Drug resistance and apoptosis in *Candida* biofilms

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Author's declaration

I declare that the work presented in this thesis has been carried out solely by myself and in accordance with the University regulations.

Signed Date

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Dedication

I should like to dedicate this work to my beloved father "Mr. Salem O. Al-Dhaheri", who has always believed in me and overwhelmed me with his continued encouragement and enthusiastic support throughout my academic career, my wonderful mother "Ayesha" for her patience, love and constant prayers, my dearest brothers and sisters, and to every member of my family.

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Abbreviations

A_{492}	Absorbance at 492nm
ABC	ATP-binding cassette
AIDS	Acquired Immune Deficiency Syndrome
ALS	Agglutinin-like sequence
AMB	Amphotericin B
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
C	Drug concentration that penetrated the biofilm
CAS	Caspofungin
CDR	*Candida* drug resistance
CF	Cystic fibrosis
CFU	Colony forming units
CSLM	Confocal scanning laser microscopy
cm	Centimetre
C_o	Drug concentration determined for the control
CVC	Central venous catheter
Da	Dalton
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substance
GDH	Glasgow Dental Hospital, Scotland UK
h	Hour (s)

HDA	Histone deacetylase
HEPES	4-2-Hydroxyethyl-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency virus
Hwp	Hyphal cell wall specific protein
INT	The integrin-like protein
L	Litre
MAPK	Mitogen activated protein kinase
MDR	Multi-drug resistance
MIC	Minimum inhibitory concentration
min	Minute (s)
ml	Millilitre
mm	Millimetre
mM	Millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MRD	Modified Robbins device
NCAC	Non C. albicans Candida species
NCCLS	National Committee for Clinical Laboratory standards
ND	Not determined
^{o}C	Degrees Celsius
OD	Optical density
OPC	Oropharyngeal candidosis
P	Probability value
PBS	Phosphate-buffered saline
PCD	Programmed cell death

PL	Phospholipase
PLA	Polysaccharide intercellular adhesion
PS/A	Extracellular capsular polysaccharide adhesin
PVC	Polyvinyl chloride
PVE	Prosthetic valve endocarditis
r.p.m	Revolutions per minute
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
Saps	Secreted aspartic proteinases
SDA	Sabouraud dextrose agar
SEM	Scanning electron microscopy
spp	Species
SR-FLICA	Sulforhodamine-Fluorescent Labelled Inhibitors of Caspases
TEM	Transmission electron microscopy
TSA	Trichostatin A
UV	Ultraviolet
v/v	Volume/volume ratio
Vit K	Vitamin K (Menadione)
Vol.	Volume
w/v	Weight/volume ratio
Wt.	Weight
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
YCA1	Yeast caspase 1
YNB	Yeast nitrogen base medium

Summary

Candida species are commonly part of the normal flora in humans; however, they are opportunistic fungal pathogens that are capable of causing a variety of infections in hospitalized and immunocompromised individuals. These infections range from superficial to systemic ones. Many *Candida* infections involve biofilm formation on the surfaces of implanted devices, such as catheters and prostheses, or host tissues. *Candida* biofilms are resistant to a range of antifungal agents in current clinical use but the basis of this drug resistance is not clear. The aim of this project was to investigate possible resistance mechanisms using two fungicidal agents, amphotericin B and caspofungin, a new drug reported to have anti-biofilm activity.

The activity of amphotericin B and caspofungin at different development phases of *Candida* biofilms was investigated *in vitro*. Amphotericin B at two times the MIC (for planktonic culture) had the least effect on *Candida* biofilms, but at a higher concentration (five times the MIC) it showed relatively high activity against biofilms of *C. parapsilosis* and *C. glabrata,* especially at the late development phase. Biofilms of *C. albicans* were more resistant to amphotericin B throughout development (except for the earliest stage) than the other *Candida* species. Caspofungin, at two times the MIC, generally exhibited a greater effect on *Candida* biofilms than amphotericin B although this was not observed with *C. parapsilosis* biofilms in some development phases. Caspofungin, at five times the MIC, was slightly less effective than at the lower concentration against *C. tropicalis* in all development phases tested. The species most susceptible to caspofungin throughout biofilm development was *C. glabrata*. In no case were biofilm cells of any *Candida* species completely killed by either amphotericin B or caspofungin.

The penetration of caspofungin through biofilms of different *Candida* species was evaluated using an *in vitro* filter disc bioassay. Caspofungin penetration through biofilms of *C. albicans* SC5314 was initially faster than *C. albicans* GDH2346; however, after 6 h drug diffusion was greater with biofilms of strain GDH2346 (70.8% of the control value). Among other *Candida* species tested, the highest drug penetration was observed with *C. glabrata* and *C. parapsilosis* (81.2% and 73.3% of the control value, respectively), while the

lowest was seen with biofilms of *C. krusei*. Biofilms of *C. tropicalis* also showed poor penetration. Exposure of biofilms of any *Candida* species to caspofungin (or amphotericin B) in this assay failed to result in complete killing of biofilm cells. However, evaluation of caspofungin activity against biofilms was complicated by the paradoxical phenomenon (reduced activity of the drug at high concentrations, above the minimum inhibitory concentration). Scanning electron microscopy revealed that caspofungin caused more structural damage to biofilm cells and matrix than did amphotericin B; the highest degree of damage due to caspofungin was observed in biofilms of *C. glabrata* and *C. krusei*.

The presence of a small number of drug-tolerant or persister cells is one possible mechanism of biofilm drug resistance. Biofilms and planktonic cells of five *Candida* species were surveyed for the presence of persister cell populations after exposure to amphotericin B. None of the planktonic cultures (exponential or stationary phase) contained persister cells. However, persisters were found in biofilms of one of two strains of *C. albicans* tested and in biofilms of *C. krusei* and *C. parapsilosis*, but not in biofilms of *C. glabrata* or *C. tropicalis*. Live-dead staining with fluorescein diacetate confirmed these results which do, however, suggest that persister cells cannot solely account for drug resistance in *Candida* biofilms.

If microorganisms exposed to antimicrobial agents undergo a type of programmed cell death or apoptosis, persisters could be variant in which this process has been disabled. Here, specific staining methods were used to investigate the existence of apoptosis in *Candida* biofilms subjected to different concentrations of amphotericin B. Caspase activity, indicative of apoptosis, was detected with SR-FLICA and D_2R fluorochrome-based staining reagents in all of these biofilms. The general inhibitor of mammalian caspases, Z-VAD-FMK, when used at a low concentration (2.5 μM), increased the viability of drug-treated biofilms up to 11.5-fold ($P<0.001\%$). Seven specific caspase inhibitors had different effects on *C. albicans* biofilm viability, but inhibitors of caspases-1, -9, -5, -3, and -2 all significantly increased cell survival (40-fold, 8-fold, 3.5-fold, 1.9-fold and 1.7-fold, respectively). On the other hand, histone deacetylase (HDA) inhibitors enhanced the activity of amphotericin B against biofilms of all three *Candida* species. Sodium butyrate and sodium valproate, for example, when added concurrently with amphotericin B, completely

eliminated biofilm populations of *C. albicans*. Overall, these results demonstrate an apoptotic process in amphotericin-treated biofilms of three *Candida* species. They also indicate that HDA inhibitors can enhance the action of the drug and in some cases even eradicate persister subpopulations, suggesting that histone acetylation might activate apoptosis in these cells.

INTRODUCTION

1 *Candida*

1.1 Background

Candida species are yeasts, or single-celled fungi, that commonly colonize different body sites such as the skin, oral cavity, oesophagus, and gastrointestinal and genital tracts (Kumamoto & Vinces, 2005; Odds, 1988; Wingard & Leather, 2004). According to Odds (1988), most people carry a single strain of *Candida* at different body sites, as part of their normal flora. However, a few individuals may harbour more than one strain or species of *Candida* at the same time; this occurs more commonly in hospitalized and immunocompromised patients (McCullough *et al.*, 1996). Under normal circumstances, *Candida* species remain as commensals and do not produce clinical disease; however, they may become opportunistic pathogens under certain conditions (Odds, 1988). Although there are about 200 species of *Candida*, it is well established that only a few are pathogenic for humans; in fact, 65% of *Candida* species are unable to grow at a temperature of 37°C (Schauer & Hanschke, 1999). Six species are most frequently isolated in human infections, namely, *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. lusitaniae* (AbiSaid, 1997).

C. albicans is the most significant and frequently isolated yeast pathogen. According to data from the US National Nosocomial Infections Surveillance System, approximately 50% of fungal nosocomial infections are caused by *C. albicans* (Calderone, 2002; Marcilla *et al.*, 1998). *C. albicans* is also the predominant species in fungal biofilm infections of medical devices (He *et al.*, 2006). *In vitro*, *C. albicans* biofilms exhibit resistance to a wide range of commonly used antifungals such as fluconazole, amphotericin B, nystatin, and ketoconazole, as well as the newer triazoles, ravuconazole and voriconazole (Chandra *et al.*, 2001a; Chandra *et al.*, 2001b; Hawser & Douglas, 1995; Kuhn *et al.*, 2002a; Kuhn *et al.*, 2002b). However, in recent years there has been a shift from *C. albicans* to non-*C. albicans Candida* species (NCAC) so that approximately half of the reported cases of Candidaemia are now caused by NCAC. Non-*C. albicans* species such as *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and the new species, *C. dubliniensis*, have been identified as important opportunistic pathogens; these species are sometimes

less susceptible than *C. albicans* to antifungal agents and may require a higher dosage for a clinical cure (Cheng *et al.*, 2005; Yucesoy & Marol, 2003) .

C. tropicalis, unlike *C. albicans*, which can be found as a commensal, is almost always associated with the development of fungal infections (Wingard *et al.*, 1979). An increase in the incidence of *C. tropicalis* has been recognized recently; it accounts for 4 to 24% of all *Candida* species isolated (AbiSaid, 1997; Price *et al.*, 1994; Wingard, 1995). With regard to its pathogenicity and virulence, data from animal models suggest that it is at least as virulent as *C. albicans* (Wingard, 1995). *C. tropicalis* has the potential to cause severe invasive disease, with multiorgan involvement in neutropenic patients, and it is associated with a high mortality rate (33-90%) (Powderly *et al.*, 1988; Wingard *et al.*, 1979; Wingard, 1995). Furthermore, this species has been found to be less susceptible than *C. albicans* to antifungal agents such as fluconazole and amphotericin B (Powderly *et al.*, 1988; Rex *et al.*, 1995; White *et al.*, 1998).

C. glabrata is the second most common cause of systemic candidosis and candiduria after *C. albicans* in the United States (Occhipinti *et al.*, 1994; Pfaller *et al.*, 1999b). It is also the NCAC species that is most commonly isolated from the oral cavities of HIV–infected patients (Sangeorzan *et al.*, 1994; Schoofs *et al.*, 1998). This species contributes to mortality in approximately 21% of pediatric patients with bloodstream infections (Fridkin *et al.*, 2006) and it is known for its decreased susceptibility to azoles (Magill *et al.*, 2006).

C. parapsilosis has become the second most frequently recovered *Candida* species from blood cultures in Europe, Latin America, and Canada, and is ranked third in the United States (Yucesoy & Marol, 2003). *C. parapsilosis* has also been shown to be the *Candida* species most commonly recovered from the hands of health workers and can adhere strongly to the surfaces of intravascular catheters and prosthetic devices (De Bernardis *et al.*, 1999; Hedderwick *et al.*, 2000; Levin *et al.*, 1998). In addition, *C. parapsilosis* has become the NCAC species most commonly isolated in neonatal intensive-care units (Rangel-Frausto *et al.*, 1999).

C. krusei is a relatively uncommon pathogen accounting for 2 to 4% of Candidaemia cases (Calderone, 2002). *C. krusei* fungaemia commonly occurs in haematology patients with severe neutropenia due to bone marrow

transplantation or leukaemia. In these patients, *C. krusei* fungaemia is associated with high mortality rates of approximately 60 to 93%, and infection is often disseminated (Abbas *et al.*, 2000; Merz *et al.*, 1986; Nguyen *et al.*, 1996). This species is known to be resistant to fluconazole (Rex *et al.*, 2000); however, some investigators have also noticed reduced susceptibility to other antifungal drugs among isolates of *C. krusei*, suggesting that *C. krusei* is a multidrug-resistant pathogen (Berrouane *et al.*, 1996; Kao *et al.*, 1999). Most recently, *C. krusei* has been implicated in disseminated disease in patients receiving caspofungin (Pelletier *et al.*, 2005).

1.2 Morphology

Colonies of *Candida* species on Sabouraud dextrose agar are normally white to cream coloured, grow rapidly and mature in 3 days at 25°C. The microscopic features of *Candida* species show species-related variations. All species grow in the logarithmic phase as oval-to-oblong, budding cells (yeasts). These cells occur singly, or in clusters or chains. Most members of the genus *Candida* also produce a filamentous type of growth involving pseudohyphae which are essentially chains of elongated yeast cells. *Candida albicans* and the closely related *C. dubliniensis* have the ability to form both pseudohyphae and true hyphae (Fig. 1). The early stage in the formation of a true hypha is known as a germ tube. In addition, *Candida albicans* and *C. dubliniensis* can also produce chlamydospores; these cells are larger and more rounded than yeast cells and their physiological status is uncertain (Calderone, 2002; Larone, 1995).

1.3 *Candida* cell wall structure

The cell wall is vital both to the biology of the yeast and to its interactions with host cells in health and disease. It protects the fungus from host defence mechanisms and it is responsible for maintaining the unique shapes that characterize fungal growth (Marcilla *et al.*, 1998). The surface of the organism is the site of the physical interactions between the fungus and host tissues that lead to adherence, and between the fungus and the immune system that lead to clearance (Cannon & Chaffin, 1999). The surface layers of the fungal cell are composed of an outer cell wall and an inner cell membrane (Aguilar-Uscanga & Francois, 2003). The cell wall is an active structure that can

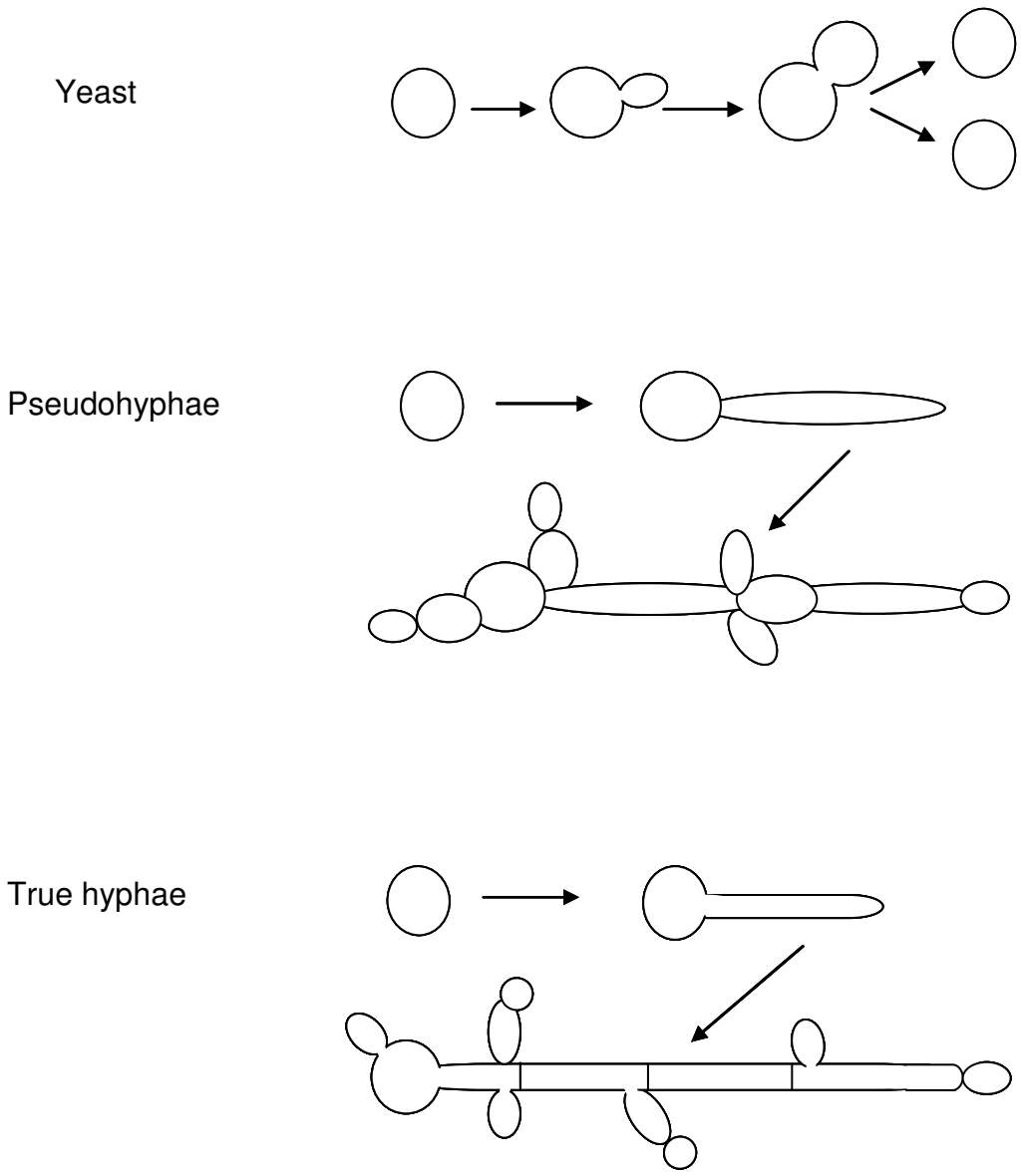


Figure 1. Growth forms of *Candida* species: yeast, pseudohyphae, and hyphae

Candida albicans can exist in three forms that have distinct shapes: yeast cells, pseudohyphae and true hyphae. Yeast cells are round to ovoid in shape and separate readily from each other. Pseudohyphae are elongated yeast cells that remain attached to one another at the constricted septation site and usually grow in a branching pattern. True hyphae are long and highly polarized, with parallel sides and septa, and no obvious constrictions between cells.

adapt to physiological changes. Its composition is unique; therefore it is an ideal target for new antifungal drugs since it is not present in mammalian cells (Marcilla *et al.*, 1998). The cell wall of *C. albicans* makes up 30% of the dry weight of the cell; about 80 to 90% of the cell wall is composed of carbohydrate, 6 to 25% of protein and 1 to 7% of lipid (Chaffin *et al.*, 1998). The carbohydrates include branched polymers of glucose (β-1,3-D-glucans and β-1,6-D-glucans), unbranched polymers of N-acetyl-D-glucosamine (chitin) and polymers of mannose (mannan), covalently bound to proteins (Calderone, 2002; Chaffin *et al.*, 1998; Marcilla *et al.*, 1998). In *C. albicans,* β-glucans account for 50 to 60% by weight of the fungal cell wall while mannoproteins account for approximately 40% of the total cell wall polysaccharide (Calderone, 2002). Glucan and chitin polymers are responsible for the rigidity of the cell wall and also for cell morphology. The cell wall polymers in *Candida* species are linked together by covalent bonds, in addition to hydrogen and hydrophobic bonds. Covalent linkages between glucan and chitin, and between mannoproteins and β-glucan, have been described. Furthermore, β-1,3 and β-1,6 glucan are also linked to proteins by phosphodiester linkages. It is believed that the fungal cell wall is composed of building blocks of mannoproteins, which are associated through glycosyl phosphatidylinositol (GPI) anchor remnants with β-1,6 glucan, which in turn is linked to β-1,3 glucan and chitin (Figure 2) (Bowman & Free, 2006; Klis *et al.*, 2001; Smits *et al.*, 2001).

1.4 *Candida* infections

Over the last few decades the prevalence of opportunistic *Candida* infections has increased. This rise is largely attributed to the extensive use of immunosuppressive and cytotoxic drugs, widespread use of broad-spectrum antibiotics (permitting fungal overgrowth), and increased use of invasive devices (catheters, prostheses, and valves) (Douglas, 2003; Kumamoto & Vinces, 2005). These opportunistic fungi are potential pathogens in patients with certain predisposing factors, e.g. patients with AIDS, organ transplants, prolonged antibiotic use, diabetes mellitus, malnutrition, and obstructive uropathy (Wainstein *et al.*, 1995). *Candida* species are capable of causing a variety of infections, ranging from the superficial to systemic (Calderone, 2002; Odds, 1988).

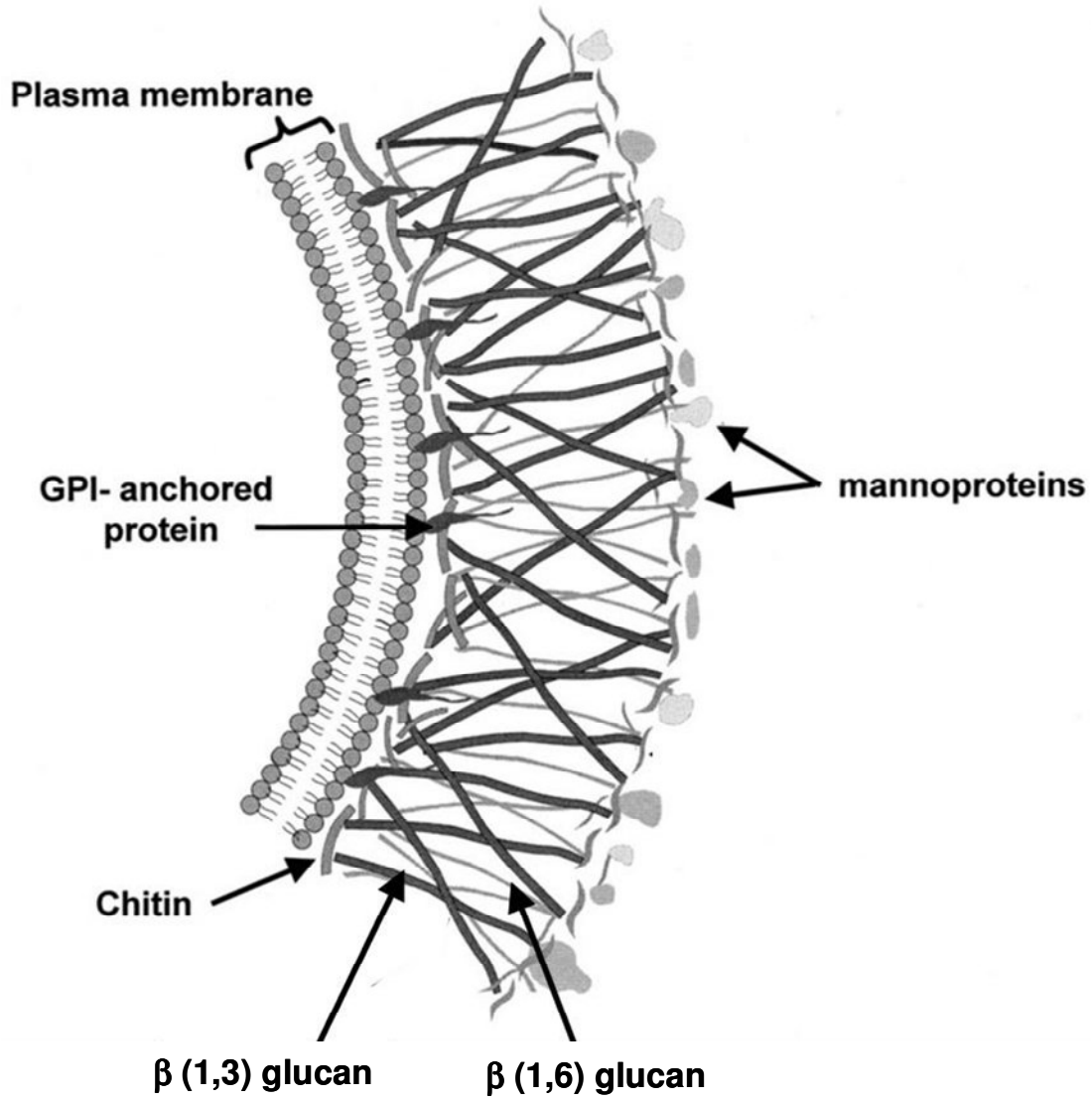


Figure 2. Schematic of fungal cell wall.

GPI, glycophosphatidylinositol. Reprinted from Selitrennikoff (2001) by permission of the publisher, American Society for Microbiology.

1.4.1 Superficial candidosis

Candida infections of the skin are referred to as cutaneous candidosis, whereas infections on mucous membranes of the mouth, oropharynx or vagina are termed mucosal candidosis. The most common superficial infections mainly take the form of mucosal candidosis: vulvovaginal candidosis or oropharyngeal candidosis (Fidel & Sobel, 1996). Vulvovaginal candidosis occurs most frequently in women of childbearing age. It has been estimated that 75% of all adult women will experience at least one vaginal infection episode during their lifetime (Odds, 1988). *Candida* species can be found as commensals in the vagina in 10 to 25% of all women (Fidel & Sobel, 1996). Predisposing factors for *Candida* overgrowth include pregnancy, antibiotic use, immunosuppressive therapy, hormone replacement therapy or uncontrolled diabetes mellitus (Arendorf & Walker, 1987; Bulad *et al.*, 2004). A study by Moreira and Paula (2006) on patients with vulvovaginal candidosis showed that *C. albicans* is the most frequently isolated species, with 90% of isolation, followed by *C. glabrata* with 6% and *C. parapsilosis* and *C. tropicalis* each with 2%.

Oropharyngeal candidosis (OPC) refers to *Candida* infection in the mouth and throat. The most common types of OPC are oral thrush and denture-induced stomatitis. Oral thrush is characterized by soft, white or cream-coloured deposits on mucosal membranes. This infection most frequently occurs in newborns, the elderly, and diabetics. In the absence of other known causes of immunosuppression, oral thrush in an adult is highly predictive of human immunodeficiency virus (HIV) infection (Dronda *et al.*, 1996). *Candida*-associated denture stomatitis is prevalent in approximately 11 to 67% of denture wearers. The upper denture provides a surface to which *Candida* can adhere. In the oral cavity, *Candida* species usually co-exist with commensal bacteria. Studies by Budtz-Jorgensen (1990) have shown that in denture plaque of patients with *Candida*-induced denture stomatitis, 93% of the plaque biofilm is composed of yeasts and the remainder comprises oral bacteria. Despite antifungal therapy to treat denture stomatitis, infection is usually re-established soon after the treatment ceases, suggesting that denture plaque may serve as a protected reservoir of *C. albicans* (Budtz-jorgensen, 1990). Resolution of the infection may sometimes occur by simply removing the dentures (Calderone, 2002).

Two forms of OPC are commonly encountered among HIV patients: pseudomembranous and erythematous candidosis. Pseudomembranous OPC consists of smooth, white, papular lesions. Erythematous OPC is characterized by smooth red patches on the hard or soft palate, oropharynx, buccal mucosa, and dorsal tongue (Becksague & Jarvis, 1993; Cheng *et al.*, 2005; Edmond *et al.*, 1999). It has been estimated that 90% of HIV patients develop OPC in various stages of their disease (de Repentigny *et al.*, 2004).

1.4.2 Invasive candidosis

Candida species now rank as the fourth most common cause of nosocomial bloodstream infections in the United States (Edmond *et al.*, 1999). Candidaemia is the most common form of invasive candidosis and usually occurs in individuals with reduced function of the immune system and those hospitalized with serious underlying conditions (Pfaller & Diekema, 2007). Despite available antifungal therapy, the rate of morbidity and mortality associated with candidaemia remains high: about 30 to 70% among cancer patients (Horn *et al.*, 1985; Viscoli *et al.*, 1999) and 26 to 75% among non-cancer patients (Cheng *et al.*, 2005; Komshian *et al.*, 1989; Phillips *et al.*, 1997). Several studies have suggested that many if not most incidents of candidaemia are catheter related (Pfaller & Diekema, 2007). The largest prospective treatment study of patients with candidaemia implicated a catheter in 72% of them (Rex *et al.*, 1994). In invasive candidosis, 60 to 75% of infections are caused by *C. albicans*. Although the incidence of non-*C. albicans* species as a cause of infection is increasing in both invasive and mucosal candidosis, *C. albicans* remains the most prevalent aetiological agent of *Candida* infection (Jabra-Rizk *et al.*, 2004; Slavin *et al.*, 2004; Wingard & Leather, 2004).

2 Virulence factors of *Candida* species

Like other pathogens, in order to establish an infection, opportunistic pathogens have to evade the immune system, survive, divide in the host environment and spread to new tissues. *C. albicans* expresses several virulence factors that contribute to pathogenesis. These factors include morphogenesis (the reversible transition between unicellular yeast cells and filamentous

growth forms), hydrolytic enzyme production, adhesion (production of host recognition biomolecules), and phenotypic switching (Calderone & Fonzi, 2001).

2.1 Morphogenesis

Morphogenesis refers to the transition between various growth morphologies. The fungal pathogen, *C. albicans,* shows considerable morphogenetic flexibility. This organism has the ability to reversibly convert between the yeast form and the filamentous form (hyphal or pseudohyphal states). This transition is thought to contribute to the ability to grow on surfaces and invade tissues (Calderone & Fonzi, 2001; Yang, 2003). Of all *Candida* species, only *C. albicans* and C. dubliniensis have the ability to undergo both types of filamentous growth and are defined as polymorphic in their growth patterns (Calderone & Fonzi, 2001). Virulence is attenuated in mutants that are unable to switch from the yeast form to the hyphal form. For example, mutant strains of *C. albicans* that were defective in germ-tube formation showed decreased tissue invasion in an immunosuppressed animal model (Riggle *et al.*, 1999). The various cellular forms of *C. albicans* can be induced by many different environmental factors, such as mammalian serum, high temperatures (37°C), and neutral pH (Brown & Gow, 1999; Brown *et al.*, 1999). Moreover, hyphae are formed in response to nutrient deprivation and filamentous growth is considered an important adaptive response that enables the fungus to forage for nutrients more effectively (Brown & Gow, 1999; Calderone & Fonzi, 2001; Palecek *et al.*, 2002).

Several signalling pathways control morphogenesis in *C. albicans*. These include the mitogen-activated protein kinase (MAPK) and Ras-cAMP signalling pathways that are thought to activate filamentous growth in response to starvation and/or serum signals (Brown, 2001; Ernst, 2000; Whiteway, 2000). The MAPK and Ras-cAMP pathways regulate the transcription factors Cph1 and Efg1, respectively (Liu *et al.*, 1994; Stoldt *et al.*, 1997). Under most experimental conditions, yeast-hypha morphogenesis is blocked in a *C. albicans cph1/cph1, efg1/efg1* double mutant. This indicates that the transduction of most environmental signals is dependent on Ras-cAMP or MAPK signalling (Lo *et al.*, 1997). Tripathi and colleagues (2002) have reported that amino acid starvation induces pseudohyphal growth in *C. albicans* and this morphogenesis

response is dependent upon CaGcn4, a functional homologue of *S. cerevisiae* Gcn4. About 30% of *C. albicans* wild-type cells (containing a functional *CaGcn4* locus) formed filamentous projections, whereas the *gcn4/gcn4* mutant cells only formed buds (Tripathi *et al.*, 2002). Another study by Wightman et al. (2004) suggested that Nim1-kinases, Gin4 and Hsl1, act as negative regulators of pseudohyphal development. Hyphal forms can be inhibited by farnesol, a quorum-sensing molecule (Hornby *et al.*, 2001). Also, down regulation of Nrg1 (a DNA-binding repressor protein) synthesis induces filamentous growth in *C. albicans* (Braun *et al.*, 2001). The hyphal and pseudohyphal forms can revert back to the yeast form but the mechanisms controlling this transition are not well understood (Whiteway & Oberholzer, 2004). Several studies have shown that most lesions in infected tissues are populated by both morphological forms, suggesting that both contribute to the development and progression of disease (Calderone & Fonzi, 2001).

2.2 Production of extracellular hydrolytic enzymes

The three most important hydrolytic enzymes produced by *C. albicans* and linked directly to virulence are secreted aspartic proteinases (SAPs), phospholipases, and lipases. Secreted hydrolytic enzymes can contribute to host tissue invasion by digesting or distorting host cell membranes, degrading host surface molecules to enhance adhesion, or digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack (Calderone, 2002).

2.2.1 Aspartic proteinases

Secreted aspartic proteinases degrade many human proteins at lesion sites; such proteins include albumin, haemoglobin, keratin, and secretory immunoglobulin A. To date, 10 different *SAP* genes (*SAP1-10*) have been identified in *C. albicans* and their proteolytic activity has been linked to tissue invasion (Naglik *et al.*, 2003). The production of SAPs is not limited to *C. albicans* only and their presence has been demonstrated in *C.tropicalis*, *C. parapsilosis*, *C. dubliniensis*, and *C. guilliermondii* (Calderone & Fonzi, 2001; Calderone, 2002). *In vitro* studies have shown that *SAP1, 2,* and *3* genes are expressed by yeast cells only, whereas expression of *SAP4, 5*, and *6* is limited to the hyphal form (Hube *et al.*, 1998; Yang, 2003). The expression of *SAP7* has

never been observed under any laboratory growth conditions. *SAP8* transcript has been detected in yeast cells grown at 25°C in a defined growth medium and *SAP9* is expressed in later growth phases (Monod *et al.*, 1998; Yang, 2003).

2.2.2 Phospholipases and lipases

The activity of phospholipases has been reported in several fungal pathogens including *Candida* species, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Birch *et al.*, 1996; Chen *et al.*, 1997; Yang, 2003). According to the specific ester bond cleaved, these enzymes have been divided into four types of phospholipases: phospholipase A (PLA), phospholipase B (PLB), phospholipase C (PLC), and phospholipase D (PLD) (Calderone & Fonzi, 2001; Yang, 2003). The major phospholipase in *C. albicans* is phospholipase B; it has both hydrolase (fatty acid release) and lysophospholipase-transacylase activities (Leidich *et al.*, 1998). Two *PLB* genes, *PLB1* and *PLB2*, have been identified and cloned; only *PLB1* has been associated with virulence. In an intravenous murine model for hematogenously disseminated candidosis, the virulence of *C. albicans* mutants lacking *PLB1* was significantly attenuated and the mutants showed a dramatically reduced ability to penetrate host cells (Leidich *et al.*, 1998). A separate study indicated that a *PLB1*-disrupted mutant of *C. albicans* was less invasive and caused reduced inflammatory response in an oral-intragastric infant-mouse model (Ghannoum, 2000). These findings suggest that phospholipase secretion may contribute to the virulence of *C. albicans*. Non-*C. albicans* species such as *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* have also been shown to secrete phospholipases, albeit at lower levels (Ghannoum, 2000).

