susceptibility phenotype include *AF3, 4, 7, 8, 15* and *17* (data not shown). The MIC_{80} of those strains matched that of the strain carrying only the empty plasmid. Some of the *AF* transporter genes however had a dramatically increased MIC_{80} and restored a wild-type drug resistance phenotype in the *pdr5* deletion background strain. *AF14* showed increased MIC_{80} to all of the azoles tested including CLT, FLC, ITC, KTC, POS, and VRC. However, *AF14* showed no resistance to non-azole antifungals such as FEN and CH indicating strong substrate specificity for the azole class of drugs (Table 4.6). The *AF14*-expressing strains also showed increased MIC_{80} to R-6G, known to be a substrate for many ABC transporters (45).

Interestingly, some strains showed a variable resistance phenotype to different azoles, suggesting a more specific substrate recognition to some aspect of the drug such as size, charge or structure. For example, *AF5* showed a strong resistance phenotype to the azoles

CLT, FLC, ITC and KTC. However, *AF5* was susceptible to the azoles POS and VRC. *AF5* and *AF10* show a similar resistance phenotype to FLC, however *AF10* shows no efflux activity against KTC, whereas *AF5* has very high activity based on the drug susceptibility results (Table 4.6).

Table 4.6. Broth Microdilution Drug Susceptibility Assay

A	BY4741 +p0	BΔ*pdr*5 +p0	BΔ*pdr*5 +AF5	BΔ*pdr*5 +AF6	BΔ*pdr*5 +AF9	BΔ*pdr*5 +AF10	BΔ*pdr*5 +AF14
CH	0.03	0.03	0.03	0.03	0.03	0.03	0.03
CLT	1.5	0.2	3.0	0.2	0.4	0.2	3.0
FEN	4.0	2.0	8.0	4.0	8.0	4.0	2.0
FLC	16	2.0	16	8.0	2.0	16	16
ITC	1.0	0.25	1.0	0.5	0.5	0.5	4.0
KTC	32	1.0	32	4.0	4.0	1.0	32
POS	2.0	0.25	0.5	0.5	0.25	0.25	0.5
R6G	200	25	200	100	200	100	200+
VRC	0.06	0.008	0.03	0.02	0.02	0.03	0.06

B	WΔ*pdr*5 +p0	WΔ*pdr*5 +AF14
AMB	4	2
BNL	256	128
CLT	0.25	2
FEN	4	4
FLC	2	16
HYGB	80	80
ITC	0.0625	0.25
KTC	2	32
MIC	0.03	0.03
R6G	3.1	50
TRB	12.5	12.5
VRC	0.03	0.25

C	ScADKan +p0	ScADKan +AF14
AMB	>4	>4
CHL	0.4	0.4
CLT	0.25	>2
DOX	0.1	0.1
FEN	1	1
FLC	0.25	>8
MET	0.01	0.25
NYT	>4	>4
PRO	2	8
RES	50	50
R6G	1.5	>3
TBC	0.01	1
VRC	0.02	1
4NQO	0.06	0.06

See Materials and Methods for drug abbreviations

Continued characterization of the differences in substrate specificities between the transporters by drug susceptibility testing to other classes of drugs and biological compounds will be done in the future. The drug susceptibility results suggest the *AF5*, *6*, 9, *10* and *14* genes are functional energy dependent ABC transporters that can compensate for the *S. cerevisiae* Pdr5 transporter to increase resistance to various drugs. These finding confirm a potential role for these *A. fumigatus* transporters in drug resistance and can begin to identify substrate specificity for efflux.

Investigating Gene Functionality with R-6G Efflux Assay

R-6G is a fluorescent substrate of yeast efflux transporters. Fluorescence-based assays of pump activity are a useful way to measure and compare the activity of ABC transporters by measuring the fluorescent signal as it fluctuates either inside the cell or as it is transported into the supernatant. Reduction of efflux activity either by non-functional pumps or by compounds that reduce pump activity are easily identified by the R-6G fluorescence assay. Other fluorescent compounds such as Nile Red and alanine β-naphthylamide could potentially be used to measure efflux activity as well (46, 47).

We used the R-6G screening assay to measure efflux activity in *S. cerevisiae* in which the individual *A. fumigatus* transporter genes were functionally expressed (Fig. 4.4). The *S. cerevisiae* host strain B-Δ*pdr5* is efflux-deficient compared to the wild-type BY4741 due to the deletion of the endogenous ABC transporter *PDR5*. The efflux-deficient strain provided a more sensitive assay for pump activity of the *A. fumigatus* genes to be analyzed in a background strongly depleted of endogenous pump activity. It also allowed a direct

comparison and contrast of the different *A. fumigatus* ABC transporters' ability to compensate for the wild-type *S. cerevisiae* Pdr5 transporter.

Heterologous expression of *AF5, 6, 9, 10* and *14* showed energy-dependent R-6G efflux activity. Figure 4.4 shows results of the R-6G efflux assay comparing the wild-type strain [BY4741] and its derived strains; B-Δ*pdr5* carrying either an empty pYES plasmid [BΔ] or a pYES plasmid with the *A. fumigatus* genes [BΔ5, 6, 9, 10, or 14]. Samples were grown overnight in galactose media to induce plasmid gene expression or glucose in which the pump expression was not induced. The assay was then performed in either an energy depleted condition (Fig 4.4A) in glucose-depleted media, or an efflux active condition (Fig 4.4B) in glucose replete media.

