Characterization and eradication of persisters in *Candida albicans* biofilms

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ABSTRACT OF DISSERTATION

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ABSTRACT

Fungal pathogens form biofilms that are highly recalcitrant to antimicrobial therapy. We report that biofilms formed by the major human pathogen *Candida albicans* exhibit biphasic killing in response to amphotericin B and chlorhexidine. Biphasic killing indicates the existence of a subpopulation of highly tolerant cells, termed persisters. The extent of killing with a combination of amphotericin B and chlorhexidine was similar to that observed when the same antimicrobials were added separately. Thus, surviving persisters form a multidrug tolerant subpopulation. Reinoculation of cells surviving killing of the biofilm by amphotericin B produced a new biofilm with a new subpopulation of persisters. This suggests *C. albicans* persisters are not mutants, but phenotypic variants of the wild type. Persisters were detected and isolated from strains defective in late stages of biofilm formation, indicating that attachment initiates persister formation. Persisters may be largely responsible for the multidrug tolerance of fungal biofilms.

Inhibition of multidrug resistance (MDR) efflux transporters and subsequent treatment with imidazole antifungals was found to kill *C. albicans* biofilms. When a panel of imidazole drugs was tested against wild type *Candida* biofilms, no killing was observed at any concentration. However, biofilms of a strain deleted in known MDR transporters were killed with concentrations as low as 50 µM clotrimazole and 200 µM miconazole. Fluconazole did not exhibit lethality, which was found to be dependent on the deletion of Cdr1p and Cdr2p transporters. The combination of disulfiram, a known Cdr1p inhibitor, and
miconazole also produced potent killing of biofilms. The combination of
imidazole antifungals and multidrug efflux inhibitors may be exploited to treat
biofilm infection.

We have also identified small molecules that kill *C. albicans* biofilms and
persisters in the presence of miconazole. A screen of 68,950 compounds
revealed 323 molecules that reduced metabolic activity of biofilms in the
presence of miconazole. Two compounds had no direct activity, but produced
over 1000-fold reduction of biofilm colony counts in combination with miconazole.
Importantly, one of these compounds, a hydrazone, completely eradicated
persisters. These small molecules could lead to the development of therapeutics
that synergize with currently available antifungals and sterilize infection.
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1.0 Background and Significance

1.1 Candida albicans biofilms

Candida albicans is an opportunistic pathogen that is a common member of the human oral and gastrointestinal microflora. There are three typical forms of infection, oropharyngeal, vulvovaginal and systemic candidiasis. Biofilms of C. albicans often form on indwelling devices, such as catheters and heart valves. Biofilm infections are essentially untreatable by antifungals and can develop into life-threatening disease with a mortality rate approaching 40%. The recalcitrance of these infections to antifungals is puzzling, since planktonic populations of the same strain are susceptible to a number of antifungals, includingazole compounds, echinocandins and amphotericin B. The focus of this review will be on Candida albicans biofilms with special emphasis on drug resistance.

C. albicans biofilms form when single cells attach to a surface and grow into microcolonies, which then merge and produce a complex 3-D structure that is held together by hyphae and an exopolymer matrix (17). The biofilm is heterogeneous containing a mixture of yeast, hyphae and pseudohyphae forms (39). Yeast attach to a surface through physical properties of the cell, such as hydrophobic and electrostatic interactions, and with the aid of adhesin proteins (110). The agglutinin-like sequence (ALS) adhesins are glycoprophosphatidylinositol anchored cell wall proteins that mediate cellular attachment to other cells and surfaces. Several genes encoding adhesins have been identified by sequence conservation and experimental evidence including, ALS1-7, ALS9-12, HWP1 and EAP1 (110). Many adhesins are redundant in function (116), yet differentially
regulated under a variety of environmental and stress conditions (4, 95). In addition, adhesin genes are subject to both genotypic and phenotypic plasticity in the form of tandem repeat recombination and epigenetic histone code modification (110). Adhesins are responsible for the ability of Candida to attach to a wide range of cell types and surfaces. Once attached, cells grow, divide and form microcolonies which merge, while adhesins stabilize the entire structure through cell-to-cell interactions (63). In wild type biofilms, a layer of predominantly hyphae forms on top of a basal layer of yeast, and the biofilm can be over 500 µm thick (9, 86). Water channels and extracellular matrix pervade the entire structure.

A number of genes have been identified that are necessary for the wild type biofilm development or maintenance described above. For example, NUP85, MDS3, KEM1, and SUV3 were identified as genes required for wild type biofilm formation since homozygous deletion caused a less opaque appearance on silicone and more turbid growth in medium in a biofilm formation assay (90). Subsequent analysis revealed all of these strains were defective in hyphae formation (85, 90). A similar screen of transcription factor mutants revealed that \( \Delta bcr1 \) produced a biofilm of greatly reduced biomass and thickness, despite the fact this strain was able to produce hyphae (76). Bcr1p was subsequently found to regulate expression of adhesins, such as ALS1, ALS3, and HWP1 (75). In contrast, Ywp1p is a negative regulator of adhesion, which may aid in biofilm cell dispersion. \( \Delta ywp1 \) caused increased attachment to surfaces and other cells, however cells overexpressing Ywp1p do still attach to surfaces, although at a
reduced level compared to wild type (36). These data suggest that any mutation that causes a defect in attachment, filamentation or growth (GCN4, (107)) will likely cause a biofilm defect. Typically, biofilm defective mutants still attach to a surface and form a congregation of cells albeit with reduced biomass. These attached cells may be less adherent and more easily washed away compared to wild type biofilms.

Biofilms have many unique phenotypic properties and gene expression patterns of biofilm cells differ dramatically compared to planktonic cells (32, 72, 115). Extracellular matrix, adhesin production and drug efflux transporters are all upregulated in biofilms (2, 63, 81). In addition, microarray analysis of biofilms points to wholesale changes in protein synthesis, metabolism (amino acid and nucleotide), transcription and cellular organization (32). For example, when gene expression patterns were followed 30 minutes to 6 hours after attachment, many genes involving sulfur assimilation and metabolism were upregulated compared to age matched planktonic controls (72). Another study compared gene expression patterns of 6, 12 and 48-hour biofilms to planktonic cells and each other (115). Again many changes in metabolism and transport were apparent in biofilms compared to planktonic cells, but few differences were detected between early and late stage biofilm metabolism (115). Taken together, these studies reveal that a common subset of genes is expressed in biofilms, different from those expressed during planktonic growth. These changes begin almost immediately after attachment to a surface. For the purposes of this review, a
biofilm will be defined as a surface attached population of cells with properties distinct from planktonic cells.

The drug resistant phenotype of biofilms supports the *sensu lato* definition described earlier. Upregulation of drug efflux pumps is detectable by RT-PCR after only 15 minutes of surface attachment and increased resistance to the antifungal fluconazole was detectable within 2 hours (68). In addition, higher order biofilm structure does not appear to be necessary for drug resistance. Analysis of morphological mutants, stuck in either yeast or hyphal form and unable to transition between the two, revealed that both morphologies were able to attach to a surface and aggregate (9). Importantly, each population displays a drug resistant phenotype compared to planktonic cells (9). Similarly, Δ*efg1* and Δ*cph1/Δefg* strains, which are defective in filamentation, produce monolayer biofilms that are resistant to both amphotericin B and fluconazole (85). Surface attachment appears to be a key factor in mediating biofilm drug resistance, since strains with severe biofilm defects continue to display increased drug resistance.

One notable exception is MCK1 characterization by Carol Kumamoto in 2005 (56). Mck1p is a contact dependent kinase required for invasive growth into agar medium. While a Δ*mck1/Δmck1* mutant is able to form a biofilm, the attached cells are remarkably susceptible to fluconazole as measured by 2,3-bis(2-methoxy-4-nitro-5-sulfophenly)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction, an indicator of metabolic activity. When MCK1 is complemented back into the deletion strain, fluconazole resistance is restored to
the wild type level (56). MCK1 appears to mediate biofilm resistance to fluconazole.

The inherent drug resistance of attached biofilm cells compared to planktonic cells has been reported for almost every antifungal and even some biocides (105). Resistance is attributed to a variety of factors, and causative or refutal evidence may only apply to specific drugs or drug classes. Therefore, an overview of currently available antifungal drugs and their mechanism of action is provided below.

1.2 Antifungal drugs

The first systemic antifungals identified were the polyenes, such as amphotericin B deoxycholate and nystatin. Polyenes act by binding ergosterol, a fungal specific component of the cell membrane analogous to mammalian cholesterol (15). Amphotericin B creates pores in the cell membrane by forming aggregates with ergosterol. Pore formation was initially inferred by measuring extracellular ion (K+) released from cells after amphotericin B treatment (1, 31). Subsequent studies used linear dichroism-FTIR and molecular modeling within membranes to suggest amphotericin B interacts with ergosterol and specifically disrupts membrane polar head groups (6, 30). Polyenes are cidal and resistance occurrence is extremely low.

The next major class of antifungals is the azoles. In vitro antifungal efficacy of benzimidazole was first reported in the 1940's (65). Azoles work by inhibiting 14-alpha lanosterol demethylase, the ERG11 gene product in Candida, leading to ergosterol depletion and growth arrest (112). First generation azoles
with an imidazole backbone, such as miconazole and clotrimazole, are still considered effective treatments for topical infection. However, systemic imidazole use was replaced with the invention of the triazoles in the 1990’s. Triazoles, including fluconazole and voriconazole, were favored over imidazoles due to increased water solubility, broader spectrum of activity, and lower toxicity (65).

The first enchinocandin, caspofungin, was licensed in 2001 and marked the arrival of another class of antifungals (101). Enchinocandins inhibit glucan synthesis, presumably by blocking the enzyme beta 1-3 D-glucan synthase, and cause defects in the fungal cell wall (21). Caspofungin is lethal to *Candida*, has an excellent safety profile and has been reported to kill biofilms *in vitro* (5).

Many currently available antifungal agents are effective against *Candida* species. Infections can range from superficial mucocutaneous candidiasis to life threatening systemic infection of the blood or organ systems. Increasingly, treatment guidelines are dependent on *in vitro* susceptibility testing (89) however, the standard measure of efficacy for antifungal agents is determination of the minimum inhibitory concentration (MIC) or the minimum fungicidal concentration (MFC) in a rapidly growing planktonic population. Indeed, this is how currently available antifungals have been validated. However, high mortality and therapeutic failure of antifungals indicates that *in vivo* systemic and biofilm infection is considerably more complex, and cannot be approximated by an exponentially growing population of the pathogen (18, 61). New drugs or therapeutic strategies are needed to combat these infections.
1.3 Proposed mechanisms for biofilm drug resistance and tolerance

Biofilms exhibit increased drug resistance compared to genetically identical planktonic cells. Several factors may be involved in this phenomenon. The exopolymer matrix of biofilms restricts penetration of immune system components (42, 111). Since antimicrobials act along with the immune system to eradicate infections in vivo, the biofilm exopolymer matrix is an important component of recalcitrance. In Candida, the matrix consists of carbohydrate, protein, hexosamine, phosphorus and uronic acid (3). Any of these components have the potential to bind antifungals, restrict access to cellular targets, and confer resistance. However, a disk filter technique showed the exopolymer matrix did not appreciably hinder penetration of fluconazole or amphotericin B through the biofilm (8, 91). In addition, biofilms that produced more matrix due to turbulence exhibited similar susceptibility to amphotericin B and fluconazole compared to static biofilms with less matrix (8). However, a similar experiment comparing growth under continuous flow (more matrix) to static biofilms reported decreased susceptibility to amphotericin B (although not fluconazole) suggesting that the matrix could be involved (2). Recently, biofilm spent medium but not supernatant derived from planktonic culture was reported to bind fluconazole (74). β 1-3 glucan, which is more prevalent in biofilms compared to planktonic cells, may bind fluconazole and contribute to resistance (74).