In comparison to proteinases and phospholipases, other secreted hydrolytic enzymes of *C. albicans* such as lipases or esterases, have been rather neglected until recently. Extracellular lipase activity of pathogenic *Candida* species was first detected by Werner (1966) and a secreted esterase was later characterized by Tsuboi *et al.* (1996). The esterase was induced by lipids such as Tween 80 and showed a high activity on α-naphthyl palmitate at pH 5.5. However, it was not able to hydrolyze triolein, tripalmitin, and α-lecithin, and it was therefore characterized as a monoester hydrolase. *C. albicans* was able to grow in media with triolein as a sole source of carbon, suggesting that other

lipolytic enzymes must exist (Sheridan & Ratledge, 1996). Subsequently, a range of lipase genes (*LIP1-10*) were identified in *C. albicans* (Hube *et al.*, 2000) and in other *Candida* species such as *C. parapsilosis*, *C.tropicalis*, and *C. krusei* (Fu *et al.*, 1997; Neugnot *et al.*, 2002). It has been found that lipase gene expression depends on the stage of infection rather than on the organ localization (Hube *et al.*, 2000).

2.3 Adhesion

One of the most important virulence factors of *Candida* species is their ability to adhere using a variety of mechanisms, permitting the yeast to anchor at a site and establish the process of colonization and biofilm formation (Cotter & Kavanagh, 2000). *Candida* species can adhere to host cells, as well as to the surfaces of medical devices, and form biofilms; this results in an increase in antifungal resistance (Chandra *et al.*, 2001a; Hawser & Douglas, 1995). There is a positive correlation between the virulence of different *Candida* species and their ability to form biofilms (Hawser & Douglas, 1994). Adhesion of the yeast form of the fungus to epithelial cells can involve several kinds of adhesin-receptor interactions, including protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions (Cutler, 1991; Staddon *et al.*, 1990). *C. albicans* and other species seem to bind and interact not only with human epithelial surfaces but also with human proteins of the blood and internal tissues (Calderone & Gow, 2002).

2.3.1 Adhesins

The components of the organism that promote host recognition and adherence are usually referred to as adhesins. The adhesins of *C. albicans* are usually polysaccharide or glycoprotein in nature (Calderone & Gow, 2002). *Candida albicans* possesses multiple adhesins and there may be more than one adhesin that recognizes a host ligand or cell (Cannon & Chaffin, 1999). Many studies *in vitro* have shown that a number of environmental signals appear to regulate the expression of *Candida* adhesins, including the availability of sugar (McCourtie & Douglas, 1984; Pizzo *et al.*, 2000), iron (Baillie & Douglas, 1998b), and temperature (Kennedy & Sandin, 1988).

C. albicans has a family of at least nine *ALS* (agglutinin-like sequence) genes that encode cell-surface glycosylated proteins. These proteins have homology with the *S. cerevisiae* α–agglutinin protein that is required for cell-cell recognition during mating (Calderone & Fonzi, 2001). In *C. albicans* and other related species, these proteins are thought to play a role in adhesion to host surfaces (Calderone & Gow, 2002). Als1p and Als5p in *C. albicans* have an adhesin function in relation to human buccal epithelial cells and fibronectin, respectively (Hoyer, 2001). Kamai and colleagues (2002) showed that Als1p has an important role in the adherence of the organism to the oral mucosa during the early stages of infection. Recently, it has been demonstrated that an *Als3*/*Als3* mutant strain of *C. albicans* had an obvious defect in biofilm formation on silicone elastomer discs; the mutant biofilm was structurally weakened and had approximately half the biofilm of a wild-type biofilm (Zhao *et al.*, 2006).

A hypha cell wall-specific protein (Hwp1) was found on the surface of germ tubes and true hyphae, but not on yeasts or pseudohyphae of *C. albicans* (Sundstrom *et al.*, 2002). Hwp1 is encoded by a gene that was originally isolated as a cDNA that encoded a hypha-specific antigen. The gene encodes an outer surface mannoprotein that is believed to be oriented with its amino-terminal domain surface-exposed and carboxyl terminus most probably covalently integrated with cell wall β-glucan (Staab *et al.*, 1996). The structure of the protein suggests that it is linked through a GPI-anchor to cell wall β-glucan (Staab & Sundstrom, 1998). In subsequent work, Staab *et al.* (1999) showed that Hwp1 functions as a substrate for mammalian transglutaminases, suggesting that the protein is involved in the formation of stable complexes with buccal epithelial cells. It has been reported that an *hwp1*/*hwp1* mutant strain of *C. albicans* was greatly impaired in its ability to form stable attachments to human buccal epithelial cells. Also, this mutant showed reduced virulence in a mouse model of systemic candidosis compared with the HWP1/HWP1 strain (Staab *et al.*, 1999).

More recently, the role of Hwp1 in *C. albicans* biofilm formation has been investigated. A study by Nobile et al. (2006) demonstrated a requirement for Hwp1 for normal biofilm formation by *C. albicans in vitro* and *in vivo*. In an *in vitro* model, a *hwp1*/*hwp1* mutant produced a thin biofilm that lacked much

of the hyphal mass found in wild-type biofilms. Further, in a rat venous catheter model, the *hwp1*/*hwp1* mutant was severely deficient in biofilm formation, yielding only yeast microcolonies (Nobile *et al.*, 2006). These findings suggest that Hwp1 is critical for adhesion and biofilm formation.

The integrin-like protein Int1 of *Candida* has been shown to have a role in adhesion and in morphogenesis. Disruption of the *INT1* gene in *C. albicans* reduced yeast adhesion to human epithelial cells by approximately 40%. Moreover, in a mouse model, the mutation in *INT1* reduced the virulence of *C. albicans* (Kinneberg *et al.*, 1999).

2.3.2 Receptors

Fibronectin was one of the first molecules to be recognized as a receptor for *C. albicans* (Skerl *et al.*, 1984). Fibronectin is an extracellular matrix glycoprotein to which a number of microorganisms adhere avidly (Pendrak & Klotz, 1995). The induction of a fibronectin-binding mannoprotein of *C. albicans* was demonstrated when cells were grown in a medium that contains 0.1% haemoglobin (Yan *et al.*, 1996; Yan *et al.*, 1998). In disseminated candidosis, the fibronectin adhesin may be responsible for the adherence of the microorganism to intravascular structures such as endothelial cells or the subendothelial extracellular matrix (Pendrak & Klotz, 1995). The presence of adsorbed fibronectin as a target protein for epithelial attachment of *C. tropicalis* was also confirmed (Bendel & Hostetter, 1993).

The complement fragment iC3b has also been implicated as a ligand associated with epithelial and endothelial cell adherence (Gustafson *et al.*, 1991). Heidenreich and Dierich (1985) were the first to describe the binding of sheep erythrocytes coated with human iC3b or C3d to germ tubes of *C. albicans*. They concluded that germ tubes had surface receptors for bound complement components that functioned similarly to the human complement receptor 2 (CR2) and the human CR3 of host defence cells in their recognition of C3d and iC3b. Later, Calderone et al. (1988) identified, in extracts of *C. albicans* pseudohyphae but not yeasts, two proteins of approximately 62 and 70 kDa that bind the C3d fragment of C3. The finding of C3 receptors, exclusively on the more pathogenic *Candida* species, is highly predictive of their involvement in disease processes (Calderone *et al.*, 1988). Jimenez-Lucho *et*

al. (1990) suggested that yeast cells of *C. albicans* and other fungi bind specifically to the glycosphingolipid, lactosylceramide. Lactosylceramide is a major glycosphingolipid in human glioma brain cells and was the only lipid to which the yeasts bound. As lactosylceramide is widely distributed in epithelial tissues, this glycosphingolipid could be a receptor for yeast colonization and disseminated disease in humans (Jimenez-Lucho *et al.*, 1990).

Lectin-like *Candida* adhesins that recognize glycosides containing L-fucose- or *N*-acetyl-D-glucosamine have been identified (Critchley & Douglas, 1987a; Critchley & Douglas, 1987b). The binding of some *C. albicans* strains to buccal epithelial cells was inhibited by fucose, but in other strains it was inhibited by *N*-acetyl-D-glucosamine or D-glucosamine, suggesting strain-specific receptors. Furthermore, fucoside-binding adhesin has been purified and shown to have an affinity for glycosphingolipid receptors carrying the H blood-group antigen, suggesting that blood group antigens may act as epithelial cell receptors for *C. albicans* (Tosh & Douglas, 1992). The purified adhesin was devoid of carbohydrate and inhibited yeast adhesion to buccal epithelial cells by 80% at an adhesin concentration of 10 µg/ml. In subsequent work, the binding of the purified adhesin (fucoside-binding protein), crude adhesin (extracellular polymeric material), and intact yeast cells of different strains of *C. albicans* to glycosphingolipid receptors was investigated by a chromatogram overlay assay. All preparations from five *C. albicans* strains bound to glycolipids carrying the H blood group antigen. However, one strain, GDH2023, showed a completely different binding pattern and bound only to glycolipids containing *N*-acetyl-D-glucosamine (Cameron & Douglas, 1996). These results confirmed earlier findings about the receptor specificity of the strains made on the basis of adhesion inhibition studies (Critchley & Douglas, 1987b).

2.4 Phenotypic switching

This phenomenon could have important implications in pathogenesis and is considered to be an attribute of virulence in *C. albicans* and other species of *Candida*; switching has been reported in *C. glabrata*, *C.tropicalis*, and *C. parapsilosis* as well as *C. albicans* (Soll, 2002). Colonies of *C. albicans* can switch among variant phenotypes including smooth, rough, star, stippled, irregular wrinkled, and fuzzy at high frequency (10^{-4} to 10^{-1}) (Slutsky *et al.*, 1985). Switching can be stimulated by low doses of UV light and the reversion

of the variant colonies (phenotypes) to the original parental phenotype occurs at high frequency (Calderone & Fonzi, 2001; Pomes *et al.*, 1985). Phenotypic switching may involve changes in the expression of cell surface antigens, enzyme production, and even drug sensitivity. However, the basic mechanism of phenotypic switching and the involvement of this phenomenon in the virulence of *C. albicans* are not clear. One of the most studied phenotypic switching processes is the white-opaque system in *C. albicans* strain WO-1 (Slutsky *et al.*, 1987). In this system, smooth, white, hemispherical colonies (white phase) can switch to flat, gray colonies (opaque phase). A number of differences exist between these two colony types, including cell shape (white cells are round-ovoid and opaque cells are elongated or bean-shaped), cell surface structure (pimples are found on opaque cells only), and germination at 37°C (by white cells only unless the opaque cells are grown on human skin epithelial cells). Opaque cells have a greater ability to colonize the skin in a mouse model of cutaneous infection and a higher frequency of mating than do white-phase cells (Kvaal *et al.*, 1999; Miller & Johnson, 2002). However, opaque cells are less virulent than white cells in a systemic mouse model (Kvaal *et al.*, 1999). Furthermore, these two phenotypes show different gene expression; *OPA1* and *SAP3* are expressed specifically in opaque cells, whereas *SAP2*, *WH11*, and *EFG1* are expressed in white cells (Srikantha & Soll, 1993). Phenotypic switching has been found to affect adhesion to human epithelial cells. The adhesion of white and opaque cells of strain WO-1 to buccal epithelium were dramatically different; white cells were significantly more adhesive than opaque cells (Kennedy *et al.*, 1988).

Recently the correlation between phenotype switching and biofilm formation has been investigated. Biofilms formed by 100% white cells of *C. albicans* were dense and composed of a mixture of yeast cells and hyphae, whereas biofilms formed by 100% opaque cells were thin and fragile, and made up almost entirely of cells that had extended conjugation tubes. However, the thickness of biofilms formed by 90% white and 10% opaque cells was twice that of biofilms formed by 100% white cells; the minority opaque cells stimulated biofilm development by white cells. This finding reveals a novel form of communication between switch phenotypes (Daniels *et al.*, 2006).

The effect of phenotype switching on biofilm formation in *C. parapsilosis* was also investigated. Cells from crepe and concentric phenotypes were pseudohyphal, whereas cells from smooth and crater phenotypes were mostly yeast-like. On polystyrene surfaces, the concentric phenotype produced up to two-fold more biofilm than the crepe and crater phenotypes. Smooth phenotypes produced the least biofilm (Laffey & Butler, 2005).

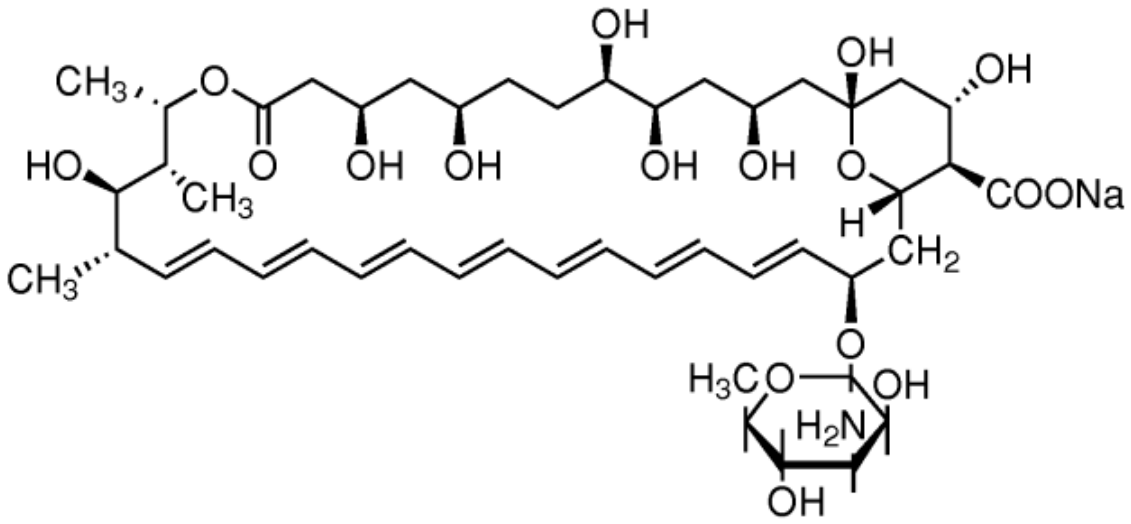
3 Antifungal drugs

The antifungal agents that are currently available for the treatment of systemic fungal infections are grouped according to their site of action in fungal cells into four major classes: the polyenes, azoles, pyrimidine analogues, and echinocandins.

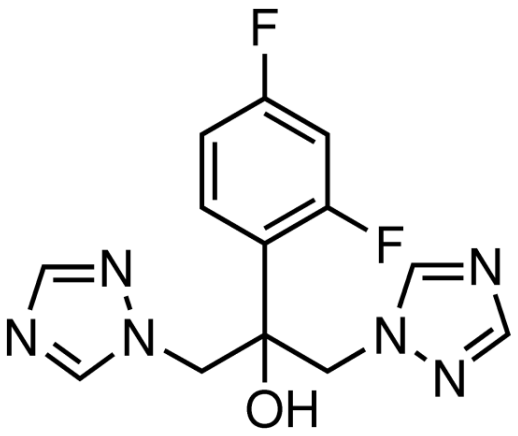
3.1 Polyenes

Polyenes are all produced by species of *Streptomyces* and include amphotericin B (Fig. 3A) and nystatin, which are considered to be fungicidal (Jabra-Rizk *et al.*, 2004; Wingard & Leather, 2004). The polyenes cause the fungal cell to die by binding to the sterol, ergosterol, in the fungal cell membrane. This binding leads to membrane disruption, increased permeability, leakage of cytoplasmic contents, and cell death (Fig. 4). Amphotericin B has a very broad spectrum of antifungal activity and it has been the drug of choice for treatment of invasive fungal infections for many years. Most fungi are susceptible to amphotericin B, including most *Candida* and *Aspergillus species* (Slavin *et al.*, 2004; Wingard & Leather, 2004). However, some species exhibit reduced susceptibility or resistance including *C. lusitaniae, C. guilliermondii*, *Trichosporon beigelii, A. terreus, A. flavus* and *Fusarium* spp. (Polak & Hartman, 1991; Slavin *et al.*, 2004; Wingard & Leather, 2004).

Frequent toxicities and a narrow therapeutic window are major limitations to the clinical use of amphotericin B (Slavin *et al.*, 2004; Wingard & Leather, 2004). Infusional toxicity occurs in approximately 50-60% of treated patients and renal failure in 80% of patients receiving a 2-week course (Slavin *et al.*, 2004). Amphotericin shows poor tolerability, especially with patients



A. Amphotericin B



B. Fluconazole

Figure 3. Chemical structures of antifungal drugs: amphotericin B and fluconazole

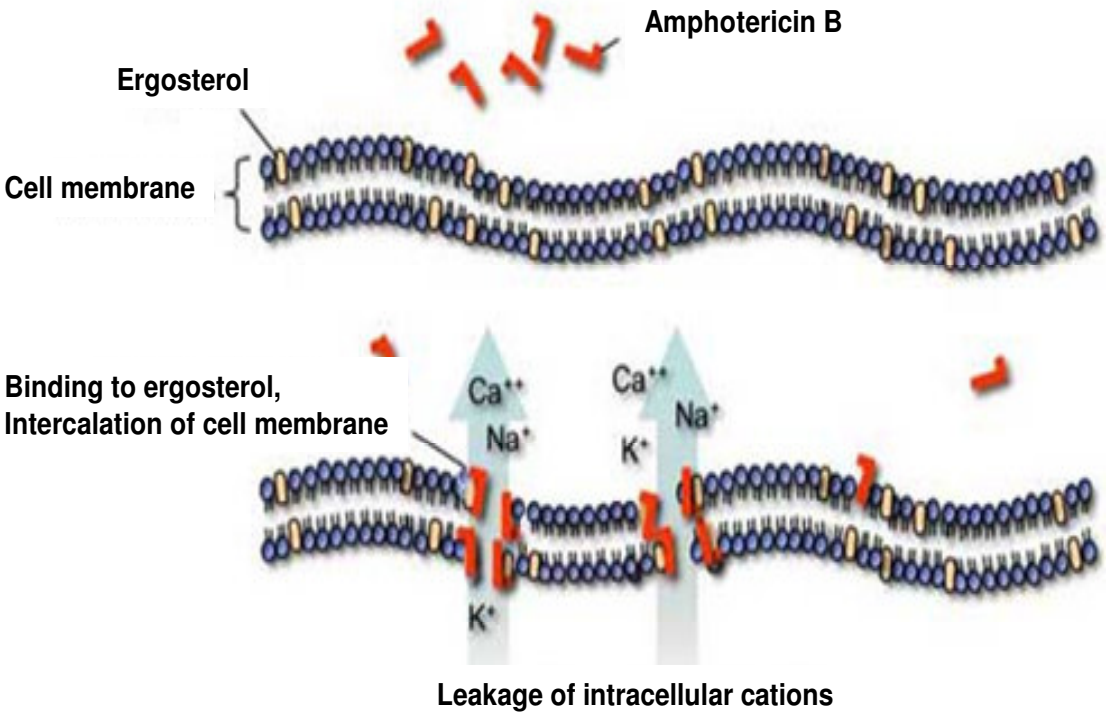


Figure 4. Mechanism of action of polyenes on the fungal cell membrane

Polyene antifungals such as amphotericin B act by binding to ergosterol in the cell membrane. This binding results in depolarization of the membrane and formation of pores that increase permeability and leakage of vital cytoplasmic components (mono- or divalent cations), eventually leading to death of the organism. Reprinted by courtesy of www.doctorfungus.org.

requiring long-term courses of doses above 0.5 mg/kg per day (Slavin *et al.*, 2004; Wingard & Leather, 2004).

Lipid formulations of amphotericin B were developed to ensure less infusional and renal toxicity, and greater tolerability; these preparations permit higher doses of amphotericin B to be used in clinical practice (Slavin *et al.*, 2004; Wingard & Leather, 2004).

3.2 Azoles

The azoles, first described in the late 1960s, are totally synthetic. This class includes the imidazoles (clotrimazole, miconazole, and ketoconazole) and the triazoles (fluconazole, itraconazole, and voriconazole) (Jabra-Rizk *et al.*, 2004; Wingard & Leather, 2004). The azoles inhibit the biosynthesis of ergosterol through their interactions with the enzyme lanosterol demethylase, which is responsible for the conversion of lanosterol to ergosterol. This leads to a reduced content of ergosterol in the fungal cell membrane and ultimately inhibition and fungal death (Fig. 5). Azole antifungal agents have a fungistatic, broad-spectrum activity against most yeasts and filamentous fungi (Slavin *et al.*, 2004; Wingard & Leather, 2004). Among the most commonly used azoles is fluconazole (Fig. 3B), available as both oral and intravenous formulations. Fluconazole is a very effective drug for the treatment of most yeast infections, particularly oropharyngeal and vaginal candidosis. Fluconazole is also recommended as a first choice for the treatment of invasive *Candida* infections in non-neutropenic patients such as solid-organ transplant patients, surgical and ICU patients, or for those with urinary tract infections due to susceptible *Candida* species. Furthermore, fluconazole can successfully treat *Candida*emia in neutropenic patients, as long as the patient is stable and the infection is not due to *Candida* species less susceptible to fluconazole (Rex *et al.*, 1994). Several species are resistant to fluconazole; almost all *C. krusei* isolates are intrinsically resistant and about 50% of *C. glabrata* isolates show reduced susceptibility to the drug (Jabra-Rizk *et al.*, 2004; Slavin *et al.*, 2004). Fluconazole is safe, with a very low incidence of side effects, and is well tolerated (Slavin *et al.*, 2004; Wingard & Leather, 2004). Furthermore, it has efficacy against moulds.

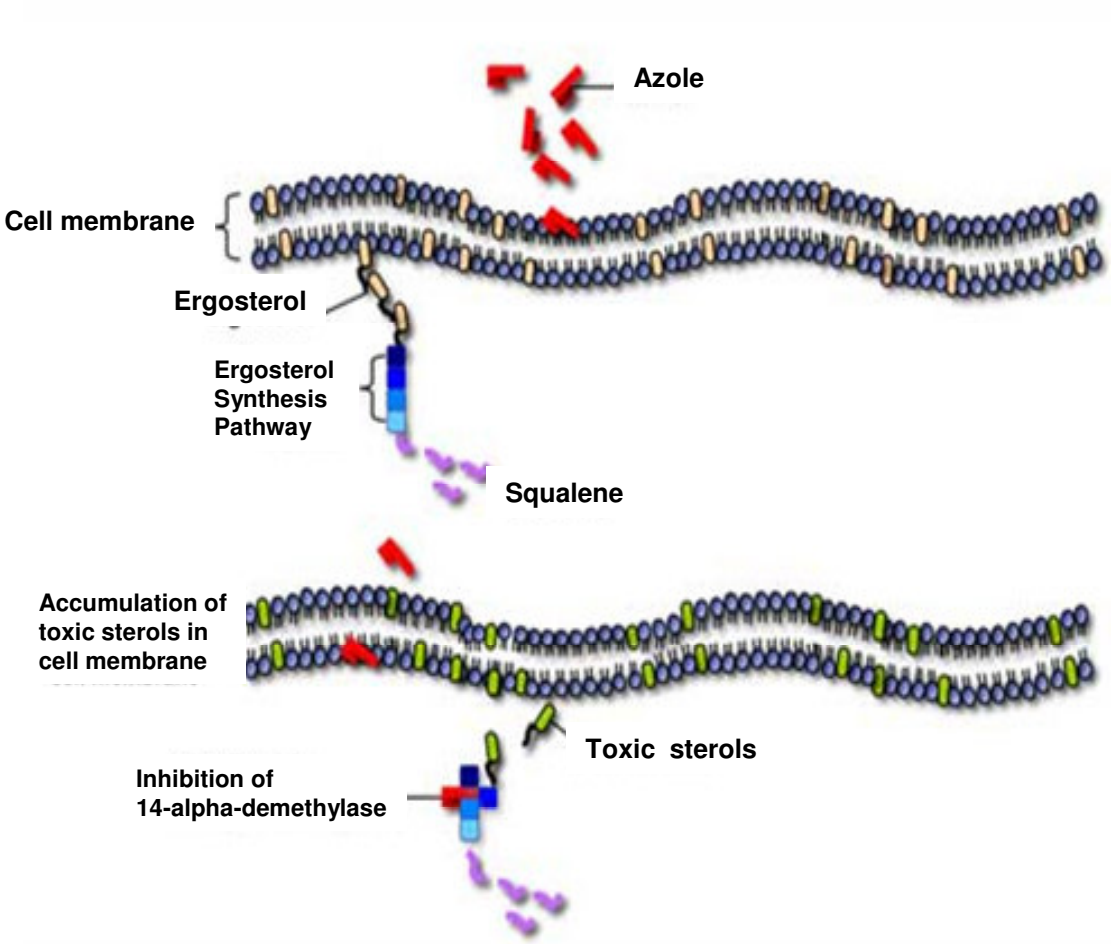


Figure 5. Mechanism of action of azoles on the fungal cell membrane

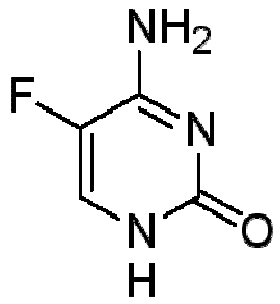
Azole antifungals inhibit the fungal cytochrome P450 14-α-lanosterol demethylase, thereby interrupting the synthesis of ergosterol. Inhibition of this critical enzyme in the ergosterol synthesis pathway leads to the depletion of ergosterol in the cell membrane and accumulation of toxic intermediate sterols, causing increased membrane permeability and inhibition of fungal growth. Reprinted by courtesy of www.doctorfungus.org.

Voriconazole is one of the newest triazoles available and it has the broadest spectrum activity of all the licensed azoles. It exhibits activity against *Candida* (including fluconazole-resistant species), *Aspergillus* and *Fusarium* species, but not against zygomycetes. Voriconazole has two toxicity effects that are not seen with other azoles. It causes photopsia, a visual disturbance, in up to 30-45% of recipients and photosensitivity, in up to 5% of patients receiving the drug. In addition to its toxicities, drug interaction is a very important issue to be considered (Jabra-Rizk *et al.*, 2004; Wingard & Leather, 2004).

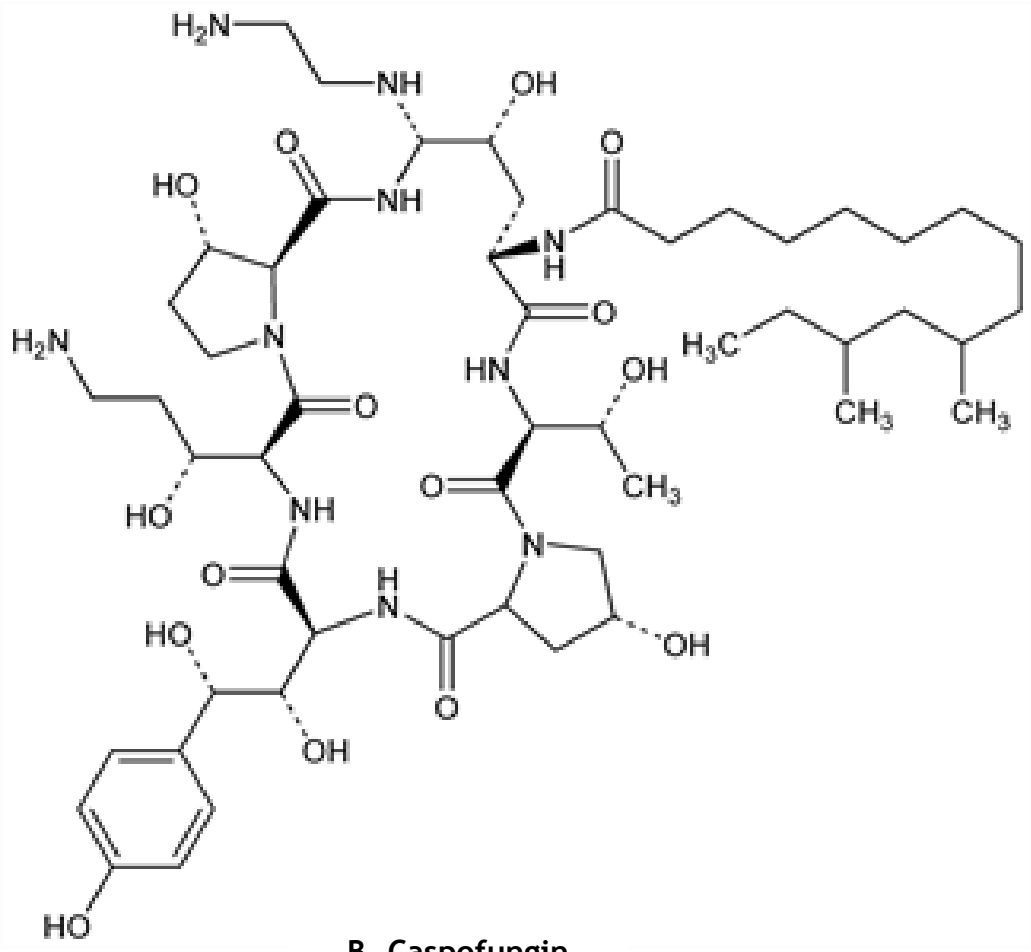
Posaconazole is an extended-spectrum triazole that was approved in 2006. It is active against *Candida* species, *Aspergillus* species, and is the only azole with reliable antifungal activity against Zygomycetes. It is also effective against *Candida* isolates resistant to itraconazole, fluconazole, and voriconazole (Greer, 2007; Keating, 2005; Torres *et al.*, 2005) and has activity comparable to fluconazole in treating oropharyngeal candidosis in HIV-positive patients (Vazquez *et al.*, 2006). Ravuconazole is the latest azole agent to undergo clinical development. It shows a broad spectrum of activity against fungal pathogens including *Candida*, *Aspergillus*, *Cryptococcus* species, and dermatophytes (Pfaller *et al.*, 2004) it also exhibits good activity against fluconazole- or itraconazole-resistant isolates of *C. albicans* and *C. dubliniensis* (Pfaller *et al.*, 1999a).

3.3 Pyrimidine analogues

5-Flucytosine (5-fluorocytosine; 5-FC) is a pyrimidine analogue that was originally developed in the 1950s as a potential antineoplastic agent and later was found to have antifungal activity (Fig. 6A). After its transport into the fungal cell, it is deaminated to the active form, 5-fluorouracil (5-FU), and ultimately leads to the disruption of DNA and protein synthesis (Fig. 7) (Polak & Hartman, 1991; Wingard & Leather, 2004). Flucytosine has a narrow spectrum of activity against *Candida* and *Cryptococcus* species (Wingard & Leather, 2004). Most moulds including *Aspergillus* spp. are resistant (Maschmeyer & Glasmacher, 2005; Slavin *et al.*, 2004; Wingard & Leather, 2004).

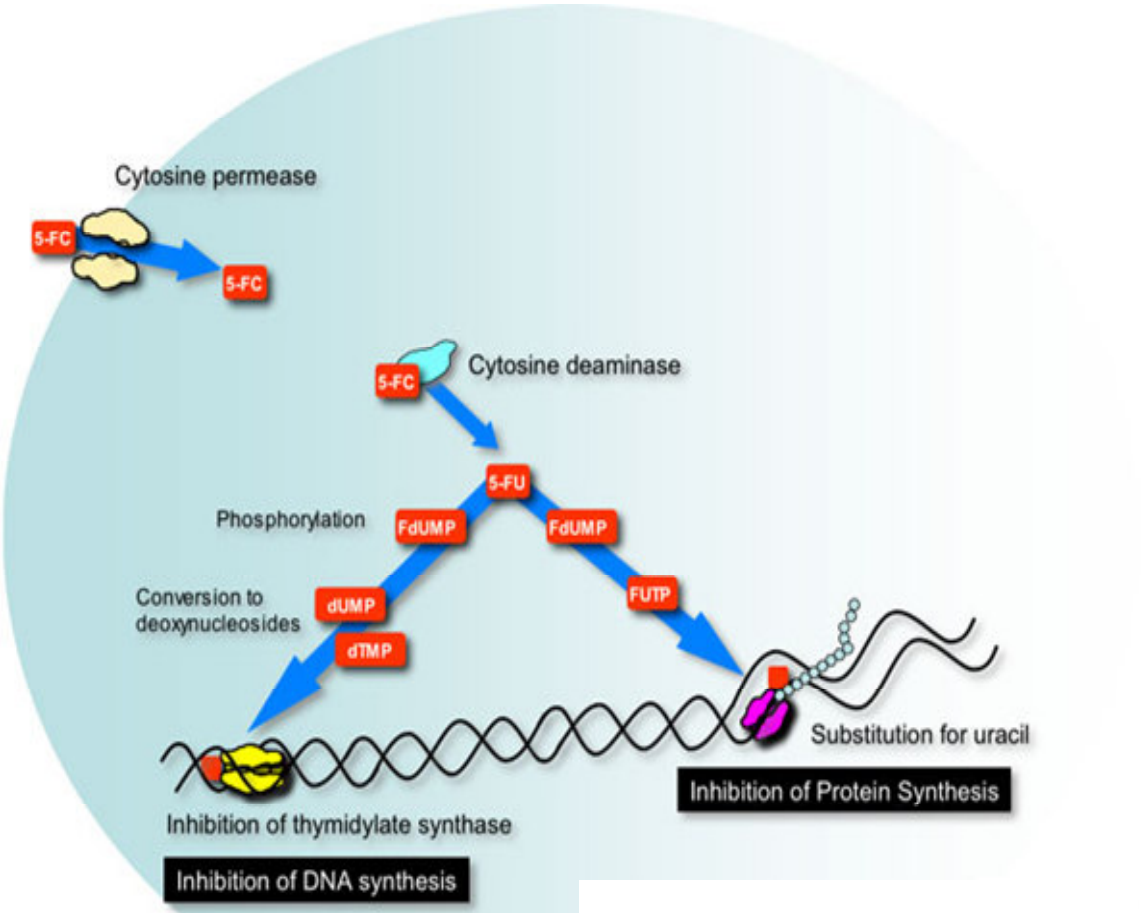


A. Flucytosine



B. Caspofungin

Figure 6. Chemical structures of antifungal drugs: flucytosine and caspofungin



5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridine; FUMP, 5-fluorouridine monophosphate; FUDP, 5-fluorouridine diphosphate; FUTP, 5-fluorouridine triphosphate; dUMP, deoxyuridine monophosphate dTMP, deoxythymidine monophosphate

Figure 7. Mechanism of action of 5-flucytosine on the fungal cell

5-Flucytosine is transported into susceptible fungal cells by a specific enzyme cytosine permease and converted in the cytoplasm by cytosine deaminase to 5-fluorouracil (5-FU). 5-FU undergoes further steps of activation and finally interacts as FUTP with RNA biosynthesis and causes miscoding and halts protein synthesis. Additionally, 5-FU is converted to dTMP which inhibits DNA synthesis by blocking the function of thymidylate synthetase. Reprinted by courtesy of www.doctorfungus.org.