In an energy-depleted state, ABC transporters are inactive and there is very little R-6G efflux from the cells into the media (Fig 4.4A). However, when ABC transporters are activated by providing glucose for energy (+GLC), the wild-type strain [BY4741] as well as the strains expressing the AF transporter genes [BΔ5, 6, 9, 10 or 14] showed dramatically increased R-6G efflux into the media (Fig 4.4B). The different *A. fumigatus* genes showed different levels of R-6G efflux activity with [BΔ9] showing the least activity of the expressed genes. The strain carrying an empty plasmid was unable to show significant R-6G efflux even in the presence of energy [BΔ].

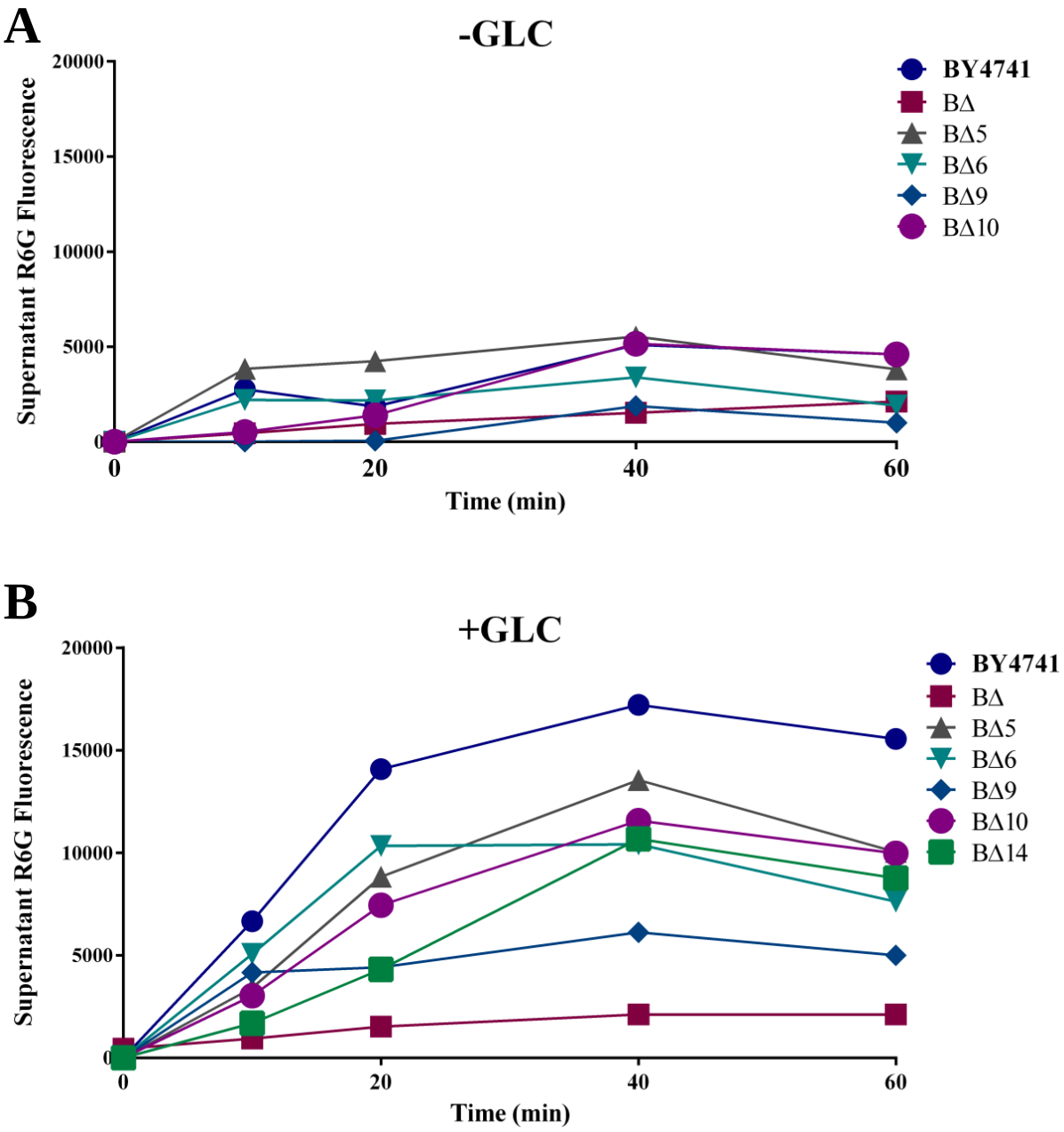

Figure 4.4. R-6G Efflux in *S. cerevisiae* BY4741 Mutant and Wild-type Strains. Plasmid expression was induced by overnight growth in galactose media. The efflux assay was then performed in either a **A)** glucose-starved (-GLC) condition in which there was little efflux from the strains or a **B)** glucose-replete (+GLC) condition in which the wild-type strain [B4741] as well as the strains expressing the AF genes [BΔ5, 6, 9, 10 or 14] showed dramatically increased R-6G efflux into the media. The strain carrying an empty plasmid [BΔ] was unable to show significant R-6G efflux even in the presence of energy.

The PDR5-inhibitor FK506 reduces energy-dependent efflux of R-6G in many *A. fumigatus* ABC transporter-expressing strains. Co-treatment of an infection with both a primary antifungal agent such as an azole and a supplemental agent such as an efflux inhibitor has been proposed as a method of combination therapy to combat drug resistant infections (26, 27, 48-50). FK506, considered to be an inhibitor of PDR5-like ABC transporters, was analyzed for its ability to inhibit the active efflux of R-6G activity in ABC-transporter overexpressing strains (Fig. 4.5) (25, 51).

