# Isolation and Characterization of Echinocandin Resistant *Candida albicans* Mutants

**By**

**Mark Henry Friesen**

A Thesis

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FACULTY OF GRADUATE STUDIES

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ISOLATION AND CHARACTERIZATION OF ECHINOCANDIN RESISTANT

*Candida albicans* MUTANTS

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MARK HENRY FRIESEN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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## List of abbreviations

| | |
|---|---|
| 5-FC | 5-Fluorocytosine |
| 5-FU | 5-Fluorouracil |
| AIDS | Acquired Immune Deficiency Syndrome |
| AM-3 | Antibiotic Media 3 |
| AMB | Amphotericin B |
| ATCC | American Type Culture Collections |
| BHI | Brain Heart Infusion |
| CAP | *Candida* Aspartyl Proteinase |
| CFU | Colony Forming Units |
| CNS | Central Nervous System |
| DMSO | Dimethylsulfoxide |
| EMEM | Eagle's Minimal Essential Medium |
| FLU | Fluconazole |
| GT | Germ tube |
| GTIC | Germ Tube Inhibition Concentration |
| GTP | Guanine Triphosphate |
| HBEC | Human Buccal Epithelial Cells |
| HIV | Human Immunodeficiency Virus |
| $ID_{50}$ | Infectious Dose 50% |
| KETO | Ketoconazole |

| | |
|---|---|
| LY | LY303366 |
| MEC | Minimum Effective Concentration |
| MFC | Minimum Fungicidal Concentration |
| MIC | Minimum Inhibitor Concentration |
| MOPS | 3-[N-Morphilino]Propane-Sulfonic Acid |
| NAG | N-Acetylglucosamine |
| NCCLS | National Committee for Clinical Laboratory Standards |
| NYP | N-Acetylglucosamine Yeast Nitrogen Base Proline Medium |
| OPC | Oropharyngeal candidiasis |
| PAFE | Post Antifungal Effect |
| R | Resistant |
| SAP | Secreted Aspartyl Proteinase |
| SDA | Sabouraud Dextrose Agar |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| SEM | Scanning Electron Microscopy |
| TEM | Transmission Electron Microscopy |
| UDP | Uracil Diphosphate |
| UV | Ultraviolet |

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# I. ABSTRACT

LY303366 (LY) is an investigational echinocandin that has demonstrated activity against *Candida* species. We attempted to isolate an LY resistant mutant by plating the *C. albicans* clinical strain Y41 at a concentration of $10^7$ CFU/plate on Brain Heart Infusion agar (BHI) plates supplemented with LY at concentrations of 1 to 40 µg/ml. After 7 days of incubation at 35°C very small colonies were observed. One colony was selected and repeatedly subcultured on BHI with 1 µg/ml LY. After 5 passages the MIC was demonstrated to be 1.28 µg/ml LY, (wild type MIC = 0.04 µg/ml, an increase of 32X). However, when 6 colonies were individually subcultured to Sabouraud dextrose agar (SDA) plates, only 3 remained resistant to LY. One of the transient mutant strains was evaluated in terms of: antifungal susceptibility (amphotericin B, 5-flucytosine, fluconazole, ketoconazole), growth, stability, and pharmacodynamic time-kill curves. There were no differences observed between parent and transient mutant strains in antifungal susceptibility, or growth patterns. Transient resistant strains reverted back to the parent MIC after 3 passages on SDA. The resistance phenotype was restored when this strain was cultured on BHI with 1 µg/ml LY. The transient resistant strain exhibit regrowth during pharmacodynamic time-kill curves at 1X MIC of LY after this strain had reverted to the wild type MIC. One of the stable mutant strains was evaluated in terms of: antifungal susceptibility (amphotericin B, 5-flucytosine, fluconazole, ketoconazole), morphology, growth, stability, pharmacodynamic time kill-curves, and germ tube formation. There were no differences observed in antifungal susceptibility, morphology, and growth patterns, between parent and stable mutant strains. Pharmacodynamic time-kill

curves were performed on both parent and stable mutant strains at 1X and 10X their respective MICs of LY. There was no difference observed in the amount of kill in either strain. In both strains the minimum concentration needed to inhibit germ tubes was 1/8X MIC. The mutant was passed 19 times and still maintained its elevated MIC. In conclusion, an in vitro mutant resistant to LY was created and demonstrated no change in susceptibility to other antifungals, morphology, growth, or germ tube formation.

# II. INTRODUCTION

## A. *Rise in prevalence of fungal infections*

Fungal infections have historically not had the dramatic impact that bacterial or viral infections have had on the human species. "There are no great pandemics; no equivalents to the plagues, potato famines, yellow fevers, cholera, and Black Deaths that have caused such devastation." [1] This may be part of the reason that medical mycology is an area that is not as well developed as other areas of microbiology. This may also explain the few number of antifungal agents relative to antibacterial antibiotics. However as one author has characterized it, there has been an "insidious epidemic of mycotic infections that has been emerging for the past 15 years." [1] For example, between the year 1980 and 1990 there was a 300% increase in the incidence of candidosis and aspergillosis nosocomial infections. [2] There are a number of reasons for the increase in fungal infections. Fungi tend to be opportunistic pathogens and hence any factor which modifies the normal defenses of the body will increase fungal infections. The biggest factor is immunosuppression stemming from a number of causes including AIDS, leukemia, cancer chemotherapy and organ transplantation. Other factors that have contributed to this increase include invasive surgery, indwelling catheterization, increased use of broad spectrum antibiotics, corticosteroid therapy and total parenteral nutrition therapy. [1] One can see how many of the factors contributing to the rise in fungal infections are iatrogenic in nature, that is they are a result of modern medical procedures. Clearly opportunistic fungal infections are a serious problem on the rise. This reality heightens the need for greater understanding of both fungal pathogens and antifungal compounds.

## B. Fungal cell

Where do fungi fit in the world of the microbes? To answer this question one must look at the taxonomy of microorganisms. The classification of microorganisms can be divided into viruses, procaryotes and eucaryotes. Viruses are obligate intracellular pathogens. The procaryotes include both the archaebacteria and the eubacteria. The eucaryotes include the fungi, protazoa, algae, and multicellular parasites. [3] Some of the differences between eubacteria and fungi are described below. Bacteria have a haploid circular chromosome with no nucleus, lack organelles, have peptidoglygan in their cell walls; and lack sterols in their cell membranes. Fungi have a diploid linear chromosome with a nucleus; contain organelles in their cytoplasm; have glucan, chitin, and mannan as their main cell wall constituents; and contain ergosterol in their cell membrane. Since fungi and mammalian cells are both eucaryotic there are fewer differences to exploit in developing antifungal agents that are not toxic to human cells. The fungal cell wall and the presence of ergosterol in the fungal cell membrane are two areas of divergence with mammalian cells that antifungal compounds can exploit. The term fungi is a kingdom classification with yeast and mold being the two main divisions within the kingdom. Yeasts are unicellular organisms that grow by budding. Molds are multicellular organisms that grow by mycelium (tube-like) formation. There are some fungi that exhibit both phases of growth under different conditions. [4]

The cell wall is an important characteristic of the fungal cell. The cell wall makes up approximately one third of the total cell weight. The cell wall of the yeast *Candida albicans* is made up of 85% carbohydrates. The majority of these are glucan, mannan, and chitin. Two thirds of the total carbohydrate content is glucan, which is a 1,6-β or1,3-β

linked polymer of glucose in *Candida albicans.* [5] However in other species such as *Cryptococcus neoformans* 1,3-α-glucan is the prevalent glucose polymer. [6] Mannan is a 1,6 D-mannose polymer that makes up 20% of the carbohydrate content and primarily appears as mannoproteins. Chitin is a 1,4 linked polymer of N-acetylglucosamine (NAG) which in the blastoconidia (bud-forming) stage has about 1% of carbohydrate content, while in the mycelial phase the chitin content rises to 10%. This has significance because the mycelial phase is traditionally associated with increased virulence (see Figure 1). [7]


**Figure 1: Blastoconidia (B), germ tube (GT) and hyphae (H) of *Candida albicans.*** [8]

The arrangement of these features in the cell wall is not completely understood. However generally the mannoproteins, mannan, and 1,6-β-glucan are thought to make up the outer layer; while 1,3-β-glucan, chitin, and some mannoprotein are thought to make up the inner layer (see Figure 2). [9] It is the mannoproteins that form the major antigenic determinant in *Candida* species. Inner layers provide the structural support for the cell. Some of this support comes from the covalent cross-linking between chitin and glucan.

Cell wall components play a large role in the morphogenesis of the fungal cell. There is clear evidence for modification of cell wall structure during blastoconidia and hyphal growth. One study which examined spheroplast regeneration found that a chitin mesh was laid down initially, upon which the other constituents were built. It was these later parts that determined the shape of the cell. [9] With regard to the mycelial phase of this species there are some clear changes in the yeast cell wall. Besides an increase in chitin there is also a change in the type of β-glucan in the cell during mycelial growth. In the mycelial phase there is an increase in the activity of 1,3-β-glucan synthase. There is a also a change in the cell wall composition, with the 1,3-β-glucan making a larger proportion of the glucan component than 1,6-β-glucan. It is interesting to note that two potential antifungal agents, echinocandins and nikkomycins, inhibit the synthesis of 1,3-β-glucan and chitin respectively. [9]

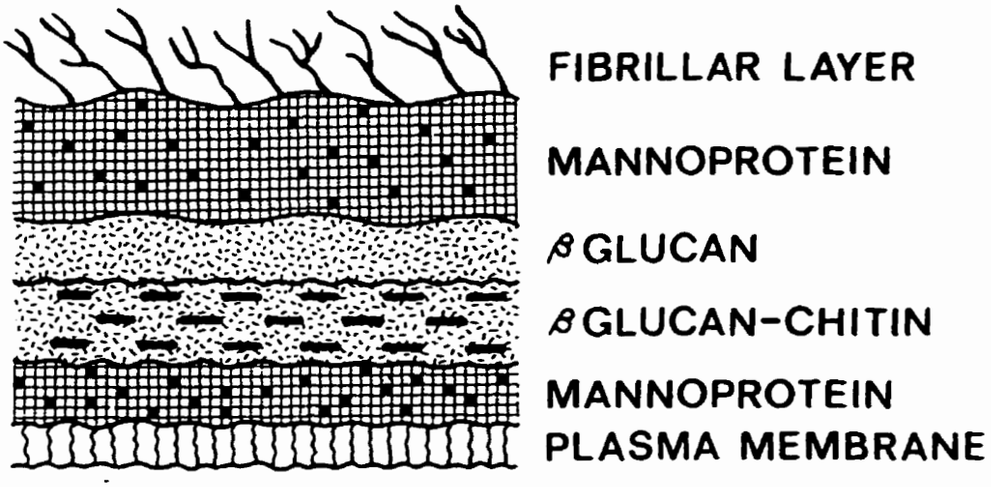



**Figure 2: Schematic of *Candida albicans* cell wall structure.** [5]

The fungal cell membrane plays an important role in the environmental control between the cytoplasm and the external environment. Its composition is approximately 80% lipids, 10% protein and 5% sugars. Its primary function is in acting as a barrier between the interior and exterior components of the cell. It is implicated in the transport of material in and out of the cell. This can occur through diffusion, passive transport, and active transport. Active transport is an energy requiring process that facilitates the influx and efflux of solutes. This is an important factor as a potential mechanism of antifungal resistance. As well the membrane provides a support for the enzymes involved in cell wall synthesis including chitin synthase, and glucan synthase. The membrane is also the target site for both the polyenes and azole antifungals. They are involved in increasing the cell membrane permeability. [5]

## C. Opportunistic fungal pathogens

The clinical manifestations of the most common opportunistic fungal infections will be examined. Firstly, *Aspergillus* species is an invasive mold that is associated with opportunistic infections. *Aspergillus fumigatus* is the species most associated with disease. [10] The most common site of infection is in the lung, where it causes pneumonia. In the lung the invading hyphae can form a fungal ball, causing bronchial obstruction and tissue necrosis. Other sites of infection include the ear, sinus, eye, CNS, and skin. [10]

*Cryptococcus neoformans* is another opportunistic fungus that has had increased prevalence in the last number of years. It has come into prominence since the emergence of the HIV epidemic. [2] *C. neoformans* is a found in the feces of pigeons and infection occurs via inhalation. [11] The major site of clinical manifestation is the CNS. Other sites of

infection include the lung, and skin. [12] The prognosis for HIV-positive patients with cryptococcosis is very poor with an average survival time of six months. [2]

Another opportunistic organism that has seen a dramatic rise in occurrence and is associated with AIDS is *Pneumocystis carinii*. [13] The organism has been embroiled in controversy in regard to its taxonomy. This organism was originally placed in the protozoan category. However genetic data now suggests that *P. carinii* belongs in the fungi kingdom. [14] Another feature of the organism that is similar to fungi is that it contains the enzyme 1,3-β-glucan synthase. This holds importance in that the enzyme is a promising potential antifungal target. This is an organism that as its name implies is associated with a clinical manifestation of pneumonia. The disease will occur in 80% of AIDS patients without chemoprophylaxis. [13] If untreated the disease follows a rapid course of respiratory insufficiency leading to death. Even with aggressive therapy the survival rate is only 30-40%. [13]

The opportunistic yeast *Candida albicans* was the focus of our study and hence will be the focus for the rest of this literature review. There are over 150 species of the genus *Candida*, with 10 species being important human pathogens. [15] *C. albicans* is known to be the most pathogenic species. [16] *Candida* species are now the fifth most common blood isolate in hospitals. [17] Oropharyngeal candidiasis (OPC) is the most common fungal infection in patients with HIV. [18] OPC will occur in more than 80% of HIV patients, and 20% of these will go on to develop esophageal candidiasis. [19] In patients with malignancies the most common site of infection is the stomach, with some involvement of the small and large intestines. The forms of candidiasis are quite varied and the classification of the clinical manifestations is divided into mucous membrane and deep

organ infections. Vaginal candidiasis is most commonly seen in diabetes mellitus, pregnancy, and broad spectrum antibiotic use. There are also a number of cutaneous syndromes associated with *Candida*, ranging from diaper rash to the *Candida* granuloma. [15] *Candida* can infect many of the major organ sites of the body including: CNS, respiratory tract, heart, urinary tract, arthrus, liver, spleen, gall bladder, and eye. Finally there is a disseminated form of the disease that can spread to multiple body sites through the blood stream. [15] Disseminated forms of the infection possess a high 55% mortality rate. [20]

## D. Virulence factors

Having considered the disease states caused by fungal organisms, and more specifically by *Candida* species the factors that contribute to virulence in this species will now be examined. Relative to pathogenic bacteria and viruses *C. albicans* is not considered particularly virulent. [21] *C. albicans* is a common commensal organism found in the mouth. In the immunocompetent person the host defense systems hold the organism in check. [21] But when a person is immunocompromised the balance is often tipped in favor of the organism and its virulence factors. [21] It must be realized that pathogenesis involves the interplay between the host and the invading organism. It is acknowledged that the immunological status of an individual plays a large part in the pathogenesis of candidiasis. In this section only the organism's contributions via its virulence factors will be investigated. There are three putative virulence factors that have been studied most extensively. These are: adherence, proteinase production, and yeast /hyphal dimorphism. [21,22]

## 1. Adherence

The characterization of the binding between *Candida* and host cell will be discussed. Currently in studying the fungal adhesins and mammalian ligands, the interactions have been divided into three categories. The first category is known as a protein-protein interaction. In this group a protein portion of the yeast mannoprotein interacts with the protein part of a host glycoprotein. [23] The mannoprotein seems to bind to the specific sequence arginine-glycine-aspartate. [22] The candidal proteins exhibit similarities to human complement receptor 3 and 4. These receptors are also known as integrins. These integrin analogs bind "fibrinogen, fibronectin, and laminin in the extracellular matrix." [24] The second category of adhesion-ligand interaction is referred to as a lectin-like interaction. [24] In this case a protein portion of a candidal glycoprotein binds to a sugar residue on the host cell. Two sugar residues have been identified: fucose, and N-acetylglucosamine. [25] This interaction could assist in explaining the correlation between candidal infection and blood type O individuals. It has been suggested that the expression of fucosylated antigen on buccal and vaginal cells in O blood group may enhance adherence. [24] The third type of interaction is known as an incompletely defined interaction. In this group the sugar moiety of mannoprotein binds to an unknown receptor. [22,24]

It would seem on an intuitive level that adherence would be important for colonization of epithelial and endothelial tissues. However the evidence for adhesion in pathogenesis is mostly correlative. [23] One classic study looked at the degree of adhesion to buccal and vaginal epithelial cells between different species of *Candida*. It was found that *C. albicans* had superior binding than *C. tropicalis*, which was better than *C. parapsilosis*. This corresponded to the relative virulence of each species. [26,27] Another study showed

that *Candida* had greater in vitro binding ability to vaginal epithelial cells from populations known to be susceptible to vaginal candidiasis. That is vaginal cells from diabetic and pregnant women were found to better bind yeast than cells from nondiabetic and nonpregnant women. [16] A spontaneous mutant with a decreased ability to adhere to epithelial cells provides some of the best evidence for a virulence role. It was found that the $ID_{50}$ in rabbits was significantly higher with the mutant than with the wild-type strain. [27] However it must be pointed out that some reports do not correlate virulence and adhesion. [26] For example, *C. albicans* isolates from pediatric burn patients were tested for virulence in a mouse model, and for adhesiveness by the standard epithelial cell adhesion assay. There was found to be no correlation between virulence and adhesiveness in this study. [28] Therefore it is important to realize that there is no one property that is the sole cause of virulence. [21]

In summary, adhesion has been shown to have an important role in the virulence of *C. albicans*. It is involved in the initial stages of infection, however other properties are needed for the propagation of the organism into tissue.

### 2. Proteinase

A detailed look at the nature of the proteinase will be entered into at this time. The enzymes that have been studied most extensively are the carboxyl acid (aspartyl) proteinases. Originally they were given the designation CAP (*Candida* aspartyl proteinase), and regarded as one enzyme. [29] However, amino acid residues did not agree with the proposed DNA gene sequence. Also, 2 phenotypes of one strain, white (W) and opaque (O), caused more confusion. Even though the W form was the more virulent one, only the O form seemed to produce CAP. [30] Four proteinase genes have now been cloned,

by different groups of scientists looking for a single enzyme. [21] The nomenclature of the proteinase has gone through a number of revisions with SAP (secreted aspartyl proteinase) being the latest designation. [31] It turns out that in the phenotype switching strain the O-type phenotype secretes SAP1 and SAP3, while the W-type secretes SAP2. [31] Although with different sequences, the number of amino acids in each enzyme is around 340, with a molecular weight of about 40 kDa. Presently, SAP2 and SAP3 are the enzymes with most closely associated with linked virulence. [26, 32]

The next characteristic of *C. albicans* with a potential virulence role is proteinase activity. It is thought that excretion of proteinase aids in host invasion by disrupting the membranes of host cells. The keratinolytic activity of the enzymes may aid invasion through the protective keratin layer on to deeper infections. Overall the proteolytic enzymes seem to play a role in the penetration and invasion of host tissue by *C. albicans*. [21,23,26] The evidence for a virulence role of proteinase is of a similar nature as the evidence for adherence. Firstly, proteolytic activity correlates with the virulence rankings of *Candida* species. *C. albicans* is the most strongly proteolytic, followed by *C. tropicalis*, followed by *C. parapsilosis*. [27] The other less virulent species do not appear to secrete proteinase. [26] Secondly, patients with candidemia have elevated levels of antibodies to proteinase. This establishes the fact that the enzyme is produced during active Candida infection. [30] Also in experimental infections of *C. albicans* proteinase has been found. [29] In all the aforementioned studies the evidence for virulence is correlative. But some of the best evidence to date involves proteinase deficient mutants. In one study experimental vaginal infections were induced in rats with both a proteinase deficient mutant and the wild-type parent. Both the intensity and longevity of infections were greater in the wild-

type. Adherence and hyphae formation were equivalent in both strains, suggesting that the only factor that was effecting virulence was the proteinase production. [33]

One of the most recent findings in regard to proteinase, is the correlation between the proteinase production of an isolate and the HIV status of the patient from which the isolate is cultured. One study looked at proteinase production in *C. albicans* isolates from HIV+ and HIV- patients. It was found that 100% of isolates from HIV+ patients produced proteinase, while only 56% of isolates from HIV- patents exhibited proteinase production. [34] Another study that examined the same issue found that all isolates showed proteinase activity, but that the activity was twice as high in isolates from HIV+ patients than from HIV- patients. Also, there was no correlation between disease stage and proteinase production. [35] However, a third study found that there was a correlation between HIV disease stage and proteinase production. Once again all isolates secreted proteinase, however there was an eight fold increase in the level of proteinase produced by isolates from symptomatic HIV patients than from non-symptomatic patients. Also, the SAP1 and SAP2 genes were found to be stable in both groups of isolates. The isolates with high proteinase levels maintained this phenotype over two years of maintenance on solid media plates , and an experimental animal model demonstrated that these isolates were more virulent than the low aspartase producers. [36] There clearly needs to be more work done to clarify the role of *C. albicans* proteinase in HIV infections. What is clear is that the immune status of the patient is not the only factor in OPC in patients, and that the virulence of the organism also plays a role in the course of this disease.

In summary, aspartyl proteinase is useful to *C. albicans* for virulence. Proteinase aids both in the invasion of tissue and breakdown of the host's defense. The enzyme works in cooperation with other factors to promote infection.

### 3. Dimorphism

When characterizing the role of hyphae formation in the pathogenesis of *C. albicans* one must look at invasiveness. The classical view is that mycelia are essential for invasion and penetration of tissue. But more recently it has been shown that *C. albicans* can invade tissue without hyphal formation. [21] So although the hyphal form has a role to play in invasion, the old view of yeast equaling commensal, and hyphae equaling pathogen, has been seen to be too simplistic to explain the whole story. [21,37] Another property of the hyphal form presents a different picture. The mycelia form is well known to adhere to epithelial cells better than the yeast form. The hyphal form would then be seen as the stable colonizer from which the infectious yeast form is propagated. [22] Also hyphae is thought to be more resistant to phagocytosis than yeast. [38] This belief has been called into question more recently. However the ability of yeast cells to germinate while inside phagocytic cells could be seen as the method by which hyphal formation enhances virulence.[23]

There is some evidence pointing to mycelial formation as an essential virulence factor. Firstly *C. albicans* the most virulent *Candida* species, is the only one to form hyphae. [5,16] In one study a spontaneous *C. albicans* mutant was found that did not produce hyphae at 37°C. It was found that fewer rats developed vaginitis when inoculated with the mutant than with the wild-type. Also the disease tended to be less severe and shorter in duration than with the nongerminative strain. Given the fact that the mutation was

spontaneous there is more evidence to believe that the only variable in the experiment was hyphae formation. [23] Another set of experiments investigated a number of mutants to assess the role morphology had on pathogenesis. It was observed that both yeast and hyphal forms could initiate renal infection, but that the hyphal form was essential for invasion of the renal pelvis. Looking at both studies together, there is some evidence for an implying virulence role for hyphal formation. [21]

*C. albicans* is the only of the *Candida* species that forms true hyphae. This means individual cells form elongated cylindrical filaments. This is in contrast to pseudohyphae which is a series of elongated blastoconidia which can be mistaken for true hyphae. A germ tube is the initial stage of true hyphae and can be observed within three hours of inoculation in a hyphae producing media. hyphal formation. [39] A number of studies have examined the effects media and antifungal agents have on *C. albicans*. [40-45] Some of the initial work in this area studied azole inhibitory effects on filamentation. [40,42] The reason for this was that researchers were trying to explain why azoles had poor in vitro activity comparison to their good clinical efficacy. Part of the explanation was sought in the possibility that the azoles were more active against the hyphal form than the yeast form of *Candida* species. One experiment used Eagle's minimal essential medium (EMEM) with amino acids and 10% fetal calf serum incubating in $CO_2$. They found that this media produced a predominance of the mycelial form but not exclusively. [40] The other disadvantage to that media was the need for $CO_2$ incubation. Therefore more study was made of hyphal producing media. A very early study had demonstrated that N-acetyl-D-glucosamine was a inducer of germ tube formation in *C. albicans*. [41] Other researchers used N-acetylglucosamine-yeast nitrogen base-proline medium (NYP) which produced

>90% hyphal culture. [42] This media consisted of $10^{-3}$ M N-acetylglucosamine, $10^{-3}$ M proline, 4.5 g NaCl, and 3.35 g yeast nitrogen base in 1 litre of water. This media was used to assay the inhibition of mycelia by ketoconazole. Growth was evaluated microscopically using a Reichert inverted microscope. The hyphae as a percentage of cells in the culture was counted. The categories used were >90% hyphal, 50-90% hyphal, and <50% hyphal. Another study used a variation of the same media to perform MICs with a number of different antifungals to assess ability to inhibit growing hyphae. [43] The variation in the media consisted of potassium phosphate 7 pH buffer in replacement of sodium chloride. One interesting study investigated polyene and azole resistant strains in regard to virulence characteristics. [44] Adherence, proteinase, germ-tube formation, and animal virulence were tested. A standard inoculum was added to sheep serum, and samples were removed at 1, 2, and 3 hours ;and the quantity of germ tube formation was determined microscopically. Variation in the amount of germ tube formation was observed among strains. [44] Another studied observed the effect cilofungin, a echinocandin antifungal, had on the ultrastructure of *C. albicans*. Germ tube formation was clearly inhibited at the MIC level or higher, with cells clumping together. [45] It must be noted that germ tube inhibition at high concentration of drug may not be indicative of a specific mechanism but rather a general inhibition of the whole cell. This is why it is important that tests be performed at subinhibitory concentrations to observe for more specific morphological effects. [39] In others studies researchers found that amphotericin B but not fluconazole was able to inhibit germ tube formation inside macrophages. [46] Given the putative relationship between dimorphism and virulence it is important to understand how antifungals effect the morphology of the cell.