3.4 Echinocandins

The echinocandins and their analogues, the pneumocandins, are semi-synthetic cyclic lipopeptides and represent a new class of antifungal agents that have a unique mechanism of action. They inhibit the synthesis of β-1,3-D-glucan, an essential component of the fungal cell wall (Fig. 8). This component is absent in mammalian cells, which increases the drug's specificity and reduces its toxicity for mammalian cells (Maschmeyer & Glasmacher, 2005; Slavin *et al.*, 2004; Wingard & Leather, 2004). Echinocandins have fungicidal activity against *Candida* spp. and fungistatic activity against *Aspergillus* spp. *In vitro,* echinocandins demonstrate excellent activity, against amphotericin B-resistant and azole-resistant *Candida* and *Aspergillus* species (Espinel-Ingroff, 2003; Maschmeyer & Glasmacher, 2005; Wingard & Leather, 2004) but have no activity against fungi that lack significant β-glucan in their cell walls, such as *Cryptococcus* and *Trichosporon* species (Espinel-Ingroff, 2003; Slavin *et al.*, 2004). The inhibition is effective and specific, and short exposure leads to cell death (Maschmeyer & Glasmacher, 2005). This class of drugs now includes anidulafungin and micafungin, as well as caspofungin. However, caspofungin (Fig. 6B) was the first echinocandin licensed for treatment of fungal infections. It has demonstrated excellent pharmacokinetic properties and exhibited good safety profiles (Chandra *et al.*, 2001a; Kuhn *et al.*, 2002a; Mukherjee *et al.*, 2005).

4 Biofilms

In Nature, biofilms are the most common form of microbial growth; microorganisms are found attached to surfaces and not as free-floating (planktonic) cells (Chandra *et al.*, 2001a; Douglas, 2003; Mukherjee *et al.*, 2005; O'Toole *et al.*, 2000). Biofilms are a preferred mode of microbial existence as they provide protection for the cells against physical forces, pH changes, chemical attacks, and offer higher chances of survival in a low-nutrient environment (Jefferson, 2004). A biofilm is defined as a community of microorganisms that develops from a single species or from multiple species of bacteria or fungi attached to a surface.

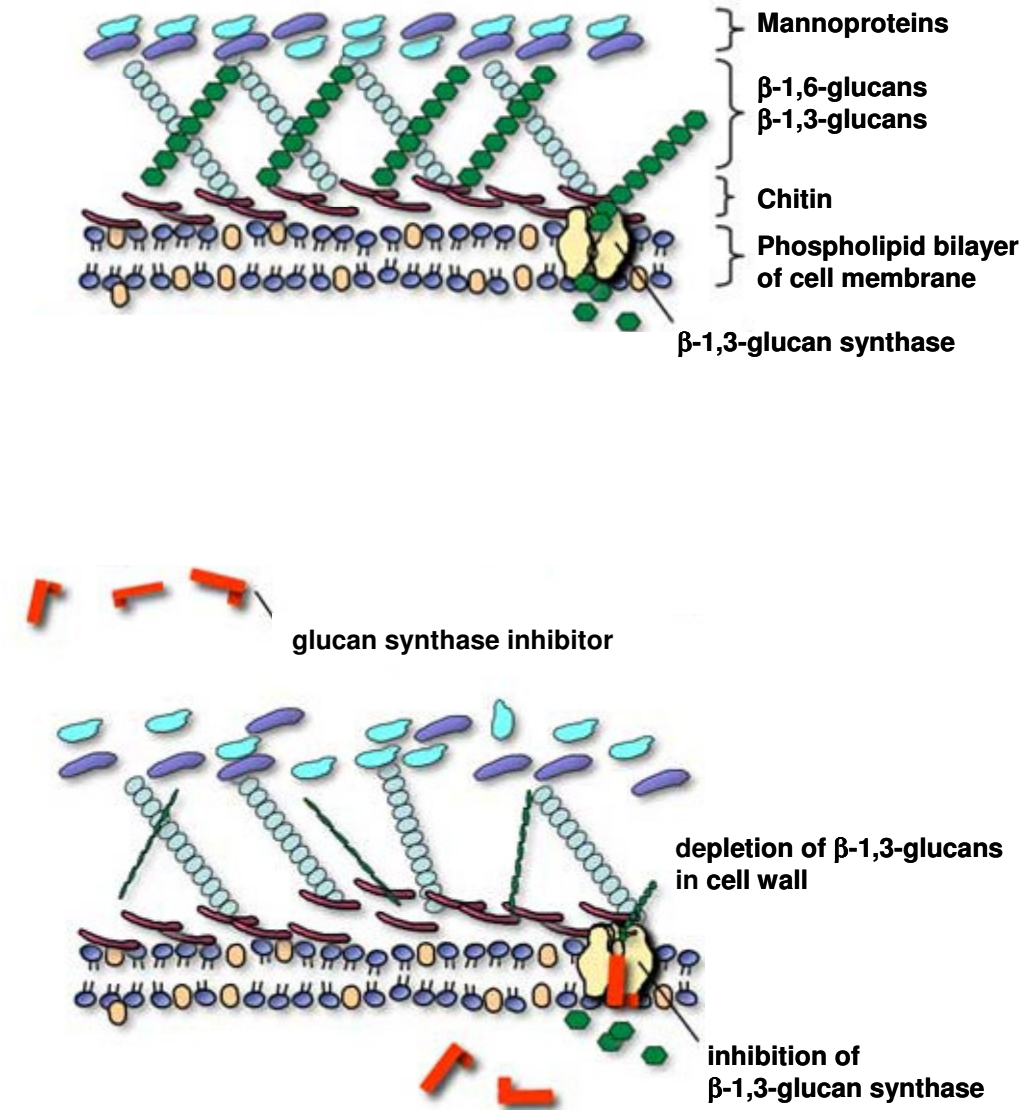


Figure 8. Mechanism of action of echinocandins on the fungal cell wall

Echinocandins inhibit the enzyme β-1,3-glucan synthase. This inhibition results in depletion of glucan polymers in the fungal cell wall, resulting in an abnormally weak cell wall unable to withstand osmotic stress. Reprinted by courtesy of www.doctorfungus.org.

These microbes are embedded in a matrix, often slimy, of extracellular polymeric material (Donlan, 2001; Donlan & Costerton, 2002; Douglas, 2003; Kumamoto & Vinces, 2005) and exhibit a distinctive phenotype with regard to growth rate and gene transcription (Donlan & Costerton, 2002; Douglas, 2003).

A large number of studies have been carried out in order to understand bacterial biofilms and their role in disease (Chandra *et al.*, 2001a; Douglas, 2003; Jabra-Rizk *et al.*, 2004; Mukherjee *et al.*, 2005). In the seventeenth century, dental plaque on tooth surfaces was the first example of what we now call a biofilm to be recognized in medical systems (Marsh, 1995). Recent estimates suggest that some 65% of all human infections involve biofilms (Dominic *et al.*, 2007; Ramage *et al.*, 2005). Many of these infections are associated with implanted medical devices such as intravascular catheters, joint replacements, endotracheal tubes and prosthetic heart valves, which can act as surfaces for biofilm growth (Donlan, 2001). These devices can easily become contaminated. The infusion fluid itself or the catheter hub may be the source of infection but, more often, organisms are introduced from the patient's skin or from the hands of health care workers. Sometimes the distal tip of the catheter is contaminated during the insertion process; alternatively, organisms from another site in the body can travel via the blood and infect the catheter (Collin, 1999; Donlan & Costerton, 2002). Biofilm microorganisms can also be found in tissues taken from non-device-related chronic infections such as cystic fibrosis, native valve endocarditis, otitis media, chronic bacterial prostatitis and periodontitis (Mukherjee *et al.*, 2005). The most common organisms isolated from catheter biofilms are *C. albicans* and a diversity of bacterial species including *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa* and *Actinobacter* species (Kumamoto & Vinces, 2005; Mukherjee *et al.*, 2005). In the United States, several million vascular and urinary catheters and tens of thousands of prosthetic heart valves are used annually (Chandra *et al.*, 2001a; Kuhn *et al.*, 2002a; Kumamoto & Vinces, 2005). Infections of catheters are not only expensive in terms of catheter replacement but may also cause a bacteraemia or fungaemia with a mortality rate of up to 40% (Cheng *et al.*, 2005; Nguyen *et al.*, 1995). The use of central venous catheters (CVCs) in current clinical practice is responsible for more than 90% of bloodstream infections (Odetola *et al.*, 2003), resulting in 10-25% mortality among these patients (Nicastri *et al.*, 2001; Veenstra *et al.*, 1999) .

4.1 *Candida* biofilms

Little attention has been given to medically relevant fungal biofilms in comparison to bacterial biofilms and their role in disease. All *Candida* species are opportunistic pathogens and their emergence as important nosocomial pathogens is related to specific risk factors. These factors include immunosuppressive therapy, antibiotic therapy, and the use of indwelling devices such as intravenous catheters (Calderone, 2002; Nucci *et al.*, 1998). Approximately 50% of all nosocomial infections are medical device-related infections and about 10% of these infections are due to *Candida* spp. (Kumamoto & Vinces, 2005). In a prospective study of catheter colonization, *C. albicans* represented the second highest colonization to invasive infection rate (Crump & Collignon, 2000). Even with current antifungal therapy, the mortality of patients with systemic candidosis can be as high as 40% (Dominic *et al.*, 2007).

Superficial *Candida* infections related to implanted devices are much less serious, but are the most frequently encountered and can be problematic (Douglas, 2003; Jabra-Rizk *et al.*, 2004; Mukherjee *et al.*, 2005). One of the most common is denture stomatitis, a *Candida* infection of the oral mucosa that is promoted by a close-fitting upper denture and is present in up to 65% of edentulous persons (Chandra *et al.*, 2001a; Jabra-Rizk *et al.*, 2004; O'Toole *et al.*, 2000).

A description of *Candida* biofilms on specific devices is presented in Table 1 (Ramage *et al.*, 2006; Kojic *et al.*, 2004). Non-device-related infections can also involve biofilms; these include *Candida* endocarditis and *Candida* vaginitis (Donlan & Costerton, 2002; Douglas, 2003).

4.2 Model biofilm systems

A variety of biofilm models have been developed by different groups of researchers to investigate the properties of *Candida* biofilms *in vitro*. Almost all of these models have been adapted from methods reported previously for bacteria.

Hawser and Douglas (1994) initially described a simple method, involving growth of adherent populations on the surfaces of small discs cut from catheters. Growth of biofilms was monitored quantitatively by a colorimetric

Table 1. Some of the implantable devices on which *Candida* biofilms develop most frequently

Device	Annual use in the United States	Infection risk (%)	Main *Candida* species
Central and peripheral venous catheters	5 million	3-8	*albicans* *glabrata* *parapsilosis*
Hemodialysis and peritoneal dialysis catheters	240 000	1-20	*albicans* *parapsilosis*
Urinary catheters	Tens of millions	10-30	*albicans*
Endotracheal tubes	Millions	10-25	*albicans*
Intracardiac prosthetic devices	400 000	1-3	*albicans* *glabrata* *parapsilosis* *tropicalis*
Breast implants	130 000	1-2	*albicans*
Prosthetic joints	600 000	1-3	*parapsilosis* *albicans* *glabrata*
Neurosurgical shunts	40 000	6-15	*albicans*
Voice prostheses	Thousands	50-100	*albicans* *tropicalis*
Dentures	> 1 million	5-10	*albicans* *glabrata*

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assay involving the reduction of a tetrazolium salt, or by [^{3}H] leucine incorporation. Subsequent *in vitro* model systems have included procedures with a variety of different acrylic strips and discs, glass slides, microtitre plates, cylindrical cellulose filters, perfused biofilm fermenters, polycarbonate membrane filters, and tissue culture flasks; these models have produced biofilms formed under both static and flow conditions (Al-Fattani & Douglas, 2004; Baillie & Douglas, 1998a; Baillie & Douglas, 1999a; Chandra *et al.*, 2001a; Honraet *et al.*, 2005; Ramage & Lopez-Ribot, 2005). Among the various systems described, the 96-well microtitre plate method permits rapid processing of a large number of samples and has therefore been widely used. It has proved particularly valuable for determination and standardization of antifungal susceptibility testing in *Candida* biofilms (Ramage *et al.*, 2001b; Ramage *et al.*, 2001c). Studies with different model systems have also identified factors affecting *Candida* biofilm development and phenotypic properties associated with this mode of growth.

More recently, two different animal models of catheter-associated *Candida* infections have been described, and visualization of the resulting biofilms formed *in vivo* has revealed structural features similar to those of biofilms formed *in vitro* (Andes *et al.*, 2004; Schinabeck *et al.*, 2004). These findings suggest that *in vitro* model systems can replicate *in vivo* events and therefore that the observations made may be clinically relevant (Ramage *et al.*, 2005).

4.2.1 Quantitative analysis of biofilm growth

Several methods have been used to measure the growth of *Candida* biofilms. Commonly used methods are: (a) uptake of radioactively labelled leucine; (b) determination of metabolic activity via reduction of tetrazolium salts by mitochondrial dehydrogenases or ferric reductase; (c) absorption of crystal violet dye, (d) determination of dry weight, (e) viable cell counts; and (f) measurement of ATP-bioluminescence (Baillie & Douglas, 1999b; Chandra *et al.*, 2001b; Hawser & Douglas, 1994; Nikawa *et al.*, 1996; Ramage *et al.*, 2001b).

4.3 Factors affecting biofilm formation

A number of environmental and growth factors can greatly influence the ability of *Candida* to form biofilms *in vitro*, including the *Candida* species, the nature of the surface colonized, the host-derived conditioning film, the liquid flow, and the type of nutrients available.

4.3.1 *Candida* species and strain

Different *Candida* species show some correlation between their ability to form biofilms and their pathogenicity when tested in the catheter disc system. Isolates of *C. parapsilosis*, *C. pseudotropicalis* and *C. glabrata* all gave considerably less biofilm growth than the more pathogenic *C. albicans* (Hawser & Douglas, 1994). Recently, it has been confirmed that *C. albicans* isolates consistently produce more biofilm *in vitro* than non-*C. albicans* isolates (Kuhn *et al.*, 2002a).

4.3.2 Nature of colonized surface

The chemical nature and properties of the colonized surface play an important role in biofilm formation (Hawser & Douglas, 1994; Mukherjee *et al.*, 2005). In the catheter disc model system, the type of material used affected biofilm formation by *C. albicans*. Biofilm formation was slightly greater on latex or silicone elastomer than on polyvinylchloride, but was significantly reduced on polyurethane or 100% silicone (Hawser & Douglas, 1994).

4.3.3 Presence of conditioning film

In vivo, catheters and other implants rapidly adsorb host proteins which form a conditioning film on the implant surface. Preincubation of PVC catheter discs *in vitro* with fibrinogen or collagen improved the formation of biofilms by *C. albicans* (Douglas, 2003; Mukherjee *et al.*, 2005). Likewise, conditioning films of serum or saliva enhanced biofilm formation on denture acrylic (Chandra *et al.*, 2001b; Nikawa *et al.*, 1996).

4.3.4 Liquid flow

In vivo, development of biofilms is frequently subjected to a liquid flow. This can be achieved *in vitro* by gentle shaking of the growing biofilm on a

catheter disc in liquid medium, to produce a flow of liquid over the surface of the cells (Hawser *et al.*, 1998). More sophisticated flow systems include biofilm formation on cylindrical cellulose filters (Baillie & Douglas, 1998b; Baillie & Douglas, 1999a), in the perfused biofilm fermenter (Baillie & Douglas, 1999b), or in a modified Robbins device (Al-Fattani & Douglas, 2006). Biofilms of *C. albicans* incubated under these conditions produce substantially more matrix material than those incubated statically.

4.3.5 Growth media and different nutrients

Nutrients play an important role in biofilm formation. The effect of different carbohydrates on *C. albicans* biofilm formation was examined and it was shown that biofilm formation reached a maximum after 48 h in a medium containing 500 mM galactose or 50 mM glucose and then declined; however, the cell yield was lower in the low-glucose medium (Hawser & Douglas, 1994). *C. tropicalis* and *C. parapsilosis* seem to form biofilms quite readily when grown in medium containing 8% glucose (Shin *et al.*, 2002).

4.4 Structure of *Candida* biofilms

The biofilm development cycle includes adhesion of planktonic cells to the surface, growth and secretion of extracellular polymers (forming the mature biofilm with mushroom-shaped microcolonies) and cell detachment (Fig. 9). The architecture of biofilms formed by *Candida* species shares several properties with bacterial biofilms (Chandra *et al.*, 2001a; Douglas, 2003; Jabra-Rizk *et al.*, 2004; O'Toole *et al.*, 2000). However, many fully mature *Candida* biofilms have a mixture of morphological forms and consist of a dense network of yeasts and filaments in a matrix of extracellular polymeric material. Some non-*C. albicans* species such as *C. glabrata* (Chandra & Ghannoum, 2004; Kuhn *et al.*, 2002a) and some isolates of *C. parapsilosis* (Laffey & Butler, 2005) form biofilms that contain yeast cells with no filaments.

Biofilm formation by *C. albicans* proceeds in three distinct developmental phases: initial adherence, followed by proliferation, and maturation over a period of 24 to 48 h (Chandra *et al.*, 2001a; Hawser & Douglas, 1994; Ramage *et al.*, 2001a). Scanning electron microscopy was shown that mature biofilms of *C. albicans* grown on catheter material consist of

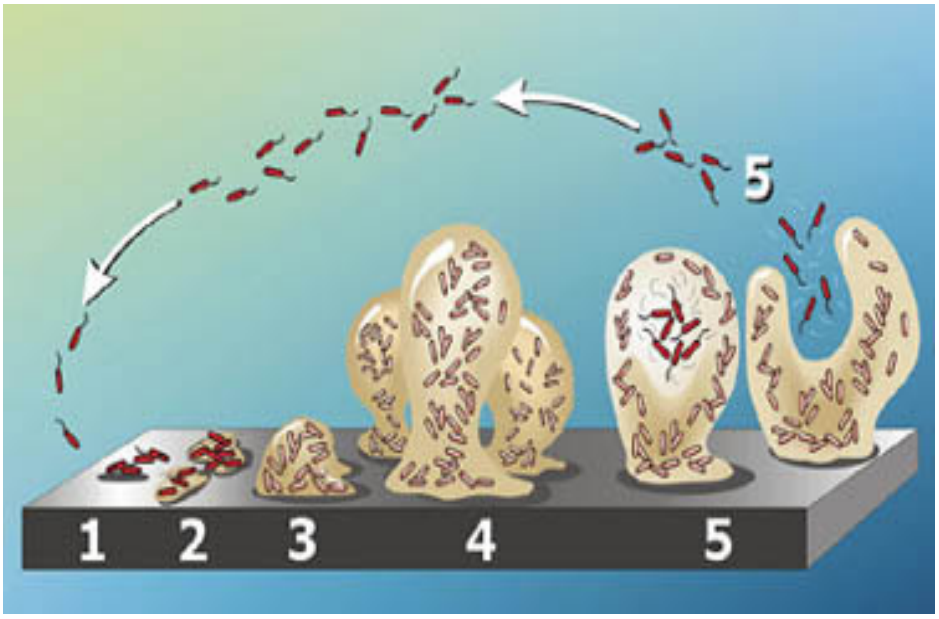

Figure 9. Different stages in the biofilm life cycle

1. Initial reversible attachment of free swimming micro-organisms to surface

2. Permanent chemical attachment, single layer, microorganisms begin making slime

3. Early vertical development

4. Maturing biofilms (mushroom-shaped microcolonies) with multiple layers and channels between the microcolonies

5. Mature biofilm with seeding / dispersal of more free swimming micro-organisms

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yeasts, hyphae and pseudohyphae arranged in a bilayer structure; there is a dense, basal yeast layer that anchors the biofilm to the catheter surface, and an overlying but more open, hyphal layer (Douglas, 2003; Kuhn *et al.*, 2002a) (Fig. 10). Subsequent studies indicated that *in vivo* biofilms are structurally similar to those described *in vitro* model except for numerous host cells including red blood cells, platelets, macrophages and neutrophils that are embedded in the matrix (Andes *et al.*, 2004; Schinabeck *et al.*, 2004).

Recent advances in confocal laser scanning microscopy (CLSM), provided the opportunity to visualize living biofilms in a fully hydrated condition. *C. albicans* biofilms were observed as three-dimensional structures consisting of microcolonies of yeasts and hyphae surrounded by a matrix of extracellular polymeric material and separated by water channels to facilitate nutrient circulation and disposal of waste products (Chandra *et al.*, 2001a; Douglas, 2003; Kumamoto & Vinces, 2005). The CLSM technique has also shown that *Candida* biofilms can range in thickness from 25 μm to more than 450 μm (Chandra *et al.*, 2001a).

Quorum sensing appears to play an important role in *Candida* biofilm formation and to date two quorum sensing molecules have been identified: farnesol and tyrosol (Alem *et al.*, 2006; Ramage *et al.*, 2002b). This strategy of cell-cell communication contributes to the biofilm's wellbeing by preventing unnecessary overpopulation and controlling competition for nutrients; it also has important implications in the infectious process, especially for dissemination and for the establishment of distal sites of infection (Nickerson *et al.*, 2006; Ramage *et al.*, 2002b). Quorum sensing systems may regulate the active detachment of cells by the production of enzymes to break down the biofilm extracellular polymeric matrix (Ghannoum & OToole, 2004; Kruppa, 2009).

The matrix composition of *C. albicans* biofilms has been studied and compared with the extracellular polymeric material collected from culture supernatants of planktonically grown organisms. Both materials contained carbohydrate, protein, phosphorus and hexosamine. However, the matrix had considerably less carbohydrate (41%), and protein (5%). It also had a higher proportion of glucose (16%) than mannose and contained galactose (Baillie & Douglas, 2000). In a recent study, the biofilm matrix of *C. tropicalis* was

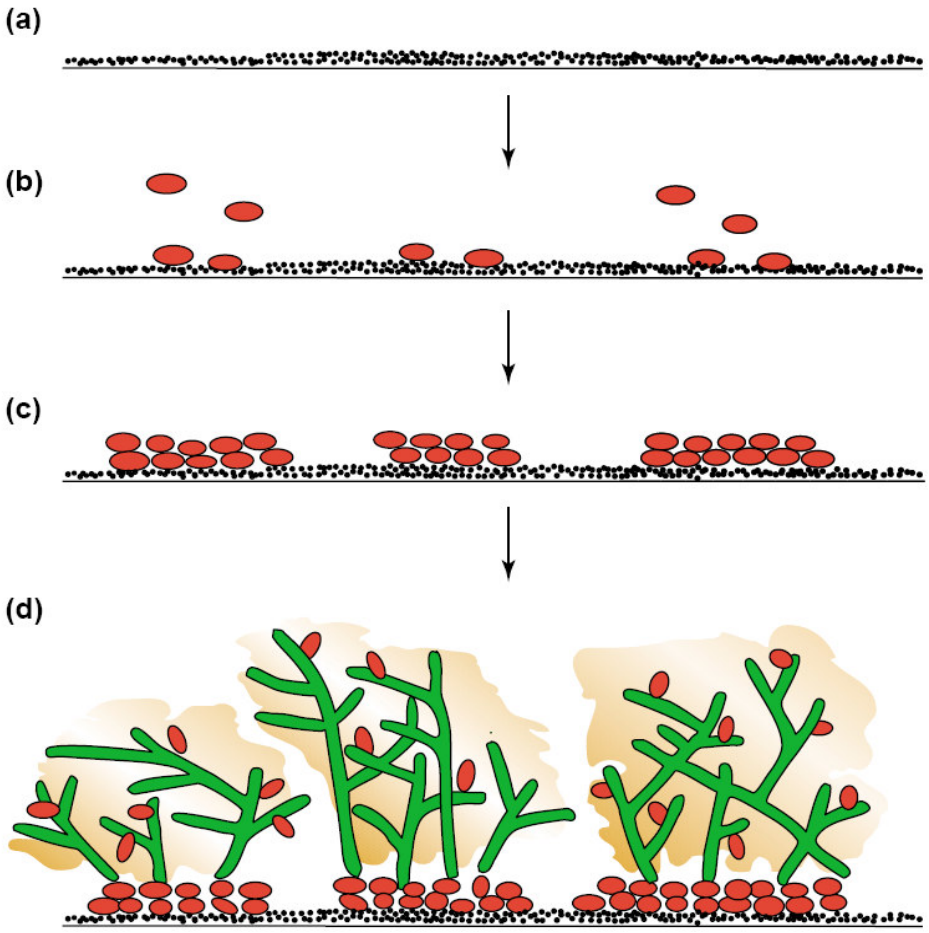


Figure 10. Stages in the formation of a *Candida albicans* biofilm on a polyvinylchloride (PVC) catheter surface

(a) Catheter surface with an adsorbed conditioning film of host proteins (black dots). (b) Initial yeast (red) adhesion to the surface. (c) Formation of the basal layers of yeast microcolonies. These anchor each microcolony to the surface. (d) Completion of microcolony formation by addition of the upper, mainly hyphal layer and matrix material (yellow) that surrounds both yeasts (red) and hyphae (green). Mature biofilms contain numerous microcolonies with interspersed water channels to allow circulation of nutrients. On other surfaces (e.g. cellulose fibres) microcolonies consisting entirely of yeast cells are produced. Reprinted from Douglas (2003) by permission of the publisher, Elsevier.

analyzed and shown to contain large amounts of hexosamine (27.4%) with smaller proportions of carbohydrate (3.3%, including 0.5% glucose), protein, and phosphorus (Al-Fattani & Douglas, 2006).

4.5 Mixed-species biofilms

Biofilms that are formed by a mixture of *Candida* species and bacteria (polymicrobial biofilms) are probably common *in vivo* (El-Azizi *et al.*, 2004). *In vitro*, various studies suggest that extensive interactions between these species occur. For example, the catheter disc model has been used to investigate mixed-species biofilms consisting of *C. albicans* and *Staphylococcus epidermidis*. Two strains of *S. epidermidis* were used: a slime-producing wild-type and a slime-negative mutant. Both strains of *S. epidermidis* showed numerous physical interactions with both yeast and hyphal forms of *C. albicans* (Adam *et al.*, 2002). Moreover, drug susceptibility studies suggested that fungal cells can modulate the action of antibiotics, and bacteria can affect antifungal activity in these biofilms. The presence of *C. albicans* in the biofilm increased the resistance of slime-negative staphylococci to vancomycin, whereas *Candida* resistance to fluconazole was enhanced in the presence of slime-producing staphylococci (Adam *et al.*, 2002).

Antagonistic interactions have been observed in mixed-species biofilms consisting of *Pseudomonas aeruginosa* and *C. albicans*. *In vitro* studies showed that *P. aeruginosa* formed a dense biofilm on *C. albicans* hyphae, and killed the fungus. However, the bacteria were unable to bind to, or kill, yeast-form *C. albicans* (Hogan & Kolter, 2002). Subsequent studies showed that *P. aeruginosa* excretes a compound structurally similar to the quorum sensing molecule, farnesol, which inhibits yeast-hyphal morphogenesis (Hogan *et al.*, 2004).

4.6 Biofilm resistance to antimicrobial agents

Microbial biofilms not only serve as a nidus for disease but are also notoriously resistant to a wide range of antimicrobial agents (Donlan, 2001; Hoyle & Costerton, 1991; Jabra-Rizk *et al.*, 2004). *In vitro*, bacterial cells in a biofilm are 10-1000 times more resistant to antibiotics than planktonic cells (Donlan & Costerton, 2002). Similar findings with *Candida* biofilms were first reported in 1995 (Hawser & Douglas, 1995). Clinically important antifungal agents, including amphotericin B, fluconazole, flucytosine, itraconozole, and

ketoconazole were tested against different *Candida* species. All these agents showed much less activity against *C. albicans* biofilms than against planktonic cells. Concentrations of antifungal agent required to reduce metabolic activity by 50% were five to eight times higher than for planktonic cells, and 30-2000 times higher than the corresponding minimum inhibitory concentrations. Biofilms of non-*C. albicans* species such as *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. glabrata* were also drug resistant (Hawser & Douglas, 1995). Subsequent studies have demonstrated drug resistance for *Candida* biofilms grown in a variety of model systems *in vitro* (Baillie & Douglas, 1998b; Chandra *et al.*, 2001a; Chandra *et al.*, 2001b; Mukherjee & Chandra, 2004; Ramage *et al.*, 2001b). Moreover, with *in vivo* animal models, *Candida* biofilms showed a resistant phenotype similar to that of biofilms formed *in vitro* (Kuhn *et al.*, 2002b).

Since antifungal therapy is often ineffective, biofilm-associated infections are problematic and removal of the infected device is recommended. However, in the case of infected heart valves, central nervous system shunts and joint prostheses, removal may result in serious implications for the patient (Jabra-Rizk *et al.*, 2004). Therefore, there is an obvious requirement for antifungal drugs that are active against this type of infection. Recently, two classes of antifungal agents, namely, lipid formulations of amphotericin B (liposomal AMB and AMB lipid complex) and the echinocandins, have shown activity against *Candida* biofilms. The mechanism behind their unique activity is unclear (Kuhn *et al.*, 2002b).

4.7 Possible mechanisms of biofilm drug resistance

The mechanisms of biofilm-associated resistance to antimicrobial agents are not fully understood. In bacteria, resistance appears to be multifactorial and may vary with the bacteria present in the biofilm and the nature of the antimicrobial agent being administered (Mah & O'Toole, 2001). With *Candida* biofilms, at least four factors have been considered to be responsible for increased resistance to antimicrobial agents. These are: a) restricted drug diffusion through the matrix of extracellular polymeric material, b) phenotypic differences resulting from low growth rate or nutrient depletion, c) surface contact-induced gene expression and d) the presence of “persister” cells (Douglas, 2003; Mukherjee & Chandra, 2004; Ramage *et al.*, 2005). Cell density

(Perumal *et al.*, 2007), membrane sterols (Mukherjee *et al.*, 2003), and cell wall glucans (Nett *et al.*, 2007b) could also play a role. To date, most evidence suggests that drug resistance in *Candida* biofilms may be due to a combination of two or more of these mechanisms.

4.7.1 Restricted drug diffusion

The production of extracellular polymeric matrix is one of the distinguishing characteristics of biofilms. It has long been supposed that this matrix might act as a barrier to the diffusion of antimicrobial agents. This was investigated in *C. albicans* by comparing the susceptibility profiles of biofilms grown under liquid flow conditions (maximal matrix production) with those of biofilms grown under static conditions (minimal matrix production). Both types of biofilm were resistant to antifungal drugs with no significant differences (Baillie & Douglas, 2000); this suggests that drug resistance is unrelated to the extent of matrix formation. However, other studies with biofilms produced under flow conditions showed that resuspended cells (which had previously lost most of their matrix) were 20% less resistant to amphotericin B than the intact biofilms (Baillie & Douglas, 1998a; Baillie & Douglas, 1998b). These results with resuspended biofilm cells were subsequently confirmed elsewhere (Ramage *et al.*, 2002a).

More recently, a filter disc assay was used to examine the penetration of antifungal drugs (flucytosine, fluconazole, amphotericin B, and voriconazole) into single- and mixed-species biofilms containing *Candida*. In single-species *Candida* biofilms, fluconazole penetration was more rapid than penetration by flucytosine. Each drug showed similar diffusion rates through biofilms of three strains of *C. albicans*. However, the rates of drug diffusion through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *C. parapsilosis* or *C. tropicalis*. In all cases, after 3 to 6 h the drug concentration at the distal edge of the biofilm was very high but failed to yield complete killing of biofilm cells. In mixed-species biofilms containing *C. albicans* and *S. epidermidis*, the diffusion of all four antifungal agents was very slow. In these experiments, the drug concentrations at the distal edges of the biofilms substantially exceeded the MIC. Thus, although the presence of bacteria and bacterial matrix material undoubtedly retarded the penetration of the

antifungal drugs, poor penetration does not account for the drug resistance of *Candida* biofilm cells, even in these mixed-species biofilms (Al-Fattani & Douglas, 2004).

Subsequently, the same research group used a different model biofilm system to investigate further the role of the matrix in biofilm resistance to antifungal agents. Biofilms of *C. albicans* were grown under conditions of continuous flow in a modified Robbins device (Al-Fattani & Douglas, 2006). These biofilms produced more matrix material than those grown statically and were significantly more resistant to amphotericin B. Biofilms of *C. tropicalis* produced copious amounts of matrix material and were completely resistant to amphotericin B and fluconazole. Mixed-species biofilms containing *C. albicans* and a slimy strain of *S. epidermidis*, when grown statically or in the modified Robbins device, were completely resistant to both drugs. On the other hand, mixed-species biofilms of *C. albicans* and a slime-negative mutant of *S. epidermidis* were completely drug resistant only when grown under flow conditions (Al-Fattani & Douglas, 2006). Overall, these results suggest that the matrix might play a significant role in biofilm drug resistance, especially when biofilms are grown under flow conditions similar to those found in catheter infections *in vivo*.

4.7.2 Slow growth rate and nutrient limitation

Biofilm cells, especially those at the bottom of the biofilm, are expected to experience some form of nutrient limitation, which in turn slows their growth rate. A slow growth rate is often associated with phenotypic changes such as alteration in cell surface composition that may affect the susceptibility of the microorganisms to antimicrobial agents. Moreover, various studies have shown that antimicrobial agents are more effective in killing rapidly growing cells (Mah & O'Toole, 2001). Therefore, growth rate could be an important modulator of drug activity in biofilms (Donlan & Costerton, 2002; Mah & O'Toole, 2001). To examine the effect of growth rate with *C. albicans*, a perfused biofilm fermentor was used to produce biofilms at different growth rates. The susceptibility of the biofilm cells to amphotericin B was then compared with that of planktonic organisms grown at the same rate in a chemostat. The results showed that biofilm cells were resistant to

amphotericin B at all growth rates tested, while planktonic cells were resistant only at low growth rates (Baillie & Douglas, 1998a). Therefore, biofilm resistance depends on some other feature of the biofilm mode of growth and is not only the result of a low growth rate.

Subsequent studies demonstrated that drug resistance is associated with an increase in the metabolic activity of the developing biofilm (Chandra *et al.*, 2001a; Chandra *et al.*, 2001b) and that drug resistance develops over time, coincident with biofilm maturation. A separate investigation by Baillie and Douglas (1998b), using the cylindrical cellulose filter model system, reported that glucose-limited and iron-limited biofilms grown at the same low rate were equally resistant to amphotericin B. In the same study, daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms. An acute disseminated infection produced by the release of such cells from an implant biofilm might therefore respond rapidly to amphotericin B treatment but the biofilm would be unaffected.