The wild-type strain [BY] and efflux-deficient strain expressing the *A. fumigatus* genes [*AF*3-*10* and *15*] were treated with the Pdr5-like transporter inhibitor FK506 to determine if this drug showed a measurable inhibitory effect on R-6G efflux. Some of the FK506-treated samples had dramatically reduced R-6G efflux compared to the untreated samples [+GLC vs +GLC +FK506] as shown in Figure 4.5A and B. Strains with high efflux activity such as BY and AF3, showed a dramatic reduction of R-6G efflux in the presence of FK506. Strains that had low efflux activity in the glucose-only conditions such as AF7 and AF9, (Fig. 4.5A), showed little effect from the FK506 treatment (Fig. 4.5B) as they were basically at a baseline level of efflux to begin with.

Interestingly, the transporters *AF*6 and *AF*10, which had a strong resistance phenotype to multiple drugs (Table 4.6A) both had a more intermediate R-6G efflux activity (Figs 4.4B and 4.5A) and were not inhibited by the addition of FK506 (Fig. 4.5B). This is possibly due to some variation at the transporter-FK506 binding site that does not allow transporter inhibition in these particular transporters. Or perhaps *AF6* and *10* do not share a certain feature of the canonical Pdr5-like transporter that FK506 usually recognizes.

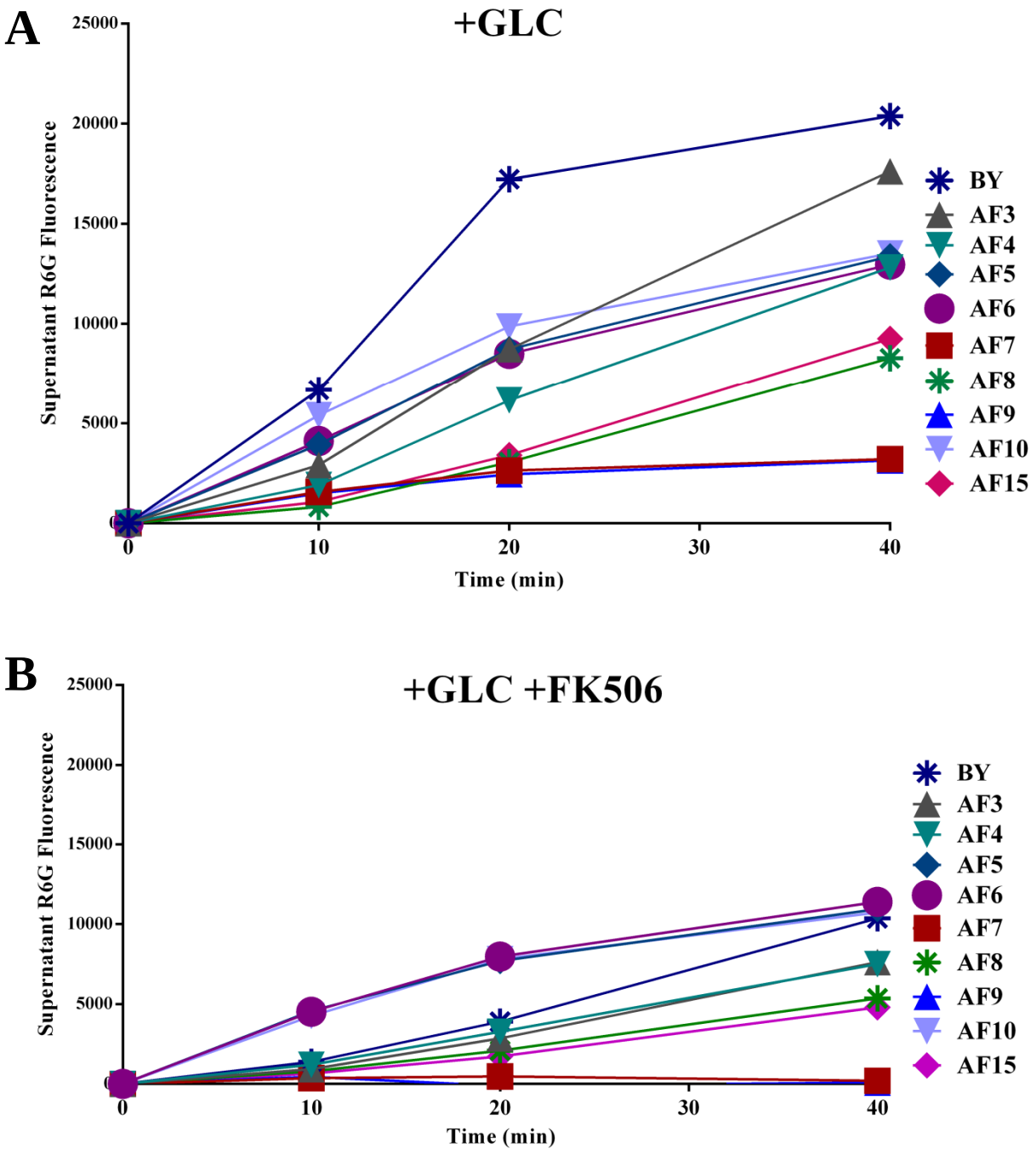

Figure 4.5. R-6G Efflux and Inhibition with the Pdr5-like Transport Inhibitor FK506. Plasmid expression was induced by overnight growth in galactose media. The R-6G efflux assay was performed in the presence of **A)** glucose-only for efflux activation **o**r **B)** glucose and FK506 treatment. Some strains were very susceptible to the inhibitory effects of FK506, while others showed very little change in efflux between the conditions.

Wild-type SC and B-Δ*pdr*5+*AF14* show reduced R-6G efflux with FLC pre-treatment. In the same assay as above, wild-type strain BY4741 (carrying an empty plasmid [BY]) and the efflux deficient strain B-Δ*pdr*5 (carrying the *A. fumigatus* gene *AF14* [BYΔ14]) were grown overnight in galactose media to induce plasmid gene expression. All samples were provided with glucose during the R-6G assay and so were efflux-active. The samples were co-treated with R-6G +/- FLC to analyze R-6G efflux, indicative of ABC transporter activity, with and without azole treatment (Figure 4.6).