Quiescence or slow growth is a factor in bacterial drug resistance. However, studies utilizing XTT and 2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1- phenylquinolinium iodide (FUN-1) demonstrated that
the majority of cells in \textit{C. albicans} biofilms are metabolically active (52-54). In fact, the increased metabolic activity of biofilms correlated with increased resistance to fluconazole and amphotericin B (17). Additionally, biofilm resistance to amphotericin B was determined to be largely independent of growth rate in a perfused biofilm fermentor setup (7).

Resistance in planktonic populations of cells may point to mechanisms involved in biofilm drug resistance. For example, genes encoding multidrug resistance (MDR) transporters \textit{MDR1}, \textit{CDR1} and \textit{CDR2} are upregulated in some fluconazole resistant clinical isolates (112). These transporters are also upregulated upon attachment of \textit{C. albicans} cells to a surface, and this accounts for the resistance of young biofilms to azole antifungals (70). However, the high level of drug resistance of mature biofilms (≥48 hour) was not affected by deletion of any of these genes, including a \textit{mdr1Δ cdr1Δ cdr2Δ} triple mutant (56, 70, 81). Apparently drug efflux is not a factor in resistance of mature biofilms to the azoles and, in addition, the echinocandins and the polyenes are not substrates of any known drug transporter.

Drug target mutation can cause resistance but is unlikely to occur universally in biofilms. Indeed, biofilm drug resistance is transient (78, 81) and genetic mutation would result in stable resistance. Drug target modification could also result in resistance and occur naturally as a byproduct of biofilm growth. Many drugs including the polyenes and the azoles, target ergosterol, an essential component of the fungal cell membrane. Compensatory mechanisms in biofilm cell membranes could account for resistance to drugs targeting ergosterol.
Indeed, a profile of membrane sterol composition revealed decreased ergosterol content in mature biofilms (56, 70). Diminished ergosterol biosynthetic gene expression (32) has also been reported in biofilms. However, cell membrane modification is unlikely to be involved in the action of echinocandins that inhibit the synthesis of cell wall β-glucan (21), or chlorhexidine, a membrane-active antiseptic that is very effective against bacteria that lack sterols.

New evidence suggests heterogeneity could cause drug resistant subpopulations of cells to pre-exist within a biofilm (38, 45, 49, 59, 102, 103, 108). This small proportion of cells could be important for the overall success of drug therapy, since biofilms are protected from immune components by the extracellular matrix in vivo. Unique subpopulations of cells within a biofilm have been documented in a variety of different circumstances. A biofilm was found to express stationary phase specific genes SNZ1 and SNO1 exclusively in a small population of yeast cells closely attached to the substrate (108). It is unknown whether these stationary subpopulations of cells have increased resistance to antifungals. However, increased resistance to amphotericin B was detected in a subpopulation of biofilm cells, which remained closely associated with the surface after most cells were washed away by increased flow rate of a perfusion chamber (49). These cells were found to have 10 fold decreased susceptibility to Amphotericin B and resistance appeared to correlate with differential expression of ergosterol and β 1-3 glucan genes ERG1, ERG25, SKN1, and KRE1 (49). Certain cells at the base of a biofilm were also reported to have increased resistance to chlorhexidine (102). A separate subpopulation of live cells was
detected within biofilms after exposure to a lethal concentration of caspofungin (54). These cells retained metabolic activity in the presence of caspofungin as reported by FUN1 staining (54). A subpopulation of *C. albicans* cells, distributed throughout the biofilm was also found to be tolerant to high concentrations of metal chelating agents (38). However, the most compelling evidence of a heterogeneous subpopulation of drug tolerant cells within a biofilm is described in Chapter 2 of this thesis.

Evidence for a multi-drug tolerant subpopulation of cells, termed persisters, within *Candida* biofilms was published in November 2006 by LaFleur et al (59). Persisters were characterized as biofilms exhibited a biphasic killing pattern in response to two microbicidal agents, amphotericin B, a polyene antifungal, and chlorhexidine, an antiseptic. The extent of killing with a combination of amphotericin B and chlorhexidine was similar to that observed with the same antimicrobials added individually. Reinoculation of cells surviving killing of the biofilm by amphotericin B produced a new biofilm with a similar subpopulation of persisters, demonstrating that the survivors were not mutants. Persisters were only present in biofilms, and analysis of biofilm defective mutants suggested attachment was sufficient for persister formation. Persisters were independent of drug efflux and could account for biofilm resistance to all known antifungals.

Failure of currently available antifungal drugs, especially in regard to biofilm infection, suggests a need for the development of new therapeutic strategies. Understanding mechanisms of biofilm heterogeneity and persister
production may lead to the development of the first antifungal drugs capable of 18 eradicating infection.
2.0 Candida albicans biofilms produce antifungal-tolerant persister cells.

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2.1 Introduction

Bacterial and fungal biofilms have many features in common. In both cases, biofilms are often formed by merging of initial founder microcolonies, are protected from the immune system by exopolymers, are composed mainly of slow-growing cells, and exhibit multidrug tolerance (58, 62). Bacterial biofilms produce dormant persister cells that are largely responsible for multidrug tolerance (16, 47, 99). Persisters are able to survive despite the presence of antibiotics at concentrations well above the minimum inhibitory concentration (MIC) and are phenotypic variants of the wild type, rather than mutants (47). Persisters are formed by all bacterial species studied, and are present at 0.1-1% in the biofilms of Pseudomonas aeruginosa, Escherichia coli, or Staphylococcus aureus, for example (47). Given the similarity to bacterial biofilms, we reasoned that fungal biofilms may similarly produce persister cells.

2.2 Abstract

Fungal pathogens form biofilms that are highly recalcitrant to antimicrobial therapy. Expression of multidrug resistance pumps in young biofilms has been linked to increased resistance to azoles, but this mechanism does not seem to
underlie the resistance of mature biofilms that are a model of *in vivo* infection. The mechanism of drug resistance of mature biofilms remains largely unknown. We report that biofilms formed by the major human pathogen *Candida albicans* exhibit a strikingly biphasic killing pattern in response to two microbicidal agents, amphotericin B, a polyene antifungal, and chlorhexidine, an antiseptic, indicating that a subpopulation of highly tolerant cells, termed persisters, existed. The extent of killing with a combination of amphotericin B and chlorhexidine was similar to that observed with individually added antimicrobials. Thus, surviving persisters form a multidrug tolerant subpopulation. Interestingly, surviving *C. albicans* persisters were only detected in biofilms, and not in planktonic exponentially growing or stationary populations. Reinoculation of cells surviving killing of the biofilm by amphotericin B produced a new biofilm with a new subpopulation of persisters. This suggests that *C. albicans* persisters are not mutants, but phenotypic variants of the wild type. Using a stain for dead cells, rare dark cells were visible in a biofilm after amphotericin B treatment and a bright and a dim population were physically sorted from this biofilm. Only the dim cells produced colonies, showing that this method allows the isolation of yeast persisters. Given that persisters only formed in biofilms, mutants defective in biofilm formation were examined for tolerance to amphotericin B. All of the known mutants affected in biofilm formation were able to produce normal levels of persisters. This finding indicates that attachment rather than formation of a complex biofilm architecture initiates persister formation. Bacteria produce multidrug tolerant persister cells in both planktonic and biofilm populations and it
appears that yeasts and bacteria have evolved an analogous strategy that assigns the function of survival to a small part of the population. In bacteria, persisters are dormant cells. It remains to be seen whether attachment initiates dormancy, which leads to the formation of fungal persisters. This study suggests that persisters may be largely responsible for the multidrug tolerance of fungal biofilms.

2.3 Materials and Methods

Yeast strains and growth conditions. Yeast strains used in this study are described in Table 2.1. Candida albicans was streaked onto yeast extract, peptone, dextrose (YPD) agar from frozen stocks (-80°C) and incubated at 37°C for 24-48 hours. When necessary (CAI4 derivatives), growth medium was supplemented with 80 µg/ml uridine. Biofilms were grown essentially as described by Ramage et al. (2001). Briefly, cells were grown overnight at 30°C in YPD liquid medium. Samples were harvested by centrifugation, washed 2X in sterile PBS pH 7.4 and resuspended in RPMI 1640 with L-Glutamine and 0.165M MOPS (BioWhittaker™). The optical density of the culture (at 600nm) was adjusted to 0.1 by dilution with RPMI 1640, which is equivalent to approximately 1 X 10^6 cells/ml. 100 µl of this suspension was aliquoted into 300 µl wells of a flat bottomed microtiter plate (Corning Costar® 3370). Plates were incubated for 48 hours at 37°C on a microtiter plate shaker (Lab-Line Instruments, model 4625) at approximately 100 rpm. Exponential and stationary cultures inoculated from
single colonies were grown in RPMI 1640 with L-glutamine and 0.165 M MOPS at 37°C for 12 and 72 hours, respectively.

**Antibiotic susceptibility testing.** Amphotericin B and chlorhexidine were obtained from Fisher Scientific and stock solutions prepared in DMSO. Caspofungin was obtained from Merck. Antifungals were dissolved in growth medium. Biofilms were washed twice with sterile PBS in order to remove non-adherent cells prior to antifungal challenge. Antifungals were then added, 100 µl per well. Biofilms were disrupted by scraping and vortexed vigorously for 30 seconds before serial dilution and plating. Microscopic observation showed that the resulting suspension consisted of clumps and individual cells in approximately equal numbers, with the size of clumps being ≤ 10 cells. Plates were incubated for 24-48 hours prior to colony counting.

100 µl samples of exponentially growing (12 hour) or stationary (72 hour) planktonic cultures were harvested by centrifugation, washed twice with PBS and challenged in microtiter plates with antifungals in 100 µl of RPMI 1640 with L-glutamine and 0.165 M MOPS growth medium. The cultures containing antifungals were incubated at 37°C for 24 hours, washed twice with PBS by centrifugation and aspiration of supernatant, and resuspended in 100 µL PBS. Cultures were then 10 fold serially diluted in PBS and plated for CFU determination.

For sequential challenge, biofilms were treated with 100 µg/ml amphotericin B or chlorhexidine as described above. After 24 hours, the medium was
aspirated and 100 µl RPMI 1640 with L-glutamine and 0.165 M MOPS containing 100 µg/ml of amphotericin B and chlorhexidine was added to each well and incubated for an additional 24 hours.

Progeny biofilms were formed by inoculating samples from antibiotic challenged, resuspended biofilms into fresh YPD and repeating the biofilm growth procedure described above.