Having looked at the evidence, characterization, and current research for each factor, it can be said that:"... no single virulence factor has been deemed essential for the pathogenicity of *Candida* in any setting, though many appear to facilitate or contribute to the infection process. The potential for opportunistic pathogenicity appears more likely to be derived from multiple low potency factors, acting in concert, in the setting of altered and reduced host resistance.". [30]

## *E. Antifungal agents*

### 1. Amphotericin B

Having examined fungal diseases that afflict the immunocompromised population, specifically candidiasis, we will now consider the chemotherapeutic agents that are being used to combat this disease. The first agent to be considered will be amphotericin B (AMB) (see Figure 3). This is a polyene antibiotic whose mechanism of action is to bind to the ergosterol in the fungal cell membrane and disrupt membrane integrity. [1] A rapid efflux of potassium ions can be detected from treated cells. [1] This drug is fungicidal and has a broad spectrum of activity. It is considered the "gold standard" when it comes to antifungal therapy. [1] It is the drug of choice in the treatment of deep organ and disseminated candidal infections.[47] It can only be administered in the IV form due to its poor absorption from the gastrointestinal tract. It is also insoluble in water hence it is administered in a colloidal suspension which is the cause of many of the adverse effects associated with this drug. These include severe fever and chills, 1-3 hours after administration. [47] Hydrocortisone is sometimes given as prophylaxis, as well as meperidine to treat the chills, and acetaminophen to treat the fever. Other side effects include headache, gastrointestinal disturbances and thrombophlebitis. Hypersensitivity reactions

including anaphylaxis are a possibility; therefore a test dose is given prior to the first dose. Decreased renal function occurs in over 80% of patients on amphotericin B. [47]

There has been much effort to decrease the toxicity of amphotericin. One approach to this problem has been deliver amphotericin in a lipid formulation. It has been found that a complex of amphotericin and liposomes allows for a selective delivery of drug to target tissues and reduces the free drug levels in the blood and kidney. This has been correlated with decreased nephrotoxicity, however there are still occurrences of fever, and chills. [48] There are three liposome products available in the United States: Amphotericin B Lipid Complex, Amphotericin B colloidal dispersion, and AmBisome. [49,50] They are a treatment alternative when either failure or intolerance to amphotericin B develops. However at this point these agents are very expensive, and still entail all the problems associated with intravenous administration.[49,50]

### 2. 5-Fluorocystine

The next drug to be examined is 5-fluorocytosine (5-FC), or flucytosine (see Figure 3). Its mechanism of action is as a prodrug which is taken into the fungal cell by cytosine permease and then deaminated to 5-flurouracil, an anticancer agent.[1] 5-FU is further metabolized to 5-fluorouridine which competes with uracil, thus inhibiting RNA synthesis. 5-FU is also converted to 5-fluorodeoxyuridylate which inhibits thymidylate synthetase therefore affecting DNA synthesis. Mammalian cells do not have the ability to deaminate 5-FC therefore making the drug selective for fungal cells. [1] 5-FC can be used to treat systemic candidiasis. However, it is given in combination with amphotericin B because of the high frequency with which resistance develops to 5-FC alone. [47] The mechanism of resistance includes defects in the enzymes used to metabolize 5-FC to its

more active forms. [51] The most serious of its adverse effects is bone marrow depression which is a dose dependent side effect. Other effects include nausea, vomiting, headache, and rash. [47]

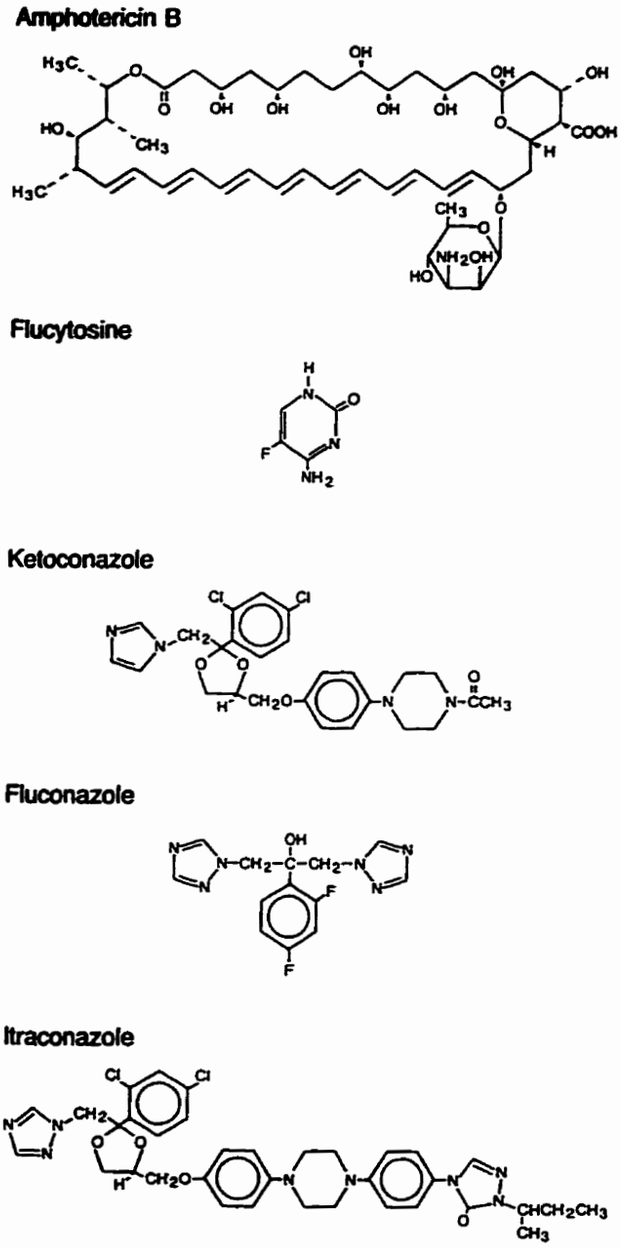



**Figure 3: Chemical structures of amphotericin B, flucytosine, ketoconazole, fluconazole, and itraconazole.** [52]

### 3. Azoles

The azoles are another group of compounds that have been found to possess antifungal properties. [52] The azoles can be subdivided into two groups: the imidazoles (ketoconazole [KETO] and micronazole), and triazoles (fluconazole [FLU] and itraconazole) (see Figure 3). The mechanism of action of these agents is to inhibit ergosterol synthesis by interfering with C-14α-demethylase (a part of the cytochrome P-450 enzyme system). The decrease in ergosterol results in increased cell membrane permeability, and inhibition of cell growth and replication. These agents are fungistatic in their activity. [52] The inhibition of P-450 system is not completely selective for fungal cells and therefore there is the potential for toxicity to mammalian cells in which the enzyme is blocked. The advantage of the newer triazoles such as fluconazole is that they have a much greater affinity for the fungal P-450 in comparison with the mammalian P-450. The therapeutic use of fluconazole is in the treatment and prophylaxis of oropharyngeal and esophageal candidiasis. There are a number of systemic candidal infections for which fluconazole is indicated including urinary tract infections, pneumonia, and peritonitis. [47] The azoles are generally well tolerated drugs. Some of the more common side effects are nausea and vomiting (<10% in ketoconazole, <5% in fluconazole), headache, rash. [52] The imidazoles because they are less discriminating between fungal and mammalian P-450 can inhibit steroidogenesis causing numerous hormonal problems. These include including: adrenal insufficiency, decreased libido, impotence, gynecomastia, and menstrual irregularities. Therefore the advantage of using the triazoles over the imidazoles is evident.

## F. Resistance to azole antifungals

### 1. Antifungal susceptibility testing

Before examining the topic of antifungal resistance the status of antifungal susceptibility testing should be reviewed. It is important to establish the reliability of susceptibility testing before one can confidently study patterns of resistance. There has been much controversy in regard to antifungal susceptibility testing. The discovery of the first antifungal agent occurred 30 years after the discovery of the first antibacterial agent. This in part explains the fact that antifungal susceptibility testing has been decades behind antibacterial susceptibility testing in development. Until the last number of years there was no standardized, reproducible testing method to assess the susceptibility of fungi to antimicrobial agents. There was as much as 50,000 fold interlabarotory variation in minimum inhibitory concentrations (MIC). Clearly there was a need for a standardized reference method that could produce reproducible interlabarotory results. There are a number of variables which could account for such wide discrepancies. The endpoint determination has a large influence on resultant MICs. The endpoint is the agreed upon point where minimum inhibition is considered to have occurred. This is relatively simple for drugs like amphotericin and echinocandins which generally produce a rapid reduction of growth over a small concentration range. For such drugs the first tube with no growth would be considered an appropriate endpoint. Azoles and 5-FC on the other hand exhibit "trailing", a gradual endpoint that would result in high MICs if interpreted in the same way as the former drugs. Therefore, with these drugs an endpoint of 80% reduction in growth would be considered a more appropriate endpoint. [53] Inoculum size and preparation could also affect results. Between a range of $10^2$-$10^6$ cfu/ml it has been shown that with

increasing inoculum size MICs increase for most drugs regardless of the test method. [53] Also a standard method for preparing the inoculum was also needed so as to cut down interlaboratory variation. Some antifungal MICs changed dramatically with changing incubation time and temperature and others hardly changed at all. [53] The media had a large part to play in susceptibility results. Components of a given media could interact with antifungal agents. For example, TRIS buffer has an antagonistic effect on the action of 5-FC.[54] The pH of a media influences the MIC of a given antifungal as well. [55] On account of the clear need for standardization between the laboratories the National Committee for Clinical Laboratory Standards formed the Subcommittee on Antifungal Susceptibility Testing. [53]

After much consultation and study, in December 1992 the Subcommittee published its document M27-P: "Reference method for broth dilution antifungal susceptibility testing of yeast ; proposed standard". [56] This standard was only for *Candida* species and *C. neoformans*. It set clear methods by which to perform antifungal testing (which will be described in detail in the Methods and Materials section), and addressed the issues mentioned in the previous paragraph. It tried to employ methods which had been found to exhibit the most reproducibility between labs. [53] It employed: macrobroth dilution testing using RPMI-1640 (pH 7) as the medium; standard inoculum of 0.5-2.5 $x10^3$ CFU, with clear procedures as how to prepare it; clear temperature and time guidelines; and methods for determining endpoints. [56] In October 1995 after feedback and revision the document M27-T: tentative standard was published. [57] One of the changes in the new document was an inclusion of a microbroth dilution method. This is important because the macrobroth test in the proposed standard is too cumbersome for a clinical lab to employ on a large

scale. Also, modification was made in the media used for amphotericin testing. It was found that Antibiotic Media 3 allowed for better discrimination between amphotericin sensitive and resistant yeast than the standard RPMI1640. [57] One of the most important questions concerning susceptibility testing is whether the test, no matter how reproducible, can be correlated to clinical outcome. The subcommittee on antifungal susceptibility testing have issued a number of statements in regards to the interpretation of MIC results. [58,59] Some of these statements are: MICs are only part of the picture when considering therapeutical options, in vitro sensitivity does not predict clinical success, however in vitro resistance often predicts clinical failure. They go on to say that in looking at: ratios of MICs to blood levels , MIC population distributions, animal models, and clinical trials, a decision ultimately has to be made concerning what one considers sensitive and resistant. In vitro fluconazole and itraconazole susceptibility to *Candida* species has been correlated to clinical outcome in HIV patients with OPC. [58,59] With fluconazole the following break-points were given : MICs of ≤8 μg/ml were sensitive MICs of 16-32 μg/ml were considered sensitive-dose dependent, and ≥ 64 μg/ml were regarded as resistant. For itraconazole the breakpoints were considered to be: sensitive ≤ 0.125 μg/ml, sensitive-dose dependent 0.25-0.5 μg/ml, resistant ≥ 1 μg/ml.

## 2. Overview of resistance

The development of fluconazole-resistant *C. albicans* is becoming an increasing problem in several patient populations. Resistance to azoles had been a rare occurrence until recently. With the emergence of HIV infection and the long term and prophylactic use of fluconazole in oropharyngeal candidiasis (OPC) there has been a considerable rise in the incidence of both in vitro and clinical resistance. [52] A recent review article found

that the clinical situations in which fluconazole resistance is seen were in two broad categories: patients with HIV, and everyone else. The authors sited 33 publications which reported resistance to fluconazole during OPC treatment in HIV patients. Five reports had too little data to evaluate and 1 defined resistance solely on the basis of MICs. The 27 remaining articles on fluconazole resistance were assessed. The two factors that seemed to correlate to resistance were: advanced AIDS, and cumulative dose of fluconazole. [60] In the typical scenario, an AIDS patient with a $CD4^+$ count $<50/mm^3$ experiences recurrent OPC which has been treated with topical agents or ketoconazole. The patient is then given repeated courses with fluconazole of 100 to 200 mg/day. This therapy is effective for a period of time (on average 13 relapses), however eventually a relapse does not respond and the MIC of fluconazole to the yeast isolate is ≈16 μg/ml. The dose is increased to 200 to 400 mg which is effective for a couple of rounds of therapy, however soon this too is not effective. A *C. albicans* strain is cultured from the patient that has a fluconazole MIC of >64 μg/ml. One study that correlated previous fluconazole therapy to resistance found that the median cumulative dose in patients who failed therapy was 10,600 mg, compared to 4,400 mg for successful treatments($p=0.001$). The largest other category in which resistance is seen is in patients with fungemia. There has been a total of 50 documented cases of resistance seen in this population. In these cases the *Candida* species found tend to be intrinsically resistant species such as *Candida krusei*. [60]

### 3. Case study

One study was particularly effective in documenting the emergence of fluconazole resistant *C. albicans* in HIV patients, and thus deserves closer attention. [19] Between October 1989 and August 1993 fungal isolates were taken from a cohort of 65 patients

with symptomatic HIV infection (mean $CD4^+$ count 68/$mm^3$). Each patient had at least one episode of OPC treated with fluconazole. In vitro resistance was defined as a MIC of ≥25 μg/ml for fluconazole. Clinical resistance was defined as failure to respond to therapy of 7.5 mg/kg/day (up to 400 mg/day) of fluconazole. Antifungal susceptibility testing was performed on 160 *C. albicans* isolates. There were 33 in vitro resistant isolates found which corresponded to clinical resistance in 9 patients (14%). One case will be discussed in greater detail. Patient B first experienced OPC in May 1988. Between this date and October 1989 he was treated with ketoconazole, miconazole, or nystatin solution for recurrent OPC. In October 1989 he first received fluconazole for OPC (100 mg orally for 5 to 10 days), which was effective treatment until April 1991. He received 100 mg/week of fluconazole as prophylaxis for OPC starting in November 1990. In May 1991 clinical resistance developed after 400 mg fluconazole was given for 7 days. From May 1991 to July 1991 itraconazole (300 mg/day) was given to effectively treat episodes. In July 1991 the patient died due to progressive Kaposi's sarcoma.

| Date of infect.(mo.yr) | Fluconazole (ug/ml) | Ketoconazole (ug/ml) | Itraconazole (ug/ml) | 5-FC (ug/ml) |
|---|---|---|---|---|
| 01.90 | 0.78 | 0.05 | 0.19 | 0.1 |
| 02.90 | 0.78 | 0.05 | 0.19 | 0.1 |
| 08.90 | 1.56 | 0.05 | 0.19 | 0.1 |
| 10.90 | 0.78 | 0.1 | 0.19 | 0.1 |
| 11.90 | 1.56 | 0.05 | 0.39 | 0.1 |
| 04.91 | 6.25 | 0.05 | 0.78 | 0.1 |
| 05.91 | 25 | 0.19 | 0.78 | 0.1 |
| 07.91 | >50 | 0.19 | 0.78 | 0.1 |

**Table 1: In vitro susceptibilities of *C. albicans* isolates from patient B.** [19]

Table 1 shows the increasing MIC of fluconazole over the course of therapy. It is also clear that there is partial cross-resistance to the other azoles, in that there was fourfold increase in the MIC's of both ketoconazole and itraconazole. There is no cross-resistance to 5-FC exhibited. Genetic studies indicate that the isolates from the individual patients are usually from the same strain suggesting that they are isogenic mutants. [19]

## 4. Mechanisms of resistance

The mechanism of resistance to azoles in *Candida* is not well understood at this time. However, there are a number of studies that suggest the mechanisms involved. [61-64] From a cellular perspective it has been proposed that one mechanism *C. albicans* uses to gain resistance to azoles is decreased permeability. [61] This decrease has been associated with changes in the phospholipid and sterol composition of the cell membrane. Another mechanism is located at the target of the antifungal azole, the cytochrome P-450 enzyme (14α-demethylase) which retains activity but demonstrates less affinity towards the azoles in some resistant strains. [62] From a molecular perspective even less is known of resistance mechanisms in *C. albicans*. There was one study performed in *C. glabrata* that showed that part of the resistance in this species was due to an upregulation of the P-450 gene resulting in increased amounts of the 14α-demethylase enzyme. [63] In one genetic study of resistance in *C. albicans* the researchers did not find an amplification of the P-450 gene, however they did find increased expression of a ATP-dependent efflux pump. [64] This suggests that azoles may be actively transported out of *C. albicans* as part of its resistance mechanism. It is clear that although some progress has been made more work needs to be done in studying the mechanisms of resistance in *C. albicans*. More recently it has been suggested that resistance to azoles occurs in stages. [65] The first stage is reduced

permeability to fluconazole, but not to itraconazole. The second stage has been suggested as an increase in expression of the P-450, and point mutations in the gene giving less affinity for the drug. At this point itraconazole would also be affected by these changes. Lastly an increased expression of multidrug efflux pump would be the final stage of resistance. This could be a possible explanation for the partial cross resistance these yeasts exhibit to itraconazole. Only some of the mechanisms of resistance are effective against itraconazole, hence the cross resistance is incomplete. [65]

## 5. Trends in resistance

### a) Cross-resistance

One of the disturbing findings in the area of fluconazole resistance is the trend toward cross-resistance to other azoles. There has been a pattern of partial cross-resistance that has emerged. Johnson and colleagues examined fluconazole and itraconazole susceptibilities to *Candida* species. [66] Among *C. albicans* isolates resistant to fluconazole, 17.5% were resistant to itraconazole. Also there was a definite trend in that isolates with high MICs to fluconazole had higher than average MICs to itraconazole. The authors suggested that itraconazole may still be effective against fluconazole-resistant *Candida*, however they cautioned that higher doses may be needed. Guennec and colleagues examined the isolates from four AIDS patients with recurrent OPC who developed clinical resistance to fluconazole and subsequently itraconazole. [67] In tracking susceptibilities of isolates, they were able to show that itraconazole MICs increased over the course of the fluconazole therapy. The patients were successfully treated with itraconazole at first however over time clinical resistance developed which correlated with

MICs to itraconazole of ≥1.56μg/ml. Clearly cross-resistance among the azoles is an alarming pattern that could threaten the future use of this class of agents.

### b) Transmission of resistant organism

Another alarming trend in this area is the transmission of fluconazole-resistant *Candida* species to new patients and previously unaffected populations. In one study it was documented through genetic typing the transmission of a fluconazole-resistant *C. albicans* strain from one AIDS patient to another. It was shown that the same strain was the cause of resistant infection in both a husband and wife. [18] Another study documented the discovery of fluconazole- resistant organism in 17 HIV-negative patients who had never received azoles previously. [68] Either, there was primary resistance to azoles in *C. albicans* which had never been shown before, or there was the possible transmission of fluconazole-resistant strains to non-HIV patients. A recent report gave the first case of fluconazole resistant *C. albicans* causing vulvovaginitis in an HIV-negative woman. [69] The isolate had MICs to fluconazole, ketoconazole, and itraconazole of > 40 μg/ml, 3.12μ/ml, and 6.25μg/ml respectively. This patient had no known contact with a reservoir of fluconazole resistant *C. albicans*. Given the increasing evidence of both nosocomial and sexual transmission of *C. albicans*, it is clear that fluconazole resistant infections could become a serious problem in many varied patient populations. [70] Clearly the ever increasing incidence of azole resistant *C. albicans* is creating a dilemma in the pharmacotherapy of candidial infections.

## G. Novel antifungal agents

### 1. The need for novel antifungal agents

Amphotericin B is the gold standard in fighting fungal infections. Low resistance is demonstrated against this agent. [47] However, amphotericin B is associated with high toxicity, which requires hospitalization at least for the first dose and can only be given in the IV form. The azoles, especially the newer agents like fluconazole, are a good alternative to the rigors of amphotericin B treatment, especially in oropharyngeal and esophageal candidiasis. However, with the increasing resistance found, the usefulness of this class of agents could be markedly compromised. [52] Therefore, there is an obvious gap in the armamentarium of antifungal agents. The need for novel antifungal agents that can be used in the case of azole resistance *Candida* infections is a clear and pressing problem.

### 2. Membrane active agents

One approach to the development of novel antifungal agents has been the development of new azole derivatives. One such novel triazole, D0870, has been the subject of numerous studies published on it. In one study the in vitro activity of D0870 was compared to ketoconazole and itraconazole against fluconazole-resistant *Candida albicans, Candida glabrata, Candida krusei,* and *Candida inconspicua.* [71] In all species the novel azole was comparable to ketoconazole, and itraconazole. In another study the D0870 in vitro activity was tested against *C. albicans* isolates from the oral cavity of HIV-positive patients. [72] Half the isolates were considered fluconazole-sensitive (MICs ≤4 μg/ml)and the other half fluconazole-resistant (MICs ≥8μg/ml). While the D0870 $MIC_{90}$ for all of the isolates combined was 0.5 μg/ml there was a clear difference in the MICs between the fluconazole sensitive and resistant groups. In the FLU-S group the MICs

ranged from ≤0.0078 to 0.125 μg/ml with a $MIC_{90}$ of 0.06 μg/ml. While in the FLU-R group the MICs ranged from ≤0.0078 to 2 μg/ml with a $MIC_{90}$ of 2 μg/ml. Clearly more work needs to done in both in vitro and in vivo studies. Development continues to occur on novel triazoles. At the 1996 Interscience Conferences of Antimicrobial Agents and Chemotherapy there were 30 posters on five new triazoles: Tak-187, SSY726, KP-103, SCH5659, and voriconazole. [73] However, the inherent problem of developing a new agent with the same mechanism of action as fluconazole is the potential for cross-resistance to develop; hence limiting the clinical usefulness of these novel agents.

### 3. Cell wall active agents

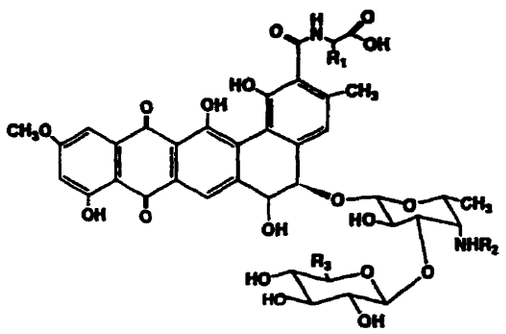
#### a) Rationale for cell wall active agents

One of the fundamental issues in developing antibiotics is the issue of selectivity. The goal is finding chemicals that have a toxic effect on the invading organisms but not on mammalian tissues. The way this is done is finding targets in the invading cell that are sufficiently different from the mammalian cells to warrant further study. This problem is made more complicated for antifungal agents by the fact that both fungal and mammalian cells are eucaryotic, thus having much more in common with each other than bacterial and mammalian cells. [4] One obvious target in the fungal cell is the cell wall. Not only do mammalian cells lack a cell wall, but the major constituents in the fungal cell wall are not found in mammalian cells. The cell wall as the target for antifungal agents also has appeal because it could be theorized that a cell wall active agent would have a similar success against fungi as the penicillin antibiotics have had against bacteria. Penicillins disrupt cell wall integrity and cause cell lysis and death in bacteria. There are three groups of agents

that are being worked on in the area of cell wall active antifungal agents: mannan-binding agents, chitin synthase inhibitors, and glucan synthase inhibitors. [74]

### b) Mannan-binding compounds

The pradimicins are a group of dihydrobenzo[α]naphthacenquinones that are known to bind mannan (see Figure 4). [74] As stated earlier mannan is one of the main carbohydrate components in the fungal cell wall and is known to play a major role in fungal adhesion. [75] The pradimicins A to E were isolated from the organism *Actinomadura hibisca*. Pradimicins A, B, and C were tested for in vitro activity against, *Candida* species, *C. neoformans*, and *Aspergillus* species A systemic murine model was also used for testing. In both cases, the pradimicins exhibited broad antifungal activity. When compared with amphotericin B and ketoconazole the pradimicins were not as potent as amphotericin but better than ketoconazole. [1,74] Water-soluble derivatives were then developed in an attempt to overcome poor oral absorption. A number of these agents have been reported to maintain good antifungal activity. [9] The mechanism of action of these agents is not completely understood at this time. However it is known that they bind to the saccharide portion of mannoprotein in a calcium dependent manner. A ternary complex seems to be formed between pradimicin, mannan, and calcium. Membrane permeability is subsequently altered. No human clinical trails have been attempted at this time. It is unclear whether these compounds will be developed further towards a therapeutic agent. [1,9,74]



| Pradimicin | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| A | $CH_3$ | $CH_3$ | H |
| B | $CH_3$ | $CH_3$ | dexylosyl-Pradimicin |
| C | $CH_3$ | H | H |
| D | H | $CH_3$ | H |
| E | H | H | H |
| FA-1 | $CH_2OH$ | $CH_3$ | $CH_2OH$ |
| L | $CH_3$ | $CH_3$ | $CH_2OH$ |
| FL | $CH_2OH$ | $CH_3$ | $CH_2OH$ |

**Figure 4: Chemical structure of pradimicins.** [74]

### c) Chitin synthase inhibitors

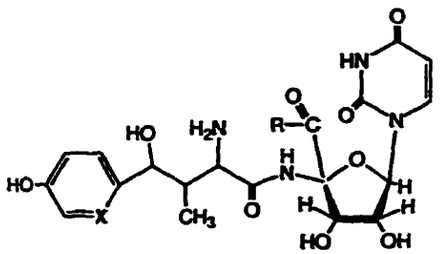
Chitin is a component of the cell wall that, even though it forms a small proportion of the wall, plays a major role in the growth and morphogenesis of *C. albicans*. Polyoxins and nikkomycins are peptido-nucleosides that mimic UDP-N-acetyl glucosamine (NAG), the building blocks of the chitin chains (see Figure 5). They are highly competitive inhibitors of chitin synthase and cause striking morphological changes in fungi eventually leading to cell lysis. [1] The polyoxins have been used for many years in the area of agricultural fungicides. Despite the promise of these agents there has been little in vivo testing of these agents. [1]

#### (1) Chitin synthase

Chitin is a linear 1,4-β linked polymer of N-acetyl-D-glucosamine (NAG) which is not found in mammalian cells. Chitin synthase is a membrane bound enzyme that catalyzes the following reaction:

$2n$ UDP-NAG→$(NAG\text{-}\beta\text{-}(1\text{-}4)\text{-}NAG)_n + 2n$ UDP.