FLC and R-6G are both thought to be substrates to Pdr5-like ABC transporters and so reduced R-6G efflux in the presence of an azole would indicate competition between the compounds for transport out of the cell. Both the wild-type strain and the strain expressing the *AF14* gene showed dramatically reduced R-6G efflux into the media in the presence of FLC [BY+GLC vs BY FLC and BYΔ14+GLC vs BYΔ14 FLC] (Fig. 4.6A). These results indicate that FLC does compete for efflux with R-6G and that the *AF14* transporter has similar activity and substrate specificity as the ScPdr5 transporter.

Farnesol and clorgyline inhibited the energy-dependent efflux of R-6G in *AF14*-expressing *S. cerevisiae*. The ability of clorgyline or farnesol to inhibit ABC drug transporters was investigated by measuring R-6G activity +/- inhibitor treatment (Fig. 4.6B). Wild-type strain BY4741 carrying an empty plasmid [BY] and efflux-deficient strain B-Δ*pdr*5 carrying the *A. fumigatus* gene *AF14* [BYΔ14] were treated with the putative ABC transporter inhibitors clorgyline or farnesol to determine if these drugs showed a measurable inhibitory effect on R-6G efflux. Both clorgyline and farnesol-treated samples had dramatically reduced R-6G efflux compared to the untreated samples [BY vs BY FAR or BY

CLORGY and BYΔ14 vs BYΔ14 FAR or BYΔ14 CLORGY]. The *AF14* transporter reversed the effect of the deleted endogenous ScPdr5 transporter and showed even greater R-6G efflux activity than the wild-type BY4741 strain. The increased activity is most likely an effect of hyperexpression of the transporter from the *GAL1* promoter in the multicopy plasmid.

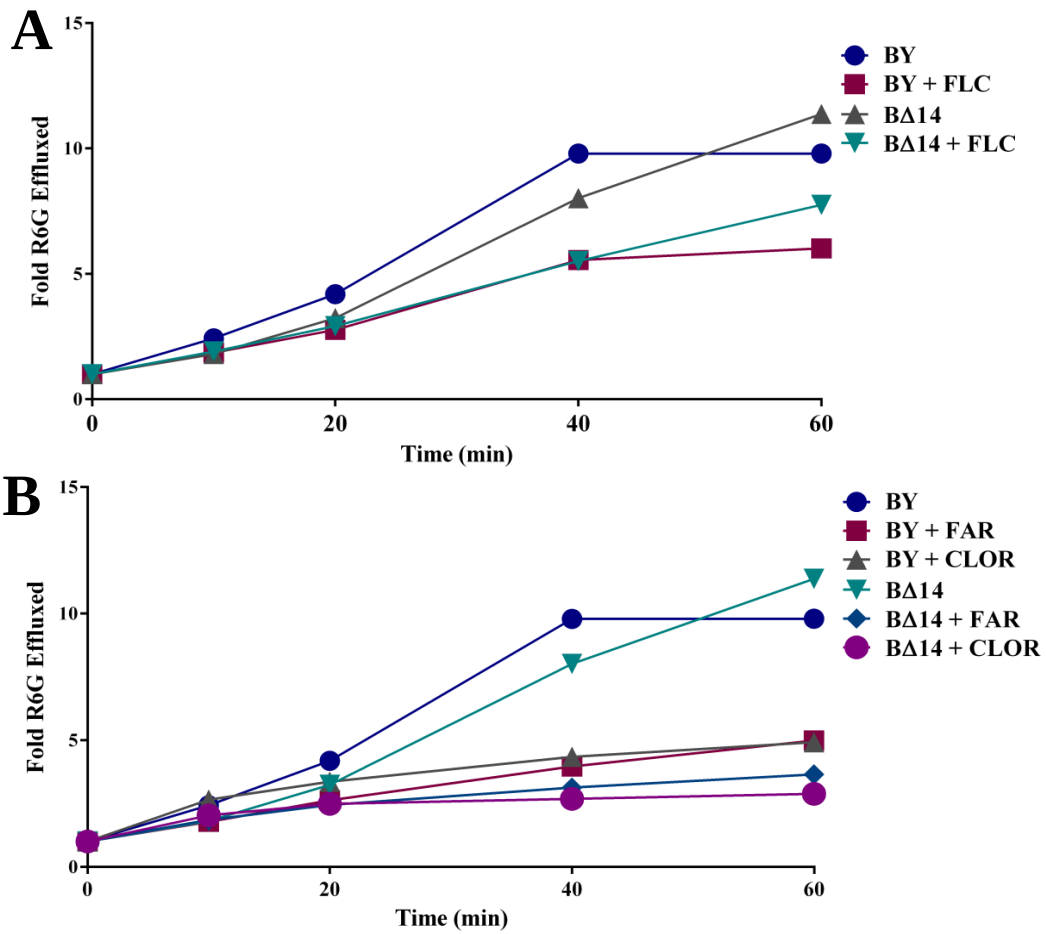

Figure 4.6. Efflux of R-6G from Wild-type and *AF14* Strain. Plasmid expression was induced by overnight growth in galactose media. The assay was performed in the presence of glucose to allow energy for transporter activity**. A)** Both the wild-type strain and the strain expressing the *AF14* gene showed dramatically reduced R-6G efflux in the presence of FLC [BY vs BY+FLC and BYΔ14 vs BYΔ14+FLC]. **B)** Both farnesol and clorgyline-treated samples had dramatically reduced R-6G efflux compared to the untreated samples [BY vs BY+FAR or BY+CLOR and BYΔ14 vs BYΔ14+FAR or BYΔ14+CLOR].