4.7.3 Surface contact-induced gene expression

When cells attach to a surface and form a biofilm, they express an altered phenotype. There is a special interest in trying to identify the genes that are activated or repressed in biofilms as compared with planktonic cells, particularly genes that might contribute to increased resistance to antimicrobial agents. Antifungal resistance in planktonic cells of *C. albicans* has been associated with the expression of two different types of efflux pump, encoded by CDR and MDR genes, respectively. Recent studies have demonstrated that both CDR and MDR genes are upregulated during biofilm formation and development. However, mutant strains lacking one or both pumps were highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth (Ramage *et al.*, 2002a). A separate study investigated the antifungal susceptibilities of biofilms developed by *C. albicans Cdr1*/*Cdr2* and *Cdr1*/*Cdr2*/*Mdr1* double and triple mutants. Biofilms formed by these mutants were more susceptible to fluconazole at an early stage of biofilm development than the wild-type strain. At later time points (12 and 48 h), all the strains became resistant to fluconazole. These studies indicate that efflux pumps are differently expressed

during biofilm formation and that they contribute to azole resistance in the early phase of biofilm formation but not in the later phases (Mukherjee *et al.*, 2003).

4.7.4 Existence of "persister" cells

A recently proposed hypothesis for the resistance of bacterial biofilms is that a subpopulation of microorganisms in a biofilm differentiates into a unique and highly protected phenotypic state in the presence of a bactericidal agent, similar to spore formation (Spoering & Lewis, 2001; Stewart & Costerton, 2001). Persisters were first noted in planktonic cultures by Joseph Bigger (1944) in one of the first studies on the mechanism of penicillin action. The persister phenomenon has recently received increased attention in the context of biofilms where persisting cells have the added protection of an extracellular matrix (Spoering & Lewis, 2001).

Persisters, which might consist of 1% or less of the original population, remain dormant (neither grow nor die) despite continued exposure to an antimicrobial drug (Lewis, 2005). These cells can withstand drug concentrations substantially above the MIC and represent special survivor cells that are phenotypic variants of the wild type, rather than mutants (Keren *et al.*, 2004b; Lewis, 2000). The immune system is able to clear any planktonic persisters whereas in a biofilm, persisters are protected by the matrix against immune cells. Biofilm persisters are formed by all bacterial species studied, and are present at 0.1-1% in biofilms of *Pseudomonas aeruginosa, Escherichia coli*, or *Staphylococcus aureus*, for example (Keren *et al.*, 2004b). Bacteria produce multidrug-tolerant persister cells in both planktonic and biofilm populations (Brooun *et al.*, 2000; Keren *et al.*, 2004b; Spoering & Lewis, 2001). A biphasic pattern of killing is a defining feature demonstrating the presence of persisters in bacterial populations; the majority of the population is rapidly killed, whereas a small fraction of the cells are unaffected even by prolonged antibiotic treatment (Brooun *et al.*, 2000; Stewart, 2002).

The existence of such cells in *Candida* species has been reported recently. Certain cells at the base of a *C. albicans* biofilm were shown to have increased resistance to the antiseptic, chlorhexidine (Suci & Tyler, 2003). In addition, small subpopulations in biofilms of both *C. albicans* and *C. tropicalis*

were found to be 4 to 533 times more tolerant of high concentrations of metal chelating agents than the corresponding planktonic cell populations (Harrison *et al.*, 2007). Increased resistance to amphotericin B was also detected in a subpopulation of *C. albicans* biofilms which remained adhering to the surface of a tubular flow cell after most cells were washed away. These cells were resistant to amphotericin B at ten times the concentration that eliminated planktonic populations, and the high level of resistance appeared to correlate with differential regulation of the ergosterol and β-1,3 glucan genes ERG1, ERG25, SKN1, and KRE1 (Khot *et al.*, 2006).

A separate study by LaFleur and colleagues (2006) demonstrated that a subpopulation of cells within *C. albicans* biofilms exhibited multidrug tolerance. In this investigation, biofilms of *C. albicans* exhibited a biphasic killing pattern in response to two microbicidal agents, amphotericin B and chlorhexidine, indicating the presence of persisters. The extent of killing with a combination of amphotericin B and chlorhexidine was similar to that obtained with individually added antimicrobials, and it was thus concluded that surviving persisters form a multidrug-tolerant subpopulation. Unlike bacterial populations, surviving *C. albicans* persisters were detected only in biofilms, and not in planktonic, exponentially growing or stationary-phase populations. Reinoculation of cells that survived killing by amphotericin B produced a new biofilm with a new subpopulation of persisters, suggesting that *C. albicans* persisters are not mutants, but phenotypic variants of the wild type. Given that persisters were produced only in the biofilm, mutants defective in biofilm formation were examined for persister production. Interestingly, all biofilm-defective mutants treated with high concentrations of amphotericin B were able to produce normal levels of persisters. This result may indicate that attachment rather than formation of a complex biofilm architecture initiates persister formation.

Mammalian cells with serious damage may undergo programmed cell death or apoptosis. In this respect, it has been suggested that treatment of microorganisms with antimicrobial agents triggers a programmed suicide mechanism, resulting in death from apoptosis (Gilbert *et al.*, 2002; Lewis, 2005). Persisters may represent cells with defective programmed cell death rather than inherent resistance to the agent (Jabra-Rizk *et al.*, 2004), i.e.,

inhibition of programmed cell death allows the cells to exhibit tolerance to antimicrobial drugs (Lewis, 2005).

5 Apoptosis

Cell death is a completely normal process in living organisms and plays a major role in physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis (Lockshin & Zakeri, 2001). The term programmed cell death was first introduced in 1964, to describe a process whereby cell death during development is not accidental in nature but follows a sequence of controlled and regulated steps leading to locally and temporally defined self destruction (Lockshin & Williams, 1964). In 1972, Kerr and coworkers coined the term 'apoptosis' to describe the processes leading to controlled cellular self destruction. Apoptosis is involved in various biological processes such as development, differentiation, proliferation/homoeostasis, regulation and function of the immune system, and in the removal of defective and therefore harmful cells (Wyllie *et al.*, 1980). Defects in apoptosis can result in uncontrolled cell proliferation (such as in cancer), autoimmune diseases and spreading of viral infections, while excessive apoptosis can cause neurodegenerative disorders, AIDS, and ischemic heart diseases (Thompson, 1995).

5.1 Apoptosis in mammalian cells

Apoptosis is characterized by cell shrinkage (the cell shows deformation and loses contact with its neighbouring cells), chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and cell fragmentation into compact membrane-enclosed structures called 'apoptotic bodies'. The apoptotic bodies are engulfed by macrophages (in mammals) or by neighbouring cells, without causing an inflammatory response (Griffin & Hardwick, 1997). Apoptosis is quite different from the necrotic mode of cell death in which the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and rupture of the cells. During necrosis, the cellular contents are released in an uncontrolled way into the cell's environment and this results in damage to the surrounding cells, inflammatory responses and,

potentially, serious health problems (Lawen, 2003; Leist & Jaattela, 2001; Van Cruchten & Van Den Broeck, 2002) (Fig. 11).

Apoptosis can be triggered by a wide variety of stimuli from outside (extrinsic inducers) or inside (intrinsic inducers) the cell. Extrinsic signals may include toxins, hormones, growth factors, or cytokines (Cobb & Schaefer, 1997; Kirby, 2004) while intrinsic signalling can be initiated by a cell in response to stress such as DNA damage (e.g. by radiation or chemotherapeutic drugs) or starvation, as well as to oxidative stress (Kaufmann & Earnshaw, 2000; Wang, 2001). The key effectors that modulate apoptosis are cysteinyl aspartate-specific proteases (caspases). Strictly defined, cell death can only be classified to follow a classical apoptosis mode if execution of cell death is dependent on caspase activity (Leist & Jaattela, 2001). These caspases exist within the cell in an inactive form and they can be cleaved to form active enzymes following the induction of apoptosis. Once the initiator caspases have been activated, a sequential activation of caspases takes place. These enzymes cleave other caspases and non-caspase substrates such as proteins of the DNA repair system, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the characteristic features of apoptosis (Earnshaw, 1999). However, there are also potential negative regulators of apoptosis including Bcl-2 family proteins and inhibitor-of-apoptosis proteins (White, 1996).

5.2 Apoptosis in yeasts

Recent studies indicate that yeasts undergo programmed cell death for several good reasons. For example, the death of aged, infertile, damaged, or virus-infected yeast cells may ensure the survival of the rest of the population (Buttner *et al.*, 2006). A large number of exogenous stimuli have also been found to induce apoptosis-like cell death in yeasts (Madeo *et al.*, 2004). These include stress stimuli such as hydrogen peroxide, acetic acid, hyperosmotic stress, ultraviolet irradiation, mating pheromone exposure, amino acid starvation, aspirin, and some antifungal drugs (Almeida *et al.*, 2008; Frohlich & Madeo, 2000; Gourlay *et al.*, 2006; Hiramoto *et al.*, 2003; Madeo *et al.*, 1997; Silva *et al.*, 2005).

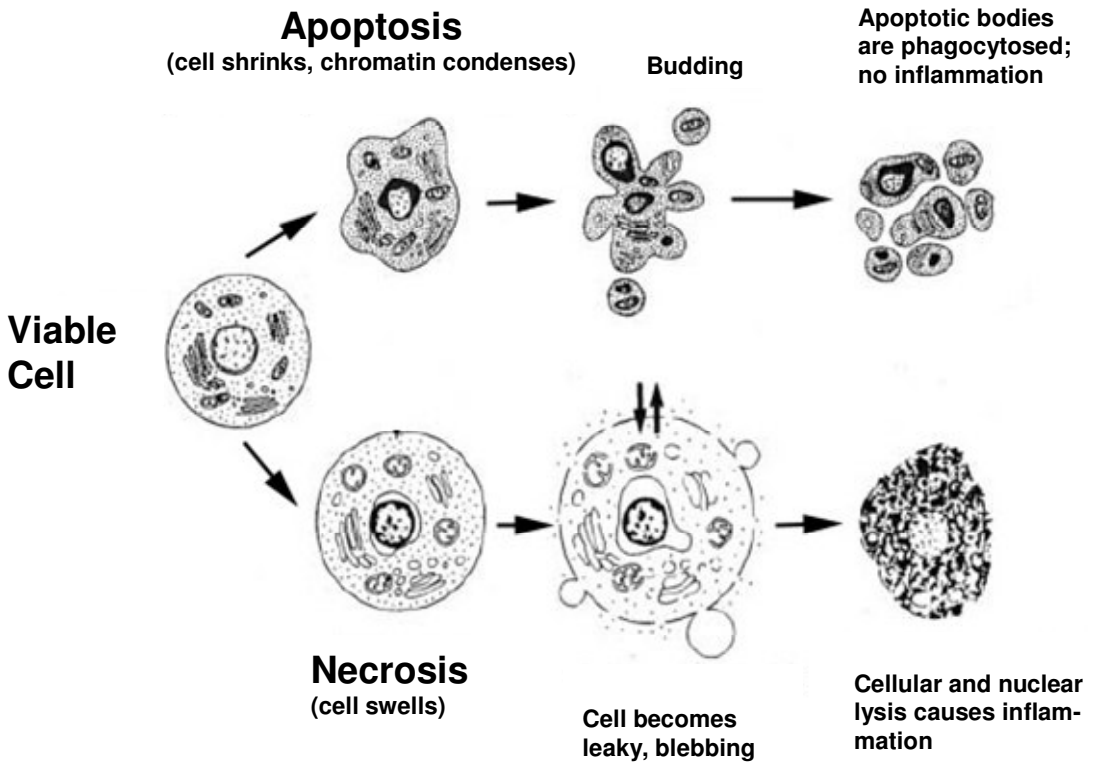


Figure 11. Hallmarks of the apoptotic and necrotic cell death process

Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies which contain organelles, cytosol and nuclear fragments. These bodies are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation. Reprinted from Van Cruchten & Van Den Broeck (2002) by permission of the publisher, John Wiley and Sons.

Phillips and colleagues (2003) reported that planktonically grown cells of *C. albicans,* when treated with low concentrations of amphotericin B or H_2O_2, exhibit cellular changes reminiscent of mammalian apoptosis. These include nuclear fragmentation, chromatin condensation, phosphatidylserine externalization, reactive oxygen species accumulation, and arrest in G2/M cell cycle phases. In *Saccharomyces cerevisiae*, one caspase-like protease (metacaspase Mca1p) has been identified that functions similarly to caspases in mammalian cells (Madeo *et al.*, 2002). Several studies have demonstrated that apoptosis in *S. cerevisiae* may be dependent on the activity of the metacaspase Mca1p (also known as MCA1 or YCA1) (Bettiga *et al.*, 2004; Khan *et al.*, 2005; Madeo *et al.*, 2002). However, in other studies, the apoptotic killing response was shown to be Mca1p-independent, indicating the presence of additional caspase-like activity in *S. cerevisiae* (Hardwick & Cheng, 2004; Hauptmann *et al.*, 2006; Herker *et al.*, 2004; Vachova & Palkova, 2005; Wissing *et al.*, 2004). More recently, Cao *et al.* (2009) have found that *C. albicans* contains a putative metacaspase CaMCA1 which shows homology to *S. cerevisiae* metacaspase. The deletion of CaMCA1 resulted in decreased caspase activity and in resistance to oxidative stress (Cao *et al.*, 2009).

Histone acetylation and deacetylation play critical roles in eukaryotic gene transcriptional regulation (Grunstein, 1997; Howe *et al.*, 1999). Acetylation is generally associated with activation, whereas lack of acetylation tends to correlate with repression; these two processes work together to achieve appropriate levels of transcription (Sterner & Berger, 2000). Histone deacetylases (HDAs) selectively regulate chromatin structure, which in turn affects the folding patterns and interactions between DNA and DNA-binding proteins (Grozinger & Schreiber, 2002; Mai *et al.*, 2007). To date, more than 50 non-histone proteins have been identified as substrates for one or another of the HDAs. These substrates include proteins with regulatory roles in cell proliferation, cell migration, and cell death (Dokmanovic *et al.*, 2007). HDA inhibitors such as valproic acid, trichostatin A, and butyric acid are known to induce apoptosis in mammalian cells (Kawagoe *et al.*, 2002). In fungi, histone deacetylases are also important regulators of many cellular functions and HDA inhibitors have been found to affect a number of processes in these organisms (Kurdistani & Grunstein, 2003; Mai *et al.*, 2007; Simonetti *et al.*, 2007; Smith & Edlind, 2002). Valproic acid induces *YCA1*-dependent apoptosis in *S. cerevisiae*

(Mitsui *et al.*, 2005). In *C. albicans*, HDA inhibitors have been reported to enhance sensitivity to azoles and other antifungal agents (Mai *et al.*, 2007; Smith & Edlind, 2002), to inhibit adhesion and serum-induced germ-tube formation (Noverr & Huffnagle, 2004; Simonetti *et al.*, 2007), and to promote colony-type switching (Klar *et al.*, 2001; Srikantha *et al.*, 2001).

6 Aims and Objectives of Research

Most hospital-acquired implant-based infections attributable to fungi are caused by *Candida albicans* and other closely related *Candida* species. These organisms are able to form adherent biofilms on the surfaces of catheters, joint replacements, prosthetic heart valves, and other medical devices. *Candida* biofilm-associated infections are resistant to a range of antifungal agents in current clinical use. As a result, antifungal therapy is often ineffective and removal of these devices is recommended. However, in the case of infected heart valves, central nervous system shunts and joint prostheses, removal may result in serious consequences for the patient. The basis of biofilm drug resistance is poorly understood. The aim of this project was to investigate possible resistance mechanisms, with particular emphasis on restricted drug penetration through the biofilm matrix and the existence of persisters in *Candida* biofilms. Research concentrated on two fungicidal agents: amphotericin B which has long been used for the treatment of systemic infections, and caspofungin, a newly licensed drug reported to show some activity against biofilms.

Specific objectives were the following:

1. To compare the susceptibility to amphotericin B and caspofungin of *Candida* biofilms at different developmental phases *in vitro*.
2. To determine the penetration of caspofungin through biofilms of different *Candida* species using a filter disc assay.
3. To investigate the presence of persister (drug-tolerant) cells in planktonic cultures and biofilms of *Candida* species by means of fluorescein staining and viability measurements.
4. To explore the existence of apoptosis in *Candida* biofilms and to determine the effect of apoptosis activators and inhibitors on drug resistance.

MATERIALS AND METHODS

1 *Candida* species

Six *Candida* isolates were used in this study. *C. albicans* GDH2346 was originally isolated from a patient with denture stomatitis at Glasgow Dental Hospital. *C. albicans* SC5314 was kindly provided by Professor Neil. A. R. Gow, University of Aberdeen, Aberdeen, Scotland. *C. glabrata* AAHB12, *C. tropicalis* AAHB73, and *C. parapsilosis* AAHB4479 were isolated from patients with line infections at Crosshouse Hospital, Kilmarnock, Scotland. *C. krusei* was obtained from a clinical specimen and came from the Regional Mycology Reference Laboratory, Glasgow, Scotland.

All isolates were maintained on slopes of Sabouraud dextrose agar (SDA; Difco) and stored at 2 to 8°C. Fresh slopes were prepared at 2-monthly intervals from long-term stocks held in 50% glycerol at -70°C.

2 Growth media

2.1 Sabouraud dextrose agar

Sabouraud dextrose agar (SDA; Oxoid; 65g/litre) was autoclaved for 15 min at 121°C. The final pH was 5.6 ± 0.2. After autoclaving and cooling to 50°C, the medium was dispensed in petri dishes or universal bottles for slope cultures. This medium was used to maintain *Candida* isolates.

2.2 Yeast nitrogen base

Yeast nitrogen base medium (YNB; Difco) was supplemented with 50 mM glucose as a carbon source. This medium was used as a standard liquid medium for growth of planktonic cells and biofilms. One litre of this medium contained of 6.7 g of yeast nitrogen base and 9 g of glucose (50 mM) and the final pH was 5.4. The medium was autoclaved at 10 p.s.i for 10 min.

2.3 YNB agar with 50mM or 200mM glucose

YNB containing 50 mM glucose was prepared as described earlier (section 2.2). Agar powder (12 g/litre; Duchefa) was added before autoclaving. The medium was dispensed into petri dishes. YNB agar with 50 mM glucose was used for drug penetration assays. YNB agar supplemented with 200 mM glucose

(36g/litre) was used for lawn production by the indicator strain during drug penetration assays and for viable cell counts.

2.4 YNB agar containing antifungal agents

Using a sterile filtration unit (Sartorius Minisart; pore size, 0.2 µm), the drug solution was filtered into culture medium (YNB agar containing 50 mM glucose) buffered with 0.165 M morpholinepropanesulfonic acid (MOPS; 34.53 g/litre; Sigma) to pH 7, and kept molten at 50°C. The medium was dispensed in petri dishes and used for drug penetration assays.

2.5 RPMI 1640 buffered with MOPS

RPMI 1640 liquid medium (with L-glutamine; Cambrex) was buffered with MOPS (34.53 g/litre; Sigma) to pH 7 at 25°C (National Committee for Clinical Laboratory Standards, 1995). The medium was then filter sterilized using a sterile filtration unit (Sartorius Minisart; pore size, 0.2 µm) and stored at 4°C. This medium was used for MIC determinations.

2.6 RPMI 1640 buffered with HEPES

RPMI 1640 liquid medium (with L-glutamine; Cambrex) was buffered with HEPES [4-(2-Hydroxyethyl) piperazine-1-ethanesulforic acid, sodium salt; 16.4 g/litre] to pH 7 at 25°C (National Committee for Clinical Laboratory Standards, 1995). The medium was then filter sterilized using a sterile filtration unit (Sartorius Minisart; pore size, 0.2 µm) and stored at 4°C. This medium was used for killing curve assays.

3 Chemicals

3.1 Antifungal agents

3.1.1 Fluconazole

Stock solutions of fluconazole (800 µg/ml; Sigma) were prepared in sterile water and filter sterilized. Small volumes of the solution were dispensed into sterile vials and stored at -20°C.

3.1.2 Amphotericin B

Stock solutions of amphotericin B (8000 µg/ml or 40 mg/ml; Sigma) were dissolved in dimethyl sulphoxide (DMSO) and filter sterilized. Small volumes of the solution were dispensed into sterile vials and stored at -20°C. Amphotericin B is light sensitive and therefore aliquots were protected in foil.

3.1.3 Caspofungin

Stock solutions of caspofungin (800 µg/ml; Merck) were prepared in sterile water and filter sterilized. Small volumes of the solution were dispensed into sterile vials and stored at -20°C.

3.2 Tetrazolium salt XTT

The tetrazolium salt, XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] was used as an indicator of cell viability. XTT is reduced by mitochondrial dehydrogenases to a brown-coloured tetrazolium formazan product. The dye formed is water soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The intensity of the dye is proportional to the number of metabolically active cells.

3.3 Live-dead cell staining

3.3.1 Fluorescein diacetate

Fluorescein diacetate (3,6-Diacetoxyfluoran; Di-*O*-acetylfluorescein; Sigma) is a fluorescent dye used to distinguish dead yeast cells from living cells. This dye specifically stains dead yeast cells with a green fluorescence.

3.4 Apoptosis inhibitors (Caspase inhibitors)

3.4.1 Z-VAD-FMK

Stock solutions (14.7 mM) of the general caspase inhibitor, Z-VAD-FMK (Calbiochem) were prepared in DMSO and stored in small aliquots at -20°C.

3.4.2 Caspase inhibitor set III

Caspase Inhibitor Set III was supplied by the manufacturer (Calbiochem) as 2 mM solutions in DMSO and consisted of: caspase-1 inhibitor VI (Z-YVAD-FMK); caspase-2 inhibitor I (Z-VDVAD-FMK); caspase-3 inhibitor II (Z-DEVD-FMK); caspase-5 inhibitor I (Z-WEHD-FMK); caspase-6 inhibitor I (Z-VEID-FMK); caspase-8 inhibitor II (Z-IETD-FMK); and caspase-9 inhibitor I (Z-LEHD-FMK). Caspase Inhibitors were stored at -20°C.

3.5 Apoptosis inducers (Histone deacetylase inhibitors)

3.5.1 Sodium butyrate

Stock solutions of sodium butyrate (1 M; Sigma) were prepared in sterile water. Small volumes of the solution were dispensed into sterile vials and stored at -20°C.

3.5.2 Sodium valproate

Stock solutions of sodium valproate (1 mg/ml; Sigma) were prepared in sterile water. Small volumes of the solution were dispensed into sterile vials and stored at -20°C.

3.5.3 Trichostatin A

Stock solutions of trichostatin A (10 mg/ml; Calbiochem) were prepared in DMSO and stored in small aliquots at -20°C.

3.5.4 Apicidin

Stock solutions of apicidin (10 mg/ml; Calbiochem) were prepared in DMSO and stored in small aliquots at -20°C.

3.6 Apoptosis detection kits

3.6.1 SR-FLICA

An SR-FLICA (Sulforhodamine-Fluorescent Labelled Inhibitors of Caspases) apoptosis detection assay (Immunochemistry Technologies) was used to detect apoptotic cells. The SR-VAD-FMK FLICA reagent comprises 3

segments: a red fluorescent label (Sulforhodamine; SR); an amino acid peptide inhibitor sequence targeted by all active caspases (VAD); and a fluoromethylketone group (FMK) which acts as a leaving group and helps form a covalent bond with the active caspase enzyme. SR-VAD-FMK irreversibly binds to many activated caspases (caspase-1, -3, -4, -5, -6, -7, -8, and -9). The FLICA reagent is supplied as a highly concentrated lyophilized powder. It is reconstituted in 50 µl DMSO forming a 150-fold stock concentrate and then diluted 1 in 5 in PBS to form a final 30-fold concentrated working solution. For best results, the working solution is used the same day that it is prepared; however, the stock concentrate can be stored at -20ºC for 6 months.

3.6.2 CaspSCREEN apoptosis detection

A CaspSCREEN^{TM} kit (Biovision Research Products, CA) was also used to detect caspase activity. The assay is based on the cleavage of $(aspartyl)_2$-Rhodamine 110 (D_2R), a reported substrate for members of the caspase family of proteases. The caspase substrate D_2R is non-fluorescent; however, upon cleavage by cellular activated caspases, the released rhodamine 110 gives rise to a green fluorescence that can be measured by flow cytometry or by using a fluorescence microscope.

3.7 Pepstatin A

Pepstatin A (Sigma) was dissolved at a concentration of 1 mg/ml in methanol containing 10 % (v/v) acetic acid. Small volumes of the solution were dispensed in sterile vials and stored at -20°C.

4 Antifungal susceptibility of *Candida* planktonic cells

4.1 Inoculum preparation

Using the NCCLS method (National Committee for Clinical Laboratory Standards, 1995), five colonies of $\geq$1 mm diameter from 24 h cultures grown on SDA plates at 37 °C were suspended in 5 ml of sterile saline (0.85% w/v). The resulting suspension was vortexed for 15 s and the cell density was adjusted to a reading of 0.5 at 530 nm using a spectrophotometer. This procedure yielded a *Candida* stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml. A working

suspension was made via a 1:50 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 medium to obtain a concentration double that of the test inoculum (1 x 10^3 to 5 x 10^3 cells/ml).

4.2 Broth microdilution

The broth microdilution method was performed according to the NCCLS guidelines (National Committee for Clinical Laboratory Standards, 1995).

4.2.1 Minimum inhibitory concentration (MIC)

The MIC assay was performed in U-shaped wells of sterile 96-well plates (Costar; Corning Inc, USA). Serial dilutions of antifungal agents (amphotericin B, caspofungin) were prepared at double the final concentration in RPMI 1640 medium buffered with 0.165 M MOPS (pH 7); the dilutions ranged from 0.06 to 16 µg/ml. The *Candida* inoculum was prepared as described previously (Section 4.1). Antifungal solutions (100 µl) were dispensed in the wells of a microtitre plate. The inoculum suspension (100 µl) was then added to each well of the microtitre plate. This results in a 1:2 dilution of both the antifungal agent and the inoculum. Control wells contained inoculum suspension and medium without drug. The plates were incubated at 37˚C for 48 h. The MIC end point for the tested drugs was defined as the lowest concentration resulting in no visible growth in the wells (100% inhibition).

4.2.2 Minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) of amphotericin B for *C. albicans* GDH2346 was determined following the method described previously (Section 4.2.1). After the MIC was read, 100 µl samples from each well at or above the MIC were transferred to duplicate SDA agar plates (90 mm diameter) and spread evenly. The plates were incubated at 37˚C and read after 48 h. The minimum fungicidal concentration (MFC) was defined as the lowest drug concentration that caused total killing of cells.

4.3 Killing curves

To further investigate the fungicidal activity of amphotericin B against *C. albicans* GDH2346, three to five colonies of *C. albicans* GDH2346 were picked from a 48-h plate culture and suspended in 10 ml of sterile distilled

water; the optical density was adjusted to 0.5 at 530 nm. One ml of this inoculum was added to either 9 ml of RPMI 1640 medium buffered with HEPES alone (control) or to 9 ml of the same buffered medium containing amphotericin B at final concentrations of one-half, one, two and four times the MIC. Cultures were then placed on a shaker and incubated at 37˚C. At time points 0, 2, 6 and 24 h following the inoculation of *C. albicans* into the solutions, a 100 µl aliquot from each test solution was removed. After 10-fold serial dilutions in RPMI, 50 µl aliquots from each dilution were spread on duplicate SDA plates. Plates were incubated at 37˚C. The total number of colony forming units (CFU) on each plate was determined after 48 h (Barchiesi *et al.*, 2005). The results were used to construct killing curves of cell survival against time for each concentration of amphotericin B tested.

5 Biofilm formation on catheter discs

5.1 Catheter discs

Discs (surface area, 0.5 cm^2; diameter, 0.8 cm) were cut from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, UK) using a metal punch (Fig. 12). The discs were sterilized by exposure to ultraviolet radiation (254nm; UVP Inc., USA) for 20 min on both sides.

5.2 Biofilm inoculum

Candida isolates were grown in YNB medium containing 50 mM glucose. Batches of medium (50 ml in 250-ml Erlenmeyer flasks) were inoculated from slopes and incubated for 24 h at 37˚C in an orbital shaker at 60 rpm. Aliquots of culture (10ml) were transferred to universal bottles and centrifuged at 3000 rpm for 4 min. Cell pellets were washed twice in 0.15 M phosphate-buffered saline (PBS; pH 7.2; Sigma) and resuspended in the same buffer. Cell suspensions were standardized to an optical density of 0.8 at 520 nm.

5.3 Biofilm formation

Catheter discs (Section 5.1) were transferred aseptically into wells of 24-well Costar tissue culture plates, with concave side facing up. A standardized cell suspension (80 µl) was applied to the surface of each disc. The cells were

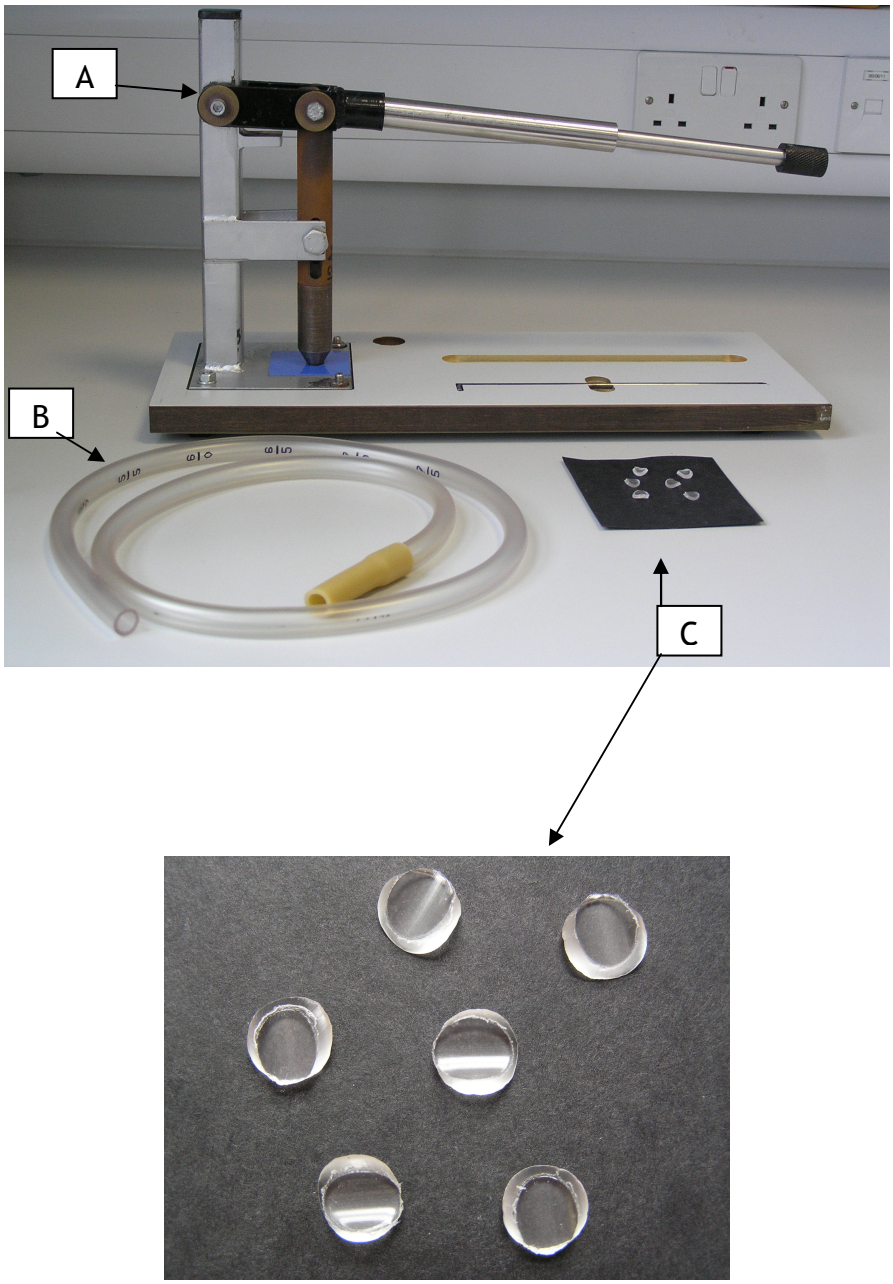


Figure 12. Metal punch device and biofilm discs.

Metal punch (A) used to cut polyvinyl chloride (PVC) discs (diameter, 0.8 cm) (C) from PVC Faucher tubes (B) for biofilm formation.

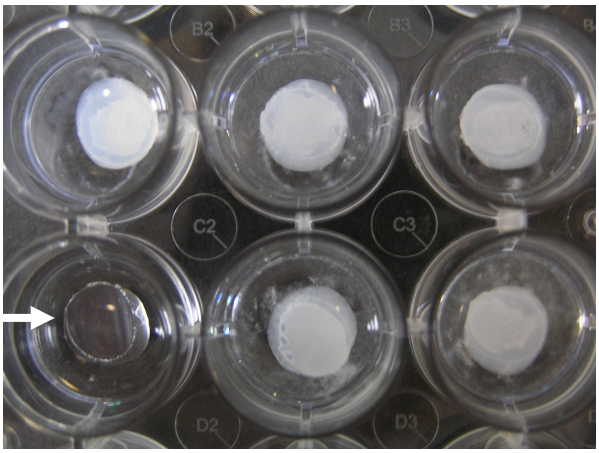
allowed to adhere to the discs for 1 h at 37°C. Nonadherent cells were removed by gentle washing with PBS (5 ml). Discs were then submerged into 1 ml YNB medium containing 50 mM glucose in wells of fresh tissue culture plates. Plates were incubated for 48 h at 37°C (Fig. 13).

5.4 Quantitative measurement of biofilm growth

Biofilm metabolic activity was measured colorimetrically using a tetrazolium salt (XTT) reduction assay. This salt is reduced by mitochondrial dehydrogenases to a brown-coloured tetrazolium formazan product, which is then determined spectrophotometrically. However, addition of the electron coupling agent menadione (Vitamin K3; 2-methyl-1,4-naphthoquinone; Sigma) is necessary. XTT solution (250 μg/ml; Sigma) was dissolved in sterile, pre-warmed PBS containing 1% (w/v) glucose and 1 ml was added to each well of fresh plates containing the biofilm discs. Menadione solution (1 mM in acetone; 4 μl) was also added to the wells to give a final concentration of 4 μM. The biofilms were then incubated for 5 h at 37°C in the dark. The solution containing XTT formazan in each well was transferred to a microfuge tube and clarified at 13000 rpm for 3 min. The supernatant was then transferred to a microcuvette for measuring formazan production at 492 nm (Fig. 14).