**Live/dead cell determination using fluorescein diacetate staining and epifluorescent microscopy.** Biofilms of strain 3153A were grown and resuspended in RPMI 1640 with L-Glutamine and 0.165M MOPS with 100 µg/ml fluorescein diacetate alone (control) or with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) for 24 hours. 1 µl samples of biofilms, scraped from the microtiter well at various time points, were placed onto a glass slide under a glass coverslip and viewed with a 100X oil immersion lens using a Zeiss Axioskop 2 plus microscope with a standard FITC filter. Photographs were taken using an AxioCam (Carl Zeiss) black and white CCD camera with an accompanying 0.63X lens using Axiovision version 4.5. Live and dead control populations of budding yeast cells were obtained from an overnight 3153A culture grown in YPD at 30°C. Samples were pelleted and resuspended in YPD with 100 µg/ml fluorescein diacetate alone or 100 µg/ml amphotericin B and fluorescein diacetate for 24 hours. Planktonic samples were viewed and photographed in a manner similar to the biofilm cells described above.
**Isolation of persisters.** Biofilms from strain MC 191 were grown and resuspended in RPMI 1640 with L-Glutamine and 0.165M MOPS with fluorescein diacetate (Fisher Scientific; 100 µg/ml) and with or without amphotericin B (100 µg/ml). Biofilms were disrupted by scraping and vortexing, and were washed 5X with PBS. Samples were analyzed using a MoFlo™ (Dako) cell sorter. A homogeneous population of cells was isolated using a gate based on forward and side scatter. Cells which passed the forward and side scatter gate were counted as single events and subsequently sorted into two distinct populations based on high and low green fluorescence. Single events from these populations were then physically sorted directly onto separate YPD agar plates in the form of a 96 well grid and were incubated for 48 hours. Photographs of the plates were taken using an Olympus Camedia C-4040 digital camera.

2.4 Results

**Detection of persisters in C. albicans biofilms.** Dose-dependent killing provides a straightforward approach to detect persisters. A biphasic killing curve revealing a subpopulation of cells that survive low doses of antimicrobials indicates the presence of persisters. Several compounds including amphotericin B, chlorhexidine, and caspofungin have been reported to kill *Candida* biofilms (54, 84, 105).

Mature, 48 hour biofilms formed in wells of microtiter plates were rinsed with fresh growth medium and challenged with antifungals for 24 hours. In parallel, antifungals were added to exponentially growing and stationary
planktonic cultures as well. Caspofungin, a β-glucan synthesis inhibitor of the cell wall had a rather limited effect on biofilms, producing ≤10 fold killing (data not shown) and was therefore unsuitable for this study.

Amphotericin B, a compound that binds to ergosterol and forms an oligomeric pore in the membrane (6), effectively killed exponentially growing and stationary cells, with little indication of surviving cells (Figure 2.1A). By contrast, a striking biphasic killing was observed in Candida biofilms with the majority of the population killed at low concentrations (but above the MIC) while the remaining cells were unaffected by higher concentrations of the drug (Figure 2.1A). About 1% of cells appeared completely invulnerable to amphotericin B, indicating the presence of persisters in the yeast biofilm. This is very different from our previous observations with bacteria, where stationary planktonic populations produce more persisters than the biofilm (99). It was also rather surprising to see complete resistance to killing by amphotericin B, which makes “holes” in the membrane.
Figure 2.1. Survival of C. albicans 3153A biofilm, exponential and stationary cells. Biofilms were cultured in RPMI medium for 48 hours, scraped, vortexed and resuspended in 100 ml PBS and plated for CFU determination. Exponential and stationary cultures were obtained by growth in the same medium. The experiment was performed in triplicate and error bars represent standard deviation. A, amphotericin B; B, chlorhexidine.

Similarly to amphotericin B, chlorhexidine, a membrane-acting antiseptic used in oral antifungal applications (11) produced a distinctly biphasic killing of the biofilm, while cells in both exponential and stationary cultures were completely eliminated (Figure 2.1B). At higher concentrations (above 100 µg/ml), killing of persisters became evident, and the biofilm was completely sterilized at 1000 µg/ml, a concentration 2 fold lower than what is commonly used in mouthwash and as a therapy for treatment of oral thrush caused by C. albicans (0.2%).

**Persister cells are multidrug tolerant.** In bacteria, persisters are dormant cells that are tolerant to multiple antibiotics. We wanted to learn whether yeast persisters were similarly multidrug tolerant. Mature, 48 hour biofilms were challenged with 100 µg/ml amphotericin B, 100 µg/ml chlorhexidine, or a combination of these two drugs. No additional killing was detected when biofilms were treated with both amphotericin B and chlorhexidine as compared to cells treated with individual antimicrobials (Fig. 2.2). Similarly, the number of persisters was essentially the same (1-3%) when biofilms were treated sequentially for 24
hours with amphotericin B and then chlorhexidine or vice versa (data not shown). These experiments indicate the presence of a single uniform persister population.

Figure 2.2. Survival of *C. albicans* biofilms challenged with amphotericin B and chlorhexidine. Biofilms were treated with 100 µg/ml amphotericin B; 100 µg/ml chlorhexidine; or a combination of the two antifungals for 24 hours. Biofilms were washed and sampled for CFU determination before and after antibiotic treatment. The experiment was performed in triplicate and error bars indicate standard deviation.

To examine the possible role of drug efflux transporters in resistance to these two antimicrobials, the susceptibility of the WT strain and a mutant lacking Cdr1p, Cdr2p, Mdr1p and Flu1p was tested. The minimal inhibitory concentrations for the wild type and the quadruple mutant DSY1024 (cdr1∆::hisG/cdr1∆::hisG cdr2∆::hisG/cdr2∆::hisG camdr1∆::hisG/camdr1∆::his
WU1::hisGflu1::hisG-URA3-hisG, (66)) were the same, 1 µg/ml for amphotericin B and 8 µg/ml for chlorhexidine. This suggests that resistance to amphotericin B and chlorhexidine is not influenced by *C. albicans* efflux transporters, and, therefore, that efflux does not contribute to persister survival.

**Persisters are phenotypic variants of the wild type.** We next wanted to learn whether cells resisting killing by drugs were phenotypic variants of the wild type, or mutants. Biofilms were treated with amphotericin B or chlorhexidine (100 µg/ml), after which they were disrupted by vortexing, washed, and reinoculated. The new biofilm, derived from persisters that survived drug treatment, was again treated with antifungals, and the procedure was repeated a total of 3 times (Figure 2.3). It is apparent that the population produced by surviving persisters is not more resistant to drugs but rather gives rise to a new persister subpopulation. If the surviving cells were mutants, we would see either complete resistance upon reapplication of the antifungal or a progressive increase in the numbers of surviving cells with each treatment cycle. Thus, we conclude that *C. albicans* persisters, similarly to their bacterial counterparts, are phenotypic variants of the wild type that arise in a clonal population of genetically identical cells.
Figure 2.3. Heritability of persister formation. Biofilms were treated with 100 µg/ml amphotericin B or 100 µg/ml chlorhexidine for 24 hours, after which they were disrupted by vortexing, washed and reinoculated in order to form new biofilms. Biofilms were sampled for CFU determination before and after antibiotic treatment. The procedure was repeated a total of 3 times. The experiment was performed in triplicate and error bars indicate standard deviation.

Isolation of persisters. Several dyes have been reported to discriminate between live and dead fungal cells including fluorescein, which was recently reported to specifically stain dead *S. cerevisiae* cells (114). Exponentially growing *C. albicans* cells, untreated or killed with amphotericin B, stained with fluorescein diacetate in the expected manner (Figure 2.4 A, B). We then stained a biofilm with fluorescein diacetate (Figure 2.4C-E). After addition of amphotericin B, there was a visible decrease in the number of live (dark) cells, and their morphology became aberrant (Figure 2.4D). After 48 hours of amphotericin B treatment, there were only a small number of unstained cells and, importantly, they appeared to have normal morphology. These cells looked like regular pseudohyphae or yeasts and were indistinguishable from morphologically normal untreated cells. This finding is similar to bacterial persisters, which look like regular cells (94).
Figure 2.4. Live-dead staining of *C. albicans* with fluorescein diacetate. Planktonic or biofilm cells were stained with 100 µg/ml fluorescein diacetate and examined by fluorescence microscopy. A, live planktonic cells; B, dead planktonic cells after treatment with 100 µg/ml amphotericin B (400X magnification). C, D, and E, biofilms (1000X magnification) of untreated control, after 18 and 48 hours of amphotericin B treatment (100 µg/ml), correspondingly.

Are the dim *C. albicans* in the amphotericin B-treated biofilm live persisters, or rather dead cells that for some reason happened to be unstained? The excellent difference observed between live and dead fluorescein diacetate-stained cells suggested that putative persisters could be sorted physically. In order to minimize size heterogeneity in the sorting process, we took advantage of the *flo8 (riv1)* null mutant strain that is defective in both hyphal and higher order
biofilm formation. This strain produces a sparse layer of attached yeast cells, and formed a normal level of persisters (Table 2.1). The absence of hyphae in this strain made it a good choice for sorting. Mature 48 hour biofilms were stained with fluorescein diacetate, disrupted, and sorted with a MoFlo instrument. The sample contained debris from disrupted biofilms, and light scattering was used to determine the predominant particle group, which represented yeast cells. This group was then isolated with a scatter gate and analyzed for fluorescent properties (Figure 2.5A). Cells representing the mean in the fluorescence distribution (Figure 2.5B) from a biofilm stained with fluorescein diacetate but not treated with an antibiotic were sorted directly onto a plate with YPD medium, and after a 48 hour incubation produced colonies. The sorting worked well under these conditions, with 8 cells producing 8 colonies (Figure 2.5D). Next, we sorted cells from a biofilm treated with amphotericin B. There was a strong shift of the bulk of the population to higher fluorescence (Figure 2.5C), reflecting the staining of dead cells with fluorescein diacetate. Two subpopulations, a bright (R2) and a dim (R4) one, were then chosen for physical sorting onto YPD plates. 96 of the sorted dim particles produced 21 colonies. This shows that a considerable part of the dim cells observed after treatment with amphotericin B were indeed live persisters. The imperfect correlation between sorted events and colonies they produce in this case may have been caused by a considerable background of debris. Indeed, the concentration of live cells is small, and debris is not likely to exhibit bright green fluorescence. Therefore, debris would be sorted and appear disproportionately represented in the dim subpopulation. Alternatively, it is
possible that some of the dim cells are actually dead. There was no growth from over 6000 sorted bright cells (Figure 2.5F).

Figure 2.5. Isolation of persister cells from a biofilm. *C. albicans* MC191 was grown as a biofilm for 48 hours in RPMI 1640 medium in microtiter plate wells. A homogenous population of cells from disrupted biofilms was obtained by applying a forward scatter and a size scatter gate as shown in A for all subsequent analyses. B, a biofilm was stained with 100 µg/ml fluorescein diacetate for 24 hours, disrupted, washed 3 times with PBS and analyzed with a MoFlo cell sorter. Single events representing individual cells were physically sorted directly on YPD medium and incubated for 48 hours (D). C, a biofilm was treated with 100 µg/ml amphotericin B, stained with fluorescein diacetate and similarly analyzed with the cell sorter. Two distinct populations were separated based on green fluorescence intensity as shown. Particles representing 96 events from the
dim population R4 were sorted onto YPD agar and incubated for 48 hours, E. Particles representing over 6000 events from R2 were sorted onto YPD agar and incubated for 48 hours.