GFP-tagging *AF14*

A. fumigatus AF14 pump shows proper membrane surface localization and expression. To visualize the plasmid induction, expression levels, and protein localization of the heterologously-expressed AF14 putative membrane transport protein, we added a GFP tag to the protein creating the pYES *AF14*-GFP plasmid.

Wild-type yeast strains carrying an empty pYES plasmid were imaged for auto fluorescence. Very little GFP signal can be seen in the wild-type control image (Fig 4.7A). The strains W-Δ*pdr*5 and BY-Δ*pdr*5 were transformed with the GAL-inducible, multicopy plasmid containing the *AF14*-GFP fusion gene and were imaged by fluorescent microscopy in plasmid-inducing conditions shown in Figure 4.7B and C. The images of the GFP-tagged AF14p confirmed that the AF14-GFP fusion protein is localized to the cell surface as evident from the rim-like staining of the cells. The GFP fluorescence could also be seen in the cytoplasm most likely as an effect of the high copy number plasmid and hyperexpression of the protein.

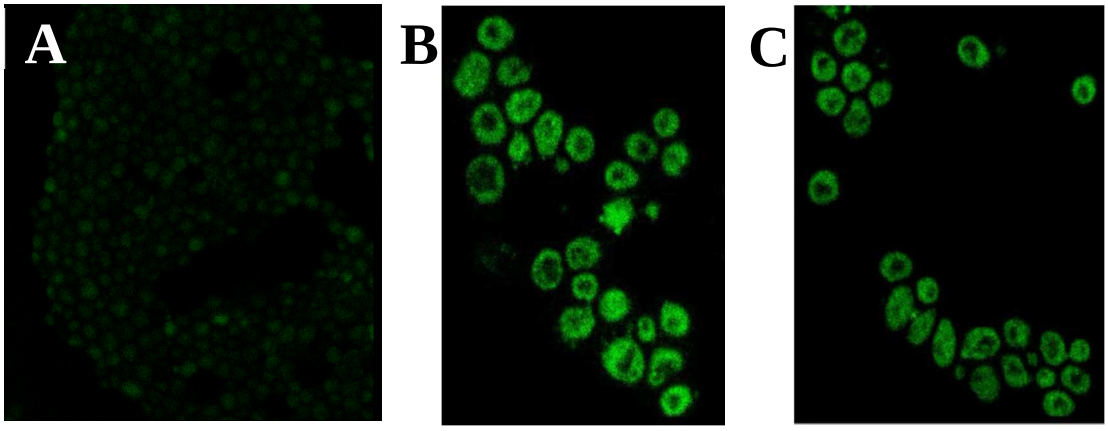

Figure 4.7. ***AF14*-GFP is Expressed and Localizes to the Plasma Membrane**. Fluorescent Microscopy of **A)** *S. cerevisiae* W303 Wild-type and **B)** W303 *AF14*-GFP expressing strain magnified. **C)** BY4741 *AF14*-GFP expressing strain magnified.

GFP tagging did not disrupt transporter function. Both E-strip susceptibility testing and R-6G efflux assay showed that the AF14-GFP protein remained functional with the GFP tag. Figure 4.8A shows that in inducing conditions (galactose) both W-Δ*pdr*5 +*AF14*-GFP (A) and BY-Δ*pdr*5 +*AF14*-GFP (B) strains have increased FLC resistance compared to the W-Δ*pdr*5 and BY-Δ*pdr*5 strains carrying an empty pYES plasmid. The *pdr*5-deletion strains showed a FLC MIC around 0.38 - 0.5 µg/ml, while the -Δ*pdr*5 *AF14*-GFP strains show FLC resistance greater than 64 µg/ml.

R-6G efflux activity is not changed by the addition of the C-terminal GFP tag on the AF14 protein as shown in Fig. 4.8C. The pattern of efflux activity in BY-Δ*pdr*5+14 and BY-Δ*pdr*5+14 GFP strains are nearly identical. R-6G efflux is greatly increased when glucose is provided in contrast to baseline R-6G efflux activity when no glucose is provided, again confirming an energy-dependent efflux mechanism.

In addition, farnesol greatly inhibits R-6G efflux from both BY-Δ*pdr*5+ *14* and BY-Δ*pdr*5+*14*-GFP expressing cells, even in the presence of glucose. Although there are examples of fusion proteins that have reduced or increased activity, misfolding, or incorrect localization due to the GFP tag, it is expected that the conserved Pdr5-like membrane transporters can be C-terminus GFP tagged and visualized without functional problems.