It has been shown that chitin synthesis in *Saccharomyces cerevisiae* is regulated by three isoenzymes (CHS1, CHS2, CHS3). The functional role of the three synthases have been analyzed. CHS1 is involved in septum and bud scar repair during cell separation. CHS2 is involved in production of chitin for the primary septum. CHS3 is responsible for chitin in the bud scar and lateral wall. [74] A recent study raised the possibility of an interesting role for chitin in virulence. The CHS3 homolog gene in *C. albicans* was disrupted and resultant mutants were tested in immunocompetent and immunocompromised mouse models. Despite no change in growth rate, or germ tube formation, the mutants were "significantly less virulent than the parental strain". [76] This could have significance in regard to agents that target chitin production. However, a similar study which disrupted the CHS2 and CHS3 separately, and tested each null mutant in a immunocompromised mouse model found no significant difference in virulence between parent and mutant strains. [77] Obviously more work needs to be done to resolve this disagreement. Another interesting study looked at the effect of a chitin synthase inhibitor on *C. albicans* binding to epithelial cells (a virulence factor for *Candida* species). It was found that there was up to a 58% reduction in the binding of yeast cells to buccal epithelial cells when the agent was administered. This is especially intriguing when one considers that chitin is found only in the inner cell wall, and would seemingly not have opportunity for binding interactions. [9]



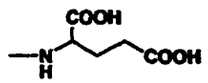
| Nikkomycin | R | X |
|---|---|---|
| Z | OH | N |
| J |  | N |
| $B_Z$ | OH | C |

**Figure 5: Chemical structure of nikkomycins.** [74]

### (2) Inhibitors

The inhibitors of chitin synthase were first discovered in 1965 in an antibiotic screen against agricultural diseases. The first compound of this class discovered was a member of the polyoxin family, which went on to be used as an agricultural antifungal. The nikkomycins were first isolated in 1976 from a soil sample taken in Nikko, Japan. Although the nikkomycins have been extensively studied, none have ever been used commercially. There have been 13 naturally occurring polyoxins and 14 naturally occurring nikkomycins described to date. [9] Polyoxins and nikkomycins are peptido-nucleosides that mimic UDP-N-acetyl glucosamine, the building blocks of the chitin chains. They are highly competitive inhibitors of chitin synthase and cause striking morphological changes in fungi eventually leading to cell lysis. Although these substances

are potent enzyme inhibitors, they have variable activity against intact organism. Part of the explanation for this is that these agents require active transport into the cell. It has been shown that peptides inhibited the activity of polyoxins in *C albicans*, suggesting the presence of a peptide transport system or permease required for transport of the drug molecule. Attempts were made to link polyoxin to other amino acids to enhance transport. However, while uptake into the cell was increased antifungal activity was reduced. [74] Despite structural similarities the nikkomycins have had greater activity against whole fungal cells. The reasons for this are believed to involve better transport into the cell because of less uptake inhibition by peptides, and nikkomycins utilize different transport systems than polyoxin. [1]

### (3) In vitro and in vivo studies

There have been a number of studies that have examined the in vitro susceptibility of fungi to these agents. Nikkomycins are highly active against the pathogenic dimorphic fungi *B. dermatitidis, H. capsulatum, C. immitiis*. It should also be noted that these pathogenic fungi contain high levels of chitin in contrast to yeast. Within the *Candida* species which have relatively low chitin levels there is a variability of susceptibility indicating that the issue is more complex than mere chitin content. For example, *C. albicans* is moderately susceptible to nikkomycin X and Z, but *Candida tropicalis* is highly resistant. Filamentous fungi like *Aspergillus fumigatus* are also highly resistant to nikkomycins, despite high chitin levels. [1,9]

There is limited in vivo testing reports of these agents against medically important fungi. In one study polyoxin D and a mixture of nikkomycin X and Z were analyzed for their activity in a murine candidiasis model. The polyoxin provided no protection as

compared to control while the nikkomycin mixture delayed but did not prevent mouse death. Overall moderate to poor in vivo activity against *Candida* species. However Nikkomycin Z has been shown to be highly effective in vivo against the dimorphic pathogenic fungi *B. dermatitidis, H. capsulatum, C. immitiis.* This efficacy correlated with good in vitro activity against these organisms. [1,9] One set of experiments provided a possible explanation as to why the nikkomycins are not more successful antifungal compounds. Gaughran and colleagues tested the effect of nikkomycin Z on the chitin synthase isozymes of *Saccharomyces cerevisiae*. They found that nikkomycin Z was inhibitory of CHS1 and CHS3 isoenzymes at low concentrations. However CHS2 was highly resistant to nikkomycin Z. This could provide an explanation for the poor activity of this class of agents. [78]

### (4) Synergy

A number of studies have investigated the possibility of synergy between the chitin synthase inhibitors and other classes of antifungal agents. The first combination examined was with the glucan synthase inhibitors. The rationale for this was based on knowledge of the yeast cell wall. There is an interrelationship between chitin and glucan; two of the major components of the yeast cell wall. It has been observed in *C. albicans* that exposure to the β-glucan synthase inhibitor cilofungin resulted in an increase in the chitin content in the cell wall. [9] These effects are thought to be an attempt on the part of the fungal cell to compensate for the inhibition of one of the major cell wall ingredients. With this in mind, an experiment was performed where combinations of nikkomycin X and Z with papulacandin B were used against *C. albicans*. Synergy between the two agents were found both in whole cells and protoplasts. [79] In another study with cilofungin and

nikkomycin Z against *A. fumigatus* dramatic results were shown. The organisms were resistant to either of the two agents alone, however when given in combination the fungi were highly susceptible to very low level of combined drugs. [80] The next combination examined was between nikkomycins and azoles. The rationale for such a combination was because of the secondary effects of azoles in disrupting the cell membrane. This could potentially disrupt the chitin synthase enzyme which is located in the cell membrane. Both in vitro synergy assays, and in vivo animal models were used in these experiments. In vitro studies showed synergy between nikkomycins and a number of azoles (including fluconazole, ketoconazole, itraconazole) against *C. albicans*, but not other *Candida* species. The in vivo experiments did not correlate with in vitro results. Synergy was confined to certain azoles and certain fixed ratios of agents. [81] It can be seen that chitin synthase inhibitors may be developed as therapeutic agents either alone or in combination with other agents.

### d) Glucan synthase inhibitors

The echinocandins are cyclic hexapeptides which exhibit noncompetitive inhibition of 1,3-β-glucan synthase. Glucan is a polymer of glucose that is the main carbohydrate component of the fungal cell wall. 1,3-β-Glucan synthase is a cell membrane enzyme that uses UDP-glucose substrate and polymerizes it to1,3-β-glucan (see Figure 6). [74]

#### (1) Glucan synthase

A standard assay for the enzyme target of echinocandins: 1,3-β-glucan synthase has been known for some time. [82] Also known for some time was that the enzyme was composed of two subunits: a catalytic insoluble membrane subunit, and a soluble GTP-

binding regulatory component. [83] There has been a large amount of effort put forth to isolate the genes of these subunits. [84-91] Almost all of the work in this area is using *Saccharomyces cerevisiae* because of its haploid nature and hence its ease of genetic manipulation. Using resistance to killer toxins of *Hansenula mrakii* a number of researcher found genes that were involved in1,3-β-glucan synthesis, (HKR-1 and KNR-4) however these genes did not encode for the enzyme itself. [84,85] Using L-733,560, a semisynthetic echinocandin, researchers isolated echinocandin resistant *Saccharomyces cerevisiae* due to a mutation in a single gene: *etg-1*. This mutation also conferred resistance to the membrane bound 1,3-β-glucan synthase enzyme subunit. The scientists proposed that this gene encoded for the catalytic subunit of 1,3-β-glucan synthase. [86] When ETG-1 was cloned a surprising result was found. The gene was identical to a concomitantly cloned gene FKS1, mutations in which caused hypersensitivity to cyclosporin. The gene encoded for a 215 kDA integral membrane polypeptide, with 16 transmembranous regions which would be consistent with the membrane bound subunit of 1,3-β-glucan synthase. Homologs of this enzyme were also detected by complementation in *C. albicans* and *Aspergillus fumigatus*. [87] A functional homolog of FKS1 named FKS2 was found in *Saccharomyces cerevisiae* that was 88% identical to FKS1. Neither gene is essential for the cell, but null mutations in both genes are fatal to the cell. [88] The transcription of FKS2 is calcineurin dependent which explains why *fks*1 mutants were sensitive to the calcineurin inhibitor cyclosporin A. There was found to be differential expression of the two enzymes dependent on the cell cycle. [88] After the initial discovery of the gene many other labs confirmed the results by their own cloning experiments. In one case papulacandin B resistant mutants were used to clone the gene PBR1, also found to be identical to FKS1. [89]

Another group isolated the protein sequence of the enzyme and worked backwards to derive GSC1 and GSC2 ( identical to FKS1 and FKS2) [90] Most recently the FKS1 homolog in *Aspergillus nidulans* has been cloned and also proposed to be the target of echinocandins the catalytic subunit of 1,3-β-glucan synthase. [91]

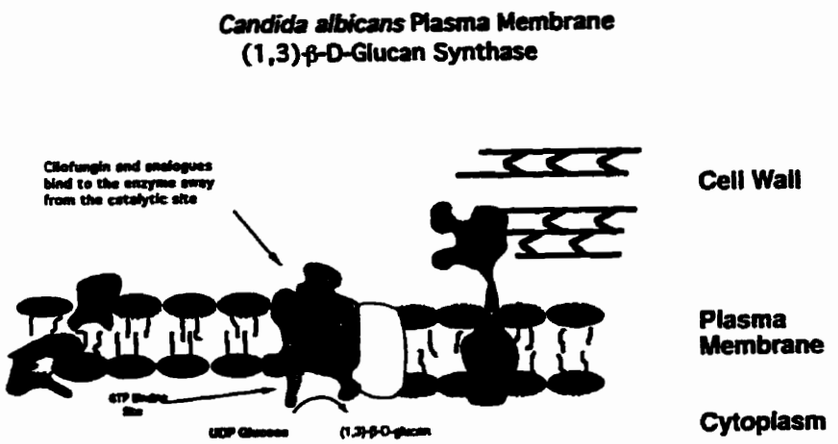
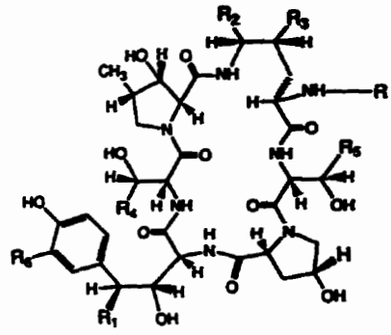



**Figure 6: Diagram of 1,3-β-glucan synthase enzyme of *C. albicans*.** [74]

### (2) Inhibitors

There are four classes of naturally occurring agents which are known to inhibit the synthesis of glucan in fungal cells. They were all discovered as a result of screening tests for antifungal agents. The first class is the aculeacins produced by the organism *Aspergillus aculeatus*. There are seven types of this lipopeptide: aculeacin A, B, C, D, E, F and G, of which aculeacin A is the major component (see Figure 7). [9] The echinocandins were discovered in 1974 and include: echinocandin B, C, and D of which echinocandin B is the primary component. The echinocandins, which are structurally similar to aculeacins,

are produced by *Aspergillus nidulans*, and *Aspergillus rugulosus* (See Figure 7). Another similar class of compounds are pneumocandins $A_0$, $B_0$, and $C_0$ derived from the organism *Zalerion arboricola.* [74] There are five papulacandins: A, B, C, D, E, which were discovered from a strain of *Papularia sphaerospere*. These compounds are structurally different from the other three classes. Their structure has been described as "spirocyclic diglycoside esterfied by two long-chain unsaturated fatty acids" (see Figure 7). [9]



| Echinocandin Lipopeptide | R | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|---|
| Echinocandin B | Linoleoyl | OH | OH | OH | $CH_3$ | $CH_3$ | H |
| Echinocandin C | Linoleoyl | H | OH | OH | $CH_3$ | $CH_3$ | H |
| Echinocandin D | Linoleoyl | H | H | H | $CH_3$ | $CH_3$ | H |
| Aculeacin A | Palmitoyl | OH | OH | OH | $CH_3$ | $CH_3$ | H |
| Mulundocandin | Me-Myristoyl | OH | OH | OH | H | H | H |
| Pneumocandins | DiMe-Myristoyl | OH | OH | OH | $CH_2CONH_2$ | $CH_3$ | H |
| FR901379 | Palmitoyl | OH | OH | OH | $CH_2CONH_2$ | $CH_3$ | $HOSO_3$ |

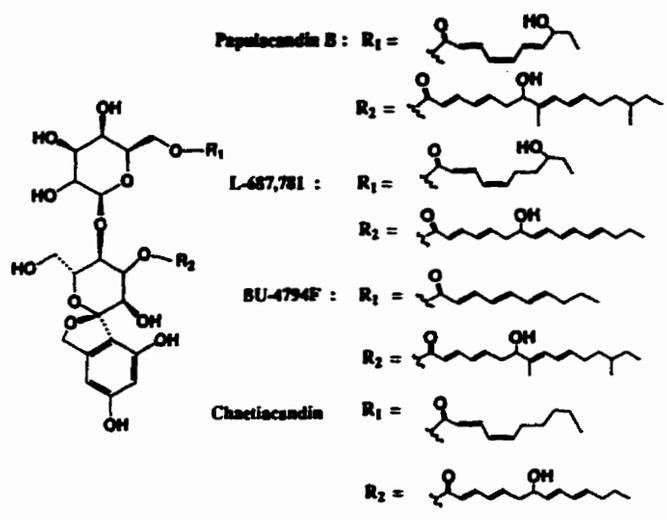
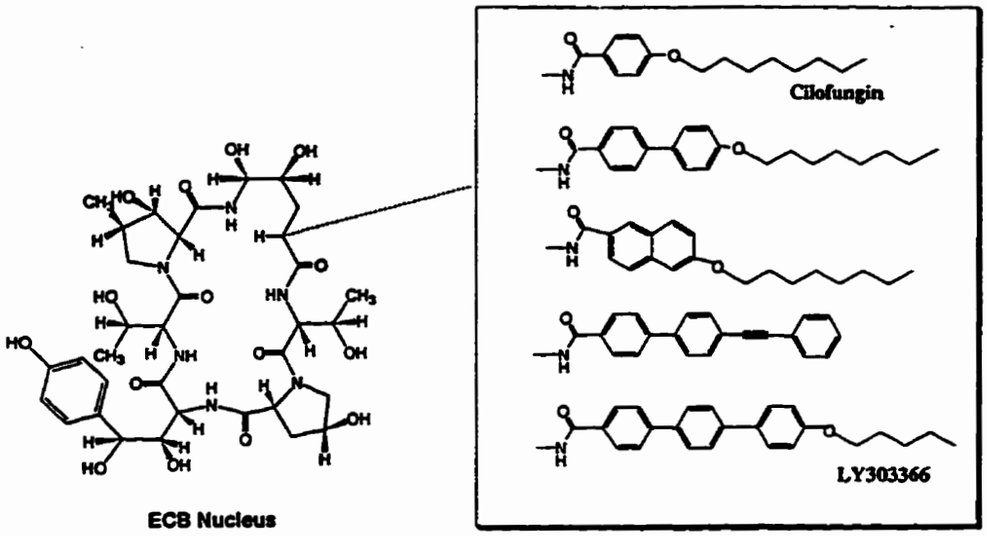



**Figure 7: Chemical structure of echinocandins and papulacandins.** [74]

The aculeacins, echinocandins, and pneumocandins share a similar structure which has been extensively studied in regard to its structure activity relationships. [74] The basic structure of these agents is a cyclic hexapeptide ring with a lipid side chain. They possess a palmotoic, linoleoic, and dimethyl-myristoic side chain for each group respectively. It has been found that neither the hexapeptide ring or the lipid side chain have any antifungal activity by themselves. Both are essential for the activity of the molecule. Synthetic substitution of amino groups of the ring were found to produce reduction in antifungal activity. This demonstrated the rigid structural requirements for activity in the peptide ring. The nature of the side chain interaction was also studied and it was found that the chain needed to be at least 12 carbons long and that the optimal activity was encountered at C-18. Chemically diverse side chains could be introduced as long as they remained linear and lipophilic. [74]

There have been two strategies that have been most successful in producing semisynthetic derivatives of the naturally occurring compounds. In one strategy the side chain of echinocandin B has been replaced with other lipophilic linear molecules. In doing so this has increased the potency and spectrum of these antifungals. Also it has prevented the lytic action on red blood cells, a toxicity of the parent compound. Two agents in this category are cilofungin and LY 303366. The linoleolic group of echinocandin B is substituted with n-octyloxybenzoic or pentyloxyterphenylcarboxoic side chains respectively (See Figure 8). These agents have good activity against *Candida* species, *Aspergillus fumigatus*, and *Pneumocystis carinii*. As stated earlier, one of the characteristics that *P. carinii* (a newly classified fungus) shares with other fungi is β-glucan in its cell wall, thus rendering it susceptible to the echinocandins [74] However,

*Cryptococcus neoformans* because it utilizes α-glucan is not susceptible to the echinocandins. Cilofungin had progressed to clinical studies that had to be halted because of acute tubular necrosis. This adverse effect was attributed to the vehicle which was 26% polyethylene glycol. [1] The newest agent studied LY303366 has greater potency, oral activity, and hopefully will avoid the adverse effects of previous chemicals when it goes to clinical trials. [74] The other approach in antifungal synthesis has been to modify pneumocandin $B_0$, not by changing the lipid chain but by modifying some of the amino groups. In doing so these compounds have increased potency and water solubility (see Figure 8). [74]



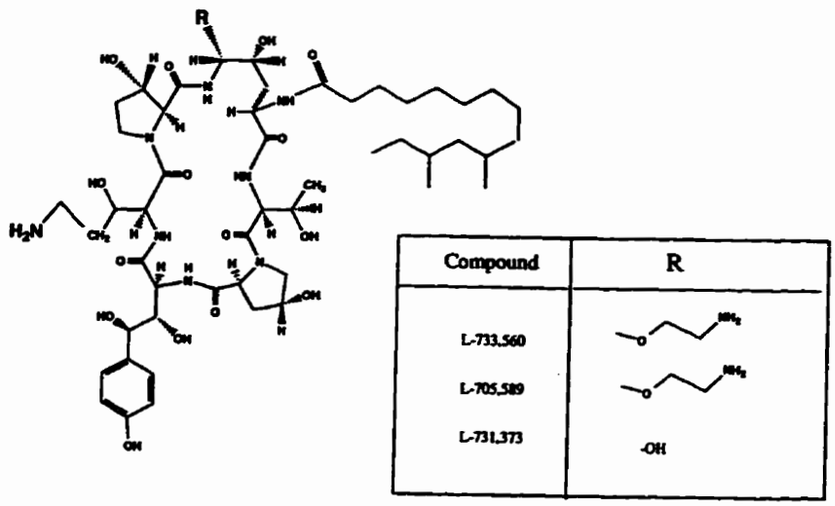




**Figure 8: Chemical structure of semisynthetic echinocandins and pneumocandins.** [74]

### (3) In vitro and in vivo studies

Having looked at the structure and target site of the echinocandins, we will review studies examining the cellular, in vitro, and in vivo effects of these agents have on fungi. Shortly after the discovery of aculeacin A, a study looked at its pharmacodynamic effect on *Saccharomyces cerevisiae* in glucose-peptone medium. It was found that there was a killing effect via cell lysis of growing cells, but there was no effect on non-growing cells. [92] This lysis was attributed to the inhibition of cell wall synthesis corresponding to a reduction of glucan incorporation into the cell wall. Around the same time papulacandin B was being studied as well. MICs were performed using an agar incorporation method and susceptibilities to most *Candida* species were lower than the polyenes or azole tested. However high MICs were found for *C. guiulloermondii, C. neoformans, and Aspergillus species.* When MFC experiments were performed the drug was deemed to have a fungistatic effect, however in broth growing cells were microscopically observed being lysed. [93] By the early 1980's the effect of papulacandin B and aculeacin A on a 1,3-β-glucan synthase assay of *Geotichim lactis* was measured. 1,3-β-glucan synthase was clearly inhibited by papulacandin B and aculeacin A. Two things of note: only the enzyme from growing cells was inhibited, and the agents had no effect on GTP induced synthesis implying they do not interact at the same site as GTP induced synthesis (see Figure 6). [94] Cassone et. al. studied the ultrastructure via transmission electron microscopy (TEM) of *C. albicans* exposed to echinocandin B in synthetic media. They observed a thinning and disorganization of the cell wall especially in the growing bud. Unaccounted for membranous bodies appeared in the cytosol close to the cell wall. [95] A similar study using aculeacin A and papulacandin B found scanning electron microscopy (SEM) images

showed cells wrinkled and clumping and TEM images showed distorted cell walls and cytoplasmic invaginations. Also germ tube formation was observed to be inhibited at subMIC concentrations of these compounds. [96] A later study utilizing cilofungin corroborated these observations. [45] By the mid 1980's standard 1,3-β-glucan synthase assays were published. [97] Soon after assays on *C. albicans* protoplast lysates that showed that cilofungin, a semisynthetic echinocandin, was a noncompetitive inhibitor of the *C. albicans* 1,3-β-glucan synthase enzyme. [98] Tang and Parr published a method of the solubilizaiton of the enzyme from the protoplasts and confirmed the noncompetitive kinetics of echinocandin inhibition. [99] In vitro susceptibilities of yeast towards cilofungin were performed using a microbroth dilution test. The $MIC_{90}$ for *C. albicans* and *C. tropicalis* was ≤ 0.31μg/ml. However *C. parapsilosis*, *C. neoformans*, and *S. cerevisiae* strains were resistant with $MIC_{90}$ of ≥ 40 μg/ml for the first two species. There was also found to be differences in MICs based on the media used. Antibiotic media-3 produced clear endpoints. While Sabouraud broth, and yeast nitrogen base broth produced partial inhibition at the AM-3 MIC with a trailing effect observed. Also, with an inoculum size of ≥ $10^5$ cfu/ml there was found to be less activity. [100] Another study demonstrated that pH had an effect on the MIC results. [55] The MICs tended to be higher as the pH decreased, however the minimum fungicidal concentrations (MFCs) were much closer to the MICs with the lower pH. Two strains which had similar results at pH 3.0 and different results at pH 7.0 were tested in a rat model and found to have similar susceptibilities. Also, there shown to be differences based on the media used. However, with inoculums between $10^2$ and $10^5$ CFU/ml and temperatures of 30 and 37°C there was no changes in MICs. Clearly the need for more study in this area was needed. Numerous studies have been performed

in animal models to test the activity of echinocandins. There has been mixed results from different labs. [9] One study which may explain the discrepancies, demonstrated in a rabbit model that cilofungin in lower dosages followed linear kinetics but in either high intermittent dosages or continuous infusion the kinetic of the drug changed and followed nonlinear saturation kinetics. It was only these higher dosages that produced significant clearing of yeast organism from organs. [101]

There has also been work done with these agents and *Aspergillus* species. Denning and Stevens determined that cilofungin was equivalent to amphotericin B in preventing death in a disseminated aspergillosis murine model. Interestingly the combination of the two agents had antagonistic effect. [102] This had been seen in an in vitro *Candida* study, [103] but an in vivo murine model of candidiasis predicted synergy between the two drugs. [104] The consequence of these drug combinations may depend on the clinical situation. In the same way as cilofungin was demonstrated to noncompetitively inhibit the glucan synthase of *C. albicans*, this was also done with *Aspergillus fumigatus*. [105] An interesting investigation studying in vitro results with *Aspergillus* species was conducted. The researchers were able to correlate the drug concentration where cell wall was clearly being disrupted using electron microscopy, and the drug concentration at which macroscopic morphological changes which could be quantified. They proposed that the use of an MEC (minimum effective concentration), the first concentration which demonstrated this morphological change, would better reflect the activity of echinocandins against filamentous fungi. [106]

An exciting development in the investigation of these agents is in regard to *Pneumocystis carinii*. As stated earlier *P. carinii* has been recently classified as a yeast

and in keeping with this finding contains 1,3-β-glucan in its cyst wall. This inspired researchers to test the susceptibility of this organism to echinocandin. Given the fact that the standard therapy of trimethoprim and sulphamethoxazole was not well tolerated in many patients this possibility was a much needed alternative. It was shown that L-671,329 (pneumocandin $A_0$) was effective in clearing *P. carinii* cysts from rat lung. A papulacandin exhibited reduced activity against the organism. [107] The most recent water soluble pneumocandin derivative L-733,560 was tested for in vitro activity using a modified microdilution adapted from NCCLS tentative guidelines (M27-P), and a disk diffusion method. This agent showed enhanced potency to *Candida* species , weak activity against *C. neoformans*, and only exhibited activity against *Aspergillus* employing the disk diffusion assay. [108] Kurtz et.al. showed that the increased antifungal activity of this agent was due to increased glucan synthase inhibition and not to a secondary effect such as disruption of cell membrane or other membrane bound enzymes. [109] Utilizing a murine model for aspergillosis, candidiasis, and cryptococcosis, Abruzzo and colleagues evaluated in vivo efficacy of L-733,560 compared with amphotericin B. L-733,560 was administered orally and interperitoneally . In aspergillosis and candidiasis L-733,560 was comparable to amphotericin in significantly increasing survival rate, and in candidiasis in sterilizing the kidney. However efficiencies were lower for L-733,560 given orally, and there was no reduction in mortality rate in the cryptococcosis model. [110] L-733,560 was tested against fluconazole sensitive and fluconazole resistant *C. albicans* using a photometric microbroth method that correlated well with the NCCLS tentative standards in reference strains. There was no significant difference in MICs between the two groups. [111]

## (4) Resistance studies

In any new class of agents it is clearly important to characterize the nature of resistance to these agents. Limited work has been done in the area echinocandin resistance. Some of the initial inquiry has been with the organism *Saccharomyces cerevisiae*. Four *Saccharomyces cerevisiae* mutants resistant to echinocandins were isolated utilizing aculeacin A and mutagens. The mutants were tested for cross-resistance to similar agents, echinocandin B was not active with these organisms. However, one strain was susceptible to papulacandin. Both the cell wall and 1,3-β-glucan synthase activity were not changed in the mutants. However, cell surface hydrophobicity was markedly reduced in 3 of the mutants. [112] In another study an induced papulacandin A resistant mutant was isolated which showed no difference in growth rates and membrane lipid content. The MIC in this mutant was 20 times higher than in the wild type. This strain was found to have mutations in the gene encoding the 1,3-β-glucan synthase enzyme. [89] A study which demonstrated spontaneous *Saccharomyces cerevisiae* resistance to pneumocandins revealed cross resistance only to similar classes of agents, and not to other antifungals. Changes in the hydrophobicity of the cell surface, and degradation of the antifungal molecule were ruled out as mechanisms of resistance. The resistance was attributed to reduced susceptibility of the 1,3-β-glucan synthase enzyme to the echinocandins, resulting from a mutation in the gene. [86] This mutant strain also exhibited a supersensitivity to nikkomycins, confirming the understanding of a close link between glucan and chitin. Echinocandin resistant mutants possibly shift some responsibilities over to chitin to overcome the effect of glucan inhibition. Drug combinations of echinocandins and nikkomycins may be a method to deal with resistance in fungi. [113] In *C. albicans* there

has also been a limited number of studies on resistance. One of the first looked at papulacandin B resistance induced by UV radiation, and found that resistance correlated to reduced inhibition of glucose incorporation into the cell wall. [114] *C. albicans* aculeacin A resistant mutants were also induced by UV light. No change in growth rate or susceptibility to agents other than aculeacin A or papulacandin B was found. There was a significant change in the lipid composition of the mutant stain. [115] *C. albicans* echinocandin resistant mutant CA-2 (induced by nitroguanidine)was studied in terms of its 1,3-β-glucan synthase inhibition to cilofungin. It was suggested that inhibition of the enzyme alone was not enough to explain the decrease in susceptibility. The 1,3-β-glucan synthase enzyme was inhibited in both parent and resistant stains for the first four hours after which time the enzyme from the resistant strain began to recover from the effect of the drug. Potential uptake or efflux mechanisms were proposed as an explanation for this observation. [116] One of the problems of the previous *C. albicans* mutant isolations is the use of mutagens which can produce multiple lesions, and would be less likely to occur outside the lab. Recently a *C. albicans* spontaneous mutant has been described. [117] Using concentrations of 2X and 8X the MIC of pneumocandin L-733,560 scientists were able isolate mutants at a rate of 0.1 to 1 in $10^7$ cfu/ml. There was no difference in growth rates or germ tube formation between parent and mutant strain. The organism was resistant to echinocandin structured drugs, but not to clinical agents including: fluconazole, itraconazole, ketoconazole, amphotericin B, and flucytosine. The sensitivity of the 1,3-β-glucan synthase enzyme to L-733,560 is reduced in the mutants stain. Virulence studies in mice demonstrated no change in virulence. While the in vitro MFC for L-733,560 was

1000 fold higher in the mutant there was only an 8-fold increase in the effective dose for treatment of disseminated candidiasis.