Persisters in biofilm-deficient strains. Given that persisters were only produced in the biofilm, it was interesting to test biofilm-defective mutants for persister production. Several mutants, e.g. strains lacking the transcription factors Efg1p and Cph1p or strains lacking the MAP kinase Mkc1p, have been reported to produce defective biofilms. A collection of such mutants was tested for persister formation. All strains tested produced biofilms under the conditions of these experiments, though their biomass in many cases appeared distinctly less as compared to the wild type. After challenge with 100 µg/ml amphotericin B, biofilms were disrupted and plated for colony count. Somewhat unexpectedly, all tested strains produced a normal level of persisters (Table 2.1). Of particular interest was strain CKY138, lacking Efg1p and Cph1p, positive transcriptional regulators of filamentation. This strain is severely defective for biofilm formation showing reduced adherence and a failure to produce an elaborate three-dimensional structure that characterizes wild type biofilms. Nevertheless, an efg1∆/cph1∆ double mutant strain gave rise to persisters. Therefore, persister formation is not dependent on formation of a complex biofilm structure. Apparently, the ability to attach to a surface, which all of these strains exhibit, is sufficient to promote persister production. This is consistent with previously published work which suggested that attachment alone is sufficient to confer
resistance to fluconazole in a young biofilm (68), and that resistance to fluconazole and amphotericin B was similar in sessile wild type and efg1Δ/cph1Δ cells as measured by XTT reduction (85).

Table 2.1. Strains used in this study and tested for persisters.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Biofilm architecture</th>
<th>Persisters</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3153A</td>
<td>wild type laboratory strain</td>
<td>Robust 3D wild type</td>
<td>+++</td>
<td>(83)</td>
</tr>
<tr>
<td>CKY357</td>
<td>CAI-4 mke1Δ::hisG/mke1Δ::hisG mke1::pCK70 (URA3)</td>
<td>Reduced filamentation</td>
<td>++</td>
<td>(56)</td>
</tr>
<tr>
<td>CAI4</td>
<td>SC5314 Δara3::Δimm434/Δara3::Δimm434</td>
<td>Robust 3D wild type</td>
<td>+</td>
<td>(56)</td>
</tr>
<tr>
<td>CKY136</td>
<td>CAI-4 efg1::hisG/efg1::hisG ade2::pDBF52 (URA3)</td>
<td>Filamentation defect; sparse monolayer of cells</td>
<td>+++</td>
<td>(35)</td>
</tr>
<tr>
<td>CKY138</td>
<td>CAI-4 efg1::hisG/efg1::hisG cph1Δ::hisG/cph1Δ::hisG ade2::pDBF52 (URA3)</td>
<td>Filamentation defect; sparse monolayer of cells</td>
<td>++</td>
<td>(35)</td>
</tr>
<tr>
<td>MC191</td>
<td>ura3Δ::Δimm434/ura3Δ::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG flo8::ARG4/flo8::HIS1 ade2::URA3/ADH2</td>
<td>Functionally defective hyphae</td>
<td>+++</td>
<td>Microbia</td>
</tr>
<tr>
<td>MC195</td>
<td>ura3Δ::Δimm434/ura3Δ::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG flo8::ARG4/flo8::HIS1 ade2::URA3/ADH2</td>
<td>Robust 3D wild type</td>
<td>+</td>
<td>Microbia</td>
</tr>
<tr>
<td>MC245</td>
<td>ura3Δ::Δimm434/ura3Δ::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG flo8::ARG4/FLO8 ade2::URA3/ADH2 HIS1/His1</td>
<td>Robust 3D wild type</td>
<td>++</td>
<td>Microbia</td>
</tr>
<tr>
<td>DAY185</td>
<td>Δara3::Δimm434/Δara3::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG flo8::ARG4/URA3 HIS1/His1</td>
<td>Robust 3D wild type</td>
<td>++</td>
<td>(90)</td>
</tr>
<tr>
<td>DAY286</td>
<td>Δara3::Δimm434/Δara3::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG</td>
<td>Robust 3D wild type</td>
<td>++</td>
<td>(90)</td>
</tr>
<tr>
<td>GKO443</td>
<td>Δara3::Δimm434/Δara3::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG suv3::Tn7-UAU1/suv3::Tn7-UAU3</td>
<td>Biofilm defect; decreased biomass</td>
<td>++</td>
<td>(90)</td>
</tr>
<tr>
<td>GKO798</td>
<td>Δara3::Δimm434/Δara3::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG kem1::Tn7-UAU1/kem1::Tn7-UAU3</td>
<td>Biofilm defect; decreased biomass</td>
<td>++</td>
<td>(76)</td>
</tr>
<tr>
<td>GKO814</td>
<td>Δara3::Δimm434/Δara3::Δimm434 arg4::hisG/arg4::hisG his1::hisG his1::hisG nup85::Tn7-UAU1/nup85::Tn7-UAU3</td>
<td>Biofilm defect; decreased biomass</td>
<td>++</td>
<td>(90)</td>
</tr>
</tbody>
</table>
Given that attachment rather than biofilm architecture appeared to be important for persister formation, it was especially interesting to test a strain deficient in Mkc1p, a recently described contact dependent kinase that affects biofilm formation and invasiveness (56). However, a strain lacking Mkc1p was able to produce normal levels of persisters (Table 2.1). Therefore, our analysis of biofilm mutants suggests that known genes are not involved in persister formation. The isolation of persisters, as described in this study, opens the possibility for obtaining their transcription profile and thus identifying genes involved in the persister phenotype.

2.5 Discussion

The discovery of persisters in bacterial biofilms helped explain the puzzling resistance of biofilms to killing (47). A similar paradox exists in the field of fungal biofilms – planktonic cells may be highly susceptible to a range of antimicrobials, while mature biofilms appear to be resistant to killing by “everything” compared to planktonic populations. A biphasic pattern of killing is a defining feature demonstrating the presence of persisters in bacterial
populations. In this study, we found that *C. albicans* biofilms exhibit this striking pattern of killing, with the majority of cells being fairly susceptible, and a small subpopulation, $10^{-3}$-$10^{-2}$ cells, highly tolerant to antimicrobials. Our analysis was limited by necessity to highly microbicidal antifungals amphotericin B and chlorhexidine, which can effectively kill slow or non-growing cells; both of these compounds are membrane acting. Persisters appeared to be completely invulnerable to amphotericin B, which was rather unexpected. It has been reported that biofilm cells have a decreased level of ergosterol to which amphotericin B binds prior to forming a pore in the membrane (70). It is possible that persisters have an even lower level of ergosterol than the bulk of biofilm cells and that other factors, such as dormancy, contribute to their survival to amphotericin B. Importantly, an experiment with the non-specific antiseptic chlorhexidine suggests that *C. albicans* persisters are not merely cells with diminished ergosterol levels. A prominent subpopulation of cells tolerant to chlorhexidine was observed as the majority of cells were killed at a low concentration of 10 µg/ml and additional killing did not occur until the concentration reached a very high dose of 500 µg/ml. At even higher concentrations, chlorhexidine was able to completely eradicate the biofilm. Interestingly, this biofilm eradication concentration of chlorhexidine, 1 mg/ml, is comparable to the 2 mg/ml concentration found in mouth wash used to treat *C. albicans* oral thrush infections. It is important to note that neither amphotericin B nor chlorhexidine is a substrate of Cdr1p, Cdr2p, Mdr1p or any known drug efflux pump. Our experiments showed that the minimal inhibitory concentration of the
wild type to these antimicrobials was the same as compared to a strain with multiple deletions in multidrug efflux transporters.

The biphasic killing produces an impression of the presence of resistant mutants in a population, and we examined the possible heritability of resistance in the surviving cells. Repeated reinoculation of persisters after amphotericin B treatment restored the original biofilm with a new persister subpopulation. This is similar to the non-heritable nature of bacterial persisters (46).

In order to observe persisters, we used fluorescein diacetate, which specifically stains dead yeast cells. After treatment with amphotericin B, most of the cells became morphologically aberrant (bloated) and then stained with fluorescein diacetate, while a small fraction of cells retained normal morphology and were dark. We then used cell sorting to isolate the dark cells. The dark cells produced colonies, while the bright ones did not. This experiment demonstrates the existence of unique survivor cells within the antifungal treated biofilm.

The production of fungal persisters by biofilm populations alone was unexpected. We did not find persisters in either exponential or stationary phase planktonic cultures of *C. albicans*. In principle, stimulation by fresh medium or regrowth could have accounted for the presence of persisters in biofilms but not in planktonic stationary cultures. In our experiments, both biofilms and stationary cultures were washed and then antibiotics were added in fresh growth medium. It is important to note that stationary yeast cells do not grow when resuspended without dilution in the same volume of fresh medium. Nevertheless, stationary phase cultures did not contain persisters; persisters were only observed in the
biofilm. It seems that the inability of a planktonic stationary culture to form persisters can not be explained by their disappearance due to stimulation with fresh medium.

Stationary cultures grown in RPMI 1640 medium produce cell aggregates, but do not form persisters. Indeed, crowding does not seem to be important – addition of quorum sensing molecules farnesol or tyrosol did not affect the numbers of persisters in biofilms (M. LaFleur, unpublished). These observations suggest that attachment to substratum is the important factor that determines persister formation in *Candida*.

In bacteria, the frequency of persisters increases sharply with the cell density of the population (46), and there are actually more persisters in a non-growing stationary culture than in a slow-growing biofilm (99). It was also surprising to find that persisters were present at normal levels in all of the presently described mutants that produce defective biofilms. These results point to attachment, rather than formation of a complex biofilm structure, as the stimulus for persister formation. At the same time, a mutant in the Mkc1p MAP kinase that is involved in contact sensing and control of the biofilm phenotype produced normal levels of persisters as well. This suggests that additional, unidentified, sensors of attachment are probably responsible for triggering a program that produces persisters.

A transcription profile of isolated persisters has recently been obtained in *E. coli*. Persisters overexpress several toxin-antitoxin modules. Induction of toxin expression from a controllable promoter caused production of persisters and
deletion of a particular toxin-antitoxin module, *hipBA*, caused a decrease in persisters in a biofilm (47). Upon overexpression, these toxins block important targets in the cell, leading to dormancy. Bacterial persisters have characteristics of dormant cells in that they are slow-or non-growing (10), lacking noticeable expression of degradable GFP indicating low levels of transcription (94). It is premature to speculate whether the drug tolerance of surviving *C. albicans* cells reported in this study is also caused by dormancy, but it is useful to note that dormancy has been documented in fungi. The ascospore of *Saccharomyces cerevisiae* is the best-studied dormant form of yeast. As a typical spore, it is highly tolerant to a wide range of noxious factors (73). *C. albicans* produces chlamydospores, a thick walled asexual form derived from hyphae (67). However, chlamydospores are not known to confer long term viability and do not appear to be present at sites of infection or in biofilms (44, 67). Another dormant form of yeast, known as quiescence, occurs after 5-7 days of incubation of *S. cerevisiae* at 30°C in complete medium (37). Quiescent cells exhibit increased thermotolerance, osmotolerance and tolerance to a variety of cell wall acting agents compared to dividing yeasts (23, 37, 79). Several genes are known to be involved in the entry into, the exit from, or the maintenance of quiescence (37). However, it is unknown whether these cells are tolerant to antifungals.