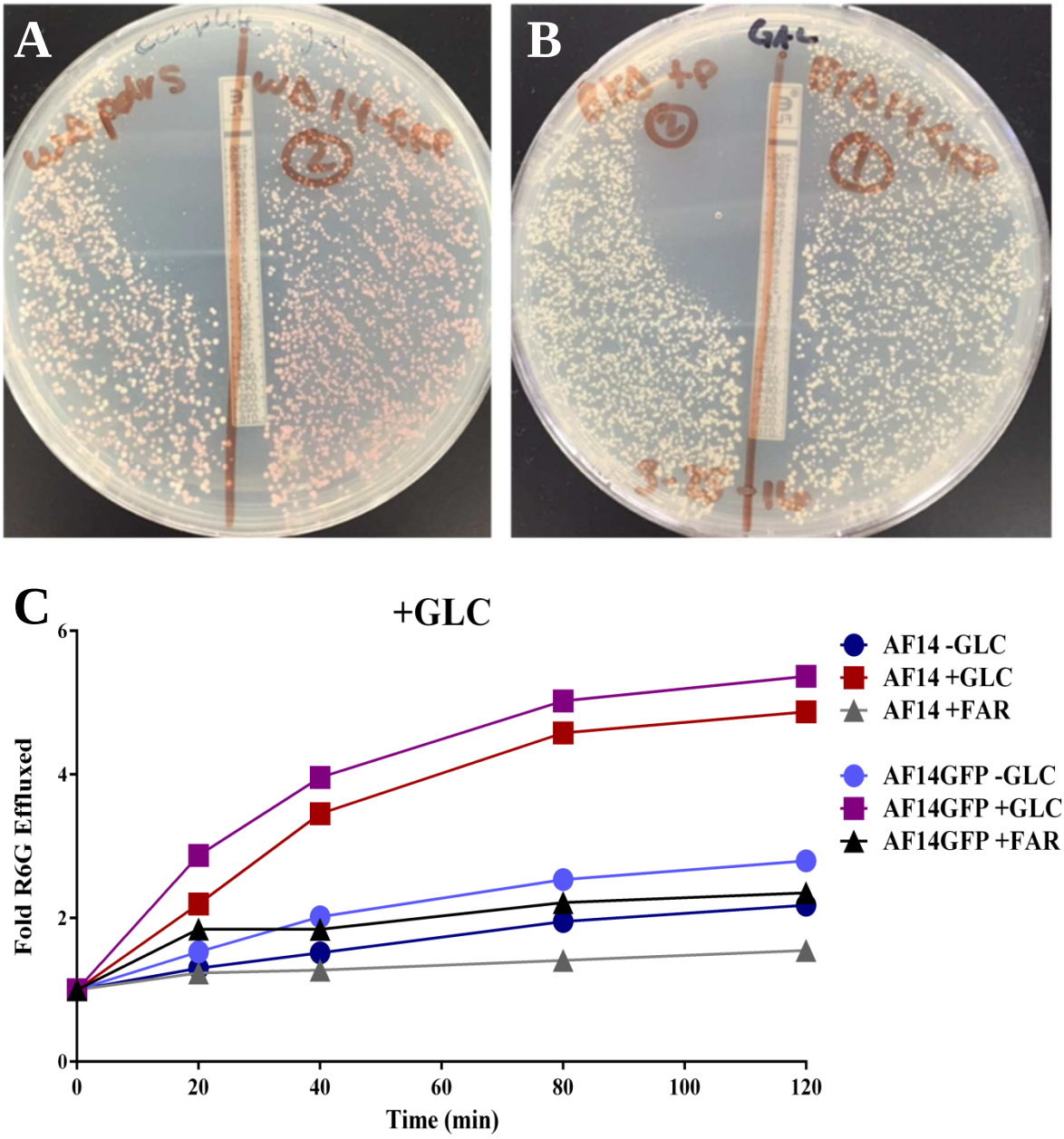

Figure 4.8. GFP Tagging Did Not Disrupt AF14 Transporter Function. E-test strips were used to compare the MIC of FLC on **A)** W303 -Δ*pdr*5 compared to W303 -Δ*pdr*5 +*AF14*-GFP strains and **B)** BY-Δ*pdr*5 compared to BY-Δ*pdr*5+*14*-GFP strains. **C)** R-6G efflux +/- glucose activation (+GLC and –GLC) and +/- treatment with the inhibitor farnesol (FAR) was compared between BY-Δ*pdr*5+*14* and BY-Δ*pdr*5+*14*-GFP strains.

Conclusions and Discussion

In many well studied yeasts and fungal pathogens, the efflux of antifungal drugs via ABC transporters constitutes a major cause of clinical multidrug resistance. This warrants a closer look at the *A. fumigatus* genome for potential ABC transporters that actively efflux antifungals. The major objectives of this study were to identify plasma membrane ABC transporters that may contribute to antifungal drug resistance in *A. fumigatus* and to characterize the substrates and efflux properties of these pumps when expressed in the model organism *S. cerevisiae*.

We first looked for evidence of energy-dependent efflux as well as intracellular accumulation of radioactively labeled FLC in *A. fumigatus* to illustrate the possibility that ABC transporters in *A. fumigatus* may play a role in azole drug resistance and that transport or intracellular accumulation of FLC is something that could be manipulated with antifungal treatments. We showed that efflux of FLC was energy dependent and cells that had glucose were able to remove the FLC at a much more rapid rate than cells starved of glucose (Fig 4.2A).

We analyzed whether the intracellular accumulation of FLC in *A. fumigatus* could be increased by preventing efflux with ABC transport inhibitors. We showed that compounds that inhibit fungal ABC transporters, such as clorgyline, could potentially be a broad-spectrum, synergistic drug that increases antifungal drug accumulation in the fungal cell when used in combination drug therapy (Fig 4.2B).

We further analyzed the potential for transport-mediated drug resistance in *A. fumigatus* by amplifying putative ABC transport genes that were actively expressed in *A.*

fumigatus. To more directly study the phenotype and efflux capability of the *A. fumigatus* proteins, we worked with the model organism *S. cerevisiae*. *S. cerevisiae* is a powerful model system that has been used extensively to study drug resistance properties in other yeasts such as *C. albicans*. We used drug-hypersusceptible *S. cerevisiae* strains lacking the main endogenous ABC transporter, Pdr5, to study the function of putative transporter genes from *A. fumigatus*.

All of the transporter genes analyzed in this study are thought to be Pdr5-like proteins based on the translated sequence and predicted protein domains. They each have 2 nucleotide binding domains and 2 transmembrane domains that each has 6 TM segments. However, the genes have many differences including number and size of introns, exon sequences, chromosome location and transcriptional regulation. Since all of the genes are placed under the control of the *GAL1* promoter, it is assumed that the expression level of each transporter is similarly overexpressed. Consequently, the difference in drug susceptibilities and efflux function must be a function of these sequence differences.