### H. Thesis objectives

We proposed to utilize large quantities of *C. albicans* strains to inoculate growth media containing inhibitory levels of LY303366 in an attempt to isolate LY303366 resistant *C. albicans* mutants. Once mutants would be obtained studies would be performed comparing the parent and mutant strains in the following areas: stability of resistance; clinical antifungal susceptibility; growth patterns; LY303366 pharmacodynamic patterns; morphology; LY303366 germ tube inhibition. This was done with the objective to better understand the nature of echinocandin resistance in *C. albicans*.

## III. MATERIALS AND METHODS

### A. Fungal strains

*C. albicans* clinical isolates Y41, Y58, and Y180 from the Clinical Microbiology Department, Health Sciences Centre, Winnipeg, Manitoba, Canada, were used during the course of this study. American Type Culture Collection (ATCC, Rockville, Md) *C. albicans* reference strain 90028 was also utilized. During the course of the study, six LY303366 resistant mutants of parent strain Y41 were isolated. The three stable mutants were labeled Y41LYRa-c, while the transient mutants were labeled Y41LYRd-f. All isolates were stored in skim milk at -80°C, maintained on Sabouraud dextrose agar (see media) incubated at 35°C.

## *B. Antifungal agents*

Drug stocks were made of antifungal agents in the following manner:

### 1. Amphotericin B

112.0 mg of amphotericin B powder (Bristol-Myers-Squib, St. Laurent, Quebec) with a potency of 0.914 mg/mg was dissolved in 10 ml of dimethlysulfoxide (DMSO, Sigma, St. Louis, MO) and allowed to self-sterilize for 30 minutes. This resulted in a stock solution of 10240 µg/ml amphotericin B which was stored at -80°C.

### 2. Fluconazole

20.05 mg of fluconazole powder (Pfizer, Kirkland, Quebec) with a potency of 1.0 mg/mg was dissolved in 2 ml DMSO and allowed to self-sterilize for 30 minutes. This resulted in a solution of 10240 µg/ml fluconazole which was made up fresh the day of each experiment.

### 3. LY303366

26.69 mg of LY 303366 powder (provided as a gracious gift by Eli Lilly, Indianapolis, Indiana) with a potency of 0.956 mg/mg was dissolved in 25 ml of DMSO or methanol (Sigma, St. Louis, MO) and allowed to self-sterilize for 30 minutes. This resulted in a stock solution of 1024 µg/ml LY 303366 which was stored at -20°C.

### 4. 5-Flucytosine

25.6 mg of 5-flucytosine powder ( Hoffman-Laroche, Missisauga, Ontario) with a potency of 1mg/mg was dissolved with heating to 56°C in 25 ml double distilled water. The solution was filtered through a 0.45 µm 115 ml volume Nalgene disposable filter

(Nalge Company, Rochester, New York). This resulted in a solution of 1024 μg/ml of 5-flucytosine which was stored at -80°C.

#### 5. Ketoconazole

25.6mg of ketoconazole powder ( Jansen-Ortho Pharmaceuticals, North York, Ontario) with a potency of 1 mg/mg was dissolved in 25 ml DMSO and allowed to self-sterilize for 30 minutes. This resulted in a stock solution of 1024 μg/ml which was stored at -80°C.

#### 6. Ciprofloxacin

29.32 mg of ciprofloxacin powder (Bayer Inc., Etobicoke, Ontario) with a potency of 873 μg/ml was dissolved in 25 ml of dd water. The solution was filtered through a 0.45 μm 115 ml volume Nalgene disposable filter. This resulted in a stock solution of 1024 μg/ml which was stored at -80°C.

### *C. Media*

#### 1. Buffered RPMI-1640

Buffered RPMI-1640 (Sigma, St. Louis, MO) was used as the liquid medium for susceptibility testing; growth-curves; time-kill curves; and postantifungal effect studies. 10.4 g of RPMI-1640, 2 g of sodium bicarbonate (Sigma, St.Louis, MO), and 34.53 g of 3-[N-morphilino]propane-sulfonic acid (MOPS, Sigma, St. Louis, MO) were dissolved in 900 ml of doubled distilled water. The pH was determined using perpHect Log R meter model 330 ( Analytical Technologies Inc., Boston, MA) and adjusted to pH 7.0 . Approximately 6 ml of 10 N NaOH was needed to adjust the pH. The medium was then made up to 1000 ml with double distilled (dd) water. The solution was then filter sterilized

with a 0.45 μm 500 ml Nalgene disposable filter (Nalge Company, Rochester, New York) and stored at 4°C.

### 2. N-acetylglucosamine yeast nitrogen base proline medium (NYP)

NYP media used to induced germ tube formation in *C. albicans* was prepared in the following manner. 39 ml of 0.2 M monosodium phosphate solution and 61 ml of 0.2 M dibasic sodium phosphate solution were mixed and diluted with distilled water to a volume of 400 ml. The pH of the solution was assessed using perpHect Log R meter model 330 ( Analytical Technologies Inc., Boston, MA), and adjusted to read 7.0 .The solution was then further diluted with sterile water to 800 ml yielding a 50 mM pH 7 phosphate buffer. To 400 ml of this buffer 1.34 g of yeast nitrogen base was added. Also 88.5 mg of N-acetylglucosamine and 46 mg of proline was added to yield 10-3 M of both compounds. Finally the solution was filter sterilized using a 0.45 μg 500 ml Nalgene disposable filter (Nalge Company, Rochester New York).

### 3. Sabouraud dextrose agar (SDA)

SDA ( Becton Dickinson, Cockeysville, MD) was used as the solid medium to culture and maintain *C. albicans* strains. 65 g of SDA was dissolved in 1000 ml of dd $H_2O$ and autoclaved at 121°C for 15 minutes. Then it was dispensed into sterile petri dishes and after cooling stored at 4°C. The pH of the media was $5.6 \pm 0.2$.

### 4. Brain heart infusion (BHI)

BHI (Difco, Detroit, Michigan) was used as the solid media for the isolation of LY303366 resistant *C. albicans*.156 g of BHI was dissolved in 3000 ml of distilled water and autoclaved at 121°C for 40 minutes. Then it was dispensed into sterile petri dishes and after cooling stored at 4°C. The pH of the media was 7.0 .

### 5. LY303366 incorporation into media

LY 303366 and ciprofloxacin was incorporated into SDA and BHI plates at a variety of concentrations utilizing two methods. Firstly, a LY303366 solution was added to the media while they were in liquid form so that the resultant plates would have a specified concentration. Secondly, a LY303366 solution was added to the surface of a solid media plate and allowed to dry. The volume of media in the plate (approximately 25 ml) was taken into consideration so that a specified concentration of LY303366 would be achieved in the plate. DMSO was also added to BHI at volumes equivalent to the DMSO added to BHI in LY303366 incorporated plates. This was as a control to test whether the vehicle (DMSO) that LY303366 is dissolved in exhibited any inhibition of *C. albicans*.

## *D. Mutant formation*

At the time of these experiment there had been minimal research performed in creating LY303366 resistant mutants in *C. albicans*. Of the work using echinocandins as the basis for mutant selection, almost all of the work had been performed on *Saccharomyces cerevisiae*, and mutagens were usually employed. [86,89,112,113] The diploid nature of *C. albicans* makes mutant selection more difficult because many resistance mutations are recessive thus necessitating mutations in both alleles. Also the use of mutagens in experiments is a disadvantage because multiple mutations may result. Therefore the procedures used for mutant selection were not as much patterned after previous yeast experiments, but rather previous work in our lab.

### 1. Broth Method

The *C. albicans* strains ATCC 90028, Y41, Y58, and Y180 were each added to 20 ml of RPMI media in flasks, and incubated for 24 hours in a 37°C shaking waterbath. After 24 hours 1 ml was taken from each broth and used to inoculate: 9 ml of RPMI with ½ x MIC of LY 303366, and 9 ml of RPMI with 0.002% DMSO. All new set of flasks were placed in the shaking waterbath at 37°C for 48 hours. After 48 hours 1 ml was taken from the ½ x MIC flask and used to inoculate 9 ml of RPMI with 1x MIC LY 303366. 1 ml from the control flask was used to inoculate another control flasks with 0.004% DMSO RPMI. The flasks were placed in the shaker waterbath at 37° C for 72 hours. After 72 hours the last step was repeated, after which viable cell counts were examined on SDA. The appearance of the broth was observed through out the experiment.

### 2. Agar Method

Two different solid media were used in these experiments: SDA, and BHI. A colony from *C. albicans* strain Y41 was used to inoculate 1000 ml of RPMI which was incubated at 35°C with agitation. After 24-48 hours the RPMI was centrifuged at 4000 rpm for 20 minutes, using Accuspin FR (Beckman, USA) and the supernatant was poured off. 10 ml of concentrated inoculum was recovered from which viable cell counts were performed. A 1000 µl and 100 µl inoculum was then pipetted on to solid media with LY 303366 incorporated into it at a concentration range of 1-40 µg/ml. These plates were incubated for up to 10 days at 35 °C. Any yeast colonies formed on these plates were repeatedly subcultured to LY 303366 incorporated solid media plates. Susceptibility testing for LY 303366 was performed on colonies from both the original and subsequent

plates. If in vitro resistance was observed from colonies on LY 303366 seeded plates, colonies were subcultured to solid media without LY 303366. To assess stability of resistance susceptibility testing was performed on colonies from these plates. The resistant mutant isolates were stocked in skim milk at -80°C.

## E. Stability

The six subcultures with the initial resistance phenotype (Y41LYRa-f) were repeatedly passed on SDA and incubated at 35 °C for 48 hours. Susceptibility testing was performed on each of these subcultures. This procedure was repeated nineteen times.Y41LYRa and Y41LYf that were stocked in skim milk at -80°C were taken out of stock and recultured on SDA. Susceptibility testing was performed on these cultures to observe stability of the mutants after the stocking procedure.

## F. Susceptibility testing

The antifungal susceptibility testing employed in these experiments was based on the NCCLS proposed reference method for macrobroth dilution antifungal susceptibility testing of yeasts (M27-P, M27-T). [56,57]

Added 1 ml of RPMI to each test tube in a series omitting the first tube. The drug stock solutions were diluted with RPMI-1640 in the following manner:

0.3 ml 10240 µg/ml amphotericin B + 2.7 ml RPMI = 1024 µg/ml amphotericin B

0.25 ml 1024 µg/ml amphotericin B + 15.75 ml RPMI = 16 µg/ml amphotericin B

0.1 ml 10240 µg/ml fluconazole + 9.9 ml RPMI = 1024 µg/ml fluconazole

1 ml 1024 µg/ml fluconazole + 7 ml RPMI = 128 µg/ml fluconazole

0.1 ml 1024 µg/ml LY 303366 + 9.9 ml RPMI =10.24 µg/ml LY 303366

2 ml 10.24 µg/ml LY 303366 + 14 ml RPMI = 1.28 µg/ml LY303366

1 ml 1024 µg/ml flucytosine + 15 ml RPMI = 64 µg/ml flucytosine

0.5 ml 1024 µg/ml ketoconazole + 15.5 ml RPMI = 32 µg/ml ketoconazole

For each of the drugs 1 ml from the diluted stock was pipetted into the first empty test tube in that drug series. 1 ml was then added to the next tube which already contained 1 ml of RPMI. This tube was then vortexed and 1 ml from the 1:2 dilution was removed and added to1 ml RPMI in the next tube of the series creating another 1:2 dilution. This procedure of 1:2 serial dilution continued until the last tube where 1 ml is removed from the tube and discarded. Hence the tubes would each contain 1 ml of a 2-fold dilution of the given drug. The concentration ranges for the antifungal drugs were as follows: Amphotericin B:0.03-16 µg/ml, Fluconazole: 0.06-128 µg/ml, LY303366: 0.00125 -1.28 µg/ml, 5-FC: 0.015-64 µg/ml, Ketoconazole: 0.004-32 µg/ml.

Three colonies from a given subcultured strain grown up on SDA at 37°C for 24-48 hours were picked off with a sterile loop and placed in universal jar with sterile water. This inoculum was measured spectrophotometrically using Spectonic 1201 (Milton Roy, Palo Alto, CA) and adjusted to achieve a tubidity equivalent to 0.5 McFarland standard. 0.1 ml was pipetted from this inoculum and diluted with 9.9 ml RPMI in a Erlenmeyer flask. 0.1 ml from this flask was pipetted into 0.9 ml (4°C) normal saline test tube. After vortexing tube 100 µl and 10 µl were each pipetted to ½ of a SDA, and streaked out over ½ plate. 50 µl from the inoculum flask was pipetted to each of the drug tubes starting with

the lowest concentration of drug and continuing to the highest. The fungal inoculum in each tube was 0.5-2.5 x $10^3$ CFU/ml. Pipette tips were changed between drugs when more than one drug is being challenged by the same organism. 50 µl of fungal inoculum was also added to a growth control tube with 1 ml of RPMI. Negative control tubes containing 1 ml of RPMI and 1 ml of RPMI and drug were also used. An NCCLS reference strain was used as a control as to the standardization of the test. All tubes were agitated prior to incubation. All tubes and colony count plates were incubated for 48 hours at 37°C. After 48 hours the tubes were examined for growth and the colony counts are read off the plates. For amphotericin B and LY 303366 the minimum inhibitory concentration (MIC) was the lowest concentration tube in which no growth is observed. For azole drugs and flucytosine the MIC was the lowest concentration tube in which a 80% reduction in growth compared to the control is observed. To carry this out 20 µl from the growth control was added to 80 µl of fresh RPMI in a new test tube. The lowest concentration of antifungal in a tube which has less or equivalent growth than the 80% diluted tube was considered the MIC. The reason for this procedure is because of the known propensity of the azoles and flucytosine to produce trailing, where a small amount of growth continued to be observed at high antifungal concentrations.

Minimum fungicidal concentration (MFC) was not part of the NCCLS guidelines but were performed in accordance with known practices. 100 µl from each clear tube in a MIC assay was taken and placed on ½ of a SDA plate and spread over the ½ plate. The plates were incubated at 35°C for 24 hours, after which time the number of colonies were counted and the CFU/ml was determined for the individual tubes.

## G. Time-growth curves

Parent strain Y41 was compared to both Y41LYRa and Y41LYRf in regards to time-growth curves. For each strain tested one colony of *C. albicans* from a SDA plate was picked off with a loop and placed in 20 ml of RPMI-1640 in an Erlenmeyer flask. The flask was incubated at 37°C in a shaker water bath for 24 hours. At 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, 24 hours 100 µl was removed from the flask and diluted through a series of 0.9 ml normal saline (4°C) test tubes. Hence in each tube the concentration of CFU/ml was repeatedly diluted 1 in 10 times. 10 µl of normal saline from each tube was placed on a ¼ of a SDA plate and streaked out to cover the full ¼ of plate. All plates were incubated at 35 °C for 24 hours. At this time the plates were taken out and colonies of yeast were counted on each section. This method is known as viable cell counts. Our limit of detection was between 20 and 200 colonies in a given section. From the number of colonies obtained at given dilution the CFU/ml of the cultures were calculated. From this information growth curves could be plotted to show the change in CFU/ml over time for a given strain .

## H. Time-kill curves

Parent strain Y41 was compared to both Y41LYRa and Y41LYRf in regards to time-kill curves. For each strain tested one colony of *C. albicans* from a SDA plate was picked off with a loop and placed in 20 ml of RPMI-1640 in an Erlenmeyer flask. The flask was incubated at 37°C in a shaker water bath for 18 hours. After this time 1 ml from the culture flask was removed and added to a new flask with 8.9 ml RPMI-1640, and 0.1 ml of antifungal at 100 times the desired concentration. The control flask contained 9.0 ml

RPMI-1640 to which the inoculum was added. Typically the final concentration of drug in the test flask was 1X and 10X the MIC for that strain. These flasks were incubated for 24 hours at 37 °C in a shaker water bath. At 0, 1, 2, 3, 4, 5, 6, 12, 24 hours 100 µl was removed from each flask and diluted through a series of 0.9 ml normal saline (4°C) test tubes. Hence in each tube the concentration of CFU/ml was repeatedly diluted 1 in 10 times. 10 µl of normal saline from each tube was placed on a ¼ of a SDA plate and streaked out with a loop to cover the full ¼ of plate. If the CFU/ml was expected to be low at a given time then non-diluted RPMI-1640 inoculum directly from the flask was added to SDA plate. 10 µl was added to ¼ plate, 100 µl to a ½ plate, and 500-1000 µl was added to a full plate of SDA. All plates were incubated at 35 °C for 24 hours. At this time the plates were taken out and colonies of yeast were counted on each section. This method is known as viable cell counts. Our limit of detection was between 20 and 200 colonies in a given section. From the number of colonies obtained at given dilution the CFU/ml of the cultures were calculated. From this information the change in CFU/ml over time could be plotted for a given drug and *C. albicans* strain.

## *I. Post antifungal effect (PAFE)*

Parent strain Y41 was compared to Y41LYRa in regards to PAFE. The PAFE experiments started in a similar manner to the time-kill curve tests. For each strain tested one colony of *C. albicans* from a SDA plate was picked off with a loop and placed in 20 ml of RPMI-1640 in an Erlenmeyer flask. The flask was incubated at 37°C in a shaker water bath for 18 hours. After this time 1 ml from the flask was removed and added to a new flask with 8.9 ml RPMI-1640, and 0.1 ml of antifungal at 100 times the desired

concentration. The control flask contained 8.9 ml RPMI-1640 (and 0.1 ml DMSO at a concentration equivalent to the amount of DMSO in the test flask) to which the inoculum was added. The final concentration of drug in the test flask was 1X the MIC for that strain. The flasks were incubated at 37°C for 2 hours. At this time the *C. albicans* cultures were centrifuged at 4000 rpm for 10 minutes using Accuspin FR ( Beckman, USA). The supernatant was decanted and the pellet was resuspended in 10 ml of RPMI-1640 (37°C). 10 µl from this suspension was diluted with 10 ml RPMI to achieve a 1:1000 dilution. The purpose of the centrifugation and the dilution was to remove the antifungal from the culture. At this point the flasks were placed back in the shaker water bath at 37 °C and allowed to incubate until turbidity was evident. At 0 and 2 hours of pre-exposure to antifungal, and at 0, 4, 5, 6, 8 hours after antifungal removal 100 µl was removed from each flask. The 100 µl was 1:10 serial diluted in 0.9 ml 4°C normal saline.10 µl of normal saline from each tube was placed on a ¼ of a SDA plate and streaked out with a loop to cover the full ¼ of plate. All plates were incubated at 35 °C for 24 hours. At this time the plates were taken out and colonies of yeast were counted on each section of plate. Our limit of detection was between 20 and 200 colonies in a given section. From the number of colonies obtained at given dilution the CFU/ml of the cultures were calculated. From this information the change in CFU/ml over time could be plotted for a given drug, and *C. albicans* strain. The PAFE was calculated as follows: PAFE= T(1log)-C(1log). The post antifungal effect is measured by the difference between the time (min) for the test culture to grow by 1 log after dilution, and the time (min) for the control culture to grow by 1 log after dilution. This is a quantification of the effect a antifungal has on a fungus after the antifungal has been removed.

## J. Morphology

The morphology of Y41 and Y41LYRa was examined by light microscopy and photographs were taken by Nikon microscope camera (Nikon, Tokyo, Japan). The pictures were taken of the yeast in normal saline at10X and 40X power. Also pictures were taken of the yeast in NYP (see germ tube inhibition) after 0 and 3 hours incubation at 35°C.

## K. Germ tube inhibition

The germ tube inhibition protocol was a combination of two previous protocols: the NCCLS proposed macrobroth antifungal susceptibility (M27-P) [56], and the germ tube induction studies of Marichal and colleagues. [42] This study looked at the *C. albicans* strains Y41 and Y41LYRa.

Added 1 ml of NYP to each test tube in a series omitting the first tube. The drug stock solutions were diluted with NYP in the following manner:

0.1 ml 1024 µg/ml LY 303366 + 9.9 ml RPMI =10.24 µg/ml LY 303366

2 ml 10.24 µg/ml LY 303366 + 14 ml RPMI = 1.28 µg/ml LY303366

1 ml from the diluted stock was pipetted into the first empty test tube in the LY303366 series. 1 ml was then added to the next tube which already contained 1 ml of RPMI. This tube was then vortexed and 1 ml from the 1:2 dilution was removed and added to1 ml RPMI in the next tube in the series creating another 1:2 dilution. This procedure of 1:2 serial dilution continues until the last tube where after vortexing 1 ml is removed from the tube and discarded. Hence the tubes will contain 1 fold dilutions of the given drug.

Three colonies from a given subcultured strain grown up on SDA plates at 37°C for 24-48 hours were picked off with a sterile loop and placed in universal jar with sterile water. This inoculum was measured spectrophotometrically and adjusted to achieve a tubidity equivalent to 0.5 McFarland standard. 100 µl from this inoculum was serially diluted to $10^{-3}$ in 4°C normal saline.10 µl and 100 µl were taken from this dilution and added to ½ plates of SDA and streaked out according to standard plating procedure. These plates were placed in the incubator at 35°C for 48 hours and colonies were counted at this time. 50 µl were pipetted from the inoculum into the series of tubes starting with the lowest concentration and continuing to the highest. A growth control consisting of 1 ml of NYP was also inoculated with 50 µl. At 3 hours incubation 17 µl from these inoculated test tubes were removed and placed in wells of the Count 10 Acryli-Slides with grids (V-Tech Inc., Pomona, CA). 3 sectors of the 9 sector grid were examined under the light microscope. Yeast cells were counted and categorized in terms of blastoconidia, germ tube, and pseudohyphae morphology. Percentage of germ tube formation from total cell was calculated for each tube. Germ tube inhibition concentration (GTIC) was determined by the tube with the lowest concentration of drug to exhibit no germ tube formation. The test tubes were incubated at 35°C for 48 hours. After 48 hours the tubes were examined and a MIC was determined as the tube with the lowest concentration to exhibit no growth.

# IV. RESULTS

## A. *Mutant formation*

### 1. Broth method

During the study, media was considered to be opaque when cloudiness was observed, while media was considered to be flocculated when particulate matter was observed. When the ½ x MIC LY303366 and control flasks were first inoculated with *C. albicans* strains 90028, Y41, Y58, Y180 the cultures were observed to be opaque. After 24 hours the growth controls were still opaque. The LY303366 test cultures were opaque as well as flocculated. The same was observed at 48 hours with some flocculation in the controls as well. At the next stage of experiments the 1 x MIC LY303366 and control flasks were inoculated with *C. albicans* from the ½ x MIC LY303366 and control flasks respectively. When first inoculated the control flask was slightly opaque, while the 1 x MIC LY303366 flask was clear. The reason for this difference in appearance could be that the ½ x MIC LY303366 flasks, used to inoculate the 1 x MIC LY303366 flasks, exhibited less growth than the control flasks. After 24 hours the control flasks were opaque, while the 1 x MIC LY303366 flasks were clear with slight flocculation. There was no changed observed at 72 hours except strain 90028 control flask exhibited heavy flocculation. When the strains were reinoculated at 1 x MIC and control the same observations were made at 0, 24, and 72 hours. In the presence of LY30366 there was clearly more flocculation in the 90028 or 41 strains than the 58 or 180 strains. The results of viable cell counts performed

at this time suggested that no regrowth occurred in flasks containing LY303366 (see Table 2). This would suggest that no resistance was selected for during these experiments.

| C.ALBICANS STRAIN | CONTROL (CFU/ML) | LY303366 (CFU/ML) |
|---|---|---|
| 90028 | $3.6 \times 10^6$ | $2.0 \times 10^3$ |
| 41 | $2.7 \times 10^7$ | $9.2 \times 10^2$ |
| 58 | $3.8 \times 10^7$ | 0 |
| 180 | $1.1 \times 10^7$ | 0 |

**Table 2: The viable cell count of 4 strains of *C. albicans* after serial passage in RPMI with (LY303366)and without (CONTROL) LY303366:**

The stains were serially passed in the following manner: 0.02 and 0 µg/ml LY303366 for 48 hours; 0.04 and 0 µg/ml LY303366 for 72 hours; 0.04 and 0 µg/ml LY303366 for 72 hours.