5.5 Viable counts of biofilm cells

This method was used to measure the number of viable cells in biofilms. Biofilm cells were harvested from the discs by scraping and vigorous vortexing, washed twice in 0.15 M PBS, pH 7.2, and resuspended in more PBS. Ten-fold serial dilutions (10^{-1} to 10^{-6}) of biofilm cell suspensions were prepared in PBS. Duplicate samples (0.1 ml) of the 10^{-5} and 10^{-6} dilutions were then spread on YNB agar containing 200 mM glucose and the plates were incubated for 24 – 48 h at 37°C. The total number of CFU was counted and this number multiplied by the dilution factor to determine the concentration of cells per ml of original sample.



Negative control

Figure 13. Costar tissue culture plate containing mature 48-h old biofilms grown on polyvinyl chloride catheter discs.

Biofilm discs were submerged in 1 ml YNB growth medium with 50 mM glucose.

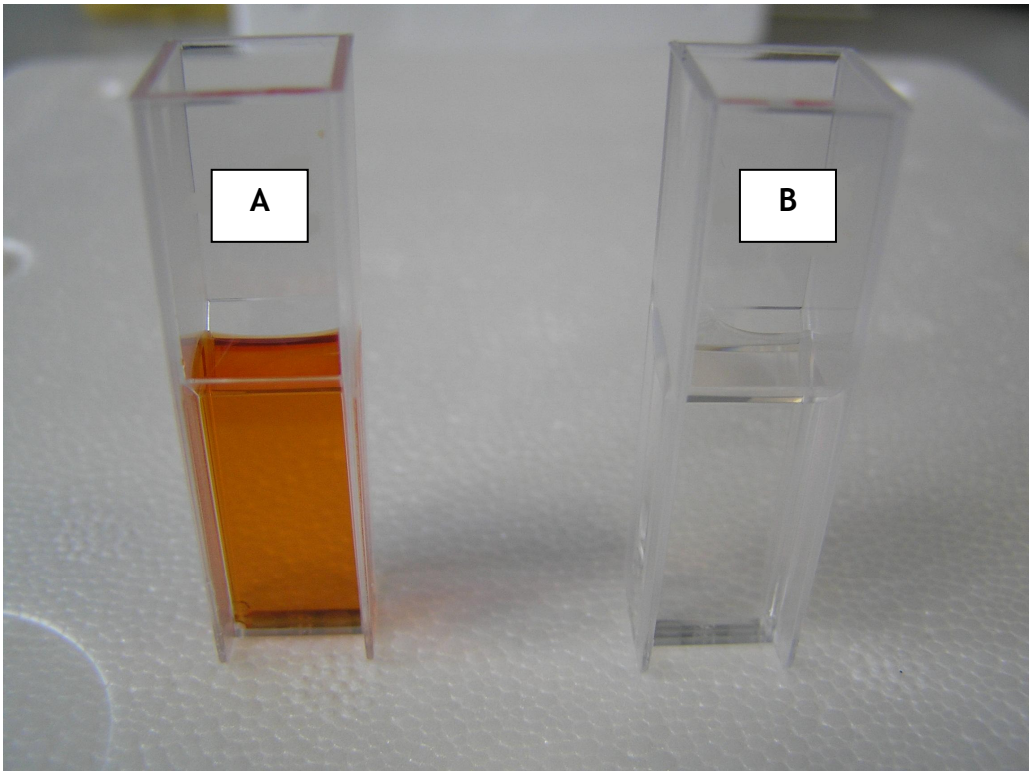


Figure 14. Tetrazolium salt (XTT) reduction assay used to measure biofilm cell viability.

Metabolically active cells convert XTT to a brown-coloured formazan product. (A) Positive sample, after incubation for 5 h at 37ºC; (B) Negative control sample, colourless.

6 Antifungal susceptibility of *Candida* biofilms

6.1 Susceptibility of biofilms on catheter discs

Stock solutions of the drugs were diluted in growth medium (YNB containing 50 mM glucose) buffered to pH 7 with MOPS buffer. Biofilms were grown on discs for 48 h at 37°C or for specific time periods and then transferred into wells containing 1 ml of buffered medium with defined concentrations of the test antifungal agents. Control discs were submerged in buffered medium free of drug. Discs were further incubated for 24 h at 37°C and the effect of the antifungal agent was measured by either the XTT reduction assay (Section 5.4) or by a viable cell count (Section 5.5).

7 Antifungal activity at different developmental phases of *Candida* biofilms

Biofilms were grown on catheter discs for 8, 17, 24 or 35 h and then submerged into 1 ml of buffered YNB glucose (50 mM) medium containing different concentrations (2 times or 5 times the MIC) of antifungal agent. These were 2.6 and 6.5 μg/ml of amphotericin B for *C. albicans* GDH2346; 1.6 and 4 μg/ml of amphotericin B for *C. albicans* SC5314; 4.6 and 11.5 μg/ml of amphotericin B for *C. tropicalis;* 2.4 and 6 μg/ml of amphotericin B for *C. glabrata;* 4.4 and 11 μg/ml of amphotericin B for *C. parapsilosis;* 0.8 and 2 μg/ml of caspofungin for *C. albicans* GDH2346; 0.6 and 1.5 μg/ml of caspofungin for *C. albicans* SC5314; 0.9 and 2.25 μg/ml of caspofungin for *C. tropicalis*; 1.4 and 3.5 μg/ml of caspofungin for *C. glabrata;* 1.6 and 4 μg/ml of caspofungin for *C. parapsilosis*. Control discs were submerged in medium free of drug. Discs were further incubated for a total of 48 h at 37°C and metabolic activities of biofilm cells were measured using the XTT assay (Section 5.4).

8 Penetration of antifungal agents through *Candida* biofilms

This penetration assay was a modification of the filter disc method for bacterial biofilms as previously described by Anderl *et al.* (Anderl *et al.*, 2000). One antifungal agent was used in this study: caspofungin. However for comparative purposes, some experiments (preparation of a standard curve for drug penetration and viability measurements) were carried out with amphotericin B.

8.1 Biofilm formation on membrane filters

The biofilm inoculum was prepared as described in section 5.2 except that the cell suspension was adjusted to an optical density of 0.2 at 600 nm. Biofilms were grown on polycarbonate membrane filters (diameter, 25 mm; pore size, 0.2 µm; Whatman) which were sterilized by exposure to ultraviolet radiation for 15 min on both sides prior to inoculation. The filters were placed aseptically on plates of YNB agar containing 50 mM glucose (Section 2.3). A standardized cell suspension (50 µl) was carefully deposited on the surface of each sterile membrane. All agar plates were incubated for 24 h at 37°C. The membrane-supported biofilms were then transferred to fresh YNB agar plates and reincubated for a further 24 h, giving a total incubation time of 48 h for biofilm formation.

8.2 Drug penetration through biofilms

A drug concentration of 60 times the MIC for *Candida albicans* GDH2346 was used in antifungal agent-supplemented YNB agar (Section 2.4), i.e., 24 µg/ml for caspofungin. After 48 h of biofilm formation on membrane filters (section 8.1), smaller polycarbonate membrane filters (diameter, 13 mm; pore size, 0.2 µm; Whatman) were sterilized by exposure to ultraviolet radiation for 15 min on both sides and then carefully placed on top of the 48-h biofilms. Paper concentration discs (diameter, 6 mm; Becton Dickinson) were also sterilized by exposure to ultraviolet radiation for 15 min on both sides and then moistened with 30 µl of growth medium prior to placement on top of the 13-mm-diameter membranes. Wetting the discs helped to prevent the capillary action of the antifungal medium through the biofilms. The whole assembly –

the biofilm 'sandwich' - was transferred to an antifungal agent-containing agar plate using sterile forceps (Fig. 15). All plates were incubated for specific time periods, i.e., 60, 90, 120, 180, 240, or 360 min at 37°C.

The amount of antifungal agent that diffused into a concentration disc through the biofilm was determined by using the disc in a standard drug diffusion assay. Plates of YNB agar containing 200 mM glucose were seeded with 150 µl of a standardized suspension of planktonic *C. albicans* GDH 2346 which was used as an indicator organism. The suspension was adjusted to an optical density of 1.0 at 520 nm prior to plating. The concentration discs were removed from the biofilm 'sandwiches' after the specified exposure time and placed on the seeded plates, which were then incubated for 24 h at 37°C. The zones of growth inhibition were measured and used to determine the concentration of active antifungal agent in the discs by reference to a standard curve prepared by using drug solutions of different concentrations but fixed volumes (Section 8.3). The assay was conducted in duplicate on two separate occasions for each *Candida* isolate tested. The control assays used for the experiment were concentration discs placed on the two-membrane system without the biofilm. To provide a normalized penetration curve, the drug concentration that diffuse through the biofilm (C) was divided by the drug concentration determined for the control (C_0).

8.3 Preparation of drug standard curves

Drug standard curves were prepared by using drug solutions of different concentrations but fixed volumes. The standard curve was constructed by plotting the log of the drug concentration used versus the diameter of the zone of growth inhibition.

Overnight planktonic cultures of *C. albicans* GDH2346 (used here as an indicator organism) were harvested by centrifugation at 3000 rpm for 5 min, washed twice in sterile PBS, and then adjusted to an optical density of 1.0 at 520 nm in PBS. Plates of YNB agar containing 200 mM glucose were seeded with 150 µl of this standardized suspension. Concentration discs were moistened with a fixed volume (30 µl) of drug solution before being transferred to the pre-seeded plates. Plates were then incubated for 24 h at 37ºC. The zones of growth inhibition were measured (in mm) at four points around each disc and

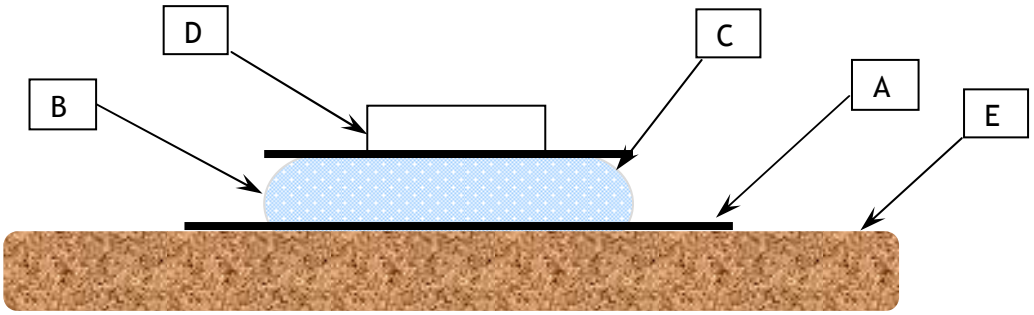


Figure 15. The experimental system used to determine the penetration of antifungal agents through biofilms.

The biofilm (B) is initially formed on a 25 mm-diameter membrane filter (A) resting on glucose YNB agar (not shown). A second, smaller filter (C) is placed on top of the biofilm, and a moistened concentration disc (D) is positioned on top of the second filter. After 48 h of biofilm formation, the entire assembly is transferred to antifungal-containing agar (E). All plates are incubated at 37ºC for time periods ranging from 60 to 360 min.

the mean value calculated. These values were plotted against the drug concentration used, to produce a standard curve.

8.3.1 Caspofungin

A standard curve of caspofungin concentration against zone of growth inhibition (distance from edge of disc, mm) was prepared as described in section 8.3 with the following drug concentrations: 4, 8, 10, 20, 25, and 30 µg/ml (Table 2 and Fig. 16).

8.3.2 Amphotericin B

A standard curve of amphotericin B concentration against zone of growth inhibition (distance from edge of disc, mm) was prepared as described in section 8.3 with the following drug concentrations: 16, 20, 30, 40, 50, and 60 µg/ml (Table 3 and Fig. 17).

8.4 Drug susceptibility of biofilms on membrane filters

After biofilm formation on 25-mm-diameter membrane filters, biofilms were capped with sterile 13-mm-diameter filters, transferred to antifungal agent-containing agar, and incubated for 6 h at 37°C (the maximum exposure period in drug penetration assays) or 24 h. A drug concentration of 60 times the MIC for *Candida albicans* GDH2346 was used in the antifungal agent-supplemented agar. The concentrations used were as follows: caspofungin, 24 µg/ml and amphotericin B, 78 µg/ml. After incubation for the specified exposure time, biofilm cells were gently scraped from the membranes with a sterile scalpel and resuspended in 10 ml of PBS. Viable cell counts were carried out as described in Section 5.5.

Table 2. Zone of growth inhibition due to caspofungin on plates seeded with *C. albicans* GDH2346 [a]

	Zone of growth inhibition (distance from edge of disc, mm) produced by caspofungin at a concentration (µg/ml) of						
Disc [b]	**2**	**4**	**8**	**10**	**20**	**25**	**30 [c]**
1	0.0	0.88	2.00	2.60	3.13	3.94	4.21
2	0.0	1.00	2.03	2.44	3.86	3.70	4.00
3	0.0	1.20	1.79	2.23	3.00	3.23	3.64
4	0.0	0.95	2.30	2.37	3.62	3.15	3.89
Total	0.0	4.03	8.12	9.64	13.61	14.02	15.74
Mean [d]	0.0	1.01	2.03	2.41	3.40	3.51	3.94

[a] Growth medium (YNB supplemented with 200 mM glucose) was seeded with 150µl of a standard suspension of *C. albicans*.

[b] Blank paper concentration disc (4 for each drug concentration) were moistened with a fixed volume (30 µl) of drug solution.

[c] Different concentrations (µg/ml) of caspofungin were used to draw the standard curve.

[d] Data are means from two independent experiments done in duplicate.

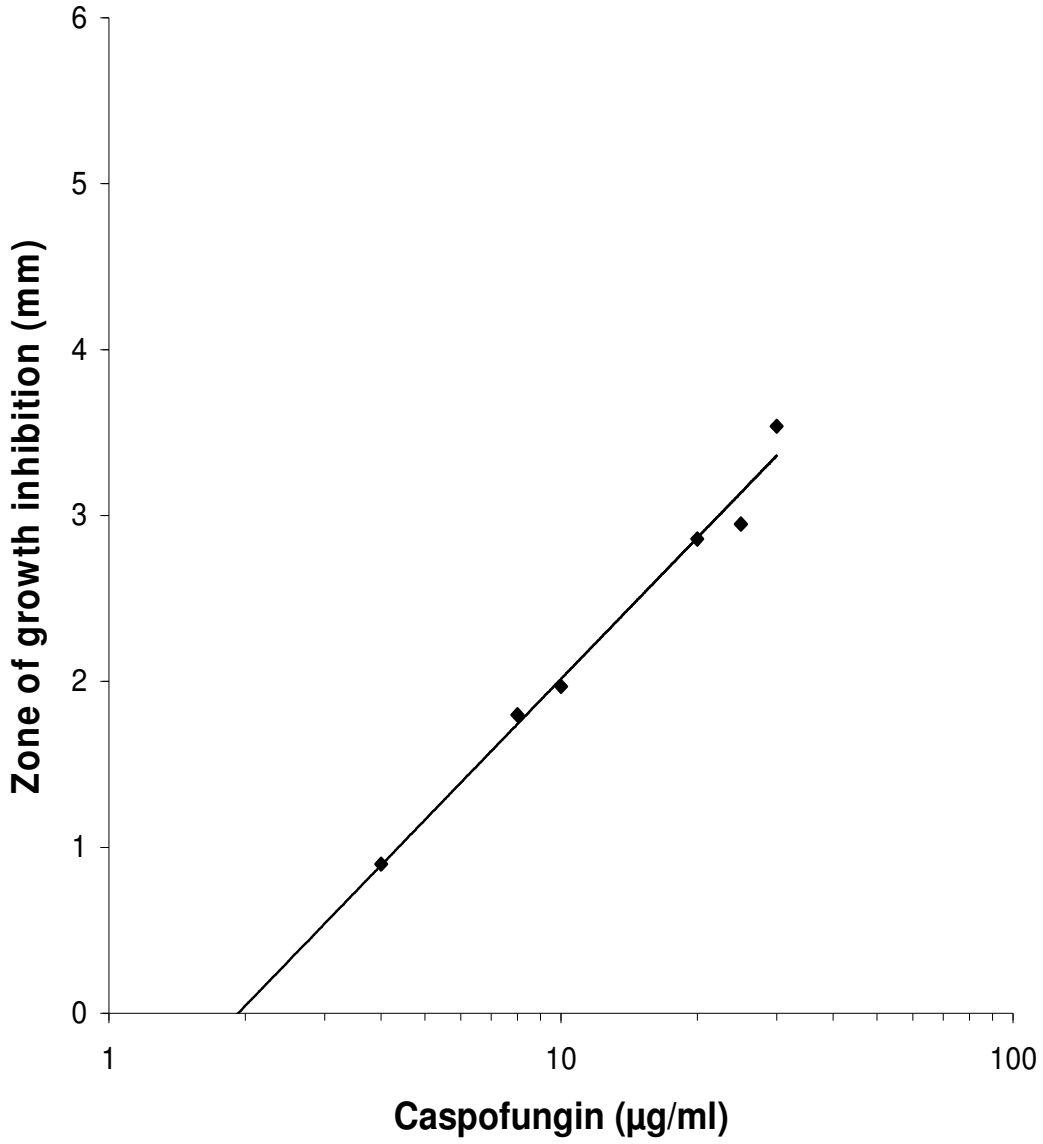


Figure 16. Standard curve for caspofungin in drug penetration assay

Table 3. Zone of growth inhibition due to amphotericin B on plates seeded with *C. albicans* GDH2346 [a]

	Zone of growth inhibition (distance from edge of disc, mm) produced by amphotericin B at a concentration (µg/ml) of						
Disc [b]	**10**	**16**	**20**	**30**	**40**	**50**	**60 [c]**
1	0.0	1.03	1.56	4.00	4.35	4.70	4.78
2	0.0	1.25	1.95	3.64	4.33	4.10	5.00
3	0.0	0.81	1.63	3.47	4.15	4.34	5.11
4	0.0	0.97	1.52	3.89	3.68	4.14	4.40
Total	0.0	4.06	6.66	15.00	16.51	17.28	19.29
Mean [d]	0.0	1.02	1.67	3.75	4.13	4.32	4.82

[a] Growth medium (YNB supplemented with 200 mM glucose) was seeded with 150µl of a standard suspension of *C. albicans*.

[b] Blank paper concentration disc (4 for each drug concentration) were moistened with a fixed volume (30 µl) of drug solution.

[c] Different concentrations (µg/ml) of amphotericin B were used to draw the standard curve.

[d] Data are means from two independent experiments done in duplicate.

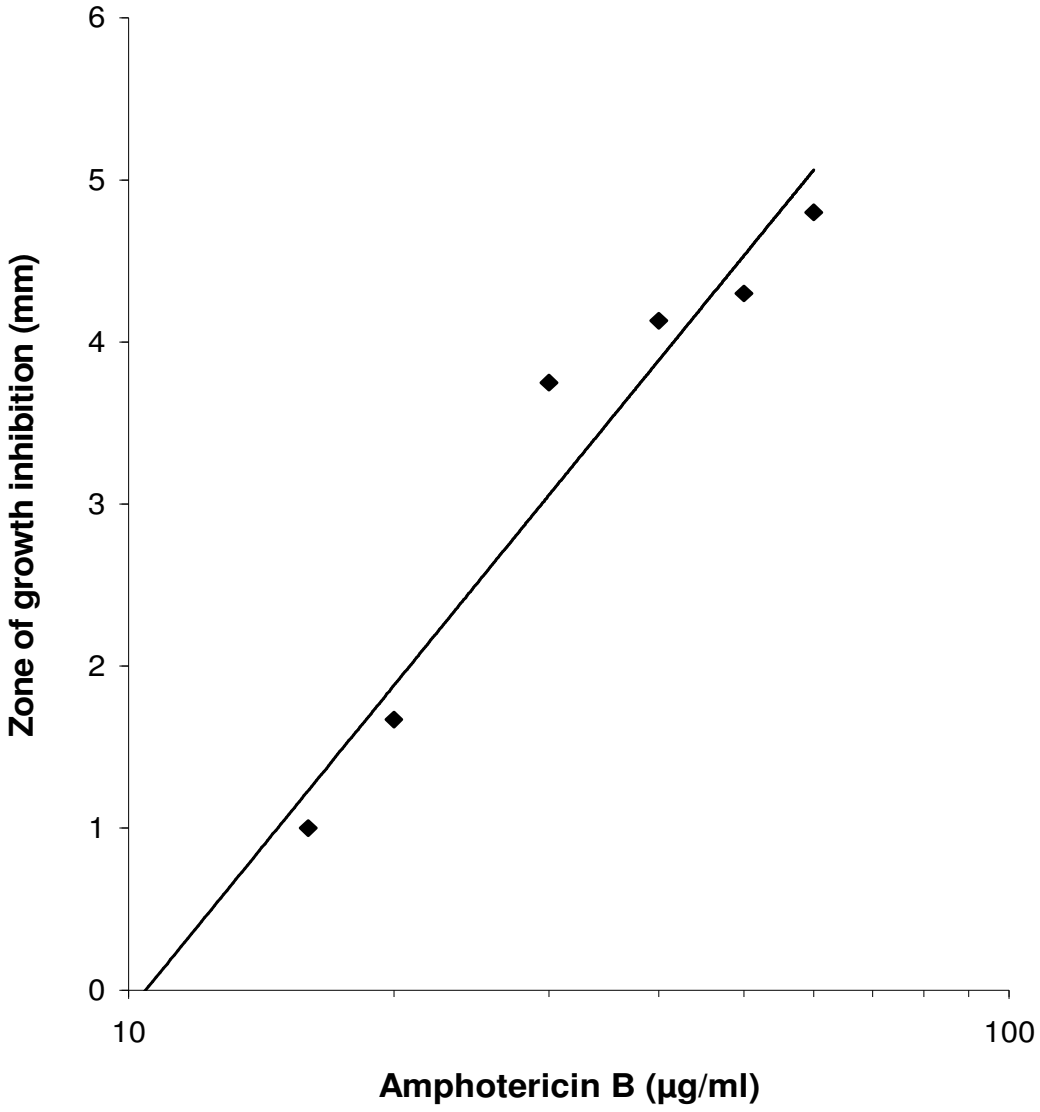


Figure 17. Standard curve for amphotericin B in drug penetration assay

9 Scanning electron microscopy of biofilms

9.1 Standard SEM air-drying procedure

Biofilms formed on polycarbonate membranes were fixed with 2.5% (v/v) glutaraldehyde in PBS (pH 7.2) for 1 h at room temperature. The biofilms were then treated with 1% (w/v) osmium tetroxide (Sigma) for 1 h, washed three times in 3 ml of distilled water for 10 min, treated with 1% (w/v) uranyl acetate for 1 h, and finally washed twice in 3 ml of distilled water . Biofilms were dehydrated in a series of ethanol solutions (30%, 50%, 70%, 90% absolute ethanol, and dried absolute ethanol) for 10 minutes each. All samples were air dried in a desiccator overnight then mounted on aluminium stubs before being gold coated with a polaron coater, and viewed under a Philips 500 scanning electron microscope.

9.2 Cationic dye procedure

To improve the preservation and visualization of matrix material in biofilms, a procedure involving cationic dyes (Erlandsen *et al.*, 2004b) was used. Biofilms formed on polycarbonate membrane filters were fixed overnight at room temperature in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4, and containing 0.15% alcian blue or 0.15% ruthenium red (cationic dyes). After primary fixation in the aldehyde mixture with cationic dye additives, the samples were rinsed in 0.15 M cacodylate buffer twice (5 min each) and immersed in 1% (w/v) osmium tetroxide in 0.15 M cacodylate buffer containing 1.5% (w/v) potassium ferrocyanide for 90–120 min. They were then washed five times with distilled water. Dehydration of the samples was achieved by immersion in an ascending ethanol series ranging, in 20% increments (30%, 50%, 70%, and 90% absolute ethanol) , followed by dried absolute ethanol, for 10 minutes each. The last step of dehydration was using 100% hexamethyldisilizane (Sigma) twice for 5 min each time. All samples were air dried in a desiccator overnight then mounted on aluminium stubs before being gold coated with a polaron coater, and viewed under a Philips 500 scanning electron microscope.

10 Persister cells in *Candida* species

10.1 Persister cells in planktonic cultures

All organisms were grown at 37°C with shaking in YNB medium containing 50 mM glucose (50 ml in 250-ml flasks). Exponential-phase cultures were incubated for 8 h (*C. krusei*, *C. albicans*, and *C. tropicalis*), 11 h (*C. parapsilosis*), or 18 h (*C. glabrata*). Stationary-phase cultures of all species were incubated for 48 h. Cells from samples (100 µl) of exponential- or stationary-phase cultures were harvested and washed twice in PBS. Washed cells were treated in microtitre plates with different concentrations of amphotericin B (5 to 100 µg/ml) in YNB glucose medium buffered to pH 7 with 0.165 M MOPS. Control cells were treated similarly with buffered medium without amphotericin B. All cell suspensions were adjusted to a concentration (approximately 10^7 cells/ml) equivalent to that of resuspended biofilms. After incubation at 37°C for 24 h, the cells were washed twice and resuspended in PBS (100 µl). Viable counts were then carried out by serial dilution and plating on YNB agar containing 200 mM glucose (Section 5.5). Assays were carried out in duplicate and done at least twice on different days.

10.2 Persister cells in biofilms

Organisms were grown in YNB medium containing 50 mM glucose and washed cell suspensions were adjusted to an optical density of 0.8 at 520 nm (Section 5.2). Biofilms were formed on PVC discs as described previously (Section 5.3). The biofilms were then treated with amphotericin B at concentrations ranging from 5 to 100 µg/ml. Mature (48-h) biofilms were transferred to fresh wells, submerged in YNB glucose medium (1 ml) containing different concentrations of amphotericin B and buffered to pH 7 with 0.165 M MOPS, and incubated at 37°C for 24 h. Control biofilms were incubated in buffered medium in the absence of amphotericin B. After incubation, biofilm cells were harvested from the discs by scraping and vigorous vortexing, washed twice in 0.15 M PBS, pH 7.2, and resuspended in more PBS (100 µl). Viable counts were then determined by the standard procedure of serial dilution followed by plating on YNB agar containing 200 mM glucose (Section 5.5). Assays were carried out in duplicate and done at least twice on different days.

10.3 Live-dead staining with fluorescein

Biofilms were grown on PVC catheter discs in YNB containing 50 mM glucose for 48 h at 37°C. Biofilm discs were then transferred into 1 ml of buffered YNB glucose (50 mM) medium plus 100 µg/ml fluorescein diacetate alone (control) or with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) and incubated for 24 h at 37°C. The biofilm cells were then scraped from the discs, vigorously vortexed, and washed three times in PBS. Cell pellets were resuspended in 300 µl PBS and viewed under a x100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1; Fig. 18). Fluorescein diacetate stains dead yeast cells with a green fluorescence while live cells remain unstained (LaFleur *et al.*, 2006).

11 Apoptosis in *Candida* biofilms

11.1 SR-FLICA apoptosis detection assay

An SR-FLICA kit was used to detect active caspase enzymes within biofilm cells. The SR-FLICA reagent was reconstituted in 50 µl DMSO, as recommended by the manufacturers, to form a stock concentrate. The concentrate was further diluted with 200 µl PBS to produce the working solution. Mature (48-h) biofilms of *C. albicans* strains GDH2346 and SC5314, *C. parapsilosis*, and *C. krusei* were submerged in buffered YNB glucose medium containing amphotericin B (50µg/ml) and incubated for 5 h or 24 h at 37ºC. The biofilms were washed gently in PBS and the cells were resuspended to a concentration of approximately 10^7/ml. SR-FLICA working solution (10 µl) was then added to 200 µl of biofilm cell suspension and the mixtures were incubated for 1 h at 37ºC in the dark. The cells were washed twice by spinning in a microfuge for 5 min using a wash buffer provided in the kit. Cell pellets were resuspended in 300 µl wash buffer and examined under a x100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1) with a bandpass filter (excitation 550 nm, emission >580 nm). Apoptotic cells with active caspase enzymes fluoresced red.

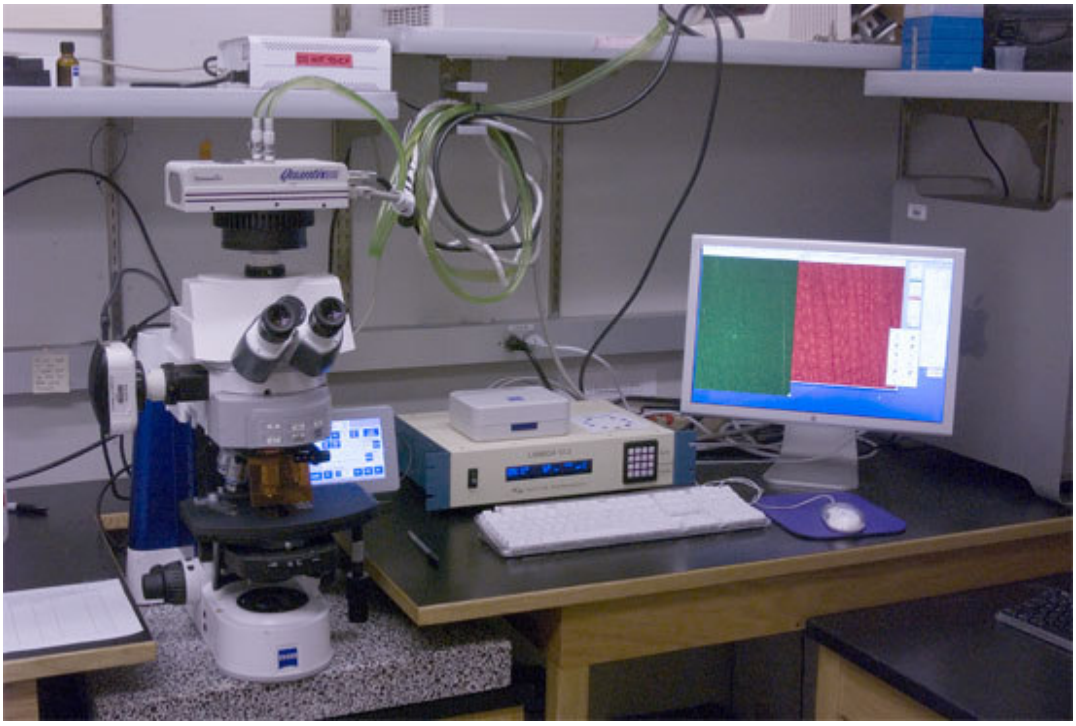


Figure 18. Fluorescence microscope (Zeiss Axioimager M1) used to examine biofilm cells stained with fluorescein diacetate, or with FLICA or D2R reagents

In addition, further investigations were carried out on the effect of pre-treating biofilm cells with unlabelled general caspase inhibitor Z-VAD-FMK before the addition of the FLICA reagent. Mature (48-h) biofilms were treated with amphotericin B (50 µg/ml) for 24 h at 37ºC and then exposed to the unlabelled general caspase inhibitor Z-VAD-FMK (2.5 µM), 1 h prior the addition of the FLICA reagent. In another experiment the unlabelled general caspase inhibitor Z-VAD-FMK was added to biofilms along with the inducer of apoptosis (i.e. amphotericin B). The biofilms were incubated for 24 h at 37ºC and then analysed by the FLICA assay.

11.2 D_2R apoptosis detection assay

A CaspSCREEN^{TM} kit was also used to detect caspase activity. Mature (48-h) biofilms were submerged in buffered YNB glucose medium containing amphotericin B (50 µg/ml) and incubated for 24 h at 37ºC. Biofilm cells were washed gently in PBS and centrifuged to give a pellet (10^5 cells) which was resuspended in D_2R incubation buffer (0.3 ml). Dithiothreitol (1 M; 3 µl) and D_2R reagent (1µl) were then added and the mixture was incubated for 45 min at 37ºC in the dark. Resuspended cells were observed under a x100 oil immersion lens using a fluorescence microscope (Zeiss Axioimager M1) with a bandpass filter (excitation 488 nm, emission 530 nm). Apoptotic cells with active caspase enzymes fluoresced green.

Further investigations were carried out on the effect of pre-treating biofilm cells with unlabelled general caspase inhibitor Z-VAD-FMK before the addition of the D_2R reagent. Mature (48-h) biofilms were treated with amphotericin B (50 µg/ml) for 24 h at 37ºC and then exposed to the unlabelled general caspase inhibitor Z-VAD-FMK (2.5 µM), 1 h prior the addition of D_2R reagent. In another experiment the unlabelled general caspase inhibitor Z-VAD-FMK was added to biofilms along with the inducer of apoptosis (i.e. amphotericin B). The biofilms were incubated for 24 h at 37ºC and then processed with the D_2R reagent.

11.3 Effect of caspase inhibitors

11.3.1 Effect on biofilm growth

To investigate the effect of Z-VAD-FMK on biofilm growth and viability, 48-h biofilms of *C. albicans* strains GDH2346 and SC5314, *C. parapsilosis*, and *C. krusei* were washed gently with PBS and submerged in YNB glucose medium (1ml) buffered with MOPS and containing different concentrations (2.5, 5, 10, and 20 μM) of Z-VAD-FMK. Control biofilms were transferred to buffered medium without Z-VAD-FMK. Biofilms were incubated for 24 h at 37ºC and then cells were harvested and their numbers determined by viable counts (Section 5.5).

Identical procedures were used to determine the effect on biofilm growth of specific inhibitors of caspases-1, -2, -3, -5, -6, -8, and -9 (Caspase Inhibitor Set III), at a concentration of 2.5 μM.

11.3.2 Effect on antifungal activity

Mature (48-h) *Candida* biofilms were submerged in buffered YNB glucose medium containing Z-VAD-FMK (2.5, 5, 10, or 20 μM) and amphotericin B (50 μg/ml). The final concentration of DMSO in assay mixtures ranged from 0.12 % to 0.26 %. Control biofilms were transferred into buffered medium containing amphotericin B only. Biofilms were incubated for 24 h at 37ºC and cell survival was determined by viable cell counts (Section 5.5).

Specific inhibitors of caspases-1, -2, -3, -5, -6, -8, and -9 (Caspase Inhibitor Set III), at a concentration of 2.5 μM, were also tested for their effects on the activity of amphotericin B (50 μg/ml) against *Candida* biofilms. The final DMSO concentration in the buffered medium was 0.25 %.

The effect of pepstatin A, an inhibitor of acid proteases (aspartyl peptidases) which are known to be produced by *Candida* species, was also tested. Mature (48-h) biofilms of *C. albicans* strains GDH2346 and SC5314 were submerged in buffered YNB glucose medium containing amphotericin B (50 μg/ml) and pepstatin A (2.5 μM). Biofilms were then incubated at 37ºC for 24 h and cell survival was determined by viable cell counts (Section 5.5). Control biofilm discs without pepstatin A were also included.