Our initial characterization of the genes encoding *A. fumigatus* putative ABC transporter genes have shown interesting differences in substrate specificity and energy-dependent efflux of R-6G (Table 4.6 and Figs. 4.4-4.6). The expression of certain *A. fumigatus* transport genes (*AF5, 6, 9, 10 or 14)* rendered the otherwise highly drug-susceptible *S. cerevisiae* host strains resistant to various drugs. Other transporter genes (*AF3, 4, 8, 15 or 17)* showed no phenotype to the drugs tested thus far (Table 4.6). Comparing drug susceptibility to a variety of azoles allowed us to tease out some differences in substrate specificities for the different transporters. Continued exploration of drug resistan

phenotypes along with comparisons of the transporters' predicted sequence and structure may illuminate important evolutionary events and even reveal potential efflux inhibitor drug targets.

The R-6G fluorescent efflux assay offered even more distinguishing phenotypes between the AF transporters. There were transporters that had strong R-6G efflux activity (*AF4, 5, 6, 10, 14*), and others that showed very little R-6G efflux (*AF7, 8, 9, 15, 17)* (Figs. 4.4 and 4.5). While the R-6G fluorescent efflux assay showed differential energy-dependent efflux between the transporters, it did not necessarily correlate with any particular drug resistance phenotype. For example the strain expressing *AF9* consistently showed a high R-6G MIC_{80} as well as a resistant phenotype to fenpropimorph and some of the azoles (Table 4.6A), however, the *AF9*-expressing strain showed very little active R-6G transport in the fluorescent efflux assay (Figs. 4.4 and 4.5). This is not unexpected as the different assays measure very different aspects of transporter function.

In addition, the response to the Pdr5-like ABC transport inhibitor FK506 was varied between the transporters, again indicating diversity among the ABC transporters even within the same organism. R6G efflux from strains expressing Sc*PDR5, AF3* or *AF4* was inhibited by FK506, while strains expressing *AF5* or *AF6* were unaffected by the R6G and FK506 co-treatment. Importantly, we have shown that transport function in yeast can be blocked through inhibitors such as FK506, clorgyline and farnesol (Figs. 4.5 and 4.6). A combination therapy using simultaneous administration of azoles with another synergistic compound, such as an efflux inhibitor, has the potential to restore fungal isolate susceptibility to treatment.

Co-treatment with antifungals that have non-overlapping toxic effects on the host,

allows for more total antifungal force to be applied without increasing the overall toxicity to the host. This is particularly important in patients that are plagued by side effects and toxicities of many common antifungals in high doses. In addition, combination treatments would help prevent the development of resistance to either of the single agents. Similarly, a combination treatment approach may be more successful against infections that include some fungal cells not inherently resistant to one antifungal agent and other fungal cells not inherently resistant to the other antifungal agent. This becomes more relevant as we learn more about microbiomes and fungal-fungal or fungal-bacterial co-infections.

The strains overexpressing the AF14 transporter showed consistent drug resistance phenotypes as well as highly active R-6G efflux and so its expression and localization was visualized with fluorescent microscopy by expressing an AF14-GFP fusion protein in *S. cerevisiae* (Fig 4.7B and C). Although a fraction also appears in cytoplasm, it is perhaps due to the hyperexpression from a galactose-inducible promoter in a multicopy plasmid. Nevertheless, even the GFP variants are functional in yeast, since they confer pronounced tolerance to azoles and R-6G drug treatment (Fig 4.8A and B). Additionally, R-6G efflux is unaffected by the GFP-tagged AF14 and remains active compared to the *S. cerevisiae PDR*5 deletion strains (Fig 4.8C).

Taken together, our work shows that ABC transporters are a remarkably diverse group of transporters that remain understudied and enigmatic in most fungal species. We will continue to use this model system of heterologous *A. fumigatus* gene expression in *S. cerevisiae* efflux-deficient strains to characterize other putative *A. fumigatus* ABC transporters for functionality and potential role in drug resistance.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The seriousness and devastating potential of fungal infections are oftentimes overlooked and disregarded by the general public, research community and funding agencies as being a concern. However, fungal infections represent a significant human health burden. Pathogenic fungi cause serious disease and death in humans, animals and plants. Hundreds of millions of people world-wide depend on vulnerable, fungal-susceptible food crops that are vital to human diet and nutrition. In reality, fungal infections pose a serious global health and food security threat and there is an urgent need to identify new fungal drug targets, develop new antifungals and proactively investigate resistance mechanisms to azoles and other antifungals.

As long as we treat fungi with drugs, it is important to understand the most basic fungal/drug interactions. Antifungal drug accumulation in the fungal cell is a balance of uptake, retention, and efflux. The goal of this research was to better characterize these specific activities of the fungal cell. In Chapters 2 and 3 we described our analysis of the mechanisms of drug uptake and retention in two diverse and important pathogenic fungi *Aspergillus fumigatus* and *Magnaporthe oryzae*. In Chapter 4 we described our analysis of the efflux of antifungal drugs by characterizing a selection of *A. fumigatus* transporter genes to determine functionality, efflux substrate specificities, and the potential for efflux-mediated drug resistance.