## 2. Agar method

### a) Sabouraud dextrose agar

All resistance isolation experiments utilized *C. albicans* strain Y41. The colony counts indicated that $1 \times 10^9$ CFU/ml was the inoculum added to the 1µg/ml LY303366 SDA plates. The LY303366 was incorporated into SDA while the SDA was in molten bulk form. No inhibition of growth was observed on these plates. Four possibilities for this observation were proposed: resistant organism had been isolated; the high inoculum overcame the inhibition of drug; the method used to add LY303366 to SDA inactivated the drug; LY303366 was inherently inactivated on SDA. To test for the possibility of resistant organism being isolated susceptibility testing was performed on yeast from the LY303366 plates and no change in MIC from original strain was observed. To test for the possibility that the high inoculum overcame the inhibition of LY303366, viable cell counts were performed using inoculums at macrobroth MIC levels: $0.5\text{-}2.5 \times 10^3$ CFU/ml on

SDA plates with and without LY303366. After 24 hours the SDA without LY303366 demonstrated a viable cell count of 530 CFU/plate. After 48 hours the SDA with LY303366 had a viable cell count of 470 CFU/plate. To test for the possibility that the LY303366 incorporation method inactivated the drug, resistance isolation was attempted using LY303366 added to the cooling agar of each individual plate, and to the solid surface of another set of plates. There was no inhibition of organisms seen with either type of LY303366 incorporated SDA when inoculated with $1.85 \times 10^7$ CFU/ml. Smaller inoculums were studied as well. A viable cell count of 600 CFU/plate was demonstrated on SDA without LY303366 while on a SDA plate where 1µg/ml LY303366 had been added to the cooling agar, 390 colonies were counted on the plate. A viable cell count of 156 CFU/plate was exhibited on SDA without LY303366 while on a SDA plate where 1 µg/ml LY303366 had been added to the solid surface, 300 colonies were counted on the plate. In each case the *C. albicans* on LY303366 SDA plate grew as smaller colonies than on SDA, but the number of colonies on each was similar. From the preceding experiments it was concluded that LY303366 was inactivated by SDA regardless of the method of LY303366 incorporation or inoculum size. Another media would need to be used to isolate LY303366 resistant *C. albicans* mutants.

### b) Brain heart infusion agar

#### (1) Initial experiments

BHI without LY303366 or DMSO, BHI with 4% DMSO, and BHI with 40 µg/ml LY303366 were inoculated with 100 µl of $10^7$, $10^5$, and $10^3$ CFU/ml of *C. albicans* strains Y41. After 48 hours the BHI without LY303366 or DMSO had yeast growth throughout

the plates. The BHI with 4% DMSO plates exhibited less yeast growth than the BHI plates, but there was still growth throughout the plates. The BHI with 40 μg/ml LY303366 had no yeast observed on any plate, however there were bacterial contaminates growing on these plates. The same observations were made at 48 hours when 1000 μl and 100 μl of 1 x $10^8$ CFU/ml Y41 were used to inoculate 20 μg/ml LY303366 BHI plates. Then 1000 μl and 100 μl of 8 x $10^6$ CFU/ml Y41 were used to inoculate 40, 20, 10, 5, 2, 1 μg/ml of LY303366 BHI plates. After 48 hours no yeast colonies were observed on any of the plates. There was a number of bacterial contaminate colonies on these plates. There were no colonies of any kind observed on the 1 μg/ml LY303366 BHI inoculated with either 1000 μl or 100 μl of 8 x $10^6$ CFU/ml. However, after 6 days of incubation at 35°C 2 or 3 small colonies were observed on both plates. Light microscopy of cells from these colonies revealed yeast-like morphology. Colonies were subcultured from these plates to BHI, SDA, and 1μg/ml BHI plates. Susceptibility testing was performed utilizing colonies from the original plates and subcultures from SDA, BHI and 1 μg/ml LY303366 BHI plates. No change in macrodilution MIC from parent strain was observed. It was found to be more difficult to reach the standardized inoculum size using cultures from LY303366 seeded plates. When the MIC tubes were left at 35°C for an extra 24 hours no change in growth was observed. There was one exception to this; on one susceptibility test performed on colonies from the original 1 μg/ml LY303366 plate after 72 hours a trailing effect was observed. At the 0.08 μg/ml tube there was a clear reduction in growth, however some growth was seen in the tubes up to and including 1.28 μg/ml. When 100 μl from the 1.28 μg/ml tube was plated on a 1 μg/ml LY303366 BHI plate yeast colonies

with a red coloration were isolated. These colonies were identified to be a Rhodotorula species. Colonies from the original 1 µg/ml LY303366 plate were passed 3 times on 1 µg/ml LY303366 BHI. After the 3 passages susceptibility testing was performed. The MIC was determined to be 0.04 µg/ml when 80% reduction was considered, and 0.16 µg/ml when 100% reduction was considered. Therefore further work was not done on this strain.

### (2) Further development

Mutant selection was attempted again by plating 1000 µl and 100 µl of $4 \times 10^8$ CFU/ml Y41 onto BHI plates with 40, 20, 10, 5, 2, 1 µg/ml LY303366. The plates with a 1000 µl inoculum contained a haze which made it difficult to observe individual colonies. These plates were incubated for 96 hours at 35°C. The plates with a 100 µl inoculums were easier to observe. After 24 hours the plates were observed for colony growth (see Table 3).

| Plate (µg/ml LY) | 40 | 20 | 10 | 5 | 2 | 1 | methanol control | BHI control |
|---|---|---|---|---|---|---|---|---|
| observation | clear | 1 colony | clear | contam.. | 1 colony | 4 (cocci) colonies | partial growth | full growth |

**Table 3: Observation of mutant isolation on LY303366 BHI plates for 24 hours at 35°C.**

After 8 days ≤ 5 small white colonies per plate were observed on the 40, 20, 10, 5 µg/ml LY303366 BHI plates. Light microscopy of cells from these colonies revealed yeast-like morphology. A colony from the 20 µg/ml plate was repeatedly subcultured on BHI plates with 1µg/ml LY303366. The colonies derived from these plates seemed to

grow better than organisms from other plates. This was the reason that these subcultures were the focus of experiments at this point in the experiments. Susceptibility testing on the these subsequent subcultures was performed (see Table 4). There was a trailing effect observed in the susceptibility results for these subcultures that did not occur in the parent strain. Therefore both 100% and 80% reduction was determined in these susceptibility tests.

| PASSAGE # | 1 | 2 | 4 | 5 | 6 | 6 REPEATED |
|---|---|---|---|---|---|---|
| LY MIC µg/ml (% inhibition) | 0.02 (100) | 0.02(80)<br>0.32(100) | 0.02(100) | 0.04(80)<br>0.64(100) | 0.01(100) | 0.02(80)<br>0.08(100) |

**Table 4: MIC results from initial *C. albicans* and subcultures grown on LY303366 BHI plates.**

The results of these tests demonstrated no change in MIC from the parent strain when the 80% endpoint was used. There was some increase in MIC when the 100% endpoint was used, but in each case the 80% reading showed no increase in MIC. This would confirm that a trailing effect was demonstrated by this strain against LY303366. However our goal was isolate markedly resistant mutants therefore further studies were not performed on this strain.

### (3) Resistant mutant isolation

The *C. albicans* strain that was isolated from the 10 µg/ml LY303366 BHI plate was repeatedly subcultured on 1 µg/ml LY303366 and 20 µg/ml ciprofloxacin BHI plates (to prevent contamination). After 5 passages on such media susceptibility testing was performed on this organism. The MIC of LY303366 on this strain was >1.28 µg/ml when determined by 100% inhibition, and 1.28 µg/ml when determined by 80% inhibition. From

this plate colonies were subcultured to BHI, and 5μg/ml ciprofloxacin in BHI. Susceptibilities were tested from both media. The organism from the BHI plate yielded a MIC of 0.08 μg/ml while the ciprofloxacin BHI plate yielded a MIC of 1.28 μg/ml (100% inhibition). At this point resistance to LY303366 had been confirmed from isolated *C. albicans*. However because the resistance was not consistently seen when the strain was grown up on different media, colonies from the BHI with LY and ciprofloxacin plate, where in vitro resistance was first demonstrated, were subcultured to a series of different media. Colonies were subcultured to BHI without antibiotic (B); BHI with 5 μg/ml ciprofloxacin (BC); BHI with 1 μg/ml LY303366 (BLY); BHI with 5 μg/ml ciprofloxacin and 1 μg/ml LY303366 (BCLY); and SDA with 5 μg/ml ciprofloxacin (SC). The parent strain Y41 was tested as a control (CON). When duplicate susceptibility testing was carried out on the organisms from these media all were shown to be resistant to LY303366, except the control (See Table 5).

| | TEST #1 | | | | | | TEST #2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Media | CON | B | BLY | BC | BCLY | SC | CON | B | BLY | BC | BCLY | SC |
| MIC (μg/ml) | 0.04 | ≥1.28 | 1.28 | ≥1.28 | ≥1.28 | ≥1.28 | ≤0.0025 | 0.32 | 1.28 | 0.32 | 0.32 | 0.64 |

**Table 5:Susceptibility testing results of *C. albicans* from various media.**

Colonies growing on various media were restreaked to their same respective media and susceptibility tests were performed again. One new strain tested was taken from *C. albicans* colonies growing on 1μg/ml LY303366 and 5 μg/ml ciprofloxacin BHI (BCLY) and streaked out on SDA (S1). The results were similar to previous experiments with the parent (CON), and strain grown on SDA (S1) demonstrating low resistance to

LY303366, while the strains grown the other media (B, BC, BLY, BCLY, SC) had high resistance (see Table 6).

| | TEST #1 | | | | | | | TEST #2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Media | CON | S1 | B | BC | BLY | BCLY | SC | CON | S1 | B | BC | BLY | BCLY | SC |
| MIC | 0.02 | 0.04 | ≥1.28 | ≥1.28 | ≥1.28 | 1.28 | 1.28 | 0.02 | 0.01 | ≥1.28 | ≥1.28 | ≥1.28 | 0.32 | ≥1.28 |

**Table 6: Susceptibility testing results of *C. albicans* from various media.**

Colonies growing on various media were again restreaked on the same media and susceptibility testing was performed. One new strain tested was taken from a colony growing on BHI and subcultured to SDA (S2). This time the results showed that even the *C. albicans* growing on SDA (S2) was resistant to LY303366 (see Table 7). For the first time *C. albicans* colonies growing on SDA without antibiotics demonstrated LY303366 resistance.

| | Test #1 | | | | | Test #2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Media | CON | S | B | BCLY | SC | CON | S | B | BC | BLY | BCLY | SC |
| MIC (μg/ml) | ≤0.0025 | 1.28 | 1.28 | ≥1.28 | 0.02 | 0.02 | 1.28 | ≥1.28 | 0.64 | 1.28 | 1.28 | 0.04 |

**Table 7: Susceptibility testing results of *C. albicans* from various media.**

All strains were cultured from their respective media onto SDA without antibiotic. SDA without LY303366 or ciprofloxacin was used to culture all *C. albicans* strains for all subsequent experiments. The strains isolated on a variety of media and subsequently maintained on SDA were labeled as indicated on Table 8. Y41LYRf resistant mutant and the subculture from this plate were stocked in skim milk at -80°C

| MEDIA | BHI | BHI WITH CIPROFLOXACIN | BHI WITH CIPROFLOXACIN AND LY303366 | BHI WITH LY303366 | SABOURAUD WITH CIPROFLOXACIN | SABOURAUD |
|---|---|---|---|---|---|---|
| Label | Y41LYRa | Y41LYRb | Y41LYRc | Y41LYRd | Y41LYRe | Y41LYRf |

**Table 8: Labels for strains isolated from various media.**

## B. Stability

### 1. Y41LYRf

| TEST# | #1 | #2 | #3 | #4 | #5 | #6 | #7 |
|---|---|---|---|---|---|---|---|
| Y41LYRf | 1.28 | 0.32 | 0.16 | 0.04 | 0.04 | 0.02 | 0.04 |

**Table 9: MIC (μg/ml) results on Y41LYRf from a series of susceptibility tests.**

The organisms which first exhibited resistance to LY303366 when grown on SDA (Y41LYRf) demonstrated a transient resistance phenotype. Table 9 shows that the MICs to LY303366 returned to parent strain levels (0.04 μg/ml) with continuous susceptibility testing of the strain. When strain Y41LYRf was taken from skim milk stock and cultured on SDA the susceptibility test yielded a MIC of 0.04 μg/ml. The parent Y41 and Y41LYRf which had reverted back to the parent MIC were plated onto BHI with 1 μg/ml LY303366.The parent Y41 did not grow on the LY303366 BHI, while the Y41LYf did grow on these plates. When susceptibility testing was performed on this culture it exhibited MICs to LY303366 of 0.32-0.64 μg/ml.

### 2. Y41LYRa-e

After the transient resistance was observed in Y41LYRf the susceptibilities of the other subcultures (Y41LYRa-e) was examined. All strains were repeatedly passed on SDA plates and susceptibility testing to LY303366 was performed. There was observed in strains Y41LYRa-e variable stability in their susceptibility to LY303366. Table 10 shows the MIC of these strains over 10 passages on SDA. Within 3 passages, 2 of the strains (Y41LYRd,e) demonstrated MICs within one tube of the wild-type strain (0.04 μg/ml). 3

other strains (Y41LYRa-c) continued to yield MICs of 0.64-10 µg/ml to LY303366. The organism were passed on SDA for a total of 19 times with no change from these results. Figure 9 displays in graphic form the stability of resistance observed in 3 strains and the return to parent strain MICs in 2 strains. After 13 passings the colonies fromY41LYRa-e were stocked in skim milk at -80°C. After 9 days the stable mutants Y41LYRa-c were taken from the skim milk stock and subcultured three times on SDA. Susceptibility testing was performed on these strains and results showed a stable MIC to LY303366 of ≥1.24 µg/ml.

| Passage # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Y41 | 0.01 | 0.04 | 0.04 | 0.01 | ≤0.0025 | N/A | 0.02 | 0.02 | 0.02 | 0.02 |
| Y41LYRa | ≥1.28 | ≥1.28 | ≥1.28 | 1.28 | 1.28 | 0.64 | ≥1.28 | 1.28 | 1.28 | 1.28 |
| Y41LYRb | 0.64 | ≥1.28 | ≥1.28 | 1.28 | 1.28 | 0.64 | 0.64 | 1.28 | ≥1.28 | 1.28 |
| Y41LYRc | 1.28 | 1.28 | 0.64 | 1.28 | 0.64 | 0.64 | 1.28 | ≥1.28 | 1.28 | 1.28 |
| Y41LYRd | 0.64 | ≥1.28 | 0.04 | 0.08 | 0.02 | ≤0.0025 | 0.04 | 0.02 | 0.04 | 0.02 |
| Y41LYRe | 1.28 | 1.28 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 | 0.02 | 0.02 | 0.04 |

**Table 10: MIC (µg/ml) results from serial passage of Y41 and Y41LYRa-e.**

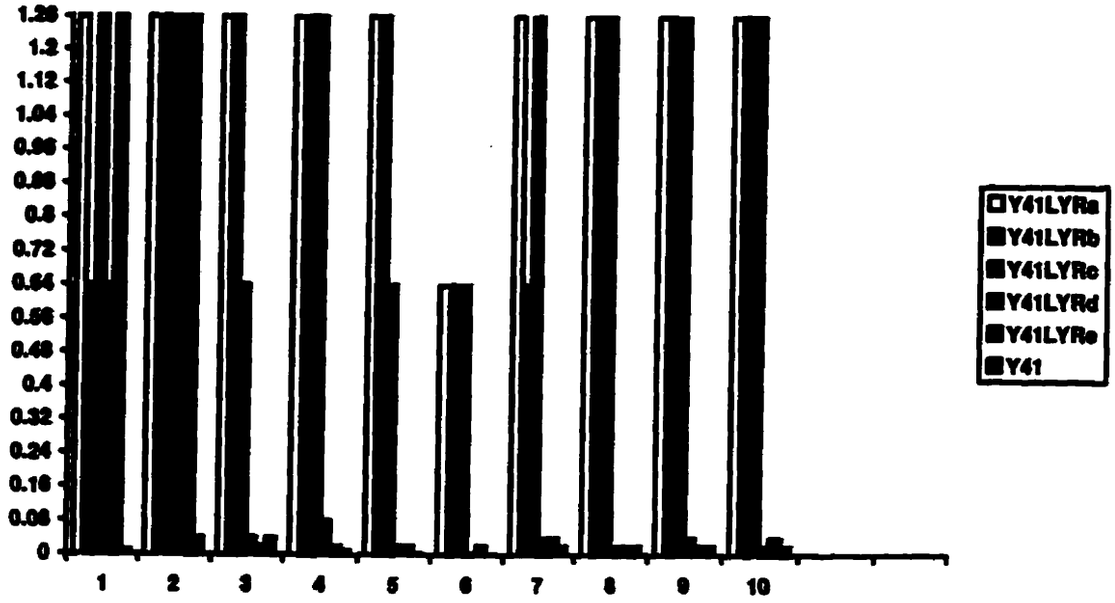



**Figure 9: Graphic display of the MICs (μg/ml) results from the serial passage of Y41LYRa-e.**

## *C. Susceptibility testing*

### 1. Y41LYRf

Susceptibility testing showed no change in MIC between Y41 and Y41LYRf for clinical antifungals (see Table 11).

| | Y41 (TEST 1/TEST 2 ) | Y41LYRf (TEST 1/TEST 2) |
|---|---|---|
| Amphotericin B (μg/ml) | 1/0.5 | 1/0.5 |
| Fluconazole (μg/ml) | 0.5/0.25 | 0.5/0.5 |
| LY303366 (μg/ml) | 0.04/≤0.0025 | 0.32/0.16 |
| 5-Flucytosine (μg/ml) | 0.125/0.125 | 0.125/0.125 |
| Itraconazole (μg/ml) | ≤0.004/≤0.004 | 0.008/≤0.004 |

**Table 11: MIC (μg/ml) results for duplicate susceptibility testing of clinical antifungals against Y41 and Y41LYRf.**

### 2. Y41LYRa

Testing examined the susceptibility of clinical antifungals against Y41 and the stable mutant Y41LYRa. There was demonstrated no change in susceptibility to antifungal agents except LY303366. All MIC differences between parent and mutant were within the NCCLS acceptable limits for reproducibility (see Table 12).

| | Y41 (TEST 1/TEST 2) | Y41LYRa (TEST 1/TEST 2) |
|---|---|---|
| Amphotericin B | 0.5/1 | 1/1 |
| Fluconazole | 0.125/0.25 | 0.25/0.25 |
| LY303366 | 0.04/0.005 | 1.28/≥1.28 |
| 5-FC | 0.125/0.125 | 0.125/0.125 |
| Ketoconazole | ≤ 0.004/≤0.004 | ≤0.004/≤0.004 |

**Table 12: MIC (μg/ml) results for clinical antifungals against Y41 and Y41LYRa.**

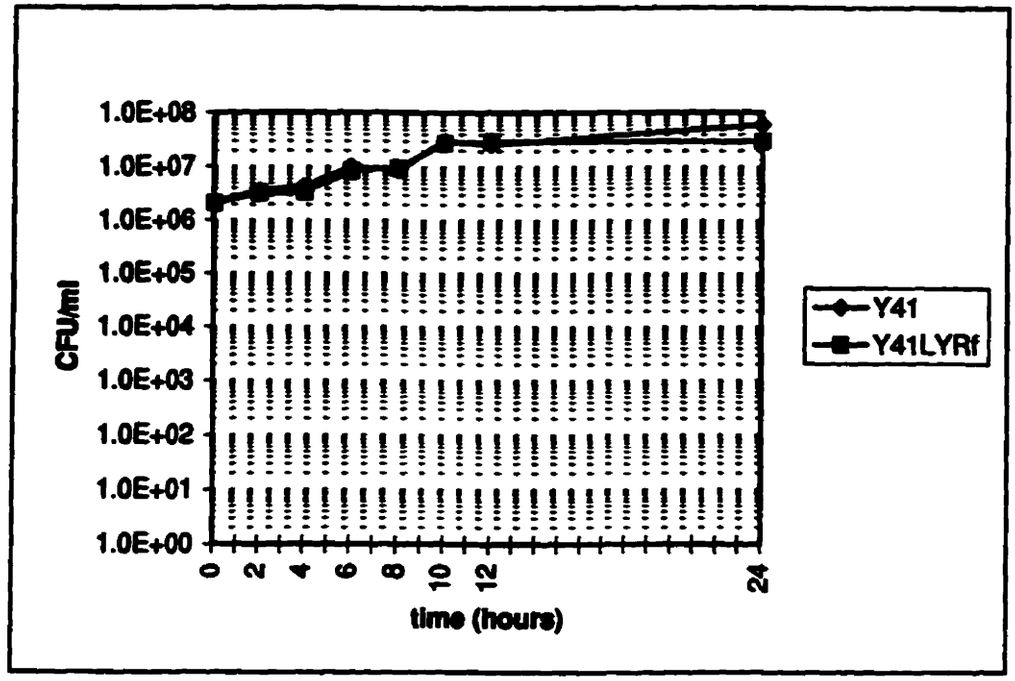
## *D. Time-growth curves*

### 1. Y41LYRf

Time growth curves revealed no difference in growth between Y41 and Y41LYRf strains (see Figure 10). Both strains had inoculums of $1x10^6$ CFU/ml and by 12 hours had reached a plateau of $\geq 1x10^7$ CFU/ml. The MIC for the Y41 and Y41LYf was 0.02 and 0.64 μg/ml respectively.

### 2. Y41LYRa

The growth curves of both organisms were similar in nature (see Figure 11). There were minor differences seen between the two strains. However there were no major differences observed in the overall growth pattern and rate of Y41 and Y41LYRa.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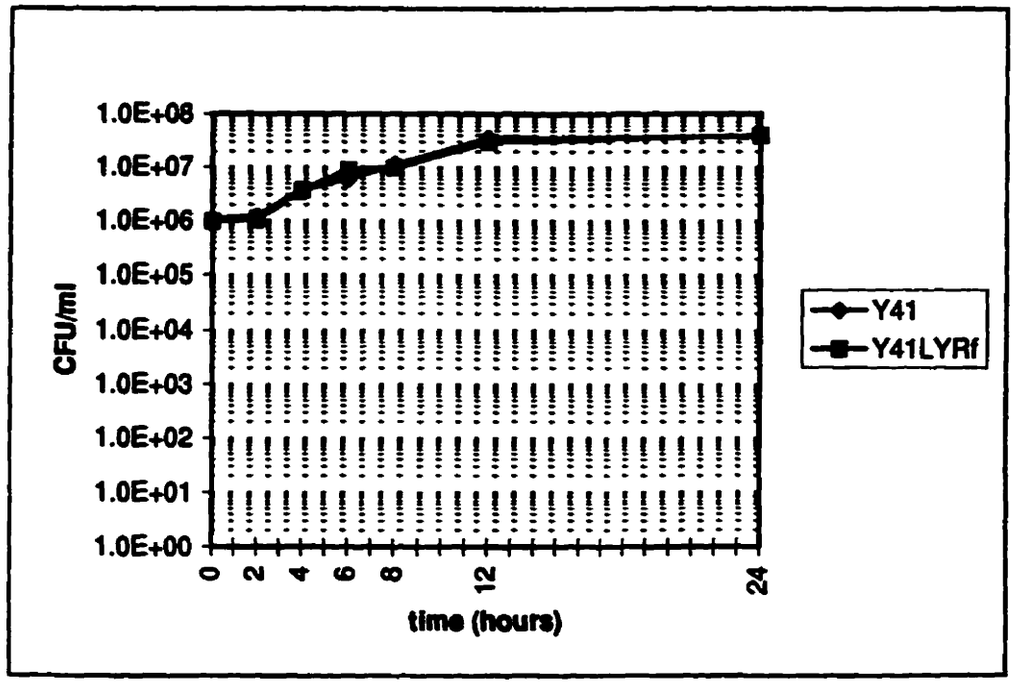
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
8
10
12
24
time (hours)
Y41
Y41LYRf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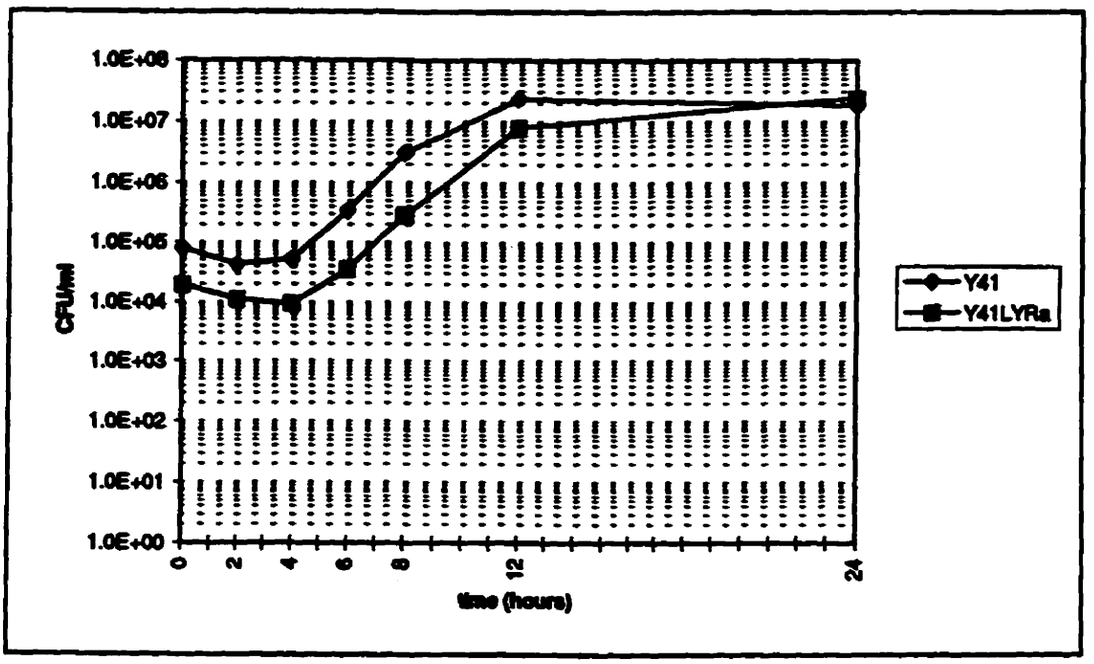
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
8
12
24
time (hours)
Y41
Y41LYRf