Future studies will show whether yeast persisters are dormant cells, and have any connection to the previously described dormant forms. For now, we may say that yeasts and bacteria have evolved an analogous survival strategy through convergent evolution that assigns the function of survival to a small part
of the population. The fact that persisters in yeast are specific to biofilms suggests they are not the result of “mistakes” such as random overexpression of non-specific proteins that may lead to stasis, as has recently been suggested for bacteria (109). The finding of a persister subpopulation in *C. albicans* will help to solve the puzzle of biofilm resistance to antifungals, and the method of isolating persisters reported in this study provides a powerful tool to search for persister genes.
3.0 Imidazole antifungals are lethal to *C. albicans* biofilms in combination with MDR inhibition.

3.1 Introduction

Persisters were characterized in *Candida albicans* biofilms since cells demonstrated biphasic killing response to increased concentrations of amphotericin B and chlorhexidine. Increased concentrations of chlorhexidine eventually overwhelmed persisters and eradicated the biofilm (Figure 2.1B). While persisters are much more tolerant compared to the bulk of the cells, they are not impervious to antimicrobial therapy.

Miconazole is an imidazole antifungal that is used for topical treatment of vulvovaginal candidiasis. Miconazole is reported to have multiple mechanisms of action and lethal effects at increased concentrations. It is of significant interest to know if increased concentrations of miconazole can be used to kill biofilms and persisters. Unfortunately, miconazole has poor water solubility, limiting its use *in vitro*. In order to overcome this limitation, we reasoned that inhibition of multidrug resistance (MDR) efflux pumps could increase the amount of miconazole available to the cells. Perhaps, the combination of MDR inhibition and subsequent treatment with miconazole can eradicate biofilms and persisters of *Candida albicans*. 
3.2 Abstract

Imidazole antifungals are known to cause growth arrest by ergosterol depletion and killing of growing cells has been reported at higher concentrations. Biofilms, on the other hand, are notoriously resistant to the azoles and no killing effects have been reported. We hypothesized the lethal action of azoles may be effective against biofilms if combined with multidrug efflux pump inhibition. This strategy could be particularly useful in treating oropharyngeal and vulvovaginal candidiasis, in which azoles can be administered at high concentrations topically. *Candida albicans* biofilms were grown in 96-well plates for 24 hours at 37°C in RPMI 1640 growth medium. Spent medium was replaced with fresh medium containing antimicrobials and biofilms were incubated for an additional 48 hours. Biofilms were subsequently washed, disrupted, diluted and plated for colony forming units. When a panel of azole drugs was tested against wild type *Candida albicans* biofilms, no killing was observed at any concentration tested. However, biofilms of a strain deleted in many known multidrug efflux transporters were eradicated at concentrations as low as 50 µM clotrimazole and 200 µM miconazole. Fluconazole did not exhibit lethality, which was subsequently found to be dependent on the deletion of the ABC transporters, Cdr1p and Cdr2p. Next disulfiram, a Cdr1p inhibitor that is used clinically to treat alcoholism, was tested for wild type biofilm killing in the presence of miconazole. The combination of disulfiram and miconazole produced potent killing of the biofilms, despite reported observations that disulfiram treatment alone produces thicker biofilms of greater biomass. Imidazole antifungals, widely regarded as static inhibitors of
growth, are thus lethal when combined with drug efflux inhibition and could be exploited to treat biofilm infection.

3.3 Materials and Methods

Yeast strains and growth conditions. Strains used in this study are listed in Table 3.1. C. albicans was streaked onto yeast extract, peptone, dextrose (YPD) agar from frozen stocks (-80°C) and incubated at 37°C for 24-48 hours. Biofilms were grown essentially as described (83) in the bottom of polystyrene 96-well microtiter plate wells. Briefly, cells were grown overnight at 30°C in YPD liquid medium. Samples were harvested by centrifugation, washed with sterile PBS and resuspended in RPMI 1640 with L-Glutamine and 0.165M MOPS (BioWhittaker™). The optical density of the culture (at 600nm) was adjusted to 0.1 by dilution with RPMI 1640, an equivalent of approximately 1 X 10^6 cells/ml. 100 µl of this suspension was aliquoted into 300 µl wells of a flat bottomed microtiter plate (Corning Costar® 3370). Plates were incubated for 24 hours at 37°C on a microtiter plate shaker (Lab-Line Instruments, model 4625) at approximately 100 rpm.
Table 3.1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF2-1</td>
<td>(\Delta ura3::imm434/URA3)</td>
<td>(29)</td>
</tr>
<tr>
<td>DSY448</td>
<td>(\Delta cdr1::hisG-URA3-hisG\Delta cdr1::hisG)</td>
<td>(93)</td>
</tr>
<tr>
<td>DSY653</td>
<td>(\Delta cdr2::hisG-URA3-hisG\Delta cdr2::hisG)</td>
<td>(92)</td>
</tr>
<tr>
<td>DSY465</td>
<td>(\Delta mdr1::hisG-URA3-hisG\Delta mdr1::hisG)</td>
<td>(93)</td>
</tr>
<tr>
<td>DSY654</td>
<td>(\Delta cdr1::hisG\Delta cdr1::hisG \Delta cdr2::hisG-URA3-hisG\Delta cdr2::hisG)</td>
<td>(92)</td>
</tr>
<tr>
<td>DSY1050</td>
<td>(\Delta cdr1::hisG/\Delta cdr1::hisG \Delta cdr2::hisG/\Delta cdr2::hisG \Delta mdr1::hisG-URA3-hisG/\Delta mdr1::hisG)</td>
<td>(70)</td>
</tr>
<tr>
<td>DSY1024</td>
<td>(\Delta cdr1::hisG/\Delta cdr1::hisG \Delta cdr2::hisG/\Delta cdr2::hisG \Delta camdr1::hisG/\Delta camdr1::hisG\Delta flu1::hisG/\Delta flu1::hisG-URA3-hisG)</td>
<td>(66)</td>
</tr>
</tbody>
</table>

Antifungal susceptibility testing. Miconazole (M3512), clotrimazole (C6019) and fluconazole (F8929) were obtained from Sigma-Aldrich and stock solutions were prepared in DMSO. Biofilms were washed with sterile PBS in order to remove non-adherent cells. Antifungals were aliquotted into fresh growth medium and then added to biofilms at the desired concentrations from 0 to 300 \(\mu\)M, 100 \(\mu\)l per well. After biofilms were exposed to antifungals for 48 hours, the medium was removed and the biofilms were washed three times with sterile PBS. The metabolic activity of biofilms was measured as described previously by adding 100 \(\mu\)l of a 10% alamar blue (Serotec) solution in PBS (88). The biofilm plates were incubated at 37°C for 4 hours and the level of alamar blue reduction
was measured using a fluorescent plate reader with an excitation and emission spectrum of 544 nm and 590 nm, respectively. Percent metabolic inhibition of biofilms was calculated by comparing drug treatments to untreated controls for each strain. Fractional inhibitory concentrations (FIC) were determined using a 50% metabolic inhibition cutoff compared to untreated biofilm controls. FIC = (concentration of drug A in combination / drug A alone) + (concentration of drug B in combination / drug B alone). An FIC < 0.5 = synergy, 0.5 < FIC > 2 = indifference, and FIC > 2 = antagonism. Biofilm colony counts were made by disruption and scraping the biofilm with a pipette tip. The mixture was transferred into an eppendorf tube along with 100 µl of PBS. Biofilms were vortexed vigorously for 30 seconds, serially diluted, and plated on YPD. Plates were incubated for 24-48 hours prior to colony counting.

### 3.4 Results

**Imidazoles are lethal to C. albicans MDR deleted biofilms.** Dose dependent killing of a biofilm is a straightforward way to measure its antifungal susceptibility. Biofilms are notoriously refractory to azole treatment, which causes inhibition of growth in planktonic cultures by blocking ergosterol production. As expected, a panel of azoles including miconazole, clotrimazole and fluconazole did not cause a reduction in biofilm colony forming units (Fig. 3.1). However, biofilms of a strain deleted in known drug efflux transporters, DSY1024 (cdr1△::hisG/cdr1△::hisG cdr2△::hisG/cdr2△::hisG camdr1△::hisG/camdr1△ ::hisG flu1△::hisG/flu1△::hisG-URA3-hisG) were killed by clotrimazole and miconazole
at concentrations as low as 50 µM and 150 µM, respectively (Fig. 3.1). Biofilms of strain DSY1024 were unaffected by fluconazole (Fig. 3.1) despite the fact that the drug is a known substrate for many of these transporters (60, 81). Miconazole and clotrimazole are reported to have secondary mechanisms of action (50, 80, 106), which may be responsible for biofilm killing. However, no miconazole or clotrimazole killing was detected in the wild type strain at any concentration tested. Presumably, higher concentrations of these drugs would saturate drug efflux transporters and result in killing similar to the efflux-attenuated strain. The failure to observe killing in the wild type strain may result from limited drug solubility and failure to overwhelm drug efflux pumps in vitro.

Figure 3.1. Survival of *C. albicans* wild type and MDR deficient biofilms challenged with azole antifungals. CAF2-1 (solid) and DSY1024 (outlined) biofilms were challenged with fluconazole (▲), miconazole (■), or clotrimazole (●) for 48 hours. Biofilms were washed and sampled for CFU following antifungal exposure. The data is representative of three independent
experiments performed in duplicate and the error bars represent standard deviations.

ΔCdr1p and ΔCdr2p are required for imidazole biofilm killing. Does disabling either a specific transporter or combinations of transporters facilitate biofilm killing by miconazole or clotrimazole? To answer this question, biofilms of mutant strains lacking either individual transporters or combinations of transporters were treated with increasing concentrations of miconazole. Alamar blue was used to measure in vitro antifungal efficacy for these experiments, due to the large number of strains and concentrations tested. Metabolic enzymes reduce alamar blue to a fluorescent product that is proportional to the number of live Candida cells (88). Only biofilms of those strains lacking both Cdr1p and Cdr2p were susceptible to miconazole according to alamar blue reduction (Fig. 3.2).
Figure 3.2. Metabolic activity of wild type and MDR deficient *Candida albicans* biofilms challenged with miconazole. Wild type and MDR deleted biofilms were grown in RPMI 1640 medium and challenged with miconazole for 48 hours and metabolic activity was assayed using alamar blue. The percent of alamar blue reduced was calculated by measuring the fluorescent signal for each strain and miconazole concentration, compared to its untreated control. The graph is representative of three independent experiments performed in duplicate and the error bars represent standard deviations.

Notably, the wild type strain and even those deleted in Cdr1p and Cdr2 individually exhibited less than a 10% decrease in alamar blue reduction at a concentration of 250µM miconazole.