The experiments described in Chapter 2 characterized drug uptake mechanisms and analyzed how cellular and environmental factors effect drug accumulation and variations in intracellular drug concentrations over time. We found that azole drugs do not accumulate in the *A. fumigatus* cell solely by passive diffusion. We used radioactively-labeled azoles to directly study azole uptake in the fungal cell under a variety of drug treatment conditions such as temperature, availability of energy and changes in pH. We characterized substrate specificity of the transporter by comparing a variety of structurally diverse compounds for competition with the radio-labeled azole. We compared drug uptake between cells of different ages and morphologies, as well as measuring drug import in a transport-mutant strain with deficiencies in the sterol uptake and biosynthesis pathway. We conclude that azole drugs are specifically selected and imported into the fungal cell by a pH-independent and ATP-independent facilitated diffusion mechanism.

Our data in Chapter 3 demonstrat that the filamentous plant pathogen *M. oryzae* also imports azoles by a facilitate diffusion mechanism that had many similarities to *A. fumigatus* drug import. While each organism is extremely specialized for their niche environment and has specialized infection mechanisms, we expect that the mechanism of azole drug import is conserved among many fungi. Contrasts between the results of the import experiments in *M. oryzae* and *A. fumigatus* revealed interesting differences that suggest differential expression, induction, or function of efflux transporters in the two organisms. Furthermore, for both *A. fumigatus* and *M. oryzae*, our azole accumulation and efflux assays, combined with the efflux inhibitor clorgyline, demonstrated the potential usefulness of combination drug therapies to ensure more azole is retained in the fungal cell.

The analysis described in Chapter 4 focused on fungal efflux mechanisms of antifungal drug treatment. We individually characterized a selection of putative ABC transporter genes from the *A. fumigatus* genome by heterologously expressing each gene in *S. cerevisiae*. We characterized the transporters' potential role in drug resistance and substrate specificities for a variety of antifungal drugs using broth microdilution susceptibility assays. We directly analyzed efflux activity of the transporters by measuring energy-dependent transport of the fluorescent dye rhodamine 6G. We also evaluated the efflux inhibitors FK506, farnesol and clorgyline for their effect on transporter activity. Importantly, we showed differences in substrate specificity, drug susceptibilities, energy-dependent efflux activity, and effect of efflux-inhibitor treatment between the different transporters. These studies illustrate the diversity, complexity and challenges of predicting and counteracting fungal drug treatment response, but also highlight the possibilities for identifying new drug targets.

Future Directions

Chapters 2 and 3 describe our preliminary investigation of drug transport across the cell membrane and accumulation inside the fungal cell. We would like to further analyze conditions that affect drug uptake and retention such as expanding the analysis of potential competitive uptake and efflux inhibitors. The next steps are to identify the proteins that are involved in drug import. Identifying the specific channel or transport protein by which this occurs is still in progress and is a major goal of our research. Determining the proteins involved in transporting azoles into the fungal cell would provide insight into how our

current drug treatments are working. Novel import proteins could potentially provide new targets for drug treatment.

In addition, we would like to use our azole import assay to compare drug import in clinical isolates and resistant strains of *A. fumigatus* and other filamentous fungi. This will help build a correlation between import and drug resistance, for example by identifying a gene or family of genes that are highly expressed in strains that import more drug. Or conversely, this may distinguish genes that are mutated or under-expressed in strains that take up very little drug.

Our Chapter 4 data on ABC transporters have provided exciting insights into the poorly studied *A. fumigatus* efflux repertoire. For these transporters, we would like to continue testing a library of antifungal drugs and compounds to characterize individual transporter substrate specificities as well as potential inhibitors of these pumps. Some of the transporters showed no phenotype to the antifungals tested and may have an entirely different set of efflux substrates such as sterols, lipids, detergents, peptides, sugars, amino acids, etc.

As the transporters are currently expressed under the control of the highly-active *GAL1* promoter, we do not know the natural expression levels and induction potential of the proteins. We would like to look at expression changes and induction upon drug treatment in *A. fumigatus* using a qRT PCR or RNA sequencing approach. Comparing expression levels of the different transporters across a range of isolates, both medical and agricultural may indicate trends that are important for future resistance threats.

Finally, transporter sequence comparisons correlated with substrate specificities, as well as mutagenesis experiments can identify amino acids important for substrate

recognition, binding, and transport. This information could be translated into potential targets for the development of efflux inhibitors.

Final Conclusions

As the trends of fungal drug resistance and multi-drug resistance rise, we are faced with challenges to develop more drugs and find new drug targets. Mammalian and fungal cells are similar enough at the individual cell level that fungal-specific drug targets are limited. Understanding and distinguishing fundamental cellular processes will help to uncover new fungal drug targets. However, the current situation necessitates that we also reconsidering drugs that are already in existence, possibly for alternative uses or as a combination therapy approach.

VITA

Brooke Esquivel attended Missouri State University from 2004-2010 where she was awarded the Missouri State University Academic Scholarship. During her time at MSU, Brooke earned a B.S. in Anthropology as well as a B.S. in Cell and Molecular Biology. She went on to obtain her M.S. in Cell and Molecular Biology from MSU and produce multiple papers from her research on nanoparticles and their potential as nucleic acid delivery agents for gene therapy.

In 2010, Brooke joined the Interdisciplinary Ph.D. program in Cell Biology and Biophysics/Molecular Biology and Biochemistry within the School of Biological Sciences at University of Missouri-Kansas City where she was awarded the UMKC Women's Council Graduate Assistant Award for multiple years, the School of Graduate Studies Research Grant, as well as the UMKC Interdisciplinary Student Council Travel Grant to attend and present her work at various scientific conferences. She was also granted a research fund from the School of Graduate Studies for her research on drug resistance in pathogenic fungi. In 2012 she earned her M.S. in Cell and Molecular Biology at which time Brooke started her dissertation work under the supervision of Dr. Theodore C. White. During her four years of research with Dr. White, she was able to co-author a book chapter as well as publish a first author paper about her work with the fungus *Aspergillus fumigatus*.