**Figure 10: Growth control curves of *C. albicans* Y41 and Y41LYRf:**

Viable cell counts were performed at 0, 2, 4, 6, 8, 12, 24 hours. CFU/ml was plotted over time in hours.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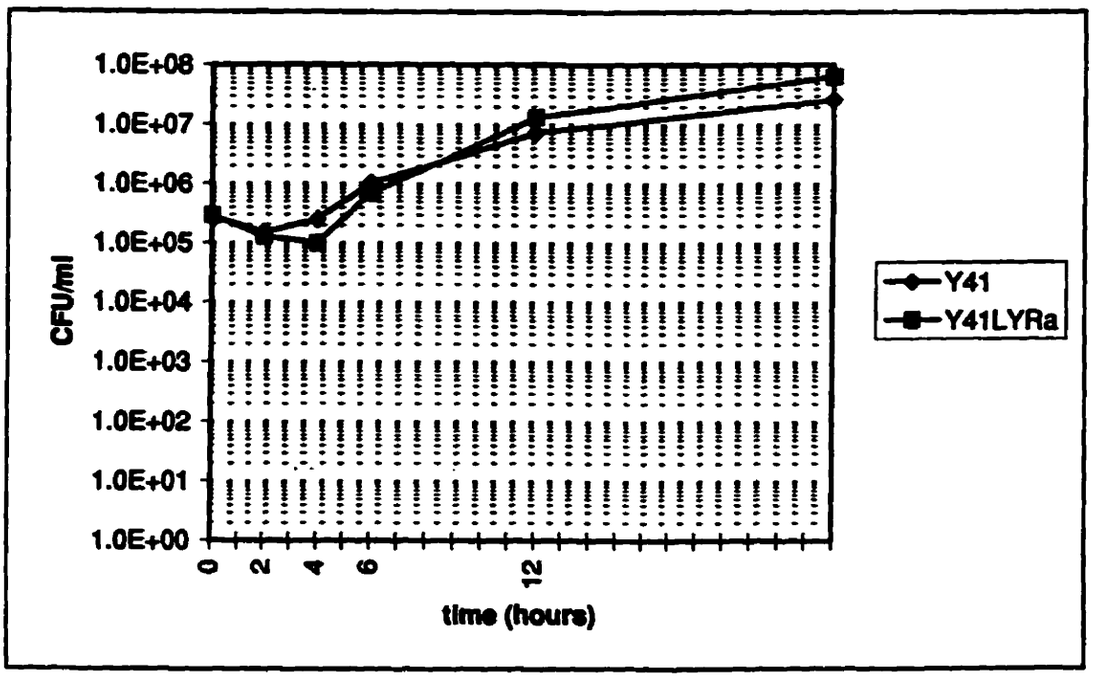
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
8
12
24
time (hours)
Y41
Y41LYRa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1.0E+06
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1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
time (hours)
Y41
Y41LYRa

**Figure 11. Growth control curves of *C. albicans* Y41 and Y41LYRa:**

**Viable cell counts were performed at 0, 2, 4, 6, 12, 24 hours. CFU/ml was plotted over time in hours.**

## *E. Time-kill curves*

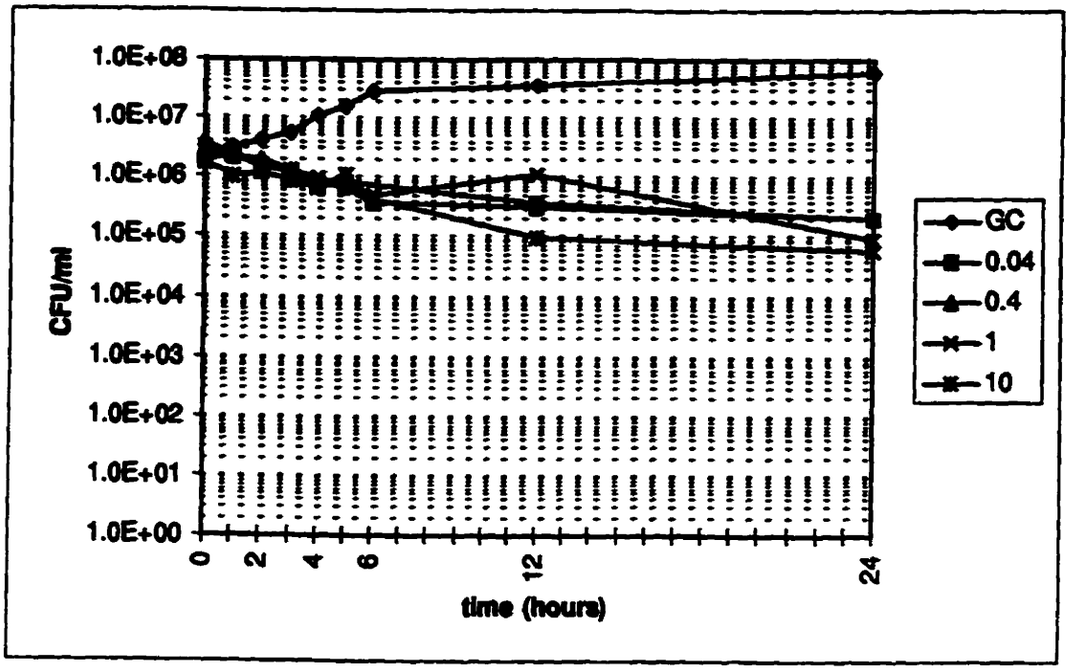
### 1. Y41LYRf

The time kill curves revealed some surprising results. When the time-kill curves were performed on Y41LYRf susceptibility tests showed that this strain had reverted to the parent MIC of 0.08 μg/ml. The concentration of LY303366 for the kill curves was 0.04 μg/ml, 0.4 μg/ml, 1μg/ml, and 10 μg/ml. For the Y41 parent strain all concentrations produced approximately the same results. At 6 hours there was a ½-1 log reduction in growth and a ½-1½ log reduction by 24 hours. For Y41LYRf the concentrations 0.4 μg/ml, 1 μg/ml, and 10 μg/ml resulted in similar kill-curves to Y41. There was a 1-1½ log reduction in growth at 6 hours and by 24 hours a 1-2 log reduction (Figure 12). However at the concentration of 0.04 μg/ml LY303366 Y41LYRf exhibited a unique kill curve pattern. In this case there was a ½-1 log reduction at 6 hours, however by 24 hours there was regrowth back to the original inoculum (see Figures 13, 14). This was especially surprising considering the low MICs this strain demonstrated at this point.

### 2. Y41LYRa

The time-kill curves of the parent Y41 were similar to what was seen in experiments with other susceptible *C. albicans* strains. At 1X and 10X MIC most killing occurred in the first 6 hours, and by 24 hours approximately 1 log of kill was achieved. The MIC of LY303366 against Y41 was 0.04 μg/ml. The MIC of LY303366 against Y41LYR was 1.28 μg/ml. Kill curves were conducted against Y41LYR at 1 and 10 μg/ml (1 and 10X MIC) with results similar to the parent strain (see Figures 15, 17). Most kill was observed in the first 6 hours with approximately 1 log of kill achieved at 24.

LY303366 demonstrated a non-dose dependent killing effect against Y41 and Y41LYRa. Kill-curves were also performed against Y41LYRa at 0.04 μg/ml LY303366 (1/32XMIC). No killing was observed at this concentration with the growth slightly less than the control curve (see Figures 15, 16).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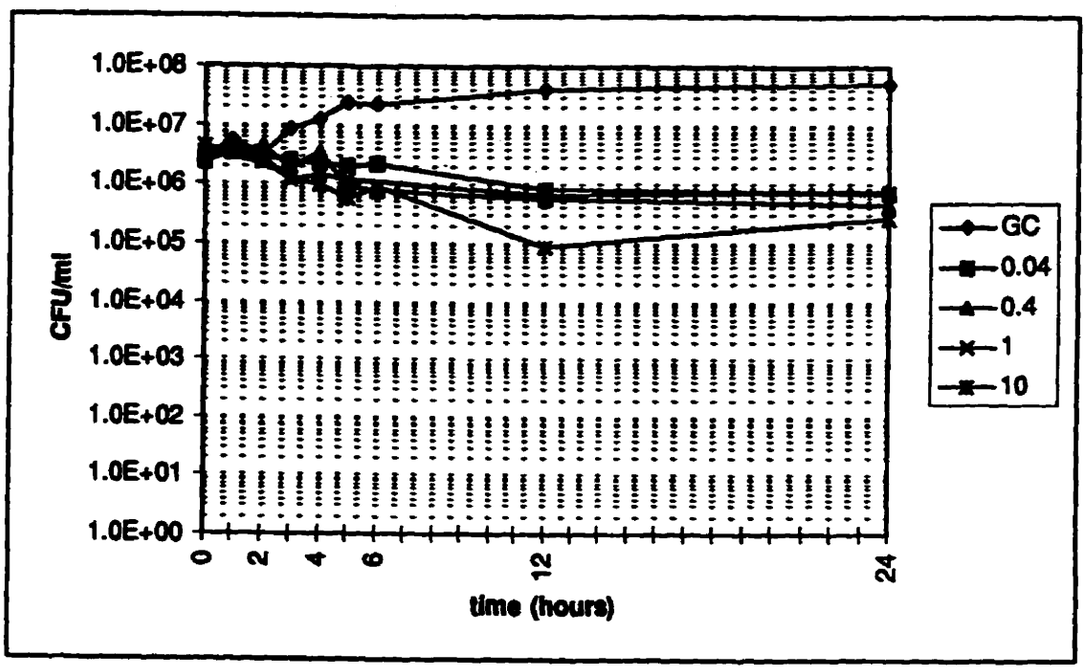
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
0.04
0.4
1
10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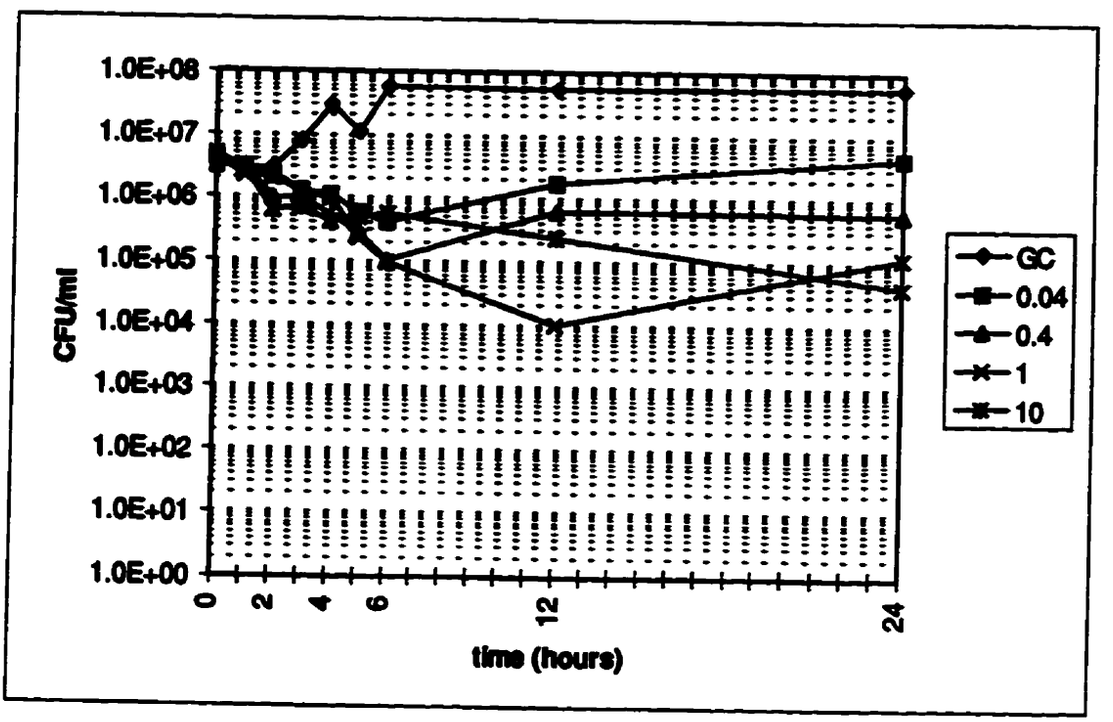
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
0.04
0.4
1
10

**Figure 12: Kill curves at 0.04, 0.4, 1, 10 μg/ml LY303366 against *C. albicans* Y41:**

Viable cell counts were performed at 0, 1, 2, 3, 4, 5, 6, 12, and 24 hours and CFU/ml was plotted over time in hours.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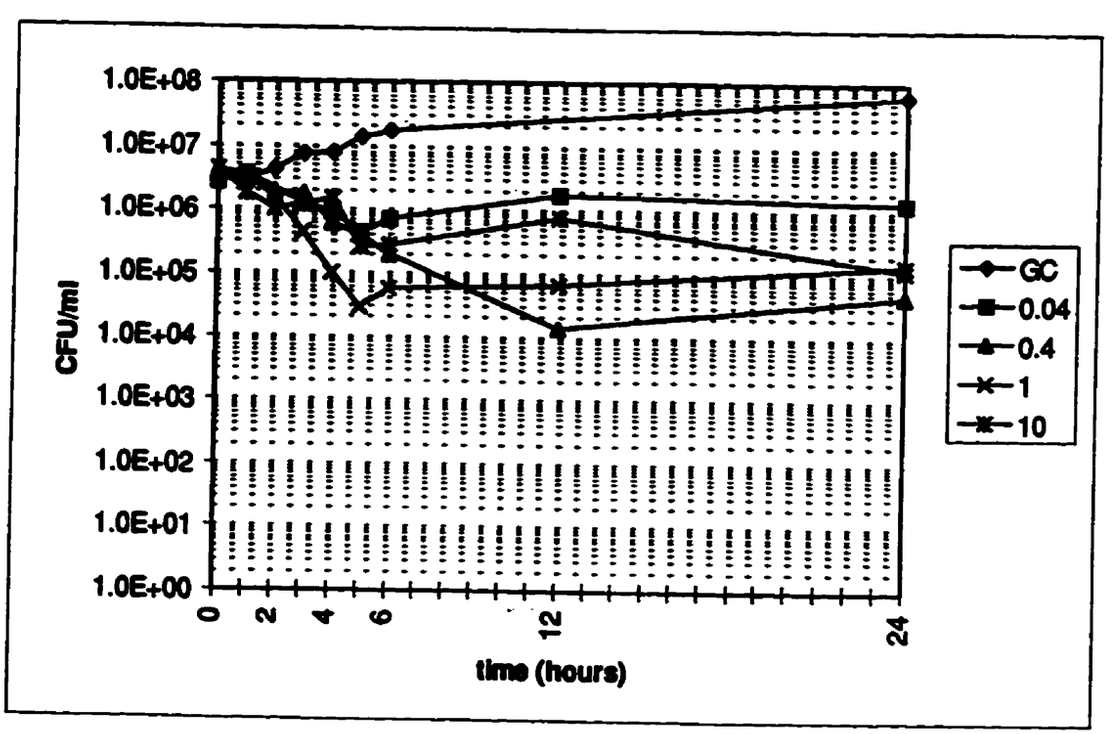
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
0.04
0.4
1
10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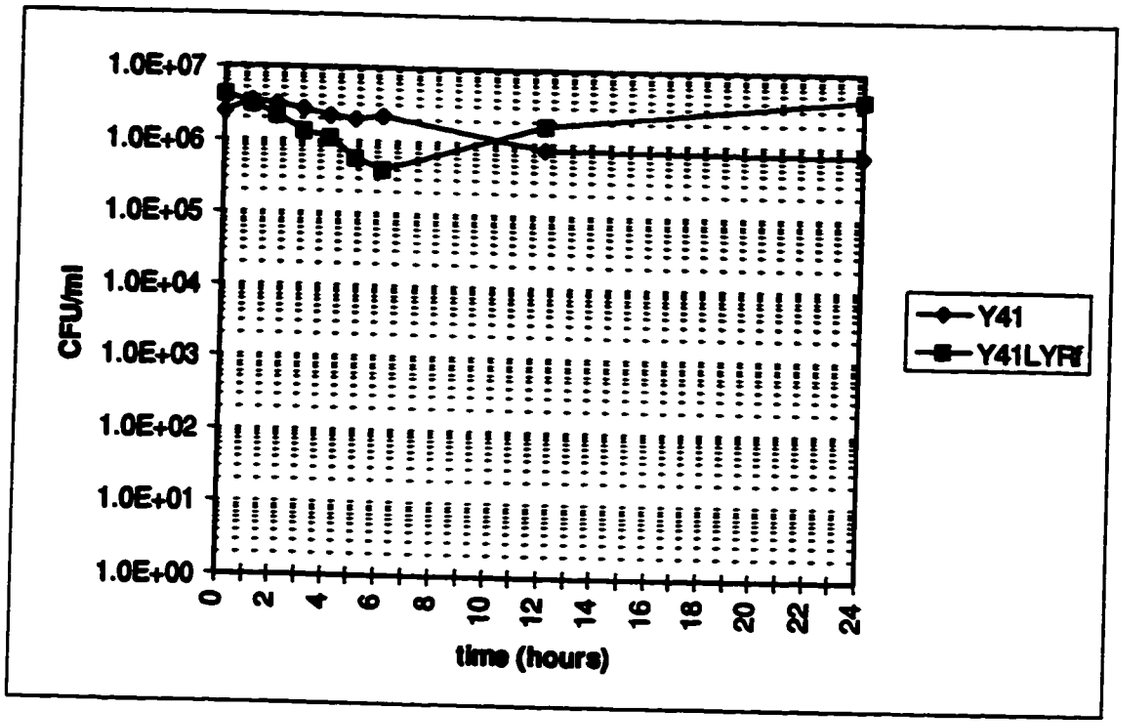
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
0.04
0.4
1
10

**Figure 13: Kill curves at 0.04, 0.4, 1, 10 μg/ml LY303366 against *C. albicans* Y41LYRf.**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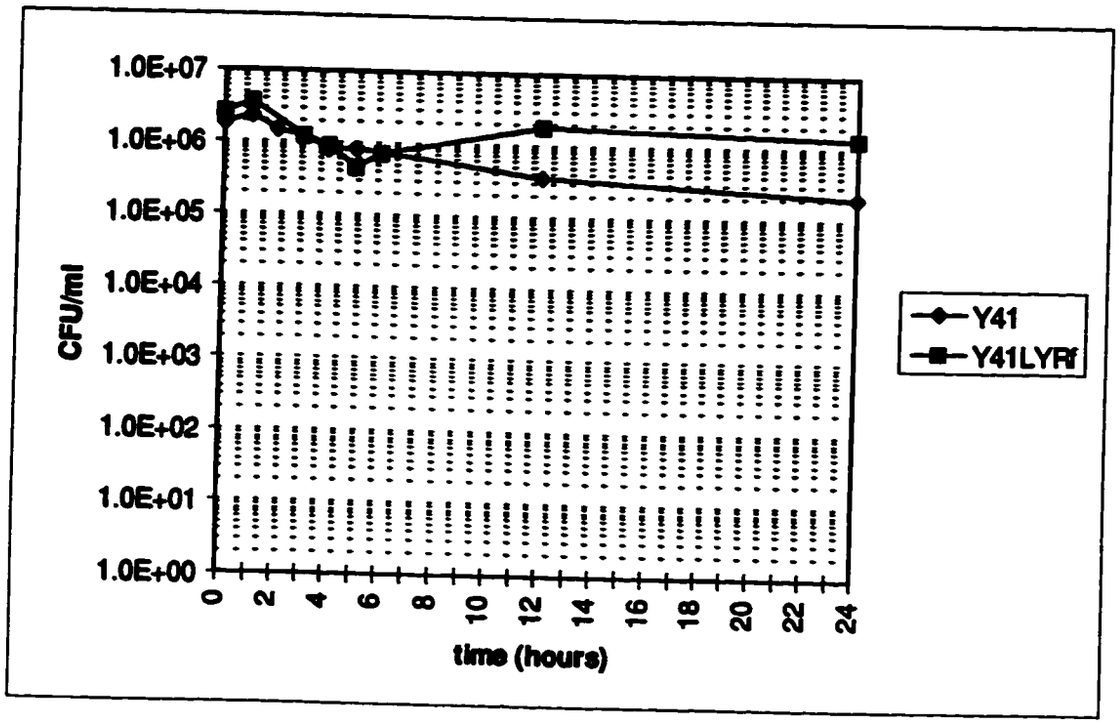
1.0E+00
CFU/ml
0
2
4
6
8
10
12
14
16
18
20
22
24
time (hours)
Y41
Y41LYRf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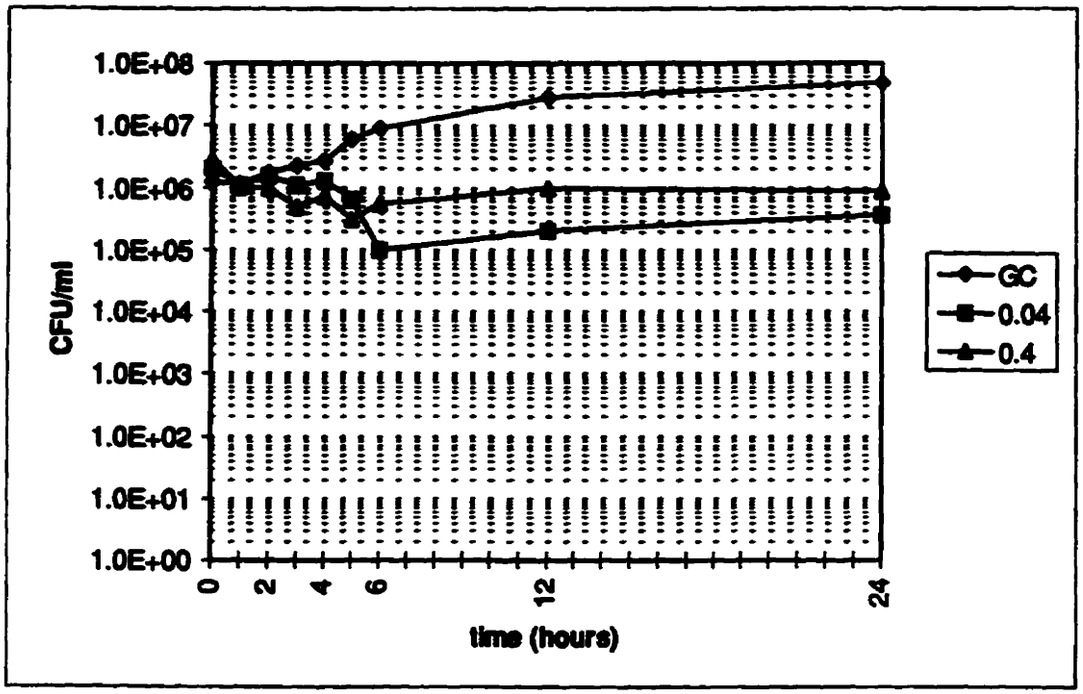
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
8
10
12
14
16
18
20
22
24
time (hours)
Y41
Y41LYRf

**Figure 14: Comparison of kill curves at 0.04 µg/ml LY303366 against *C. albicans* Y41and Y41LYRf:**

Reduction of cell count observed inY41 culture over 24 hours, initial reduction with regrowth to original inoculum observed in Y41LYRf over 24 hours.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1.0E+03
1.0E+02
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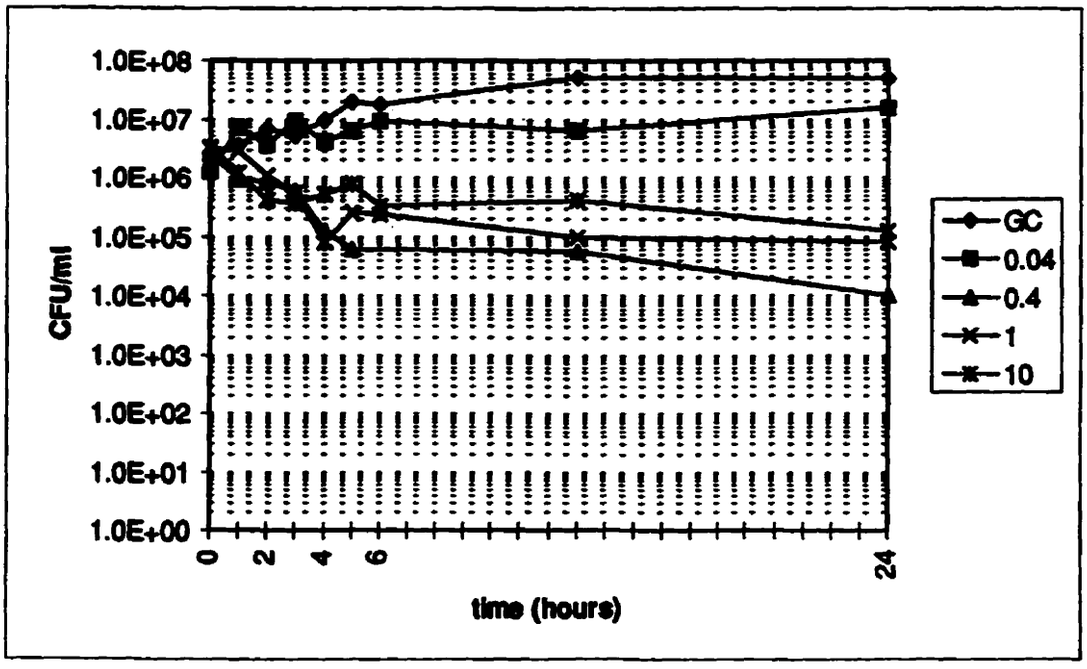
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
0.04
0.4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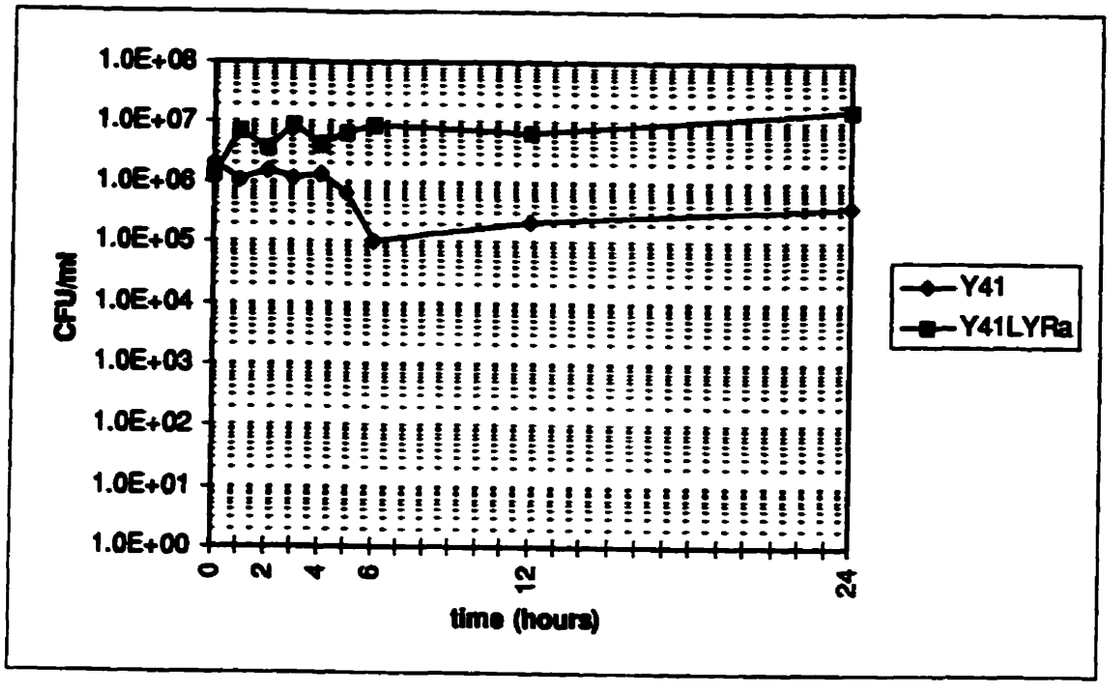
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
24
time (hours)
GC
0.04
0.4
1
10