**Combination miconazole and disulfiram biofilm killing is due to MDR inhibition.** There has been considerable interest in identifying and obtaining chemical inhibitors of drug efflux pumps (41). Such compounds synergize with conventional antimicrobials, increasing their efficacy and preventing resistance. Disulfiram, used clinically to treat alcoholism, is also a Cdr1p inhibitor in *C. albicans* (96). We wanted to learn whether disulfiram synergizes with miconazole and kills wild type biofilms. Disulfiram was not lethal to biofilms alone, but caused a significant reduction in CFU when combined with miconazole (Figure 3.3).
Figure 3.3. Survival of CAF2-1 and DSY654 (Δcdr1, Δcdr2) biofilms challenged with miconazole alone or miconazole and a fixed concentration (300 µg/ml) of disulfiram (CAF2-1) for 48 hours. Biofilms were washed and sampled for CFU following drug treatment. The data is representative of three independent experiments performed in duplicate and the error bars represent standard deviations.

Increased concentrations of miconazole combined with a fixed concentration of disulfiram (300 µg/ml) caused a reduction in CFU, which mimicked the action of miconazole alone on a strain deleted in both ΔCdr1p and ΔCdr2p (Fig. 3.3). However, disulfiram has been reported to have direct antifungal activity (48), so it was important to test whether disulfiram was acting through MDR inhibition. Therefore a checkerboard assay was modified to test the antimicrobial synergy of miconazole and disulfiram against biofilms for the wild type and strain DSY 654 deleted in Cdr1p and Cdr2p. In this experiment, miconazole was diluted 2-fold
along the y-axis of a microtiter plate and disulfiram was diluted 2-fold along the 50 x-axis to produce unique combinations of compounds in each well. After antimicrobial exposure, biofilm metabolic inhibition was measured using alamar blue. The fractional inhibitory concentrations (FIC) were calculated using a 50% metabolic inhibition as an activity cutoff. In the wild type strain, an FIC of 0.375 was indicative of synergy, while a higher FIC of 1.0 in DSY654 biofilms indicated indifference. The lethal action of disulfiram and miconazole on biofilms is thus concluded to be due to disulfiram inhibition of drug efflux transporters.

3.5 Discussion

The discovery of imidazole killing of *Candida albicans* biofilms when combined with MDR inhibition is surprising considering azoles are widely regarded as static inhibitors of growth. High concentrations of miconazole have been reported to be lethal, but only for exponentially growing populations of cells (12). Yet, in this study we report miconazole had a drastic effect on biofilms when combined with MDR inhibition. One explanation might involve the fact that imidazole antifungals are reported to have multiple mechanisms of action in addition to ergosterol depletion, such as the generation of reactive oxygen species (50), inhibition of mitochondrial ATPase (80) and deleterious effects on the actin cytoskeleton (106). These secondary effects may cause biofilm killing when drug efflux transporters are inhibited, since ergosterol depletion alone is growth inhibitory. In this study, wild type biofilms were completely protected from increasing imidazole concentrations by the action of MDR transporters. In theory, such transporters could be saturated and overwhelmed by increased drug concentrations.
However, imidazoles have poor water solubility (104) — it is likely the efflux pumps of wild type biofilms were not saturated as they appeared completely invulnerable to increased concentrations of the drug. In contrast, when drug efflux was inhibited genetically (knockout) or chemically (MDR inhibitor), increased imidazole concentrations exerted their lethal effects. High concentrations of fluconazole did not kill biofilms, even combined with MDR inhibition. Interestingly, the newer triazole antifungals, such as fluconazole, have been optimized through medicinal chemistry for increased water solubility and specificity for alpha-14 lanosterol demethylase (65). It is possible the lethal effects of the imidazoles have been unintentionally sacrificed as a result of the chemical optimization strategies for the newer triazoles. It may prove interesting to test other triazoles, such as ravuconazole and posaconazole, for biofilm killing in the presence of MDR inhibition.

The therapeutic use of an imidazole antifungal and a MDR inhibitor to kill biofilms may be possible if a suitable MDR inhibitor is found. Data presented in this study revealed that inhibition of both Cdr1p and Cdr2p is necessary for biofilm killing. These transporters exhibit considerable homology and substrate overlap, however substrate specificity has also been demonstrated (33). For example, disulfiram and enniatin have been reported to be potent inhibitors of only Cdr1p and not Cdr2p (60, 96). Data from this study suggests a much higher concentration of disulfiram (300 µg/ml) is needed to additionally inhibit Cdr2p and cause biofilm killing. Although disulfiram was reported to have direct activity on exponentially grown *C. albicans*, the concentration of disulfiram used in this study
(300 µg/ml) did not have any effect on *Candida* biofilm survival as measure by CFU counts. In addition, the loss of miconazole and disulfiram potentiation in strain DSY 654 (Δcdr1, Δcdr2) as measured by the biofilm checkerboard assay suggests potentiation is the result of MDR inhibition and not direct activity. Currently, there are no known inhibitors of Cdr2p that are potent, specific and commercially available. However, it was recently reported that low concentrations (µg range) of milbemycin derivatives alpha 20, 25 and beta 11 inhibit *Candida* Cdr1p and Cdr2p when these MDR pumps are overexpressed in *Saccharomyces cerevisiae* (60). These compounds have been requested and will be tested for biofilm killing in the presence to miconazole. If a suitable MDR inhibitor is found, subsequent studies will be undertaken to determine whether surviving persister cells are present after combinational therapy with miconazole. Data presented in this work suggest that drug efflux is an important factor limiting imidazole lethality of biofilms.
4.0 Screen for small molecule miconazole potentiators that kill *C. albicans* biofilms and persisters.

4.1 Introduction

Vaginal yeast infections are contracted by essentially 100 percent of females at some point in their lives. These infections are caused primarily by *C. albicans* and most cases are easily treatable with azoles, but 8-9% resist therapy, producing a chronic relapsing disease (97, 98). The standard measure of efficacy for antifungal agents is determination of MIC or MFC in a rapidly growing planktonic population. Indeed, this is how currently available antifungals have been validated. It is becoming increasingly apparent that *in vivo* infection is considerably more complex, and cannot be approximated by an exponentially growing population of the pathogen (18, 61). Non-growing, or slow growing populations are considerably more tolerant to antimicrobials than growing cells (18). We recently discovered that upon attachment, *C. albicans* forms a small subpopulation (~1%) of persister cells that are completely tolerant to currently used systemic antifungals, and resemble well-characterized dormant persisters formed by pathogenic bacteria (47, 61, 94, 99, 100). For *Candida*, attachment to surface is an important step in fungal pathogenesis, including vaginitis (22, 34). In addition, biofilms contain both persisters and stationary cells, and represent a common form of many fungal infections (8, 22, 25, 34, 43, 51, 55, 57, 58, 64, 69, 71, 82, 87). Adherence of *C. albicans* to epithelial cells could produce persisters,
and account for the recalcitrance of the infection to antimicrobials. It is also possible that, in a manner similar to infections of indwelling devices, adhered cells will grow into a biofilm which restricts penetration of immune system components, further complicating treatment. Currently, high doses of topically applied miconazole (Monistat®) are used as an alternative to systemic fluconazole treatment for vaginitis. Chlorhexidine, a membrane-acting antiseptic, has considerable side effects and is poorly tolerated (113), especially at levels necessary to eliminate persisters in vitro (59). Fluconazole has no microbicidal activity against *C. albicans*, while miconazole is able to kill growing planktonic cells. However, miconazole does not have a microbicidal effect on biofilms or persisters. This could account for the limited efficacy of Monistat® in treating relapsing vaginitis.

The most effective antifungals such as miconazole, amphotericin B and caspofungin are completely inactive against *C. albicans* persisters (59), suggesting that it is unlikely to discover a single “sterilizing” compound. A molecule that disables the mechanism of formation or maintenance of quiescent cells could synergize with a conventional antimicrobial and lead to biofilm eradication.
4.2 Abstract

*Candida albicans* is an opportunistic pathogen, which frequently colonizes implanted medical devices such as central venous catheters and heart valves as a biofilm. Biofilms are essentially untreatable and infection requires device removal. It was previously reported that a subpopulation of drug tolerant persister cells forms within biofilms upon attachment and likely contributes to antimicrobial failure. We have identified small molecule potentiators of the antifungal miconazole, which together kill biofilms and persisters, while biofilms are completely resistant to miconazole alone. This is the first validated high-throughput screen of *Candida albicans* biofilms. The screen used a metabolic indicator alamar blue as a measure of biofilm survival and hits were validated by counting colony forming units (CFU). Of 68,950 compounds, 323 reduced metabolic activity of biofilms in the presence of miconazole by more than 25 percent. 24 compounds with the highest activity were tested for biofilm killing alone and in the presence of miconazole. Two compounds were identified that had no direct activity against exponential or biofilm cells, but produced over 1000 fold reduction of biofilms CFU in combination with miconazole. Importantly, one of these compounds, a hydrazone, completely eradicated biofilms and persisters. These small molecules could lead to the development of therapeutics that enhance the activity of currently available antifungals and sterilize infection.
4.3 **Materials and Methods**

**Yeast strains and growth conditions.** Yeast strains used in this study are *Candida albicans* CAF2-1 (\(\Delta\text{ura3::imm434/URA3}\)) and *Saccharomyces cerevisiae* BY4742 (MATalpha his3 \(\Delta\) 1 leu2 \(\Delta\) 0 lys2 \(\Delta\) 0 ura3 \(\Delta\) 0). *C. albicans* and *S. cerevisiae* was streaked onto yeast extract, peptone, dextrose (YPD) agar from frozen stocks (-80°C) and incubated at 37°C or 30°C for 24-48 hours. Biofilms were grown essentially as described by Ramage et al. (2001). Briefly, cells were grown overnight at 30°C in YPD liquid medium. Samples were harvested by centrifugation, washed 2X in sterile PBS pH 7.4 and resuspended in RPMI 1640 with L-Glutamine and 0.165M MOPS (BioWhittaker™, East Rutherford, N.J). The optical density of the culture (at 600nm) was adjusted to 0.1 by dilution with RPMI 1640, which is equivalent to approximately 1 \(\times 10^6\) cells/ml. Either 100 µl or 30 µl of this suspension was aliquoted into wells of a flat bottomed microtiter plates (Corning Costar® 3370 or Corning Costar® 3310). Plates were incubated for 48 hours at 37°C.

**Pilot screen for miconazole potentiators.** Corning Costar® 3370 microtiter plate wells were seeded with 100 µl *C. albicans* strain CAF 2-1 diluted in RPMI 1640 growth medium. Biofilms were grown for 48 hours at 37°C, washed with PBS pH 7.4 and resuspended in a final volume of 100 µl RPMI 1640 containing 10 µg/ml miconazole. Individual compounds from the commercially available chemical library ChemBridge DiverSetE (San Diego, CA) were then added at 10 µg/ml to the wells. Plates were incubated for 24 hours and XTT with 1% PMS (Sigma-X4751) was added at 1 mg/ml to each well. After 4 hours of
incubation at 37°C, reduction of XTT was monitored by recording the optical density at 690 nm (subtracted from OD450) with a microtiter plate reader. Hits were identified when XTT reduction was inhibited by more than 30% of the negative control (miconazole no compound) values.