11.4 Effect of histone deacetylase (HDA) inhibitors

A variety of histone deacetylase inhibitors (sodium butyrate, sodium valproate, apicidin, and trichostatin A) were tested against different *Candida* isolates (*C. albicans* strains GDH2346 and SC5314, *C. parapsilosis*, and *C. krusei*). The effect of HDA inhibitors on biofilm growth and viability was investigated at two stages: at time zero of biofilm formation, and after 48 h of biofilm formation. The effect of HDA inhibitors was determined by the XTT reduction assay (Section 3.2) and/or viable cell counts (Section 5.5). XTT assays were carried out in triplicate and done twice on different days. Viable cell counts were performed twice, in duplicate.

11.4.1 Effect on biofilm growth

11.4.1.1 Addition at time zero of biofilm formation

A standardized cell suspension (80 µl) was applied to each PVC disc, incubated for 1 h at 37ºC, and washed gently with PBS to remove non-adherent cells. Each disc was then submerged in 1ml YNB glucose medium buffered with MOPS and containing different concentrations of HDA inhibitors (2, 8 or 32 µg/ml; 2, 8, or 32 mM for sodium butyrate). Discs were incubated for 48 h at 37ºC for biofilm formation. The final concentration of DMSO in all cases was less than 0.5 %. Control biofilms were transferred to buffered medium without HDA inhibitor.

11.4.1.2 Addition to mature biofilms

Mature (48-h) biofilms of *Candida* isolates were washed gently with PBS and submerged in YNB glucose medium (1 ml) containing different concentrations of HDA inhibitors (2, 8 or 32 µg/ml; 2, 8, or 32 mM for sodium butyrate). Biofilms were incubated for a further 24 h at 37ºC. Control biofilms were transferred to buffered medium without HDA inhibitor.

11.4.2 Effect on antifungal activity

The effects of HDA inhibitors on the activity of amphotericin B and fluconazole against *Candida* species were assessed under two sets of conditions. First, mature (48-h) biofilms grown in the presence of different concentrations of HDA inhibitors (2, 8 or 32 µg/ml; 2, 8, or 32 mM for sodium

butyrate) were washed gently with PBS and submerged in 1 ml buffered YNB glucose medium containing different concentrations of antifungal agents (10 or 50 µg/ml). Discs were then incubated for further 24 h at 37ºC. Secondly, mature (48-h) biofilms grown in HDA inhibitor-free medium were washed gently with PBS and submerged in 1 ml buffered YNB glucose medium containing different concentrations of antifungal agents (10 or 50 µg/ml) and HDA inhibitors (2, 8 or 32 µg/ml; 2, 8, or 32 mM for sodium butyrate). The final concentration of DMSO in the medium was less than 0.5 %. Discs were then incubated for further 24 h at 37ºC. Control biofilms were incubated in medium without HDA inhibitor, or without both HDA inhibitor and amphotericin B.

RESULTS

1 Activity of amphotericin B and caspofungin on planktonic cells of *Candida* species

1.1 Minimum inhibitory concentration of both drugs for various *Candida* species

Antifungal susceptibility testing was performed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) recommendations (National Committee for Clinical Laboratory Standards, 1995). The MIC endpoint was defined as the lowest concentration of the drug that produced optically clear wells (100% inhibition). Amphotericin B and caspofungin drug concentrations initially ranged from 0.06 to 8 μg/ml, plus 16 μg/ml for caspofungin (Tables 4 and 6). The concentration range for each drug was then expanded to reach an exact endpoint (Tables 5 and 7). The planktonic MICs of five *Candida* isolates are summarised in Table 8. The majority of the isolates were relatively resistant to amphotericin B; normally endpoints for *Candida* species are tightly clustered between 0.25 and 1.0 μg/ml (National Committee for Clinical Laboratory Standards, 1995). The MICs ranged between 0.8 and 2.3 μg/ml, with *C. albicans* SC5314 being the isolate most susceptible to amphotericin B, at an MIC of 0.8 μg/ml. However, all five isolates showed greater susceptibility to caspofungin; the MICs for this antifungal agent ranged from 0.3 to 0.8 μg/ml (Table 8).

1.2 Paradoxical effect of caspofungin

The paradoxical effect consists of reduced activity of the drug at high concentrations, above the minimum inhibitory concentration. This phenomenon has been reported recently for caspofungin by several different research groups (Arikan *et al.*, 2005; Ostrosky-Zeichner *et al.*, 2003; Ramage *et al.*, 2002c; Stevens *et al.*, 2004; Stevens *et al.*, 2005). In this study, five *Candida* isolates were screened at caspofungin concentrations up to 16 μg/ml by the broth microdilution method. Three isolates only demonstrated the paradoxical effect at different concentrations of the drug; these were *C. albicans* (both strains) and *C. tropicalis*. The MICs for all three isolates were $\leq$ 0.45 μg/ml. However, reduced activity of caspofungin was observed at high drug concentrations: at 4

Table 4. MIC determinations for amphotericin B against planktonic cells of different *Candida* species: drug concentrations of 0.06 to 8 μg/ml

Isolate	Growth at amphotericin B concentration (μg/ml) of:*								
	0	0.062	0.125	0.25	0.5	1	2	4	8
C. albicans GDH2346	4+	4+	4+	3+	2+	2+	0	0	0
C. albicans SC5314	4+	4+	4+	3+	2+	0	0	0	0
C. tropicalis AAHB73	4+	4+	4+	4+	3+	2+	1+	0	0
C. glabrata AAHB12	4+	4+	4+	4+	3+	1+	0	0	0
C. parapsilosis AAHB4479	4+	4+	4+	4+	4+	3+	1+	0	0

* 4+ = no reduction in turbidity relative to control; 3+ = slight reduction in turbidity; 2+ = prominent decrease in turbidity; 1+ = slightly hazy; and 0 = optically clear well. ND= not done. Assays were performed two or three times in duplicate.

Table 5. MIC determinations for amphotericin B against planktonic cells of different *Candida* species: drug concentration range expanded from 0.5 to 2.6 μg/ml

Isolate	Growth at amphotericin B concentration (μg/ml) of:*																				
	0	0.5	0.6	0.7	0.8	0.9	1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2	2.1	2.2	2.3	2.4	2.6
C. albicans GDH2346	4+	2+	ND	ND	ND	ND	2+	2+	0	0	0	0	0	0	0	0	0	0	0	0	0
C. albicans SC5314	4+	2+	1+	1+	0	0	0	0	ND	0	ND	0	ND	ND	ND	0	0	0	0	0	0
C. tropicalis AAHB73	4+	3+	ND	ND	ND	ND	2+	2+	2+	2+	2+	2+	2+	2+	2+	1+	1+	1+	0	0	0
C. glabrata AAHB12	4+	3+	2+	2+	2+	2+	1+	0	ND	0	ND	0	ND	ND	ND	0	0	0	0	0	0
C. parapsilosis AAHB4479	4+	4+	4+	4+	4+	4+	3+	2+	ND	1+	ND	1+	ND	ND	ND	1+	ND	0	0	0	0

* 4+ = no reduction in turbidity relative to control; 3+ = slight reduction in turbidity; 2+ = prominent decrease in turbidity; 1+ = slightly hazy; and 0 = optically clear well. ND= not done. Assays were performed two or three times in duplicate.

Table 6. MIC determinations for caspofungin against planktonic cells of different *Candida* species: drug concentrations of 0.06 to 16 μg/ml

Isolate	Growth at caspofungin concentration (μg/ml) of:*									
	0	0.062	0.125	0.25	0.5	1	2	4	8	16
C. albicans GDH2346	4+	4+	2+	1+	0	0	0	1+	2+	2+
C. albicans SC5314	4+	4+	3+	1+	0	0	0	0	1+	1+
C. tropicalis AAHB73	4+	4+	3+	1+	0	0	0	2+	2+	2+
C. glabrata AAHB12	4+	4+	3+	3+	2+	0	0	0	0	0
C. parapsilosis AAHB4479	4+	4+	3+	3+	3+	0	0	0	0	0

* 4+ = no reduction in turbidity relative to control; 3+ = slight reduction in turbidity; 2+ = prominent decrease in turbidity; 1+ = slightly hazy; and 0 = optically clear well. ND= not done. Assays were performed two or three times in duplicate.

Table 7. MIC determinations for caspofungin against planktonic cells of different *Candida* species: drug concentration range expanded from 0.5 to 2.6 μg/ml

Isolate	Growth at caspofungin concentration (μg/ml) of:*																				
	0	0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65	0.7	0.8	0.9	1	2	3	3.5	4	5	6	7	8
C. albicans GDH2346	4+	1+	1+	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	1+	2+	2+	2+	2+
C. albicans SC5314	4+	1+	0	ND	0	0	0	ND	0	ND	0	0	0	0	ND	ND	ND	0	ND	ND	ND	1+
C. tropicalis AAHB73	4+	1+	1+	1+	1+	0	0	0	0	0	0	0	0	0	0	1+	2+	2+	2+	2+	2+	2+
C. glabrata AAHB12	4+	3+	3+	ND	3+	3+	2+	ND	1+	ND	0	0	0	0	ND	ND	ND	0	ND	ND	ND	0
C. parapsilosis AAHB4479	4+	3+	3+	ND	3+	3+	3+	ND	3+	ND	2+	0	0	0	ND	ND	ND	0	ND	ND	ND	0

* 4+ = no reduction in turbidity relative to control; 3+ = slight reduction in turbidity; 2+ = prominent decrease in turbidity; 1+ = slightly hazy; and 0 = optically clear well. ND= not done. Assays were performed two or three times in duplicate.

Table 8. Summary of the MICs of amphotericin B and caspofungin for planktonic cells of different *Candida* isolates

Isolate	MIC (µg/ml)	
	Amphotericin B	Caspofungin
C. albicans GDH2346	1.3	0.4
C. albicans SC5314	0.8	0.3
C. tropicalis AAHB73	2.3	0.45
C. glabrata AAHB12	1.2	0.7
C. parapsilosis AAHB4479	2.2	0.8

to 16 μg/ml, 8 to 16 μg/ml and 3 to 16 μg/ml for *C. albicans* GDH2346, *C. albicans* SC5314 and *C. tropicalis*, respectively (Tables 6 and 7).

2 Fungicidal activity of amphotericin B against planktonic cells of *C. albicans* GDH2346

The *in vitro* fungicidal activity of amphotericin B was measured for planktonic cells of *C. albicans* GDH2346 by two methods (Barchiesi *et al.*, 2004; Barchiesi *et al.*, 2005; Di Bonaventura *et al.*, 2004): assays for the minimum fungicidal concentration and time-kill curves.

2.1 Minimum fungicidal concentration

After the minimum inhibitory concentration of amphotericin B for *C. albicans* GDH2346 was determined, 100 µl samples from each well at or above the MIC were plated on to SDA agar plates and incubated for 48 h at 37ºC. The minimum fungicidal concentration (MFC) was defined as the lowest drug concentration that caused total killing of cells. The minimum inhibitory concentration of amphotericin B for *C. albicans* GDH2346 was 1.3 µg/ml; the MFC occurred at the MIC end point.

2.2 Time-kill curves

To investigate the fungicidal activity of amphotericin B against *C. albicans* GDH2346, three to five colonies were picked from a 48-h plate culture, suspended in sterile distilled water, and the optical density was adjusted to 0.5 at 530 nm. An aliquot (1ml) of the adjusted suspension was added to 9 ml of growth medium plus the appropriate amount of drug. Amphotericin B was used at concentrations of one-half, one, two, and four times the MIC. The suspensions were placed on a shaker and incubated at 37ºC. At time points 0, 2, 6, and 24 h, 100 µl of the suspension was removed, diluted in growth medium, and plated on to SDA plates. The number of CFU on each plate was determined after 48 h of incubation. Amphotericin B at 0.5 times the MIC exhibited some fungicidal activity (55% killing after 24 h), whereas complete fungicidal activity against *C.*

albicans was observed at a drug concentration equal to the MIC. Amphotericin B at 4 times the MIC was fungicidal after 2 h of incubation while drug concentrations equal to, or double the MIC were fungicidal after 6 h (Fig. 19).

3 *In vitro* activity of amphotericin B and caspofungin at different developmental phases of *Candida* biofilms

Previous studies of *C. albicans* biofilm formation (Chandra *et al.*, 2001a; Hawser & Douglas, 1994) showed that it occurs in several distinct developmental phases, resulting in a highly heterogeneous architecture of well-defined cellular communities enclosed in a thick polysaccharide matrix. These phases are: (i) early phase (0 to 11 h) involving the initial attachment of yeast cells to the disc material, (ii) intermediate phase (12 to 30 h) involving aggregation of the yeast cells and the formation of hyphae, and (iii) maturation phase (31 to 72 h) involving the production of the extracellular matrix.

In this investigation, the effects of three factors on drug resistance were examined: i) the *Candida* species; ii) the developmental stage of the biofilm; and iii) the drug concentration. *Candida* biofilms were grown on polyvinyl catheter discs. Different concentrations of amphotericin B and caspofungin (two times and five times the MIC for each isolate) were introduced at different stages of biofilm development (after 8, 17, 24, and 35 h). The efficacy of both drugs was measured after 48 h using the tetrazolium XTT reduction assay.

3.1 8-h *Candida* biofilms

For most *Candida* isolates, biofilms in the early stage of development (i.e. 8-h biofilms) were relatively resistant to amphotericin B at a concentration of two times the MIC, whereas at five times the MIC, metabolic activity was reduced significantly ($P < 0.01$; Fig. 20). However, amphotericin B at both concentrations was highly effective against *C. albicans* SC5314, with a 99% reduction in metabolic activity. On the other hand, caspofungin at two times the

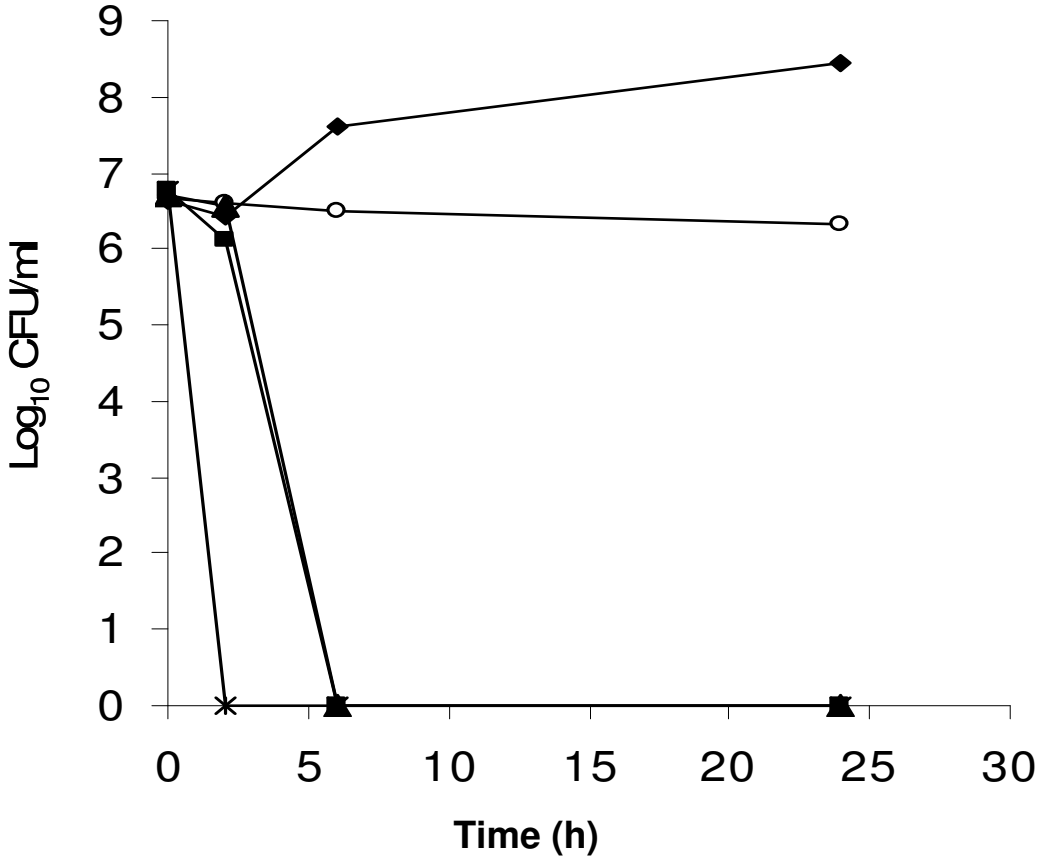


Figure 19. Time-kill study conducted against *C. albicans* GDH2346 planktonic cells

Amphotericin B was used at concentrations equal to 0.5, 1, 2, and 4 times the MIC. Control (♦); 0.5X MIC (o); 1X MIC (▲); 2X MIC (■); 4X MIC (×). Results are from one experiment, carried out in duplicate.

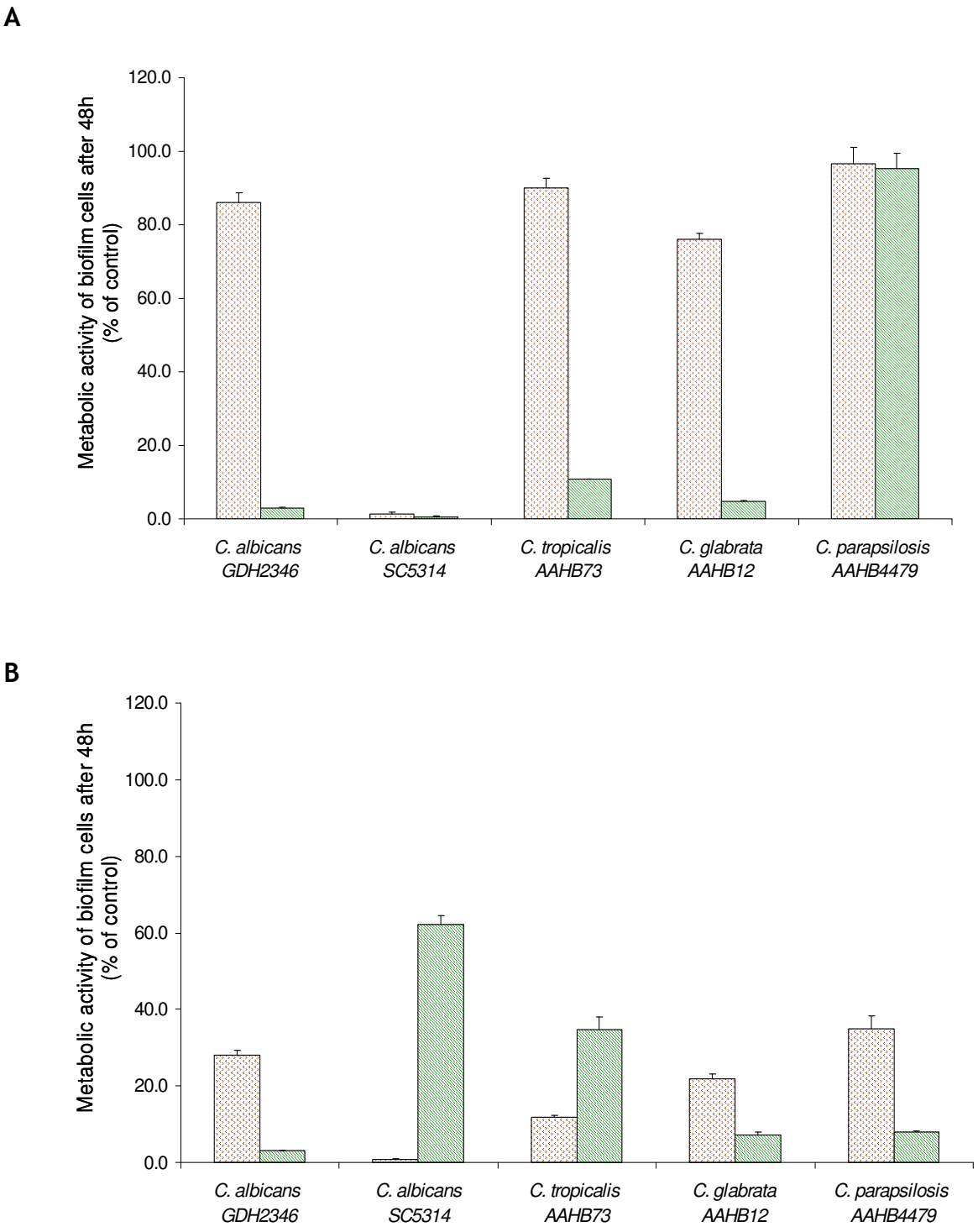


Figure 20. Effect of amphotericin B ( ) and caspofungin ( ) at 2x (A) or 5x (B) the MIC on 8-h *Candida* biofilms. Data represent the mean ± standard error for one experiment carried out with three replicates.

MIC showed higher activity than amphotericin B, except with *C. parapsilosis* biofilms where there was little effect. However, at the higher concentration, caspofungin was less effective against *C. albicans* SC5314 and *C. tropicalis* than amphotericin B. Also, the higher concentration of caspofungin was less effective at reducing the metabolic activity of *C. albicans* SC5314 and *C. tropicalis* biofilms than the lower drug concentration.

3.2 17-h *Candida* biofilms

With the exception of *C. albicans* SC5314, biofilms grown for 17 h were more susceptible to amphotericin B at twice the MIC than biofilms grown for 8 h (Fig. 21). However, at this stage of biofilm development, biofilms were more resistant to the higher drug concentration. Caspofungin, at both concentrations, exhibited a greater effect than amphotericin B. Biofilms were more resistant to caspofungin at a concentration of twice the MIC than the biofilms grown for 8 h, with the exception of *C. parapsilosis* biofilms which were more sensitive. The greatest reduction in metabolic activity was observed with the higher drug concentration, with the exception of *C. tropicalis* biofilms.

3.3 24-h *Candida* biofilms

Increased resistance to both antifungal drugs was observed in 24-h old biofilms of some *Candida* species (Fig. 22). At this stage of biofilm development, amphotericin B at the lower concentration had little effect on the metabolic activity of both *C. albicans* strains but showed greater activity against biofilms of *C. parapsilosis*, compared with earlier stages of development. Biofilms of both *C. albicans* strains also showed increased resistance to the higher drug concentration than at the earlier stages. Caspofungin was more effective at inhibiting biofilm growth than amphotericin B, except with *C. parapsilosis* biofilms. Caspofungin was more effective at the higher concentration than at the lower concentration, except with *C. tropicalis* biofilms. Its greatest effect was against *C. glabrata* biofilms.

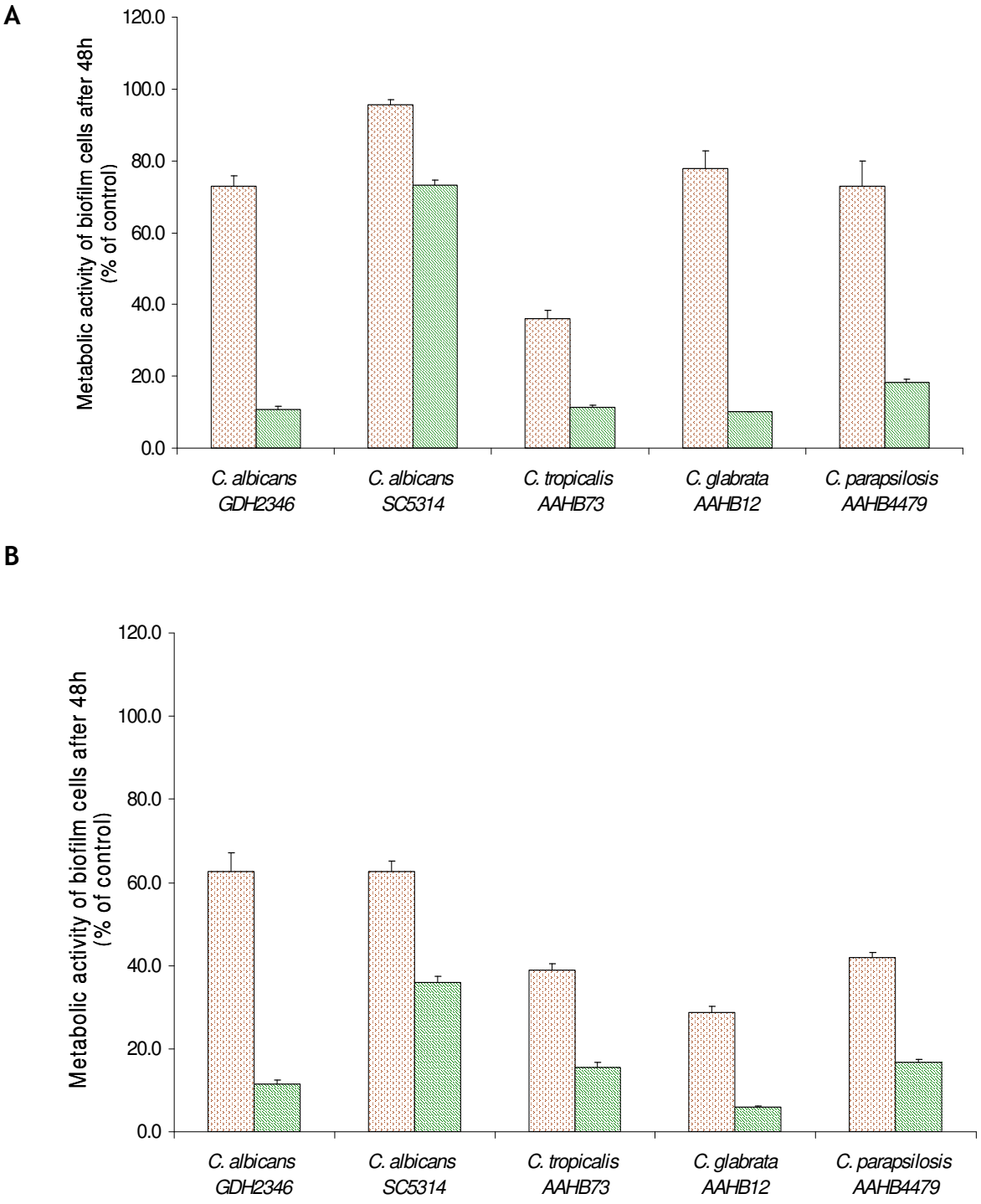


Figure 21. Effect of amphotericin B (▨) and caspofungin (▨) at 2x (A) or 5x (B) the MIC on 17-h *Candida* biofilms. Data represent the mean ± standard error for one experiment carried out with three replicates.

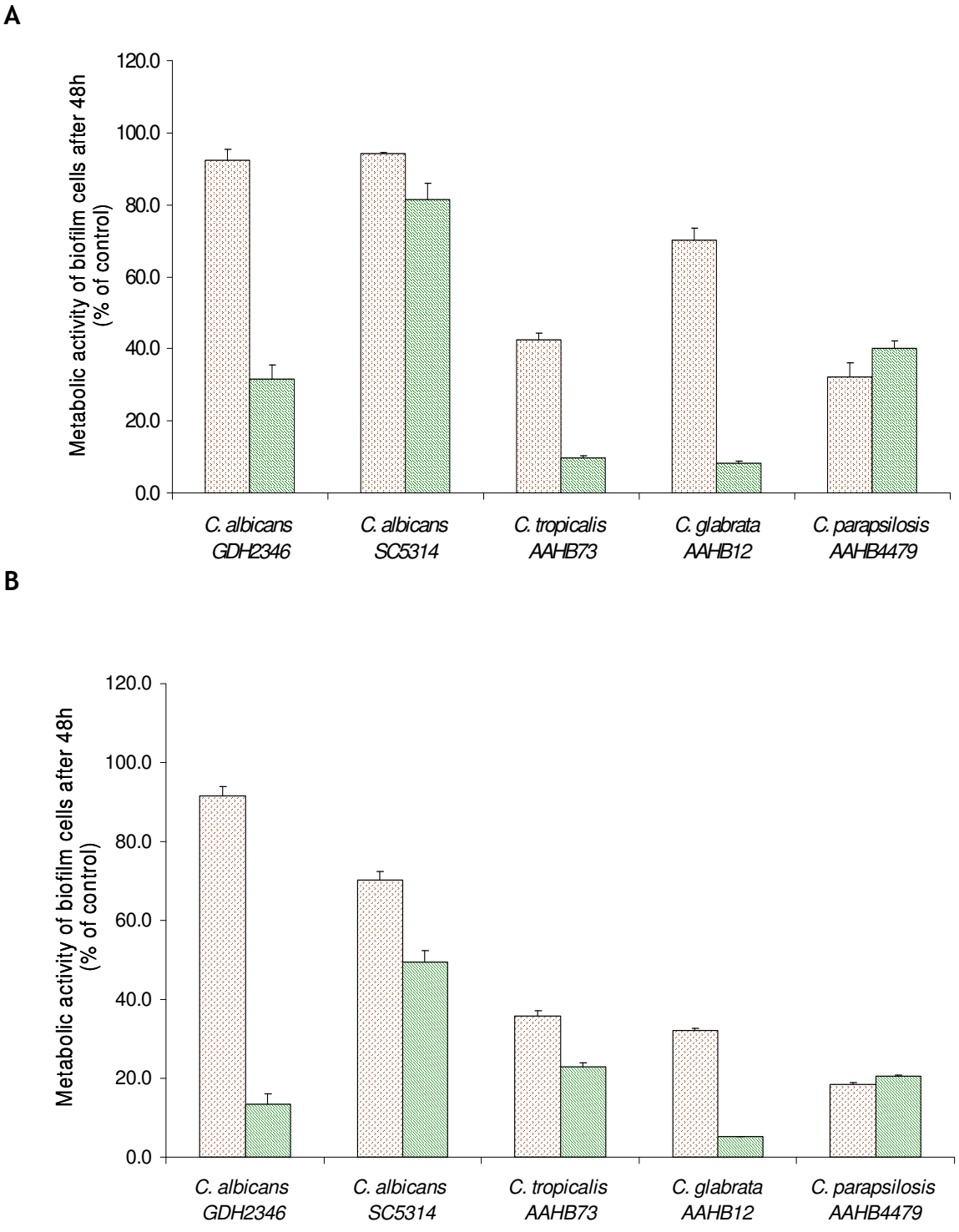


Figure 22. Effect of amphotericin B (▨) and caspofungin (▨) at 2x (A) or 5x (B) the MIC on 24-h *Candida* biofilms. Data represent the mean ± standard error for one experiment carried out with three replicates.

3.4 35-h *Candida* biofilms

Mature (35-h) biofilms showed a susceptibility pattern similar to that of the 24-h biofilms. Both antifungal drugs were least effective against *C. albicans* SC5314. Caspofungin at the lower concentration was more effective than amphotericin B against biofilms of *C. albicans, C. tropicalis*, and *C. glabrata,* but not *C. parapsilosis*. However, caspofungin at the higher concentration showed more activity than amphotericin B against *C. albicans* GDH2346, *C. tropicalis*, and *C. glabrata,* but not against *C. albicans* SC5314 and *C. parapsilosis*. At this phase of development, all species showed increased resistance to caspofungin, at five times the MIC, as compared with 17-h and 24-h biofilms. Moreover, some *Candida* biofilms were more resistant to caspofungin at a concentration of five times the MIC than to the lower concentration; this was observed with biofilms of *C. albicans* SC5314 and *C. tropicalis* (Fig. 23).

3.5 Overall conclusions

In summary, amphotericin B at two times the MIC had the least effect on *Candida* biofilms. However, when the drug concentration was increased to five times the MIC, the effect of amphotericin B increased. Amphotericin B at the higher concentration showed relatively high activity against *C. parapsilosis* and *C. glabrata* biofilms, especially at the late development phase (Fig. 23B). Biofilms of both strains of *C. albicans* were more resistant to amphotericin B throughout development (except for the earliest stage) than the other *Candida* species.

Caspofungin, at two times the MIC, generally exhibited a greater effect on *Candida* biofilms than amphotericin B although this was not observed with *C. parapsilosis* biofilms in some development phases (i.e. 24-h and 35-h biofilms). Caspofungin, at five times the MIC, was slightly less efficacious than at the lower concentration against *C. tropicalis* in all development phases tested (Figs. 20-23) and against *C. albicans* SC5314 in some phases (Figs. 20 and 23). In no case were biofilm cells of any *Candida* species completely killed by either amphotericin B or caspofungin, both of which are fungicidal agents, at either concentration used. The species most susceptible to caspofungin (at both concentrations)

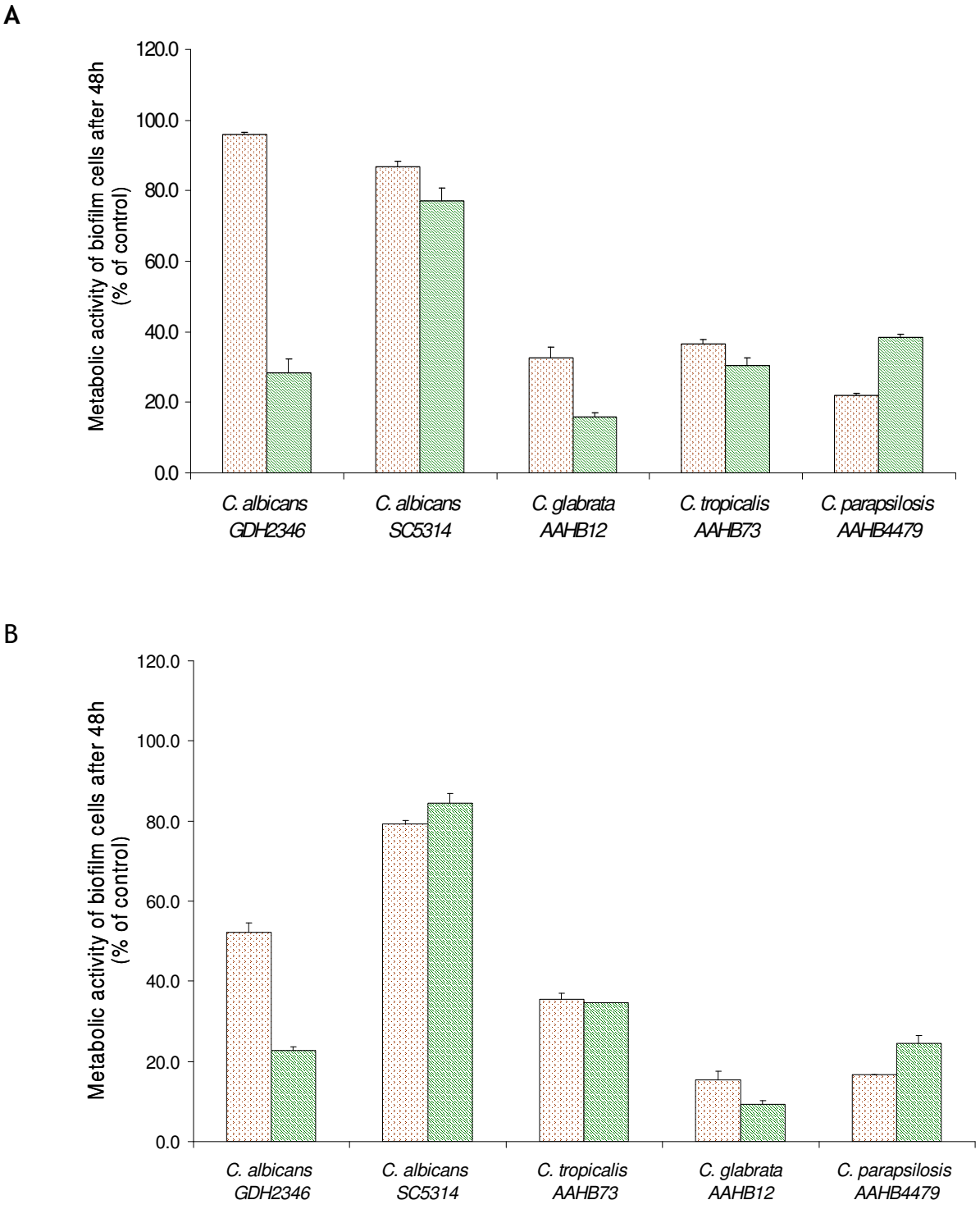


Figure 23. Effect of amphotericin B (▨) and caspofungin (▨) at 2x (A) or 5x (B) the MIC on 35-h *Candida* biofilms. Data represent the mean ± standard error for one experiment carried out with three replicates.

throughout biofilm development was *C. glabrata* (Figs. 20-23); *C. albicans* GDH2346 was susceptible in the early phase (Fig. 20).