**Figure 15: Kill curves at 0.04, 0.4,( 1, 10 μg/ml) LY303366 against *C. albicans* Y41 and Y41LYRa:**

Viable cell counts were performed at 0, 1, 2, 3, 4, 5, 6, 12, and 24 hours and CFU/ml was plotted over time in hours. The top graph depicts the kill curves of 0.04 and 0.4 μg/ml LY303366 againstY41. The bottom graph depicts the kill curves of 0.04, 0.4, 1, 10 μg/ml LY303366 against Y41LYRa.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1.0E+03
1.0E+02
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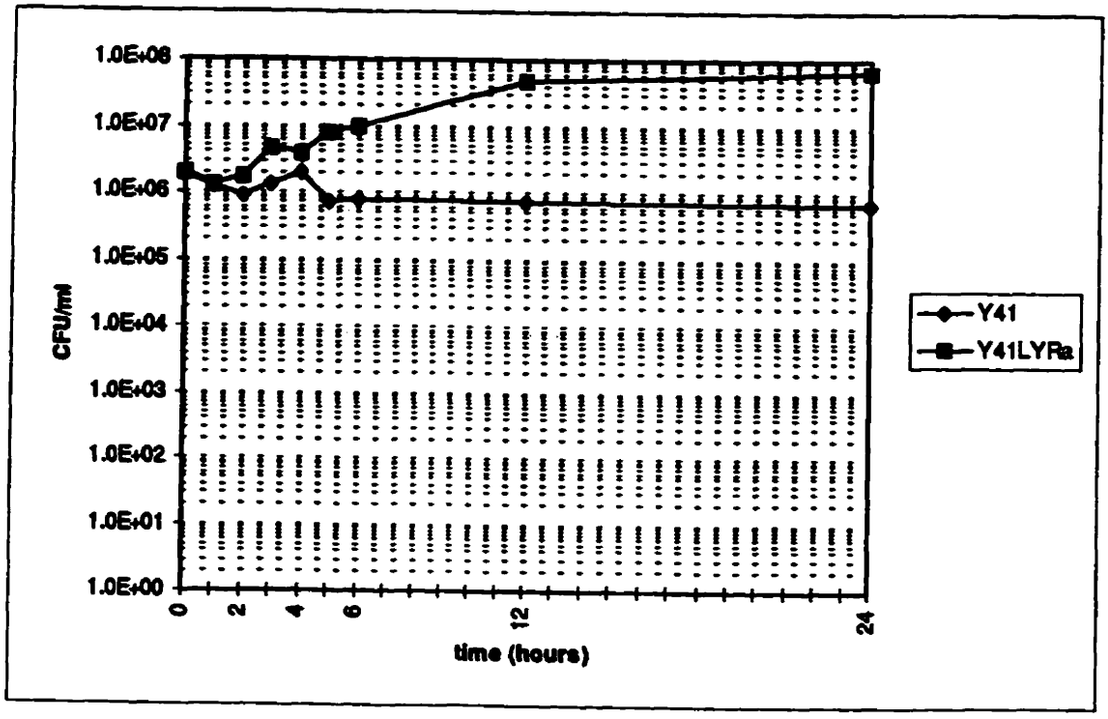
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
Y41
Y41LYRa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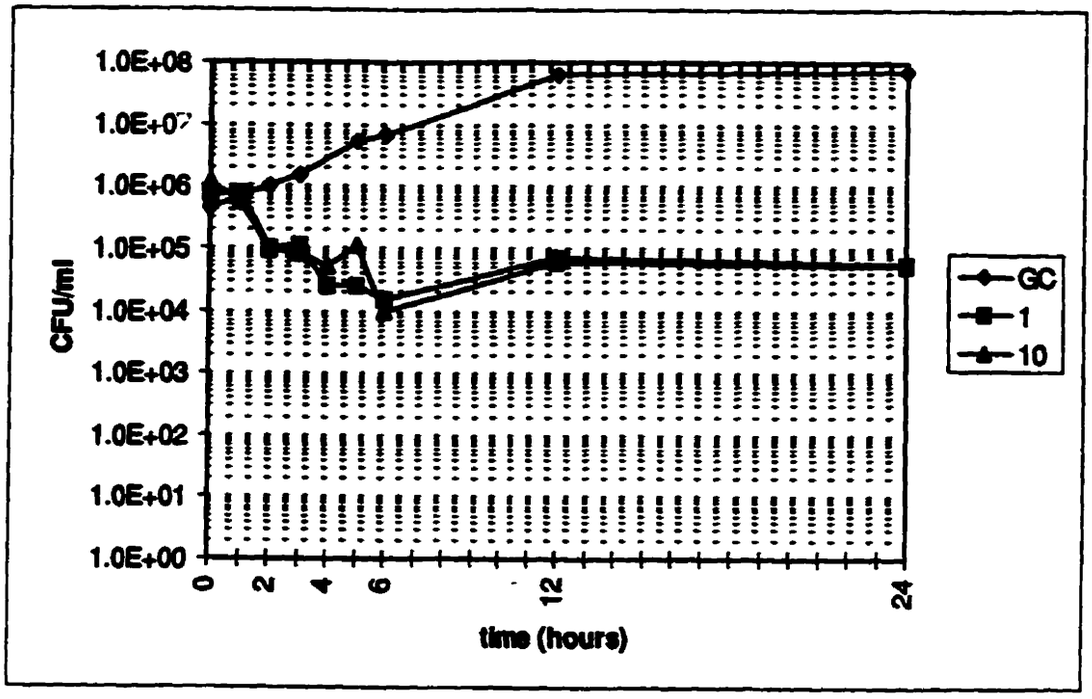
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
Y41
Y41LYRa

**Figure 16: Comparison of kill curves at 0.04 µg/ml LY303366 against *C. albicans* Y41 and Y41LYRa:**

**Reduction of cell count observed in Y41 over 24 hours, while increase in cell count observed in Y41LYRa over 24 hours.**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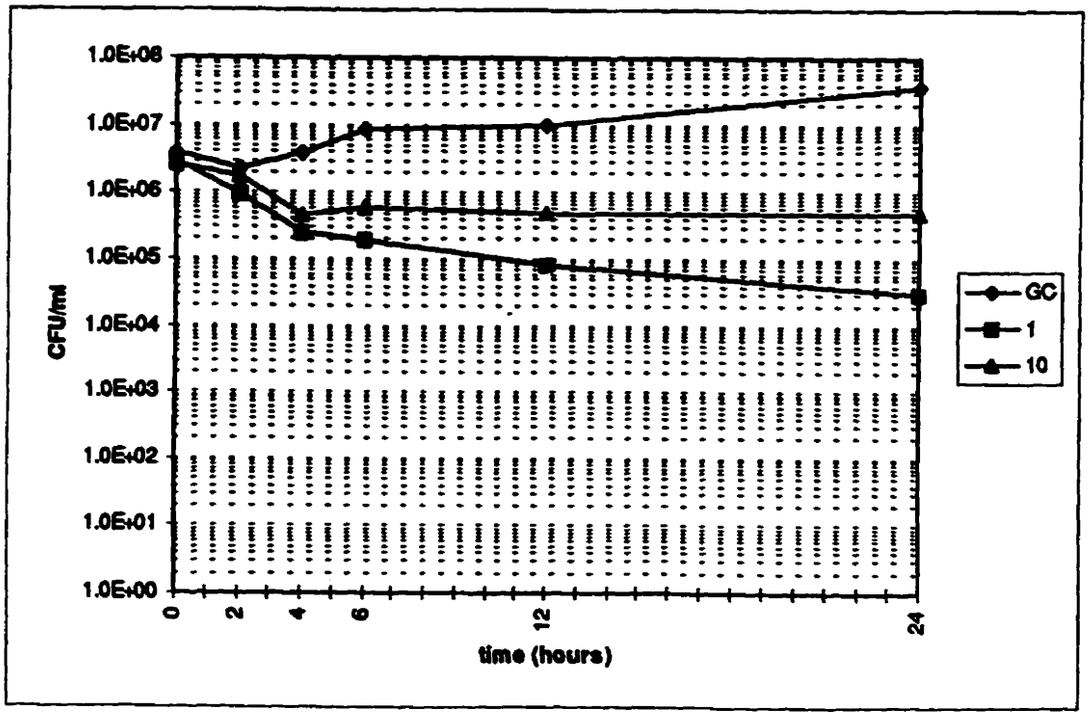
CFU/ml
0
2
4
6
12
24
time (hours)
GC
1
10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1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
2
4
6
12
24
time (hours)
GC
1
10

**Figure 17: Kill curves at 1 and 10 μg/ml LY303366 against *C. albicans* Y41LYRa.**

**Post Antifungal Effect**

Both Y41 and Y41RLYa exhibited a PAFE to LY303366 at MIC concentrations. The organism that had been exposed to drug, consistently required more time to grow by one log than the control organism (see Figures 19, 20).The Test #2 Y41LYRa growth control grew by just under 1 log in 6 hours and plateaued. The time to 1 log for the growth control was considered to be 6 hours. The PAFE of LY303366 on Y41 and Y41RLYa was similar (see Table 13).

| | TEST #1 | TEST #2 | AVR ± SD |
|---|---|---|---|
| Y41 PAFE | 71 min | 104 min | 87.5 ± 23.33 |
| Y41LYR PAFE | 83 min | 47 min | 65 ± 25.46 |

**Table 13:PAFE results on *C. albicans* strains Y41 and Y41LYRa.**

Sample calculations: PAFE= T(1log)-C(log); T(log)=360 min + ($1.5x10^4$ cfu-$2.4x10^3$ cfu)/($4.6x10^4$ cfu-$2.4x10^3$ cfu) x 120 min=395 min; C(1log )= 300 min + ($1.8x10^4$-$9.9x10^3$/$3.0x10^4$-$9.9x10^3$) x 60 min=324 minutes.; PAFE=395 min-324 min=71 min.

## *F. Morphology*

### 1. Blastoconidia

There was no discernible difference in appearance between both Y41 and Y41LYRa in saline solution utilizing light microscopy. Both had the classical oval blastoconidia appearance in this solution (see Figure 21).

### 2. Germ tube formation

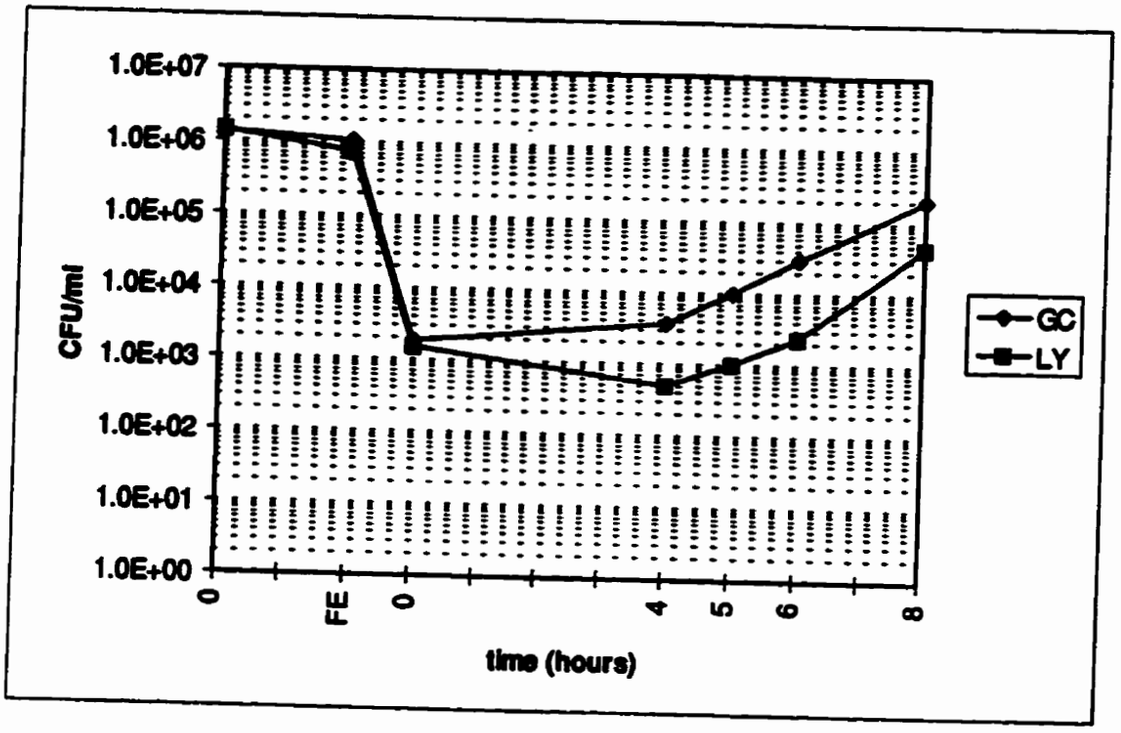
Both parent and mutant strain produced the characteristic germ tube in NYP media as observed utilizing light microscopy (see Figure 22).

## G. Germ tube inhibition

The results of the MIC susceptibility testing performed using the protocol described in the germ tube inhibition section of Materials and Methods correlated with results obtained using the NCCLS reference standard . After 48 hours the LY303366 MICs observed for Y41 and Y41LYRa were 0.04 µg/ml and 1.28 µg/ml respectively. Germ tubes were produced in the control broth and broth with low concentrations of LY303366. However inhibition of germ tube formation was seen as the concentration of drug increased (see Figure 22). There was a fixed ratio observed between the GTIC and MIC in both strains. In all three sets of experiment the GTIC of LY303366 was 1/8 x MIC. Both the parent and mutant strain exhibited this effect (see Table 14).

| | MIC µg/ml LY303366 | GTICµg/ml LY303366 |
|---|---|---|
| Y41 | 0.08 | 0.01 |
| | 0.04 | 0.005 |
| | 0.04 | 0.005 |
| Y41LYRa | ≥1.28 | 0.32 |
| | 1.28 | 0.16 |
| | 1.28 | 0.16 |

**Table 14: Comparison of MIC and GTIC of *C. albicans*Y41 and Y41LYRa.**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1.0E+03
1.0E+02
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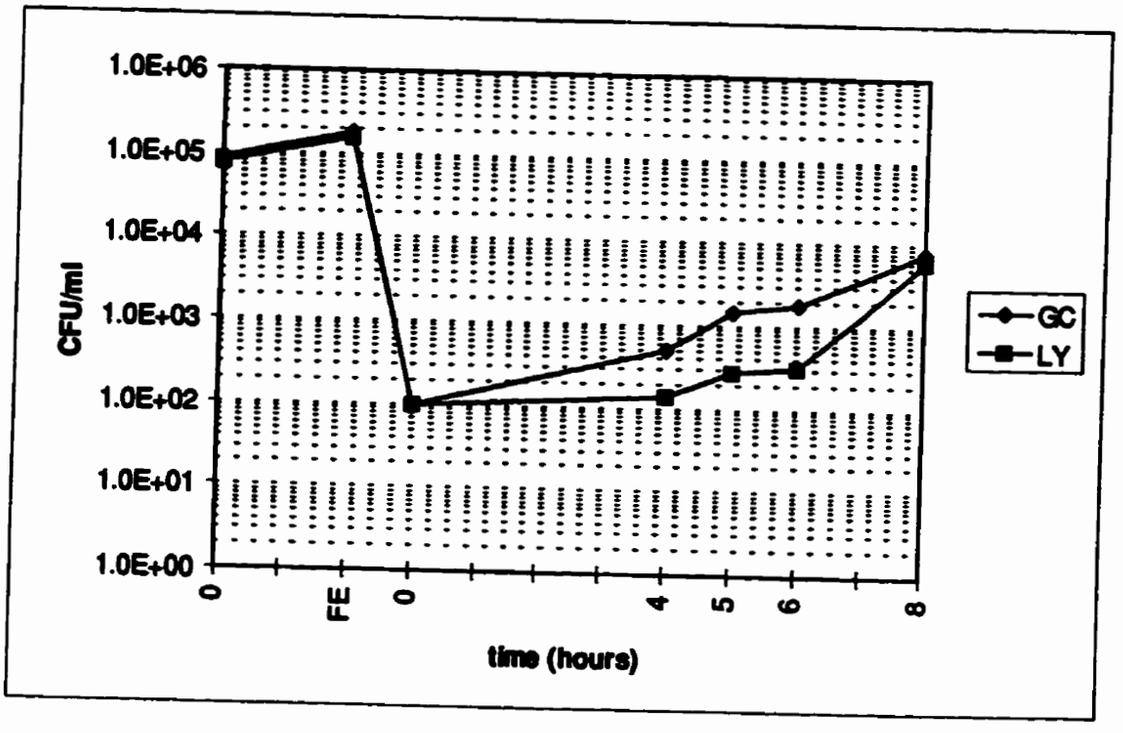
1.0E+00
CFU/ml
0
FE
0
4
5
6
8
time (hours)
GC
LY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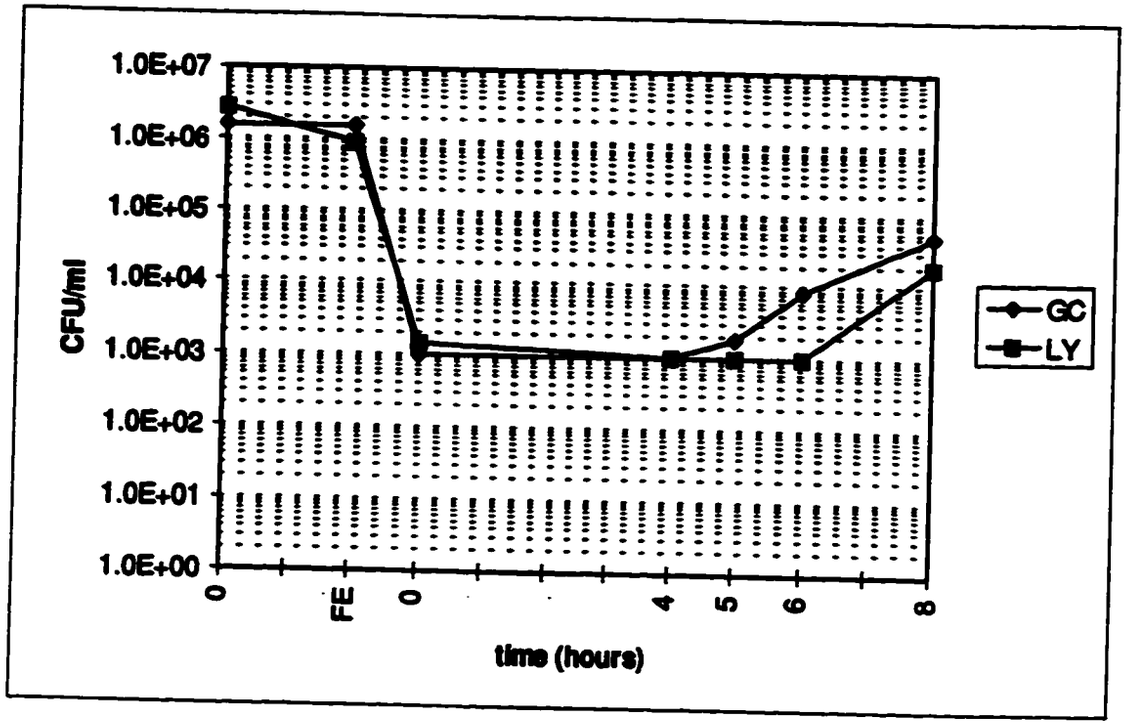
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
FE
0
4
5
6
8
time (hours)
GC
LY

**Figure 18: Post-Antifungal Effect at 0.04 μg/ml LY303366 for *C. albicans* Y41:**

After two hours of incubation at 35°C both control and test broth were centrifuged at 4000 rpm for 10 minutes and dilution 1:1000 to remove all drug from cultures. Viable cell counts were performed at 0 and 2 hours after drug exposure, and 0, 4, 5, 6, 8 hours after drug removal.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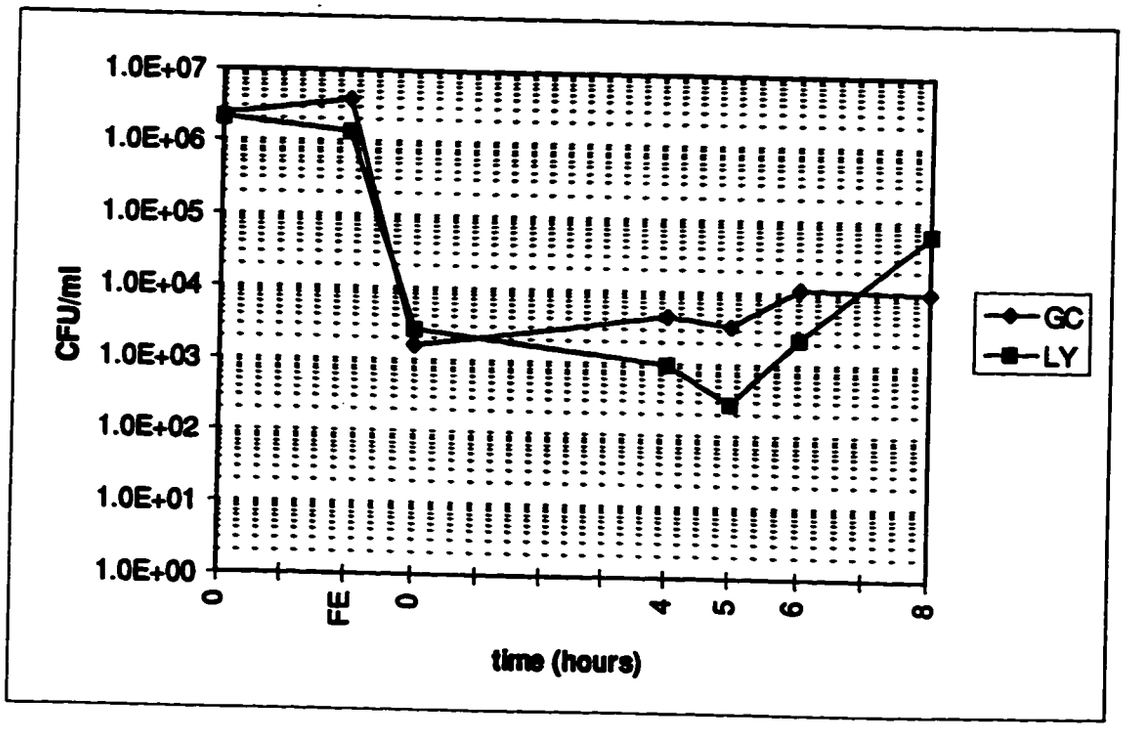
FE
0
4
5
6
8
time (hours)
GC
LY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1.0E+05
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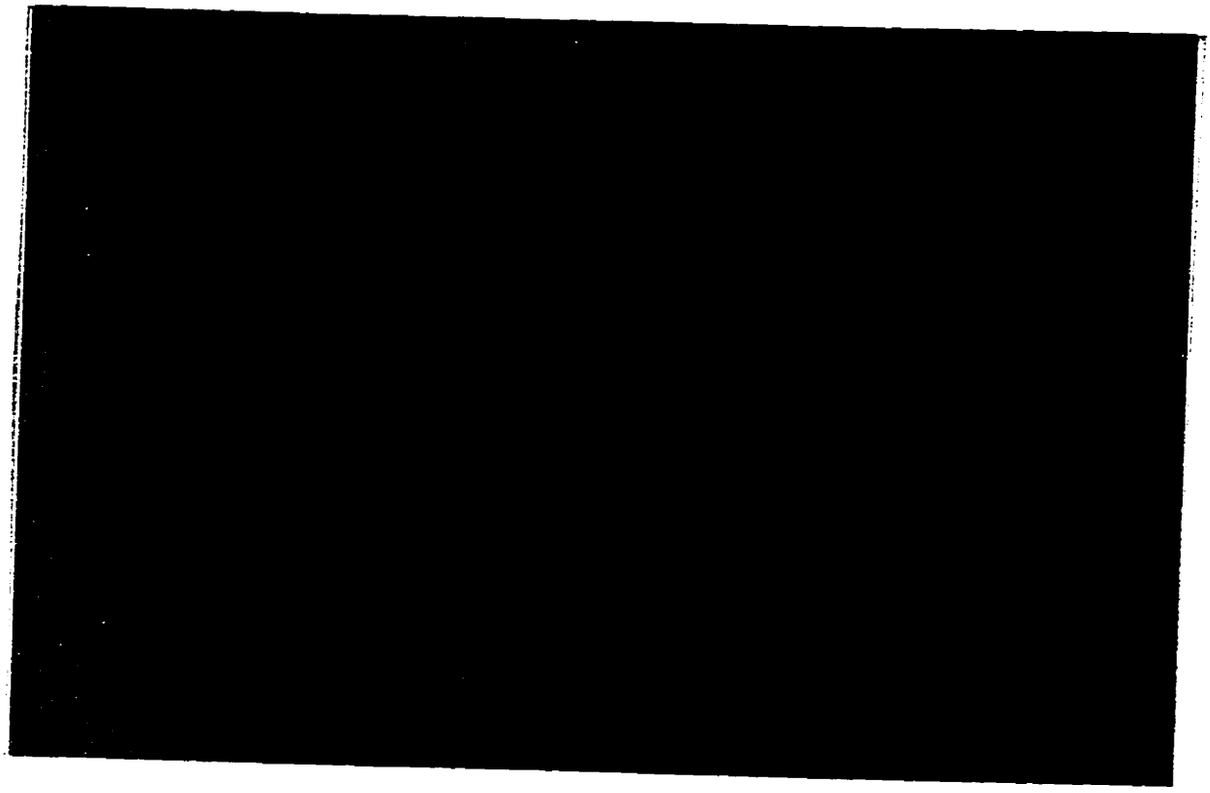
1.0E+03
1.0E+02
1.0E+01
1.0E+00
CFU/ml
0
FE
0
4
5
6
8
time (hours)
GC
LY

**Figure 19: Post-Antifungal Effect at 0.04 μg/ml LY303366 for *C. albicans* Y41LYRa:**

After two hours of incubation at 35°C both control and test broth were centrifuged at 4000 rpm for 10 minutes and dilution 1:1000 to remove all drug from cultures. Viable cell count were performed at 0 and 2 hours after drug exposure, and 0, 4, 5, 6, 8 hours after drug removal.