**HTS screen for miconazole potentiators.** The HTS screen was performed at the Institute of Chemistry and Cell Biology Longwood at the Harvard Medical School in Boston, MA. Detailed descriptions of liquid handling and pin transfer equipment, as well as compound library information can be obtained at http://iccb.med.harvard.edu/. Corning Costar® 3310 microtiter plate wells were seeded with 30 µl *C. albicans* strain CAF 2-1 using a WellMate (Matrix Technologies, Hudson, NH) microfill apparatus. Biofilms were grown for 48 hours at 37°C. Spent medium was removed from the biofilms by dumping and replaced with 30 µl of fresh RPMI 1640 medium containing 100 µg/ml miconazole nitrate (Sigma M3512). 100 nl of compounds (5 mg/ml in DMSO) were transferred to the biofilms using an Epson Robotics (Carson, CA) platform. Biofilms were incubated for an additional 48 hours at 37°C. Medium containing miconazole and compounds was removed by dumping and replaced with phosphate buffered saline containing 10% alamar blue (AbD Serotec, Kidlington, UK). Plates were incubated for at least 6 hours at 37°C and fluorescent intensities were measured using an EnVision (Perkin Elmer, Waltham, MA) plate reader with 535 nm excitation and 590 nm emission filters with attenuators. Hits were scored based on the percent inhibition compared to the average negative control fluorescent intensity for each plate, which contained 100 µg/ml miconazole alone and no
compounds. Each compound plate was screened in duplicate and wells from both plates had to score greater than 75%, 50%, or 25% inhibition in order to be scored as strong, medium, or weak hits, respectively.

**Biofilm antibiotic susceptibility testing.** Miconazole nitrate was obtained from Sigma Aldrich (Saint Louis, MO) and stock solutions prepared in DMSO. AC15 and AC16 were obtained from chemical suppliers Asinex (Moscow, Russia) and Enamine (Kiev, Ukraine) respectively, and dissolved in DMSO. Biofilms were washed twice with sterile PBS in order to remove non-adherent cells prior to antifungal challenge. PBS was replaced with 100 µl medium containing antifungals and/or compounds at the appropriate concentrations. After 48 hours of incubation at 37°C, biofilms were disrupted by scraping, transferred to an eppendorf tube, and vortexed vigorously for 30 seconds before serial dilution and plating. Plates were incubated for 24-48 hours prior to colony counting.

**Agar diffusion assays.** Sterile filter disks were placed on top agar containing 1X10⁶ CFU/ml exponentially growing *S. cerevisiae* cells. Filter disks were soaked in 15 µg of miconazole (mic), and/or 30 µg potentiators, while DMSO levels were identical for each disk (4.5 µl). Plates were incubated for 24 hours at 37°C and then photographed. Horizontal and vertical diameters of each zone of inhibition were measured and averaged (shown) using Axiovision software.

**MDR inhibition assay.** 2-fold serial dilutions of fluconazole (Sigma-F8929) were made with YPD medium, and YPD medium containing 10 µg/ml
An equal volume of exponentially growing *C. albicans* strains CAF2-1 or DSY1024 was added to the fluconazole dilutions so final concentrations of cells was $1 \times 10^3$ and compound was 5 µg/ml. Plates were incubated overnight at 37°C and endpoint determinations were made both visually and using optical density measured with a spectrophotometer at 600nm.

### 4.4 Results

**Pilot screen for miconazole potentiators.** Given that known antifungals are inactive against persisters (with the exception of high levels of the antiseptic chlorhexidine), we reasoned that a combination of a compound disabling persister formation with a conventional antifungal would lead to eradication of the infection. To test this hypothesis, we developed a screen for potentiators of miconazole, the active component of Monistat®, a leading topical therapeutic for vaginitis.

The rationale of the screen is to add compounds from a chemical library to biofilms in the presence of miconazole (which has no killing activity against biofilms), and measure the effect on *C. albicans* cells. This primary screen does not discriminate between directly acting compounds and those that potentiate miconazole. Subsequent validation of primary hits by a checkerboard assay (see below) then allows identification of synergistically acting compounds. In order to identify compounds that are active against all forms of cells including biofilms and persisters, the screen was developed against a biofilm population. In order to make the screen compatible with high-throughput approaches, biofilms were
grown in microtiter plates and reduction of a vital dye XTT, commonly used to monitor yeast viability, was used as the quantitative readout.

Wild type *C. albicans* treated with miconazole alone at 10 µg/ml provided a negative control, which showed good XTT reduction, indistinguishable from biofilms with no miconazole. This concentration is over 300 fold higher than the MIC of exponentially growing cells (0.03 µg/ml), yet is completely innocuous to the biofilm (the concentration of miconazole in Monistat is 20 mg/ml). Miconazole was added to test wells at 10 µg/ml, and then compounds from the ChemBridge library were added to the same wells at 10 µg/ml. After 4 hours of incubation, the plates were transferred to a microtiter plate reader to quantify the amount of XTT reduction (Fig. 4.1).

Figure 4.1. Pilot screen for miconazole potentiators. Microtiter plate wells were seeded with wild type *C. albicans* strain CAF 2-1. Biofilms were grown for 48 hours at 37°C, washed with PBS pH 7.4 and resuspended in a final volume of 100 µl RPMI containing 10 µg/ml miconazole. Individual compounds from ChemBridge DiverSetE were then added at 10 µg/ml to the wells. Plates were
incubated for 24 hours and XTT (Sigma-X4751) was added at 1 mg/ml to each well. After 4 hours of incubation at 37°C, reduction of XTT was monitored by recording the optical density at 690 nm (subtracted from OD450) with a microtiter plate reader. Hits were identified when XTT reduction was inhibited by more than 30% of the control values.

The pilot screen of 5,000 compounds produced 32 hits, with a hit rate of 0.64%. Upon re-evaluation, 13 of the 32 were confirmed, giving a rate of verified hits of 0.26% (false positive rate 0.38%). A checkerboard assay was then performed to examine possible synergy between miconazole and the hits. An example of this analysis is given in Fig. 4.2 with compound AC9. A clear synergy can be seen and the inhibition of metabolic activity as reported by XTT was evident with AC9 at concentrations as low as 3.1 µg/ml in the presence of 12.5 µg/ml miconazole.

Figure 4.2. C. albicans biofilm checkerboard assay with miconazole and compound AC9. 100 µg/ml miconazole (2X dilution in each subsequent well, y-
axis) and 100 µg/ml compound AC9 (2X dilution, x-axis) were added to mature 62 48 hour wild type biofilms and incubated for an additional 24 hours in the presence of the drugs. XTT was added to each well at 1 mg/ml and biofilms were incubated at 37°C for an additional 4 hours. Biofilms in wells above and to the right of the dashed line (appearing clear) have OD₄₅₀ – OD₆₉₀ values less than 0.240 and are metabolically inhibited, compared to wells (red) outside of the dashed line with OD₄₅₀ – OD₆₉₀ >0.240.

Importantly, miconazole or AC9 alone did not inhibit metabolic activity at any concentration tested. One of the hits had direct activity. The fact that only 1 out of 13 hits had direct activity is encouraging since the screened was aimed at finding miconazole potentiators. We intentionally performed the primary screen at a rather low concentration of test compounds, 10 µg/ml. This, together with the high level of biofilm resistance, strongly decreased the probability of finding compounds with direct activity.

Next, the concentrations of miconazole and AC9 that showed activity according to the checkerboard assay was used to test the ability of the hit compounds to potentiate biofilm killing by miconazole. The single compound that showed direct activity had no effect on killing of the biofilm cells either alone or in the presence of miconazole. This is not surprising – as we have mentioned, it is probably unrealistic to find single-acting compounds (apart from antiseptics and disinfectants) that will kill a biofilm or persister cells, since no known antifungals have this capability. One of the 12 compounds, AC9 ((2-pyridinyldiazenyl)-1-
naphthol) showed killing in the presence of miconazole. Complete eradication of the *C. albicans* biofilm was observed in the presence of 200 µg/ml AC9 and 100-200 µg/ml miconazole (Fig. 4.3). Importantly, neither miconazole nor AC9 alone had any killing activity against the biofilm. This to our knowledge is the first example of biofilm eradication by compounds that are not generally toxic antiseptics. This result demonstrates the feasibility of developing a synergistic therapy capable of eradicating, rather than merely suppressing, biofilm infections. This is an important proof-of-principle for the proposed approach, and more potent potentiators have been identified in a subsequent HTS screen.

Figure 4.3. Killing of *C. albicans* biofilms by miconazole and AC-9. Wild type mature biofilms were challenged with 100 µg/ml miconazole, 200 µg/ml compound AC-9, or a combination of the two for 24 hours. Biofilms were washed, scraped, resuspended in PBS, vortexed for 30 seconds and plated for colony forming units. The dashed line indicates the limit of detection.
Based on the literature and the structure of AC9, it is tempting to speculate that this compound chelates metal ions. Indeed, metal chelators such as ferrozine, are known to cause increased membrane fluidity and azole drug susceptibility against exponentially growing *Candida* (Prasad, 2006). Given the relative high concentrations of AC9 (200 µg/ml) required for complete biofilm killing, and the fact that some metal chelators are toxic to mammals, a larger high-throughput screen was undertaken to find more potent miconazole potentiators.

**High-throughput screen for miconazole potentiators.** The encouraging results of this pilot screen enabled the development of a HTS aimed at discovering potentiators of miconazole with increased potency. The biofilm screen was adapted to a 384 well format, and alamar blue was used as the metabolic indicator replacing XTT, which was used in the pilot screen. Alamar blue produced more consistent results and a higher signal to background ratio compared to XTT. The general screening diagram is shown in Figure 4.4.
Figure 4.4. Schematic of biofilm screen for potentiatators of miconazole. 1) Biofilms were formed by seeding 30 µl of *C. albicans* OD600= 0.1 in RPMI 1640 medium into 384-well plates. 2) Plates were incubated for 48 hours at 37°C and then the spent medium was replaced with fresh medium containing 100 µg/ml miconazole. The biofilms remain attached to the bottoms of the plate wells. 3) Unique compounds from the chemical libraries were pin transferred at a final concentration of 17 µg/ml into individual wells of the microtiter plate. 4) Biofilms were incubated for an additional 48 hours and the medium was replaced with PBS containing 10% alamar blue. 5) Plates were incubated at 37°C for an
additional 6 hours and alamar blue reduction was measured with a fluorescent plate reader with an excitation of 544 and an emission at 590 nm respectively. In order to measure the reproducibility of the HTS screen, a Z' was obtained by using the following formula:

\[
Z' = 1 - \frac{3SD_+ + 3SD_-}{I_{Ave} - Ave_{Ave}}
\]

Where:
- \(SD_+\) = positive control standard deviation
- \(SD_-\) = negative control standard deviation
- \(Ave_+\) = positive control average
- \(Ave_-\) = negative control average

In the Z' experiment, negative control biofilms were treated with 100 µg/ml miconazole, while positive control biofilms were exposed to 100 µg/ml miconazole and 50 µg/ml chlorhexidine. Chlorhexidine is an antiseptic and the only compound known to effectively kill biofilms and persister cells (Fig. 2.1). The Z' was calculated by measuring the change in fluorescence produced by the reduction of alamar blue by metabolic enzymes using a fluorescent plate reader. A Z' of 0.80 was obtained and indicated a good, highly reproducible HTS. Another advantage of alamar blue is that increased fluorescence is accompanied by a prominent color change, from blue to red. A typical screening plate from the HTS is shown in Figure 4.5.
Figure 4.5. A HTS plate screened for miconazole potentiators. *C. albicans* was inoculated into RPMI 1640 medium and dispensed at 30 µl per well into a 384 well plate. After 48 hours of incubation at 37°C, the spent medium was replaced with fresh medium containing 100 µg/ml miconazole. Test compounds were then introduced at 17 µg/ml by pin transfer into duplicate plates. After an additional 48 hours of incubation at 37°C, the medium was replaced with PBS containing 10% alamar blue. The photograph was taken after 6 hours of incubation at 37°C; two hits are apparent.