4 Penetration of antifungal agent (caspofungin) through *Candida* biofilms

This penetration assay was a modification of the filter disc method described previously for bacterial biofilms. Biofilms were grown on polycarbonate membrane filters resting on YNB agar plates containing 50mM glucose for 48 h. A second smaller filter was placed on top of the 48 h biofilm and finally a moistened concentration disc was positioned on top of the second filter. The whole assembly was then transferred to antifungal containing agar. Plates were incubated at 37°C for specific time periods (60, 90, 120, 180, 240, or 360 min). Drug concentrations were selected based on their ability to produce large zones of growth inhibition in control assays for drug penetration; caspofungin was tested at a concentration of 60 times the MIC for *C. albicans* GDH2346 (i.e. 24 µg/ml). The amount of antifungal agent that diffused out of the agar and through the biofilm 'sandwich' to the concentration disc was determined by using the disc in a standard drug diffusion assay. The diameters of zones of growth inhibition were measured and used to determine the concentration of active antifungal agent in the discs by reference to a standard curve prepared using drug solutions of different concentrations but fixed volumes.

4.1 Penetration of caspofungin through biofilms of *C. albicans* strains

Although caspofungin is a large polypeptide molecule with a molecular mass of 1213 Da, it penetrated *Candida* biofilms well. Different levels of penetration were demonstrated with biofilms of different *Candida* species.

4.1.1 Susceptibility of *C. albicans* to caspofungin

The MIC of caspofungin for planktonic *C. albicans* GDH2346 is 0.4 µg/ml, as determined previously (section 1.1). In this assay, biofilms of *C. albicans* were challenged with 60 times the MIC of caspofungin (i.e. 24 µg/ml).

4.1.2 Caspofungin penetration through biofilms of *C. albicans* GDH2346

Caspofungin penetration through *C. albicans* GDH2346 biofilms was zero after 60 min but increased gradually to 28 % of the control value after 90 min. Within 360 min, the drug concentration (C) at the distal edge of the biofilm had reached 70.8 % of the control value (C_0) (Fig. 24).

4.1.3 Caspofungin penetration through biofilms of *C. albicans* SC5314

Diffusion of caspofungin through biofilms of *C. albicans* SC5314 was also zero after 60 min but then rose gradually to 38.6 % after 90 min. After 360 min, it had reached 57.8 % of that of the control value (Fig. 25).

4.2 Penetration of caspofungin through biofilms of non-*C. albicans* species

4.2.1 Caspofungin penetration through *C. glabrata* AAHB12 biofilms

Caspofungin penetration through *C. glabrata* biofilms was rapid, reaching 54.8 % of the control value after 60 min. Within 360 min it had reached 81.2 % of the control value (Fig. 26).

4.2.2 Caspofungin penetration through *C. parapsilosis* AAHB4479 biofilms

The rate of penetration of caspofungin through *C. parapsilosis* biofilms was also fast, reaching 39.4 % of the control value after 60 min. After 360 min, it had reached 73.3 % of the control value (Fig. 27).

4.2.3 Caspofungin penetration through *C. tropicalis* AAHB73 biofilms

Caspofungin diffusion through biofilms of *C. tropicalis* was zero after 60 min but rapidly reached 25.6 % of the control value after 90 min. It then levelled

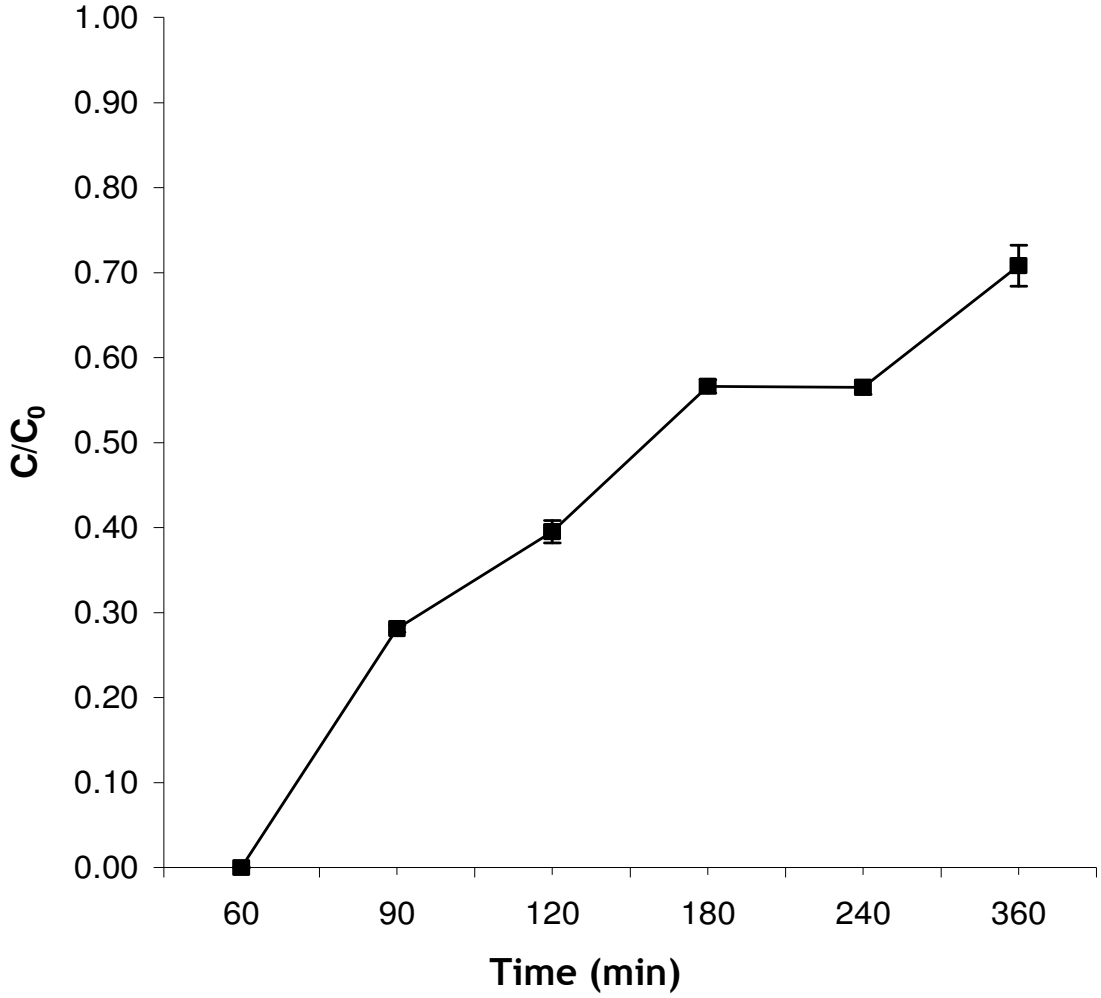


Figure 24. Penetration of caspofungin through biofilms of *C. albicans* GDH2346 with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

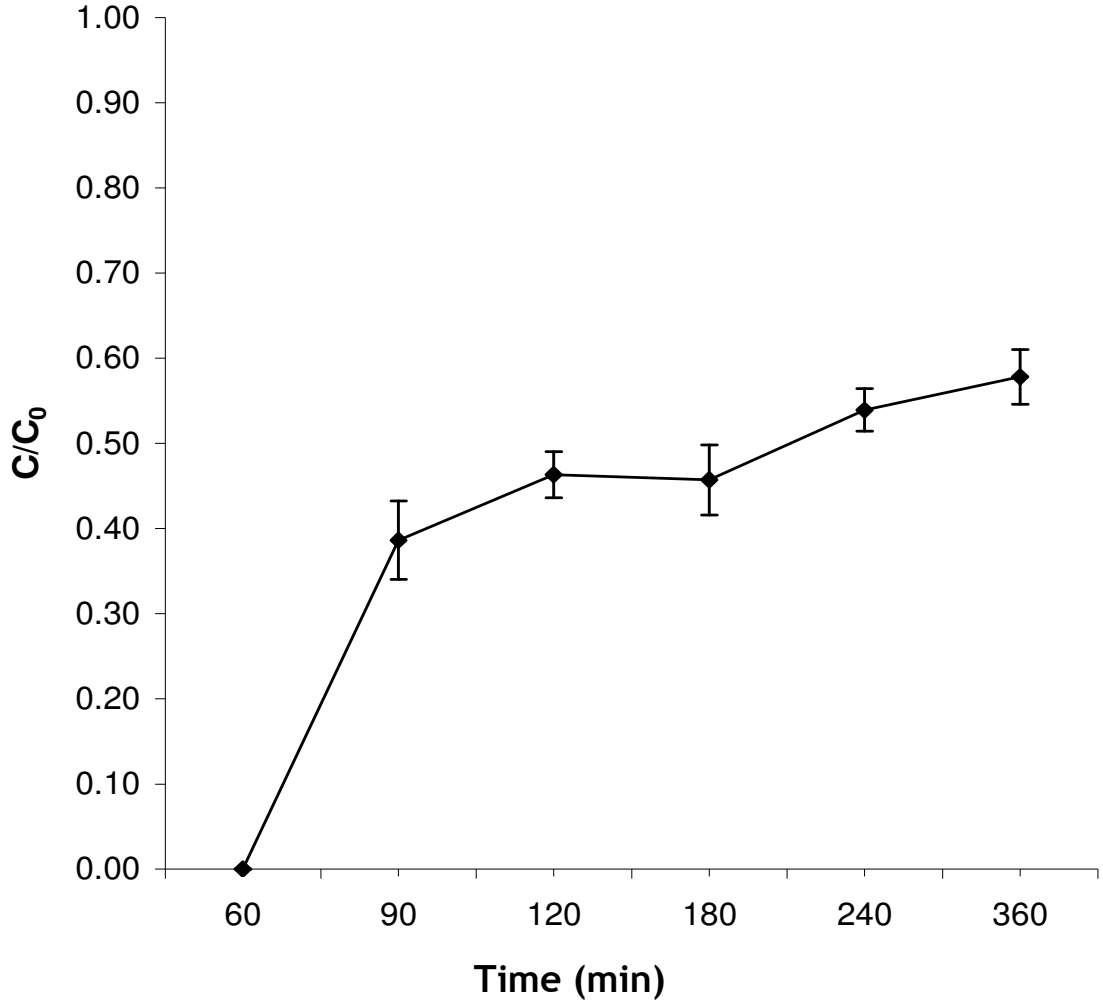


Figure 25. Penetration of caspofungin through biofilms of *C. albicans* SC5314 with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

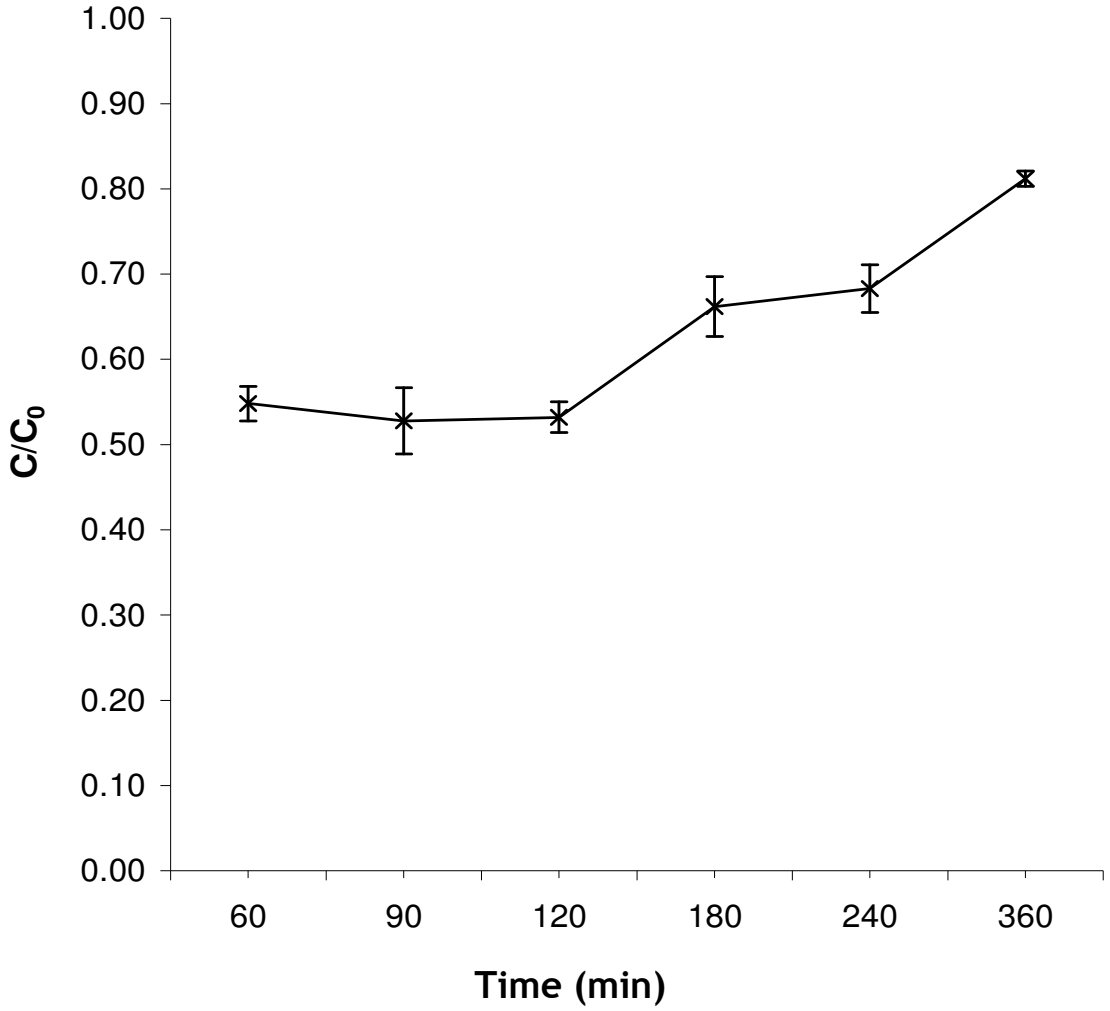


Figure 26. Penetration of caspofungin through biofilms of *C. glabrata* with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

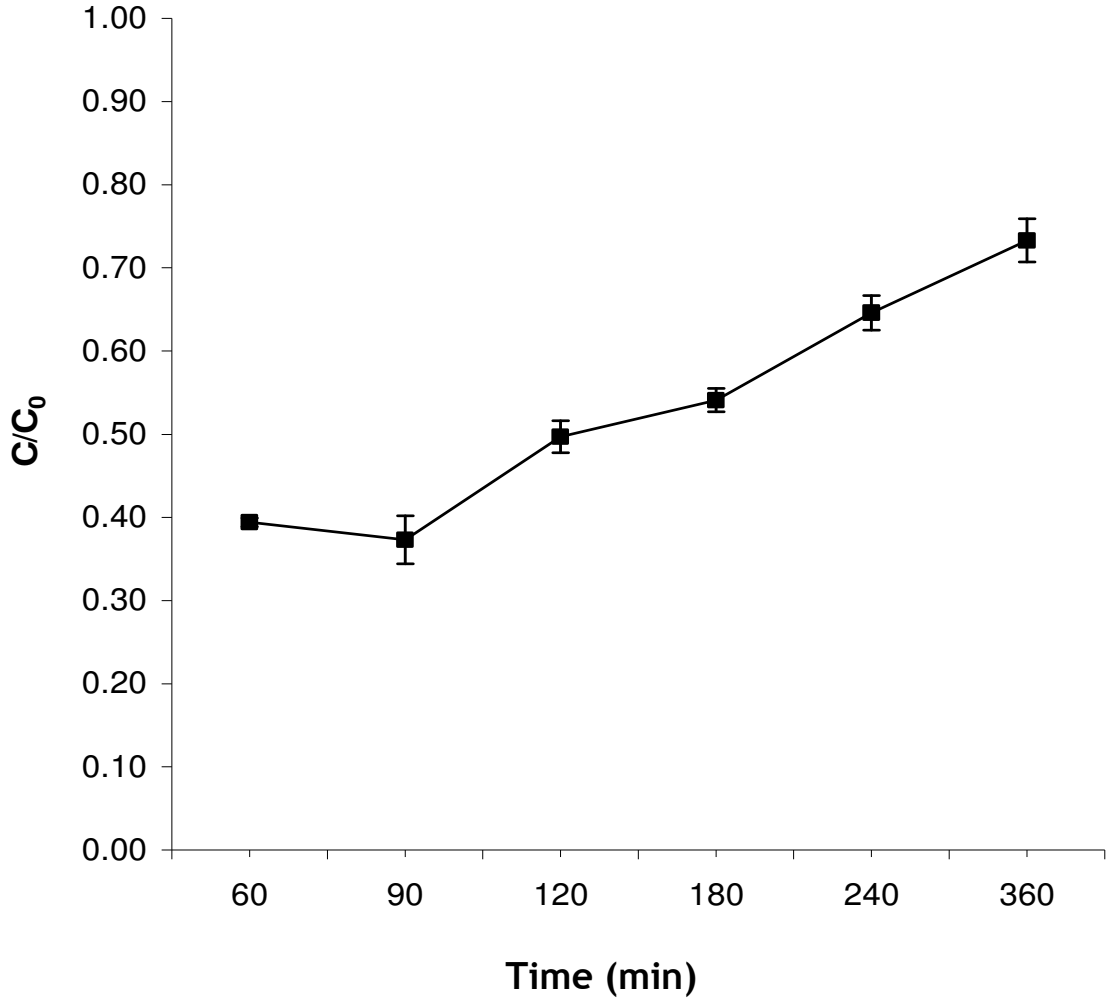


Figure 27. Penetration of caspofungin through biofilms of *C. parapsilosis* with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

off but increased again to reach 49.9 % of the control value after 360 min (Fig. 28).

4.2.4 Caspofungin penetration through *C. krusei* (Glasgow) biofilms

There was zero drug penetration through *C. krusei* biofilms after 90 min. After 120 min, the caspofungin concentration had risen rapidly to 34.6 %. Within 360 min, it had reached 44.2 % of the control value (Fig. 29).

4.3 Comparison of caspofungin penetration through biofilms of different *Candida* species

Both *C. albicans* GDH2346 and *C. albicans* SC5314 biofilms showed zero diffusion of caspofungin after 60 min of exposure (Fig. 30). Subsequently, drug diffusion was initially faster through biofilms of strain SC5314 but then levelled off. After 360 min, drug penetration was greater (70.8% of the control value) with biofilms of strain GDH2346.

The initial rate of drug penetration after 60 min through biofilms of *C. glabrata* AAHB12 and *C. parapsilosis* AAHB 4479 was faster than that seen with the other species (54.8 % and 39.4 % of the control value, respectively) (Fig. 31). The slowest diffusion was through *C. krusei* biofilms; it was zero after 90 min and then increased rapidly to 34.6 % after 120 min. Biofilms of this species showed the lowest drug penetration overall (44.2 % after 360 min). Biofilms of *C. tropicalis* were also penetrated poorly, with zero penetration after 60 min and then a gradual increase to 49.9 % of the control value after 360 min. The highest drug penetration overall was observed with *C. glabrata* and *C. parapsilosis*, at 81.2 % and 73.3 % of the control value after 360 min, respectively (Fig. 31).

4.4 Effect of antifungal agents on the viability of biofilm cells

After incubation of biofilms sandwiched between the two membranes on antifungal agent-containing agar, as in the penetration assay, the effects of antifungal agents on the viability of biofilm cells were assessed. Biofilms were incubated for 6 h (the time period during which drug penetration was determined) or 24 h at 37°C and viable counts were then carried out. Antifungal agents (amphotericin B or caspofungin) were present at concentrations similar to

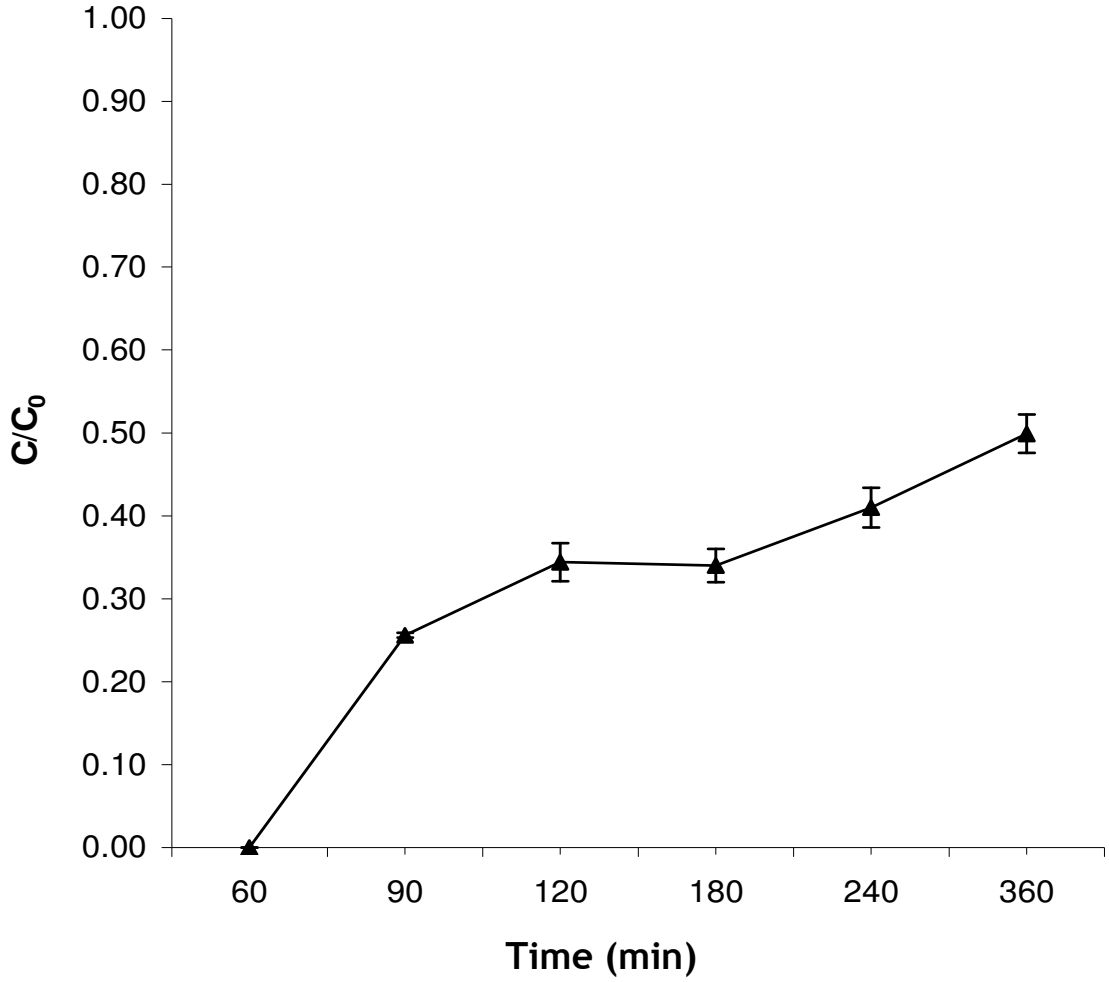


Figure 28. Penetration of caspofungin through biofilms of *C. tropicalis* with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

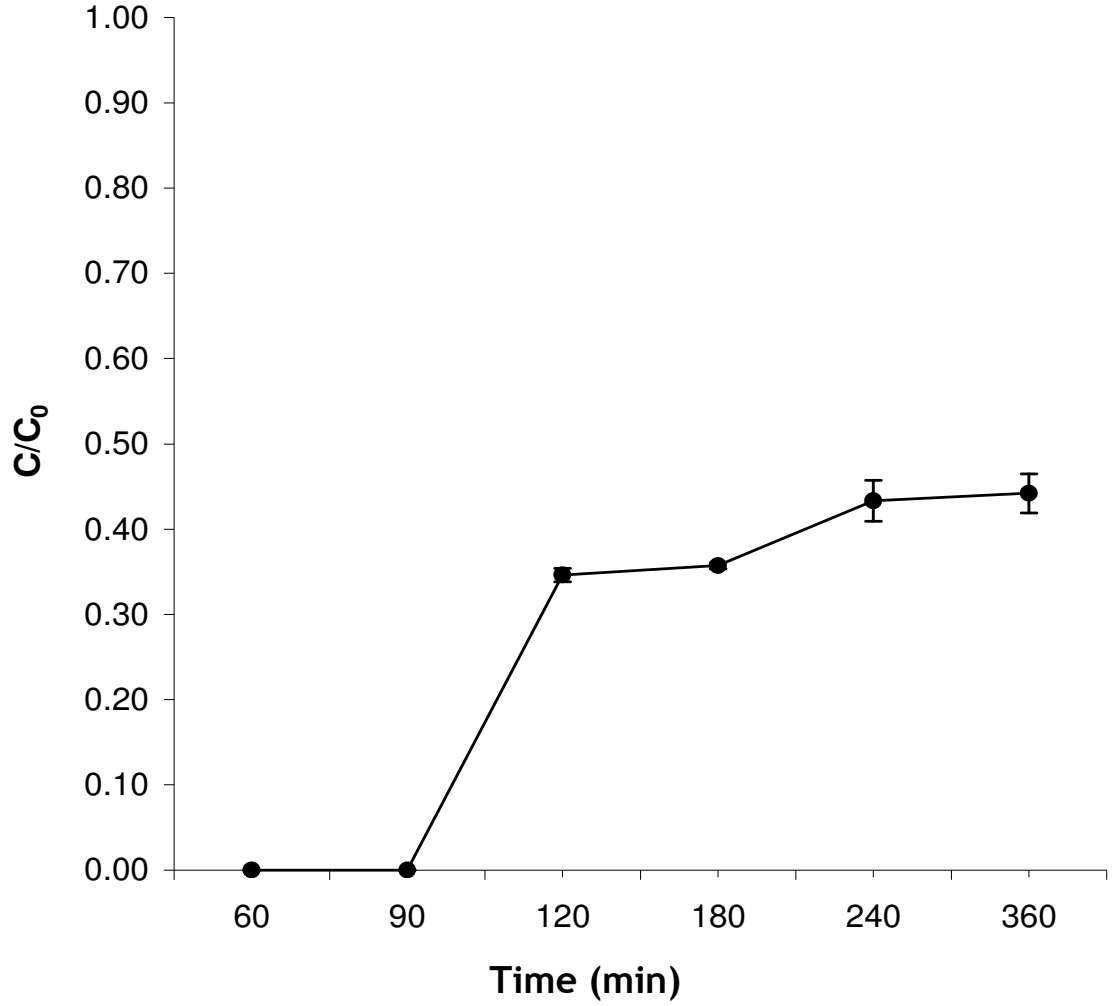


Figure 29. Penetration of caspofungin through biofilms of *C. krusei* with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

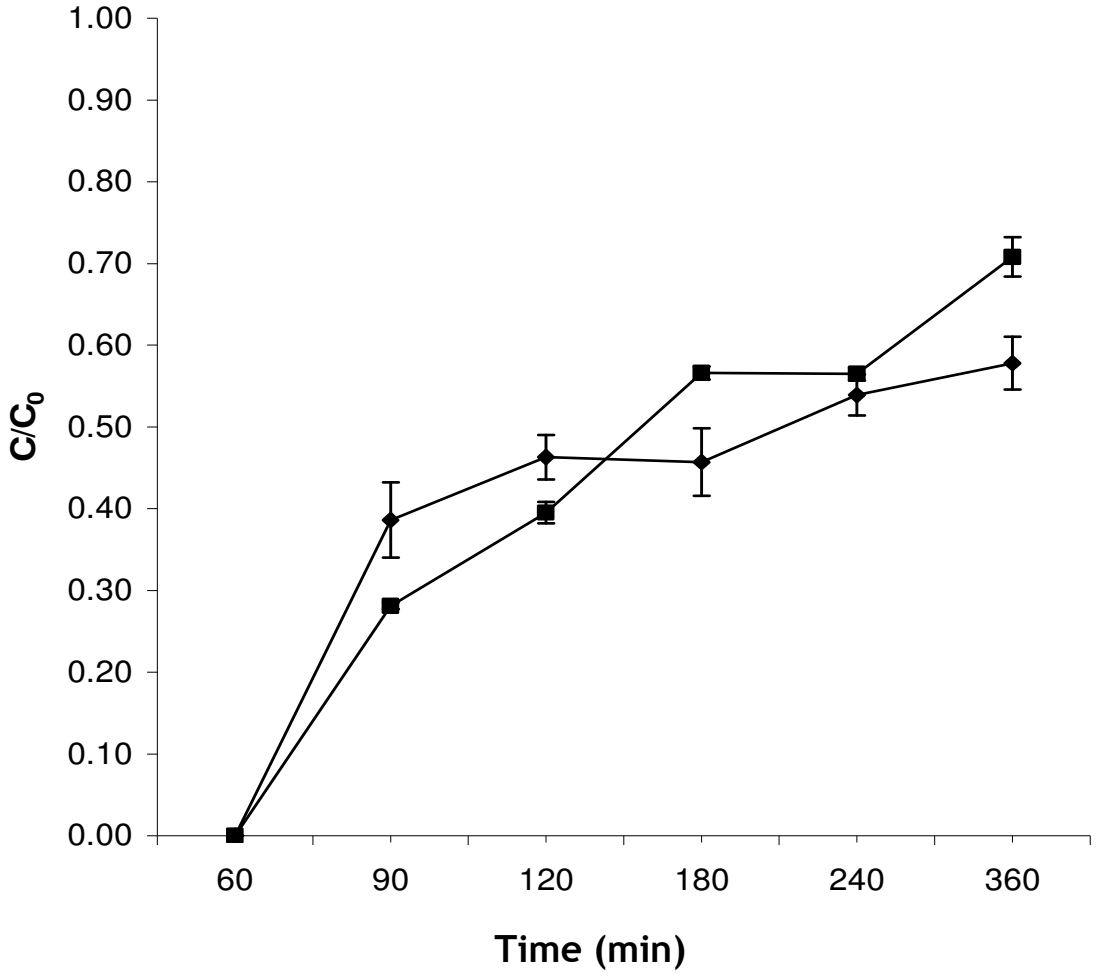


Figure 30. Penetration of caspofungin through biofilms of *C. albicans* GDH2346 (■) and *C. albicans* SC5314 (♦) with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

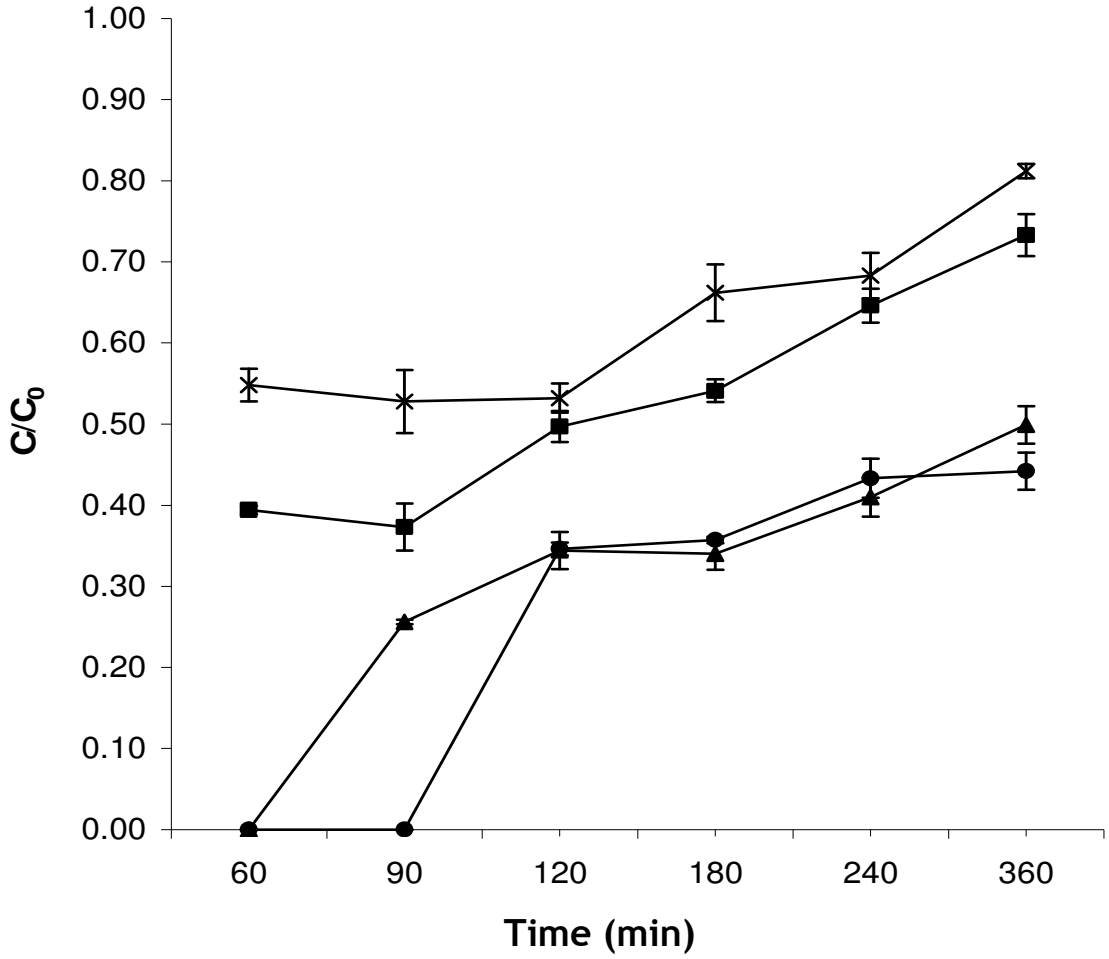


Figure 31. Penetration of caspofungin through biofilms of *C. glabrata* (×), *C. parapsilosis* (■), *C. tropicalis* (▲), and *C. krusei* (●) with time

The drug concentration that diffused through the biofilm (C) was divided by the drug concentration for the control (C_0). Error bars indicate the standard errors of the means for two independent experiments carried out in duplicate.

those in the drug penetration assay (i.e. 60 times the MIC of *C. albicans* GDH2346).