**Figure 20: Light microscope photography of Y41 and Y41LYRa in saline solution:**

The top photograph depicts parent strain Y41 *C. albicans* cells in saline solution . The bottom photograph depicts mutant strain Y41LYR *C. albicans* cells in saline solution.



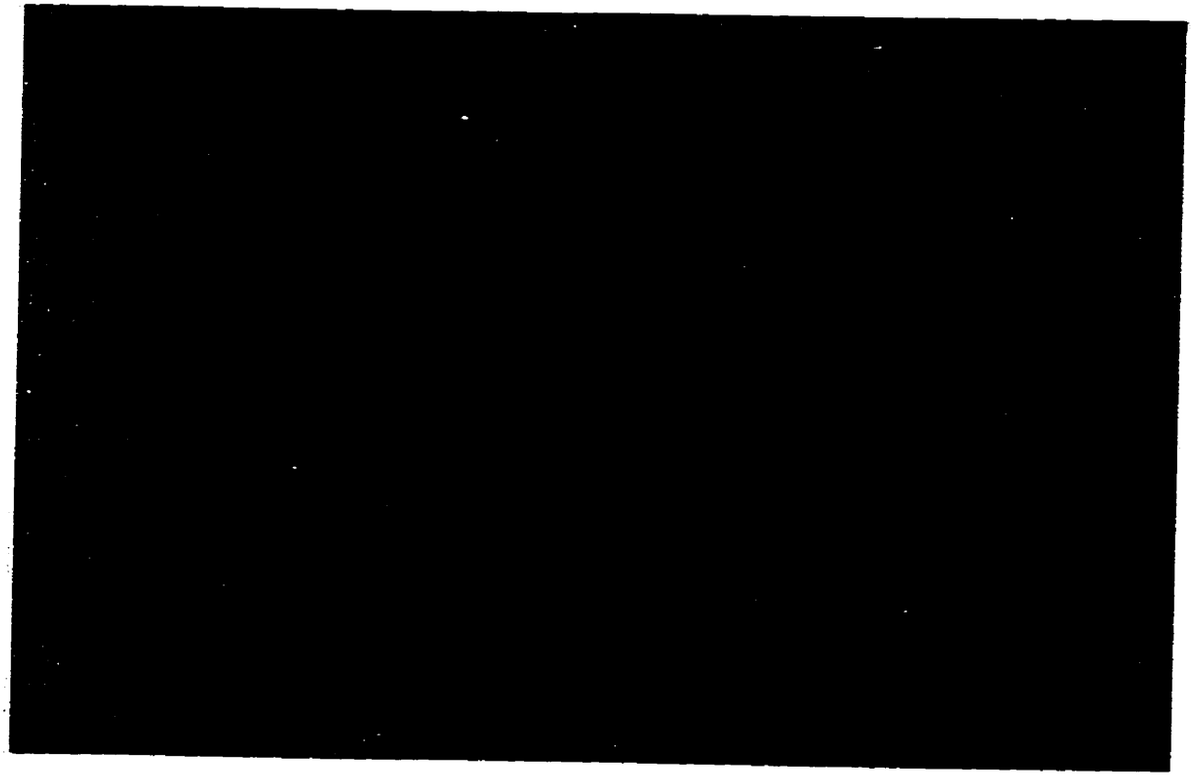


**Figure 21: Light microscope photography of germ tube formation in Y41 and Y41LYRa:**

The top photograph shows germ tube formation in parent *C. albicans* stain Y41 after 3 hours at 35°C in NYP. The bottom photograph shows germ tube formation in *C. albicans* stain Y41LYRa after 3 hours at 35°C in NYP.

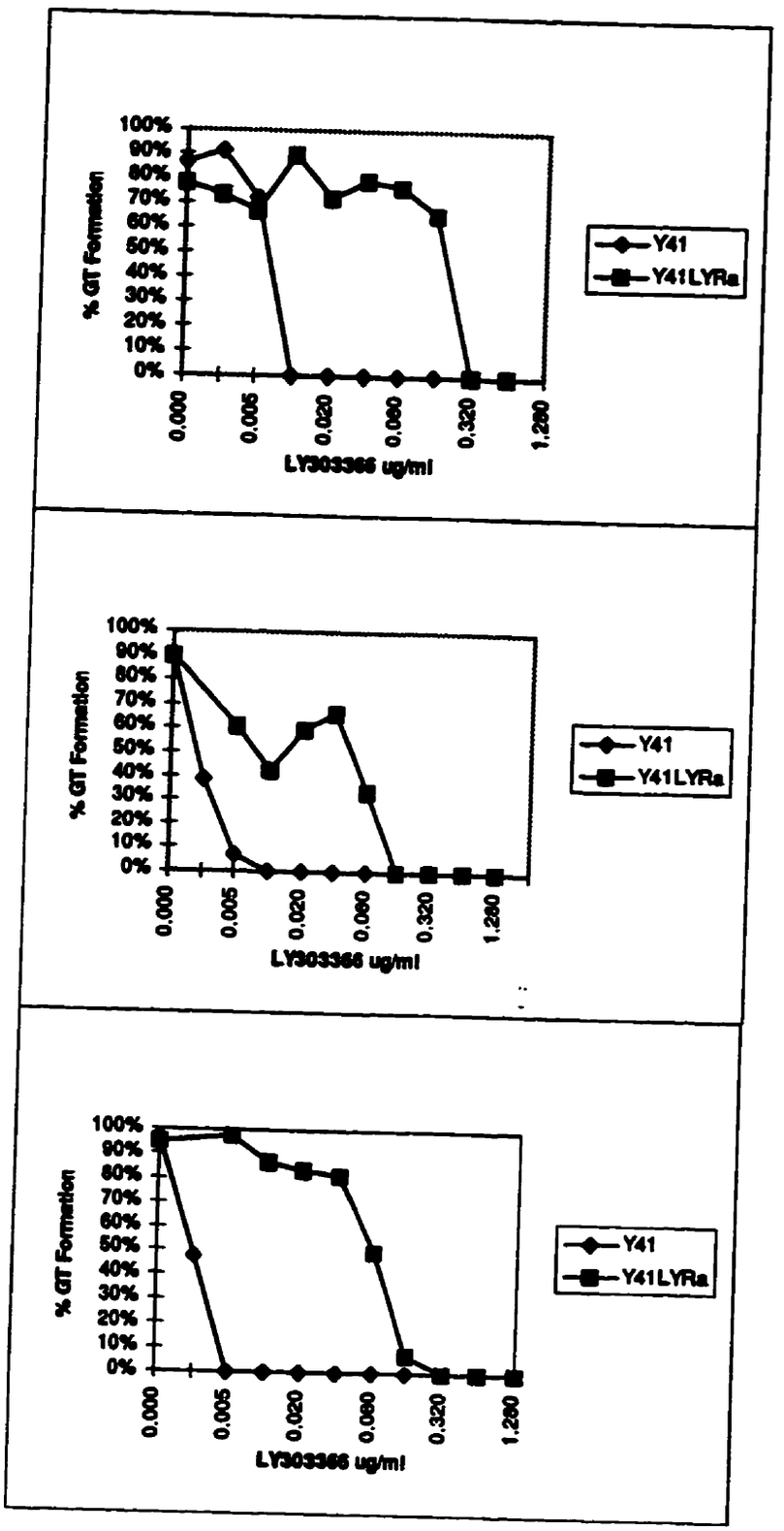

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% GT Formation
0.000
0.005
0.020
0.080
0.320
1.280
LY303366 ug/ml
Y41
Y41LYRa

**Figure 22: Germ tube inhibition assays of LY303366 against Y41 and Y41LYRa:**

After 3 hours of incubation at 35°C in NYP 15 µl from each tube was examined through the accucheck grid 10 slide using 10X power light microscope. The number of germ tubes, pseudohyphae, and blastoconidia were measured on 3 grids. These graphs display the of germ tubes formed as a percentage of the total number of cell counted for each NYP broth tube.

# V. DISCUSSION

## A. Introduction

There has been a dramatic rise in fungal infections in the last decades. [2] Some of the reasons for this increase have been the emergence of HIV infection, and the use of chemotherapeutic agents in patients with malignancies. [1] Both of these factors result in immunocompromised populations. There is a limited number of antifungal agents available to combat these infections. The gold standard for many fungal infections: amphotericin B, is only available in intravenous form and is associated with a high level of toxicity. [1,47] The azoles are fungistatic in nature and fungal resistance to these agents is an emerging problem. [52] There is a clear need for novel antifungal agents to be developed. Echinocandins are a new class of agents that inhibit the cell wall synthesis of fungal cells. [74] LY303366 is an echinocandin antifungal that is being developed for clinical use. [118] Resistance to antifungal agents is a serious problem as seen with the azoles and 5-flucytosine. [47,52] Therefore it is important to investigate potential resistance to LY303366 at an early stage of its development and use.

## B. Creation of LY303366 resistant mutants is achievable

There is limited material that examines echinocandin resistance in *C. albicans* or even *Saccharomyces cerevisiae*. Of the work that has been performed much of it has employed the use of mutagens to induce resistance. [89,112,114,115,116] This decreases the likelihood that these mutations would be observed in clinical isolates. Few studies have been published involving the examination of spontaneous echinocandin resistant mutants,

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and of them none have utilized LY303366 as the selection agent. [86,117] We proposed that exposing large inoculums of *C. albicans* to high concentrations of LY303366 in vitro for prolonged periods of time would select for LY303366 resistant *C. albicans* mutants. The first attempt at mutant isolation utilized RPMI-1640 as the media to conduct the experiments. Turbidity in LY303366 media would indicate the growth of yeast in the presence of LY303366. This would indicate that resistant mutant selection had occurred. This was not observed during these experiments. When the viable cell count was determined there were little to no cells in the LY303366 media. It is unclear as to why no resistance was isolated using RPMI-1640. This may be due to the inability to isolate individual colonies from liquid media. Also the duration of exposure to LY303366 may have not been long enough for resistance to be isolated.

The second attempt at mutant isolation utilized SDA as the selection media. No inhibition of *C. albicans* was observed using LY303366 with this media. An interaction between the SDA and LY303366 seemed to inactivate the drug on the plate. It is not clear at this time what factor caused this inactivation but one possibility is the pH of the media. SDA has a pH of $5.6 \pm 0.2$. It has been shown that pH is a factor in the susceptibility of *C. albicans* towards cilofungin, another analog of echinocandin B. [55] As much as a 64 fold increase in inhibitory concentrations has been observed as pH decreased from 7.4 to 3.0 . Therefore the lower pH of the SDA media could be a factor in the inactivity of the LY303366 in this media.

The next media utilized was BHI which has a pH of 7.0 . LY303366 in this media did inhibit *C. albicans* growth almost entirely. After 8 days at 35°C $\leq 5$ small colonies were observed growing on BHI plates with LY303366. The surviving colonies were

smaller in size than the parent strain suggesting that the mutants were not completely resistant to LY303366 . Colonies from these plates were subcultured and susceptibility tests were performed. However some subcultures did not exhibit any change in MIC to LY303366. It is not clear why colonies growing on BHI with 1 μg/ml LY303366 yielded MICs of 0.04 μg/ml. The answer may lay in the trailing effect demonstrated in these isolates. A small quantity of organism was able to survive in the presence of LY but overall LY inhibition of the organism did not change. Other researchers have observed this phenomenon with echinocandins in certain media. [100] One subculture did exhibit a 32 fold increase in LY303366 MIC (1.24 μg/ml from 0.04 μg/ml). It is not clear whether this resistance was present in the first colonies from the selection plate because the susceptibility testing was not performed on these colonies until they had been passed on LY303366 1 μg/ml plates 5 times. The reason why the test was not performed earlier was because the colony size was very small and it appeared that there were not enough cells to form an inoculum. As the colonies were subcultured repeatedly they appeared less inhibited by LY303366 as indicated by the colony size. This would suggest that the ongoing exposure to LY303366 was affecting the response of the yeast to the antifungal. From one BHI plate with LY303366, 6 colonies were subcultured that demonstrated resistance when grown on SDA plates in the absence of LY303366 (Y41LYRa-f). This was the first documentation of the selection of LY303366 resistant *C. albicans* mutants. These results validate our hypothesis that with a large number of *C. albicans* organisms exposed to a high concentration of LY303366 over a prolonged period of time that selection for resistance organisms would be achievable. There are a number of differences between the experiments isolating the echinocandin resistant *C. albicans* mutant via L-

733,560 and the present experiments. [117] The media used for their isolation was yeast-extract-peptone dextrose with 100 μg/ml of adenine and uridine instead of BHI in our study. 2x MIC of the echinocandin was used as the concentration in the media whereas between 25x and 1000x MIC was used in our study. Their plates were incubated at 30°C for 2 days whereas our plates were incubated for 8 days at 37°C. The difference in time required for incubation may be a function of the higher concentrations of echinocandin that were utilized in our isolation experiments. They found the mutation frequency to be 0.1 to 1 x $10^{-7}$ cfu which was consistent with our results of 1 x $10^{-7}$ colonies of spontaneous mutations. Susceptibility testing performed by this group was not the NCCLS proposed standards but a similar test utilizing yeast nitrogen base. With this method they ascertained a 100-fold increase in MIC to L-733,560 between the mutant and parent stains in comparison to the 32 fold change in MIC seen in our mutant strain. [117] In summary it is possible to isolate *C. albicans* LY303366 resistant mutants in the laboratory, however it is a difficult task to achieve.

### *C. LY303366 resistant mutant stability is variable*

From one BHI plate with 1μg/ml LY303366, 6 subcultures were isolated that demonstrated resistance when grown on SDA plates in the absence of LY303366. Of these 6 cultures, 3 maintained their reduced susceptibility over 20 passages (Y41LYRa-c), while the other 3 reverted back to their original susceptibility (Y41LYRd-f). Clearly there was a mixed culture on the LY303366 BHI plate from which resistance was detected originally. One type of culture had transient resistance which reverted to wildtype sensitivity when passed on SDA plates without LY303366 more than 2 times. However

colonies from revertant mutants were able to be cultured on 1 μg/ml LY303366 BHI plates, whereas the parent strain was unable to grow in the presence of that concentration of LY303366. When susceptibility testing was performed on the cultures grown on 1 μg/ml LY303366, elevated MICs to LY303366 (0.32-0.64 μg/ml) were observed again. Therefore these *C. albicans* strains were able to exhibit a decreased sensitivity to LY303366 when grown in the presence of LY303366. These strains seemed to lose this phenotype shortly after being grown in the absence of LY303366. This would suggest that the mechanism of resistance in these strains is a regulatory change that is induced in the presence of LY303366. One such change would possibly be an increase in the expression of the target enzyme 1,3-β-glucan synthase enzyme. The other set of mutants Y41LYRa-c demonstrated a more stable resistance pattern. After 20 passages on SDA media these organisms continued to demonstrate a MIC to LY303366 of 1.24 μg/ml. This would suggest that the mechanism of resistance in these strains would be of a more stable genotypic nature than the transient resistant mutants. One potential such mechanism could be a change in the 1,3-β-glucan synthase gene that makes the LY303366 target site less susceptible to the drug. In summary resistant mutants to LY303366 demonstrate variable stability in maintaining their phenotype.

### *D. Evaluation of transient LY303366 resistant mutant Y41LYRf*

MIC susceptibility testing demonstrated no change between wildtype and mutant in MICs to amphotericin B, fluconazole, 5-flucytosine, and ketoconazole. The MIC to LY303366 during these tests was decreasing but still higher than the parent MIC. Given the divergent mechanisms of action of these antifungal agents their lack of cross resistance

was not surprising. The growth control curves indicated very similar growth as the parent strain. This would indicate that an increase or decrease in growth rate is not involved as a mechanism of resistance of the transient strain. The results of the time-kill experiments support the hypothesis of an inducible resistance pattern. The LY303366 MIC of Y41LYRf at the time of the kill-curve experiments had reverted to the wildtype level of 0.04 µg/ml. The 0.04 µg/ml LY303366 kill curve for Y41LYRf exhibited a ½-1 log kill by 6 hours but regrowth at 12 and 24 hours (see Figures 14,15). This would suggest that in response to the presence of LY303366 a regulatory change occurs that confers LY303366 resistance to this mutant. This is consistent with the result of Y41LYRf's ability to grow on 1µg/ml LY303366 BHI after its MIC has returned to 0.04 µg/ml LY303366. Given that our study was directed at isolating a stable mutant no further work was done on the transient mutants. In summary transient resistant mutants demonstrated no change in antifungal susceptibility profile, or growth pattern. After it had reverted to the sensitive phenotype, the mutant did demonstrate a change in regard to pharmacodynamic activity of LY303366 at 1 x MIC.

### *E. Evaluation of stable LY303366 resistant mutant Y41LYRa*

This stable mutant demonstrated no significant difference in MIC with the parent strain Y41 against the clinical antifungals: amphotericin, fluconazole, 5-flucytosine, ketoconazole. This was expected given that the other agents target either the cell membrane (AMB, FLU, KETO) or nucleic acids (5-FC) while LY303366 targets the cell wall. These results demonstrate that no cross resistance to other common classes of agents is observed in echinocandin resistance. This has positive implications for the clinical

setting; yeast that are resistant to echinocandins will still be susceptible to other antifungal agents. This result is consistent with the work on the pneumocandin-resistant *C. albicans* mutant which was resistant to aculeacin, echinocandin B, and pneumocandin, but not to amphotericin B, itraconazole, flucytosine, and fluconazole. [117] This would indicate that the mechanism of resistance is specific rather than general. A general mechanism such as a decrease in the permeability of a cell membrane, or an efflux pump could affect the susceptibility of multiple classes of agents. However a mutation in the echinocandin target site would be a specific change that would confer resistance to only echinocandin agents. This would be consistent with other researchers findings. Enzymatic studies on the glucan synthase enzyme of pneumocandin resistant *C. albicans* showed that the isolated enzyme was resistant to the inhibitory effect of the echinocandin. [117] Also in *Saccharomyces cerevisiae* pneumocandin resistant mutant tests were performed that ruled out cell surface hydrophobicity change or degradation of the pneumocandin agent as the mechanism of resistance. [86] In a further study the resistance mutations were found to be in the gene encoding the 1,3-β-glucan synthase enzyme. [87]

The time-kill results were similar to other results seen with LY303366 and *C. albicans*. Both the mutant and parent exhibited a 1 log non-dose dependent kill at 1x, and 10X their respectively LY303366 MIC. Both the mutant and the parent exhibited the same rate and extent of killing of *C. albicans* in this kill curve model. The mutation in Y41LYRa did not affect the pharmacodynamic profile of LY303366 at 1X and 10 X MIC. This is also the case in regard to the postantifungal effect of LY303366 at 1 X MIC. LY303366 exhibited a postantifungal effect of a little more than an 1 hour in both strains at 1X MIC. The PAFE was slightly reduced in Y41LYRa . However the

pharmacodynamic profile of Y41LYRa at 0.04 μg/ml LY303366 (1/32 x MIC) exhibited no reduction in growth but rather an increase in growth slightly below the growth control. These results would confirm the resistance demonstrated in susceptibility testing. The kill curve experiments highlight the difference in the stable and resistant mutants. At 0.04 μg/ml LY303366 the stable mutant showed minimal reduction in growth, while the transient mutant showed initial reduction in CFU/ml with regrowth occurring after 6 hours exposure. This would confirm the hypothesis of Y41LYRf possessing an inducible resistance, with Y41LYRa possessing a more stable resistance with reduced susceptibility to LY303366. The growth of the mutant and parent strain is similar in nature.

Examination of the morphology of both the parent and mutant strain revealed no observable difference between the two strains. This would indicate that the changes in the mutant are not of a nature as to change the ultrastructure of the cell as observed by light microscopy. The overall growth and morphology of the mutant cell is not changed from the parent strain. This is consistent with the results found in the pneumocandin resistant mutant. [117] The lack of difference in germ tube formation is an initial indication that the mutant is no less virulent than the parent. This can be surmised because hyphal formation is a putative virulence factor of *C. albicans*. This result is consistent with the murine virulence studies of the pneumocandin resistant mutant which demonstrated no difference in virulence between the parent and mutant strains. [117] The germ tube inhibitory activity of LY303366 is consistent in both parent and mutant strain. At LY concentrations of 1/8 x MIC germ tube inhibition was observed in both strains. Both the MIC and GTIC are 32 fold higher in the mutant strain than the parent strain for LY. Therefore the GTIC tests confirms the results observed during susceptibility testing. It is of interest to note that LY

inhibits germ tube formation at subinhibitory concentrations. This may have significance clinically because of the association between hyphal formation and increased virulence. [7] The transition to the mycelial phase of yeast is accompanied by an increase in 1,3-β-glucan synthase activity. [9] Given the fact that yeast hyphae are more dependent on 1,3-β-glucan synthase than budding yeast, it is logical that hyphae would be more sensitive to inhibitors of 1,3-β-glucan synthase than blastoconidia. However it must be remembered that susceptibility test results are a function of conditions of testing. [59] Therefore the difference observed between MIC and GTIC in the same strain may be merely a function of the different test parameters. Germ tube inhibition studies demonstrated that LY303366 exhibited the same germ tube inhibitory characteristics in both sensitive and resistance strains in relation to their respective MICs. In summary stable resistant mutant exhibited no change in growth; morphology; clinical antifungal susceptibility profile; LY303366 pharmacodynamic profile at 1x, 10x MIC; in comparison to the parent strain.

## *F. Future study*

There are a number of areas of further study which could be conducted on the LY303366 resistant mutants. Firstly, the genes which contain the resistance mutation could be cloned and sequenced to determine the exact nature of the mutation. The wildtype gene where the echinocandin resistance mutation occurred in *Saccharomyces cerevisiae* was cloned and sequenced in a number of studies. [87] However the *C. albicans* echinocandin resistance gene has not been cloned or sequenced at this point in large part due to the increased complexity of the *C. albicans* genome. Secondly, studies could be performed on the activity of LY303366 on the glucan synthase enzyme of both the parent and mutant strains. When this was done with the glucan synthase enzyme from the

pneumocandin resistant *C. albicans* mutant, it was demonstrated that the mutant synthase was four fold more resistant to pneumocandin than the wildtype. [117] This did not correspond to the 100 fold increase in the MIC of the mutant to pneumocandin. But the result did show that the mutant's glucan synthase enzyme did exhibit resistance to pneumocandin. Thirdly, the antifungal susceptibility profile could be widened to determine the activity of other agents to our echinocandin resistant mutant. These could include: clinical agents (nystatin, itraconazole) or other cell wall active agents. One area of particular interest in this regard would be the chitin synthase inhibitors. The *Saccharomyces cerevisiae* echinocandin resistant mutant exhibited a supersensitivity to nikkomycin Z. [113] This result as well as studies reporting synergistic activity of echinocandins and nikkomycins suggest that this area of study could lead to clinically relevant developments. [78,79] Lastly, further experiments employing in vitro pharmacodynamic and in vivo models to analyze the LY303366 resistant *C. albicans* mutant could be performed. In vitro modeling that mimics the pharmacokinetics of LY303366 in the human body could be employed to observe its activity against the mutant in this situation. Animals studies could analyze the virulence and susceptibility of the mutant in vivo. When the pneumocandin resistant mutant was tested in vivo it was found that the strain was fully virulent requiring 10 fold the effective dose of LY303366 than the parent strain. [117] This was significantly lower than the 100 fold difference in MIC observed and would suggest a lack of correlation between their in vitro and in vivo results. In summary future work with LY303366 resistant *C. albicans* mutant could be conducted in genetic, enzymatic, in vitro, and in vivo areas of study.

## G. Summary

In summary, studies were conducted that demonstrated the feasibility of the isolation of laboratory derived LY303366 resistant mutants. The stability of the mutant resistance phenotype was variable. Some of the mutants reverted to the parental MIC after a few passages in the absence of LY. Others maintained their resistant phenotype over 20 passages in the absence of LY303366. The transient resistance mutant exhibited no change in antifungal susceptibility profile or in growth rate. It did possess the ability to regain a measure of resistance to LY303366 when grown in the presence of LY303366. The stable resistance mutant exhibited no change in morphology, growth rate, antifungal susceptibility profile, or LY303366 pharmacodynamic profile. Further study could be conducted on the LY303366 resistant mutant on a genetic, enzymatic, in vitro, and in vivo level.

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