Approximately 70,000 compounds were screened in duplicate as shown in Fig. 4.6. The screen produced 6 strong hits (inhibition of alamar blue reduction >75%) and 52 medium hits (50% < inhibition > 75%), which were examined further. The details of the screen results are given in Table 4.1.
Figure 4.6. Metabolic activity of biofilms challenged with miconazole and small molecules from chemical libraries. *C. albicans* was inoculated into RPMI 1640 medium and dispensed at 30 µl per well into a 384 well plate. After 48 hours of incubation at 37° C, mature biofilms adhering to the bottom of the wells were evident, and the spent medium was replaced with fresh medium containing 100 µg/ml miconazole. Test compounds were then introduced at 17 µg/ml by pin transfer into duplicate plates. After an additional 48 hours of incubation at 37° C, the medium was replaced with PBS containing 10% alamar blue. After 6 hours of incubation at 37° C, alamar blue reduction was measured with 535nm excitation, 590nm emission with a fluorescent plate reader. Hits were determined based on percent inhibition as compared to the negative control (biofilms + miconazole, no test compound) columns in each plate. Results from compound plate #1738 are shown as a representative example. Indicated are results from duplicate plates A and B; a strong and a weak hit; cutoffs for 50% and 75% inhibition.
Table 4.1 Hit summary of the HTS for miconazole potentiators. Conditions as described in legend, Fig. 4.4. Strong (S) hits had greater than 75% inhibition, while medium (M) hits had greater than 50% inhibition and weak (W) hits had greater than 25% inhibition.

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<th>M</th>
<th>W</th>
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The most potent hits were examined for their ability to kill biofilms in the presence of miconazole (100 µg/ml), by measuring colony count. Compound AC15 completely eradicated biofilms at concentrations as low as 40 µg/ml and AC16 produced 3 logs of biofilms killing at a concentration of 10 µg/ml (Figure 4.7). The structures of these compounds are listed in Figure 4.8. Importantly, these compounds had no activity against biofilms alone, and even more impressively,
had no activity on growth of \textit{C. albicans} at concentrations as high as 250 \textmu g/ml when measured by a standard broth microdilution assay.

Figure 4.7. Killing of \textit{C. albicans} biofilms by miconazole and AC15 (A) or AC16 (B). Wild type mature biofilms were challenged various concentrations of compound AC15 or AC16, with or without a fixed concentration of miconazole (100 \textmu g/ml) for 48 hours. Biofilms were washed, scraped, resuspended in PBS, vortexed for 30 seconds and plated for colony forming units.
It was interesting to learn whether compounds AC15 and AC16 were potentiating miconazole due to inhibition of Multi-Drug Resistance (MDR) efflux pumps. MDRs are known to extrude azole antibiotics and their overexpression leads to clinically significant drug resistance (60). Fluconazole is a well-studied substrate of \textit{C. albicans} MDRs, and a MDR inhibitor is expected to potentiate fluconazole in a MIC assay. Table 4.2 shows the MIC of the wild type to fluconazole was unchanged in the presence of AC15 and AC16. By contrast, the MIC of fluconazole for strain DSY1024, deleted in MDR transporters was 0.078 µg/ml. Similarly, a known inhibitor of \textit{C. albicans} MDRs, enniatin B (19, 20), increased susceptibility to fluconazole from 2.5 to 0.156 µg/ml. These data suggest that AC15 and AC16 are not MDR inhibitors. The absence of fluconazole potentiation also suggests that AC15 and AC16 do not target known azole synergistic
pathways, such as calcineurin and HSP90 (19, 20, 40). AC15 and AC16 potentiate the action of miconazole but have no activity on their own. It seems that they target pathways necessary for survival in the presence of miconazole, and importantly the synergy between miconazole and AC15 is sufficient to eliminate persisters.

Table 4.2. Testing MDR inhibition by AC15 and AC16.

<table>
<thead>
<tr>
<th>MIC fluconazole (µg/ml)</th>
<th>Wild type</th>
<th>ΔMDR</th>
<th>Enniatin B</th>
<th>AC15</th>
<th>AC16</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.078</td>
<td>0.156</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

It was also interesting to test whether AC15 and AC16 potentiate miconazole against exponentially growing *Saccharomyces cerevisiae* cells in an agar diffusion assay. Figure 4.9 shows that the zones of inhibition due to miconazole increased from 25 mm to over 28 mm in the presence of either AC15 or AC16. Again, AC15 and AC16 lack direct activity and no zones of inhibition were caused by the presence of either compound alone.

Figure 4.9. *S. cerevisiae* agar diffusion susceptibility to miconazole, AC15 and AC16. Sterile filter disks were placed on top agar containing $1 \times 10^6$ CFU/ml exponentially growing *S. cerevisiae* cells. Filter disks were soaked in 15 µg of
miconazole (mic), and/or 30 µg potentiators, while DMSO levels were identical for each disk (4.5 µl). Plates were incubated for 24 hours at 37°C and then photographed. Horizontal and vertical diameters of each zone of inhibition were measured and averaged (shown) using Axiovision software.

Discussion

The discovery of persisters, which form in C. albicans biofilms upon attachment, raised the possibility that they contribute to antifungal drug failure and recurrent infection. One way to test this hypothesis is to develop therapeutics, which eliminate persisters. Since currently available antifungals fail to kill persisters, it is unlikely to find a single nontoxic agent, which does. Therefore, a screen was developed for small molecules that kill biofilms and persisters in combination with the antifungal miconazole. Miconazole was chosen since it is used to treat vulvovaginal candidiasis of which 8-9% of women suffer from recurrent infection (97, 98). In addition, Candida attachment to the vaginal epithelium is a prerequisite for pathogenesis and may cause persisters to form.

In this study, we report a small molecule that eliminates biofilms and persisters in the presence of miconazole as measured by colony counting. The fact that the compounds identified in this study had no activity against growing cells of C. albicans or S. cerevisiae suggests that they have a specific mode of action and are likely to be non-toxic to mammalian cells as well. Future directions involve testing these compounds alone and together with miconazole in a mouse model of vaginal Candida infection (26-28). The concentrations of these
compounds that completely eradicated biofilms *in vitro* can likely be achieved *in vivo* since drug treatments are administered topically for this model of infection.

Currently the mechanism of action for AC15 and AC16 are unknown. A nonspecific mechanism of action, such as membrane or DNA damage, is unlikely for compounds, which do not have direct activity. Since the compounds do not potentiate fluconazole, they do not appear to act by targeting MDR transporters or calcineurin. Taken together, these compounds may act on a currently unknown pathway important for survival in the presence of miconazole. The fact the miconazole potentiation also occurs in *S. cerevisiae* may be advantageous in determining the mechanism of action of each compound. A variety of methods have been used to find previously unknown drug targets in *S. cerevisiae* including fitness profiling of haploid deletions, haploinsufficiency screening of essential genes, and microarray studies (13).

Increased resistance and antimicrobial drug therapy failure suggests a new strategy is needed and therapeutics should be made to specifically target recurrent, persistent and biofilm infection. This high-throughput whole cell based *C. albicans* biofilm screen was designed in order to meet this goal. While this study used miconazole and targeted recurrent vaginal infection, the basic approach of the screen can be applied to other drugs and difficult to treat infection. For example, candidemia currently has a mortality rate approaching 40%. A similar, biofilm screen could be developed using a systemic antifungal such as the voriconazole. Future studies will focus on the mechanism of action of
the small molecule potentiators identified in this screen and the role of persisters in recurrent infection.
5 Conclusions

There is no treatment currently available for fungal biofilm infection. Clinical guidelines recommend device removal in order to avoid progression of systemic disease, which has a mortality rate approaching 40%. Biofilms are difficult to treat since they are recalcitrant to antifungals and isolated from host immune components. Most antifungals are static inhibitors of growth. These drugs require interactions with the immune system to clear infection are not likely to be useful in treating biofilms. However, even the most effective lethal antifungals, such as amphotericin B and caspofungin, cannot cure biofilm infection.

Biofilm resistance to antifungal drugs has been attributed to many factors. However, none can explain recalcitrance to all existing antifungal agents. The discovery of a persister cell population, which survives high concentrations of amphotericin B and chlorhexidine, suggests persisters could contribute to therapeutic failure for many drugs. Persisters were characterized by biphasic killing in response to increased antifungal dosage. Persisters are phenotypic variants, which form upon attachment to a surface. Future studies will seek to determine the molecular mechanisms responsible for the formation of persister cells.

Persisters eventually succumbed to increasing concentrations of chlorhexidine, a membrane-acting antiseptic. This result suggests it might be possible to kill biofilms and persisters with certain antifungals, if appropriate concentrations are achieved. To test this hypothesis, imidazole antifungals were
combined with MDR inhibition and found to produce potent killing of biofilms. The imidazoles may be useful in treating biofilm infection if a suitable MDR inhibitor, which blocks both Cdr1p and Cdr2p, is found.

In parallel, a systematic approach was undertaken to find small molecules that kill biofilms and persisters in combination with miconazole. These molecules may specifically disable persister formation or maintenance, act as MDR inhibitors or by some other mechanism. A screen of approximately 70,000 compounds produced two lead molecules, one of which completely eliminated biofilm persisters in the presence of miconazole. Importantly, these compounds had no growth inhibitory activity on exponentially growing cells, suggesting they specifically synergize with miconazole and are non-toxic.

The screen for miconazole potentiators, which eliminate persisters, is the first known example of a validated high-throughput screen of *Candida* biofilms. The screen demonstrates the feasibility of specifically targeting and completely eradicating biofilm infection. Future studies will be aimed at determining the mechanism of action of these compounds. Compounds that target genes responsible for persister formation or maintenance could provide important insight regarding the molecular mechanism of persister formation.

In short, the characterization of persisters, has made a significant contribution regarding the mechanism of *Candida* biofilm drug resistance (14, 24, 38, 77). A novel high-throughput screen of *Candida* biofilms has been designed, optimized, implemented and validated. The screen was based on both the
characterization of persisters and novel efforts made in an attempt to kill biofilms using an imidazole antifungal combined with MDR inhibition.
REFERENCES