4.4.1 Amphotericin B

Amphotericin B at 60 times the MIC (78 µg/ml) showed relatively poor activity against biofilms of all five *Candida* species (Table 9). *C. glabrata* and *C. parapsilosis* biofilms were the most affected, with cell viabilities of 3 % and 43 %, after 6 h, and 0.1 % and 7 % after 24 h, respectively. The two *C. albicans* strains were the least susceptible, retaining 48 % viability after 24 h. Biofilms of *C. tropicalis* and *C. krusei* had intermediate viabilities of 38 % and 36 %, respectively, following drug treatment for 24 h (Table 9).

4.4.2 Caspofungin

In general, *Candida* biofilms were less susceptible to caspofungin than to amphotericin B when treated at high drug concentrations (Table 9). After 6 h of exposure to caspofungin at 24 µg/ml (60 times the MIC), biofilms of all species showed a similar effect, with 67 to 76 % viability. Biofilm cells of *C. glabrata* were the most susceptible to caspofungin after 24 h of exposure, with 32 % viable cells. However, biofilms of this species were even more susceptible to amphotericin B (Table 9). Overall caspofungin treatment, like amphotericin B treatment, failed to result in complete killing of biofilm cells.

The paradoxical effect of caspofungin (reduced activity at high concentrations) was observed in previous work with planktonic cultures of some species, i.e. *C. albicans* (both strains) and *C. tropicalis,* but not *C. parapsilosis* or *C. glabrata* (Section 1.2). Therefore, biofilm cells of all six *Candida* isolates in this study were examined further for the paradoxical phenomenon. The biofilms were challenged with 5 or 10 times the MIC of caspofungin (2 or 4 µg/ml, respectively). The paradoxical effect was demonstrated in biofilms of *C. glabrata* and *C. parapsilosis* after either 6 h or 24 h (*C. parapsilosis* only) exposure time (Table 10). A caspofungin concentration of 4 µg/ml had a lesser effect on *C. glabrata* than a concentration of 2 µg/ml after 6 h of exposure ($P<0.001$). Similarly, biofilms of *C. parapsilosis* were more susceptible ($P<0.006$) to a concentration of 2 µg/ml than to one of 4 µg/ml after either 6 h or 24 h

Table 9. Viability of biofilm cells of *Candida* spp. after exposure to amphotericin B (78 µg/ml) or caspofungin (24 µg/ml) for 6 or 24 h *

Organism	Viability (%)			
	Amphotericin B (78 µg/ml)		Caspofungin (24 µg/ml)	
	6 h	24 h	6 h	24 h
C. albicans GDH2346	65.6 ± 1.8	48.0 ± 0.5	71.3 ± 3.6	44.9 ± 2.7
C. albicans SC5314	54.3 ± 1.5	48.2 ± 1.3	75.9 ± 1.6	42.0 ± 2.2
C. tropicalis AAHB73	57.1 ± 1.5	38.3 ± 2.4	73.8 ± 2.7	49.7 ± 2.7
C. glabrata AAHB12	2.8 ± 0.7	0.1 ± 0.1	70.5 ± 2.8	32.2 ± 1.5
C. parapsilosis AAHB4479	43.1 ± 1.7	6.6 ± 0.9	75.9 ± 3.3	52.6 ± 1.5
C. krusei Glasgow	63.7 ± 2.8	35.6 ± 1.8	66.6 ± 2.6	58.5 ± 2.1

* Viability is expressed as percentage of that of control cells. Data represent the means + standard errors for two experiments carried out in duplicate. All results were significantly different at $P \leq 0.006$ from that of control.

Table 10. Viability of biofilm cells of *Candida* spp. after exposure to caspofungin (2 or 4 µg/ml) for 6 or 24 h *

Organism	Viability (%)			
	Caspofungin (2 µg/ml)		Caspofungin (4 µg/ml)	
	6 h	24 h	6 h	24 h
C. albicans GDH2346	84.8 ± 3.2 [b]	44.3 ± 1.9 [a]	79.5 ± 3.2 [a]	43.7 ± 1.8 [a]
C. albicans SC5314	77.4 ± 3.0 [a]	39.7 ± 3.7 [a]	76.7 ± 2.0 [a]	36.8 ± 2.0 [a]
C. tropicalis AAHB73	79.3 ± 2.8 [a]	62.3 ± 4.4 [a]	57.9 ± 3.4 [a]	49.9 ± 2.1 [a]
C. glabrata AAHB12	47.7 ± 3.2 [a]	11.1 ± 0.5 [a]	59.9 ± 2.5 [a]	7.9 ± 0.7 [a]
C. parapsilosis AAHB4479	77.1 ± 3.0 [a]	61.2 ± 4.5 [a]	83.9 ± 3.9 [a]	70.4 ± 3.0 [a]
C. krusei Glasgow	91.6 ± 4.5 [c]	58.4 ± 3.7 [a]	65.4 ± 3.8 [a]	38.0 ± 4.0 [a]

[*] Viability is expressed as a percentage of that of control cells. Data represent the means ± standard errors for two independent experiments carried out in duplicate.

[a] Value significantly different at $P \leq 0.006$ from that of control

[b] Value significantly different at $P < 0.012$ from that of control

[c] Value not significantly different at $P > 0.087$ from that of control

exposure (Table 10). The paradoxical phenomenon complicates the evaluation of caspofungin activity against biofilms. Nevertheless, caspofungin clearly showed high activity at low concentrations against biofilms of several *Candida* species.

4.5 Scanning electron microscopy of *Candida* biofilms before and after penetration by antifungal agents

Membrane-supported biofilms were fixed and processed for scanning electron microscopy (SEM) according to the method described by Erlandsen *et al*. (2004) who had used this procedure previously to investigate the presence of bacterial glycocalyx. Here, the presence of acidic polysaccharides and the effects of two antifungal drugs (amphotericin B and caspofungin) on biofilm structure and morphology were investigated. After air drying, biofilms were more easily detached from the membrane filter and this allowed examination of three areas: (i) the top surface of the biofilm; (ii) the bottom surface of the biofilm (biofilms were turned upside down); and (iii) the membrane-attached basal region of the biofilm. Using cationic dyes (alcian blue or ruthenium red) in the primary fixation significantly improved the visualization of exopolysaccharides in the biofilm samples. Procedures with either dye gave good preservation of cell morphology and matrix material. However, with the exception of *C. tropicalis* and *C. krusei* biofilms, ruthenium red treatment produced a weakly held biofilm that broke easily into pieces. Because of this, all the biofilms shown in micrographs presented here were stained with alcian blue.

4.5.1 Effects of amphotericin B and caspofungin on biofilm structure of different *Candida* species

The effects of amphotericin B (78 µg/ml) and caspofungin (24 µg/ml) on the structure of the biofilms were studied. The areas of the biofilm were examined as described above.

4.5.1.1 The top surface of the biofilm

SEM observations of the top surface of the biofilm showed that the untreated control biofilm was, in some cases, covered with a slime-like material in which yeasts and filaments were completely embedded. This type of material was visualized in abundance in *C. tropicalis* and *C. krusei* biofilms (Fig. 32, E1

and F1). The other species failed to exhibit the slime-like material and only relatively sparse amounts of matrix material were seen (Figs. 32, A1, C1, D1 and 33, A1-D1). Biofilms formed by *C. albicans* GDH2346 consisted of a dense network of yeast cells only (Figs. 32 and 33, A1), whereas those of *C. albicans* SC5314 consisted of both yeast and filamentous forms (Fig. 32, B1). *C. glabrata* and *C. parapsilosis* formed biofilms containing densely packed yeast cells (Figs. 32 and 33, C1 and D1); in the case of *C. parapsilosis*, some of the cells were rather elongated (Figs. 32 and 33, D1).

After 24 h of exposure to amphotericin B, the top surface of the biofilms seemed largely unaffected and the cells retained the morphology of those found in the controls (Fig. 32, A2, B2, C2, D2, and F2), although in *C. tropicalis* biofilms the slime-like material was less evident (Fig. 32, E2). Caspofungin, on the other hand, affected the morphology of *C. parapsilosis* biofilms; some cells were damaged and appeared to be spherical and enlarged compared with those found in the control (Fig. 33, D2). Moreover, following caspofungin treatment, *C. tropicalis* and *C. krusei* biofilms contained less matrix material and were devoid of the slime-like component (Figs. 33, E2 and F2). However, caspofungin did not appear to affect the top surface of *C. albicans* biofilms (Figs. 33, A2 and B2), nor that of *C. glabrata* biofilms (Fig. 33, C2).

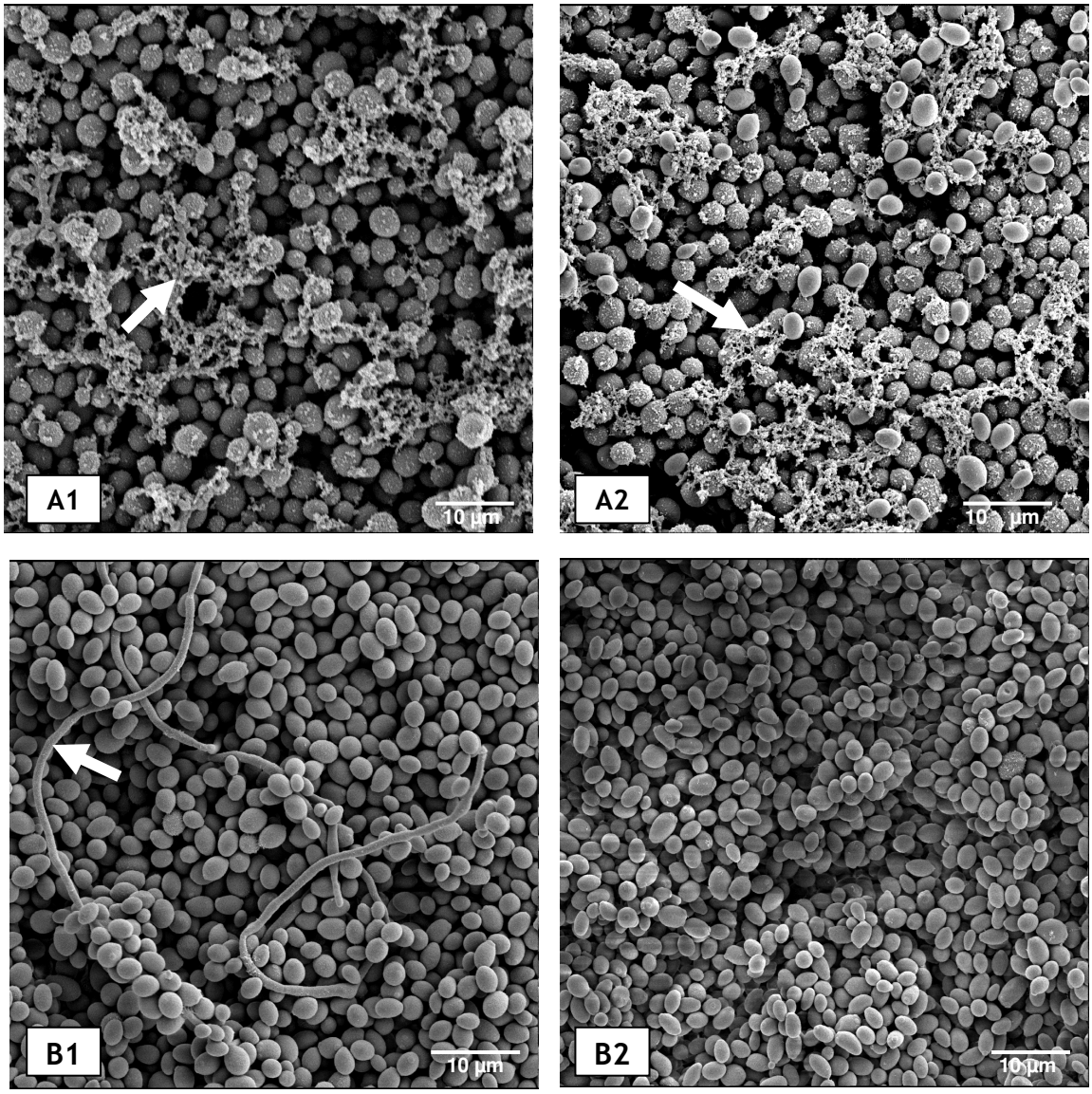


Figure 32. Scanning electron micrographs of the top layers of biofilms of *Candida* species exposed to amphotericin B

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with amphotericin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of amphotericin B (78 µg/ml) (2). Arrows in A indicate matrix material; arrow in B1 indicates hyphae.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

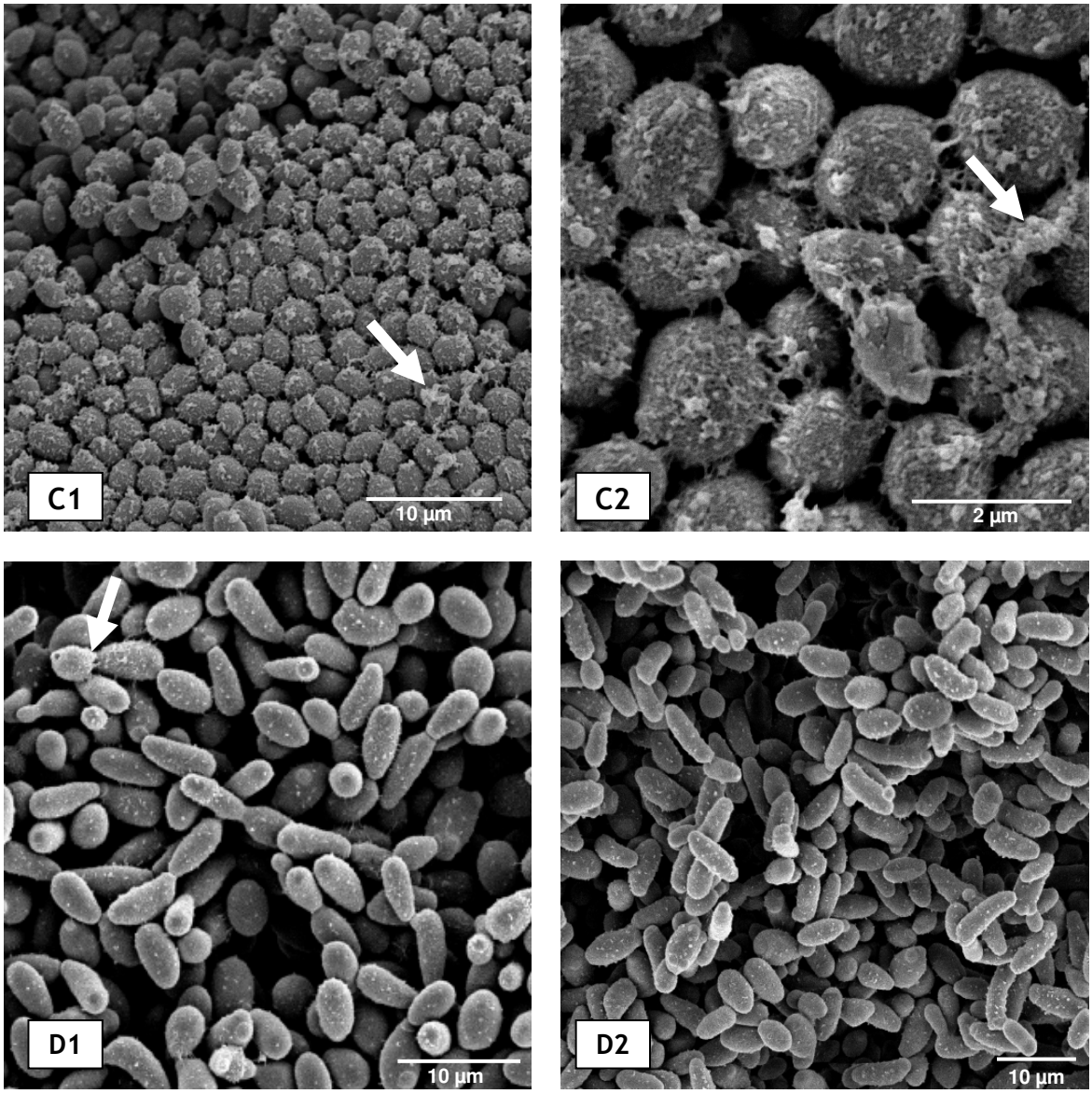


Figure 32

(**continued**)

Arrows indicate matrix material

C. *C. glabrata*

D. *C. parapsilosis*

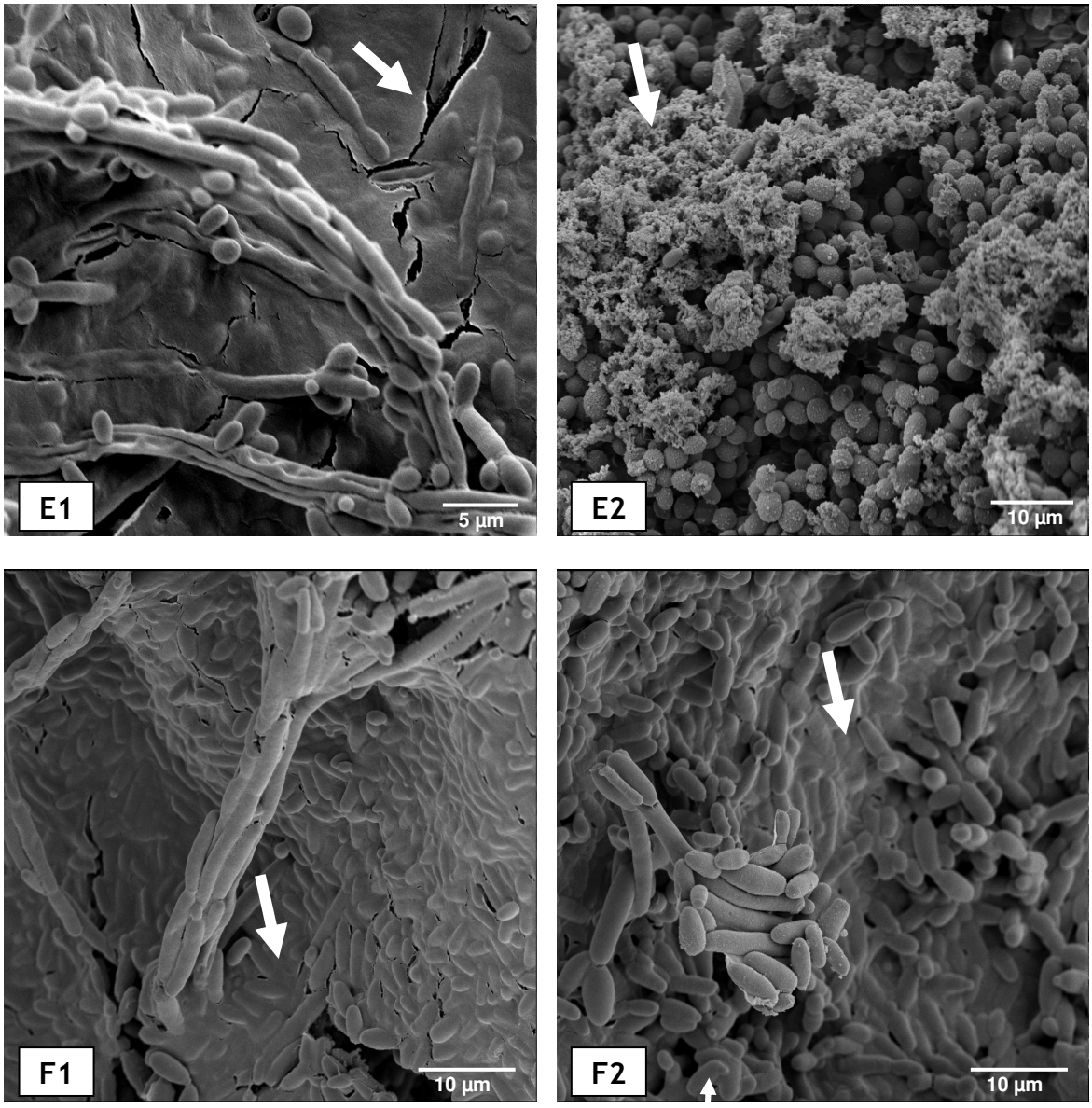


Figure 32

(continued)

Arrows in E1, F1 and F2 indicate slime-like matrix material; arrow in E2 indicates matrix material

E. *C. tropicalis*

F. *C. krusei*

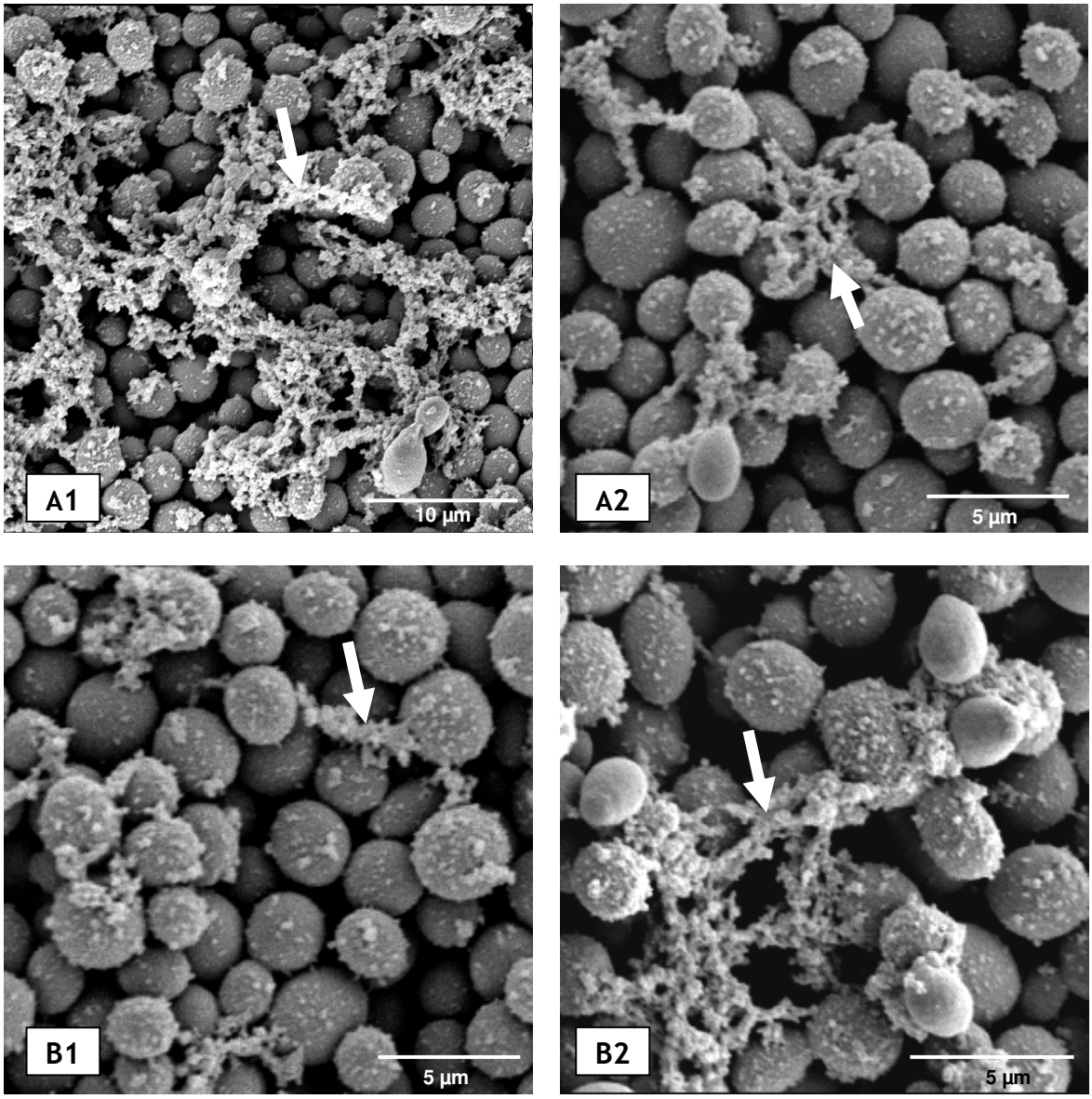


Figure 33. Scanning electron micrographs of the top layers of biofilms of *Candida* species exposed to caspofungin

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with caspofungin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of caspofungin (24 µg/ml) (2). Arrows indicate matrix material.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

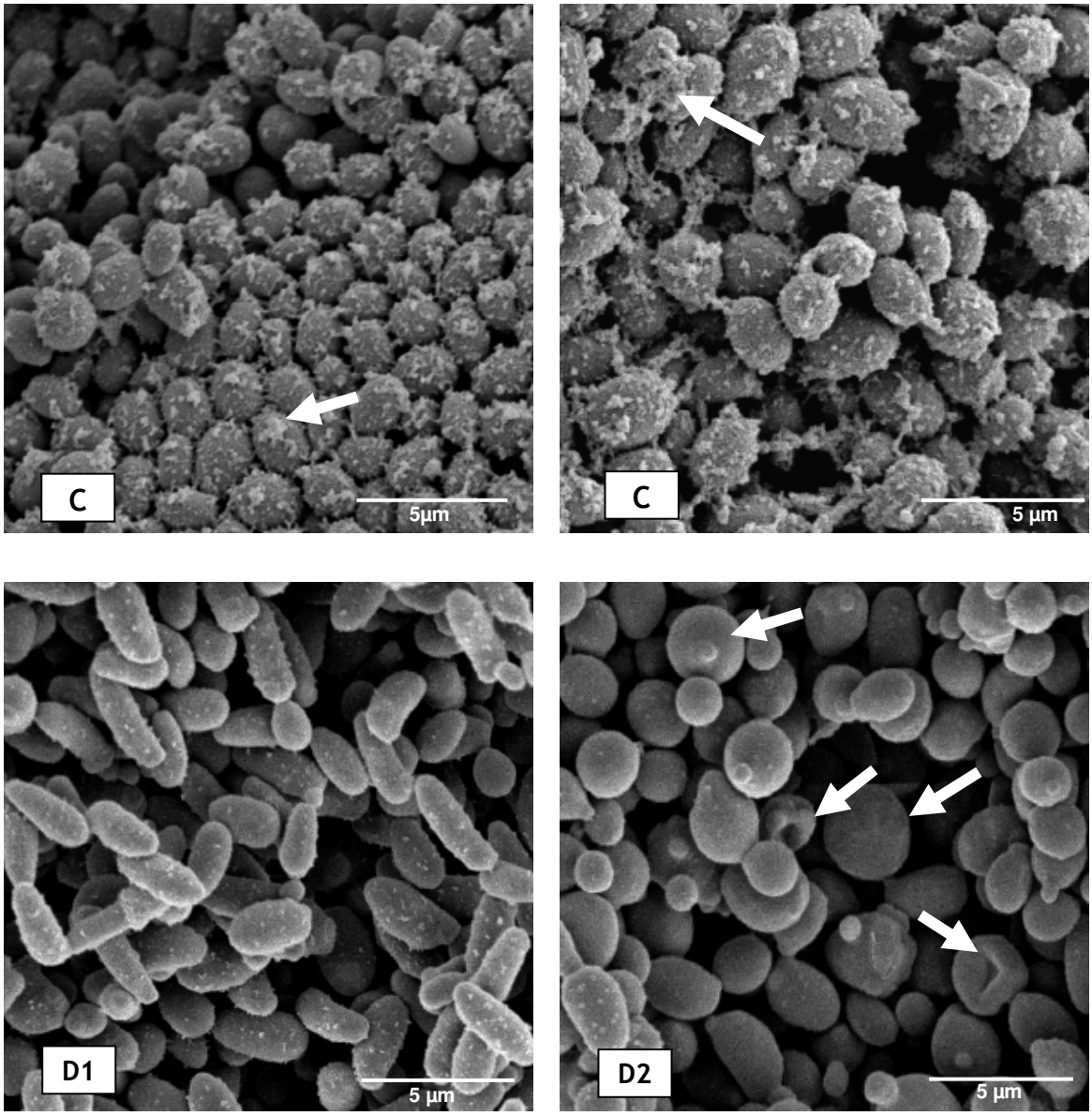


Figure 33

(**continued**)

Arrows in C1 and C2 indicate matrix material; arrows in D2 indicate spherical, enlarged, and damaged cells.

C. *C. glabrata*

D. *C. parapsilosis*

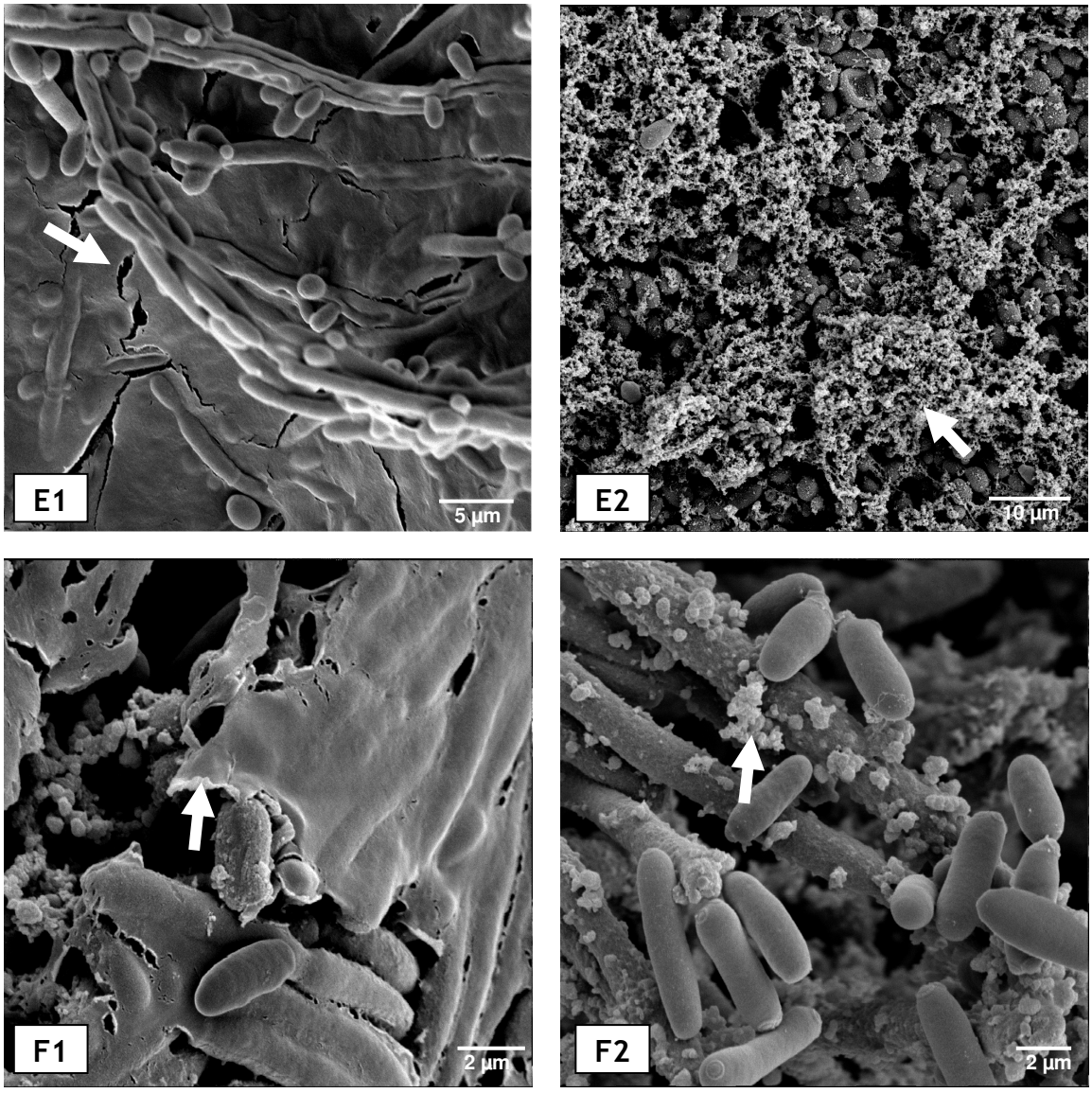


Figure 33

(**continued**)

Arrows in E1 and F1 indicate slime-like matrix material; arrows in E2 and F2 indicate matrix material

E. *C. tropicalis*

F. *C. krusei*

4.5.1.2 The bottom surface of the biofilm

The bottom surface of the untreated control biofilms showed different types of matrix material. These appeared as strands (Figs. 34, C1 and 35, F1) attached to the cell surface and forming a complex meshwork between cells, or as slime-like material (Figs. 34, D2 and 35, E1). Globule-like matrix particles were also seen on cell surfaces (Figs. 34, A1, B1 and 35, A1, B1).

After 24 h of amphotericin B treatment, some damage to the biofilm structure, at the bottom surface, was clearly seen in *C. krusei* biofilms; the meshwork structure was almost completely destroyed (Fig. 34, F2) and relatively little matrix remained. Amphotericin B also caused elongated cells in biofilms of *C. krusei* to become shorter and more spherical (Fig. 34, F2). In contrast, no change in biofilm structure or cell morphology was apparent in biofilms of the other *Candida* species (Fig. 34, A2-E2).

Caspofungin caused much more damage to the bottom surface of the biofilm. Severe destruction of the bottom surface meshwork structure was observed with biofilms of *C. glabrata*, *C. tropicalis,* and *C. krusei* (Figs. 35, C2, E2, and F2). In addition, many yeast cells in the biofilm had lost their surface matrix coat; this was visualized clearly with *C. albicans* and *C. tropicalis* (Fig. 35, A2, B2, and E2). Caspofungin also caused cell rupture in biofilms of *C. tropicalis* and *C. krusei* (Fig. 35, E2 and F2). However, no such changes were apparent with biofilms of *C. parapsilosis* (Fig. 35, D2).

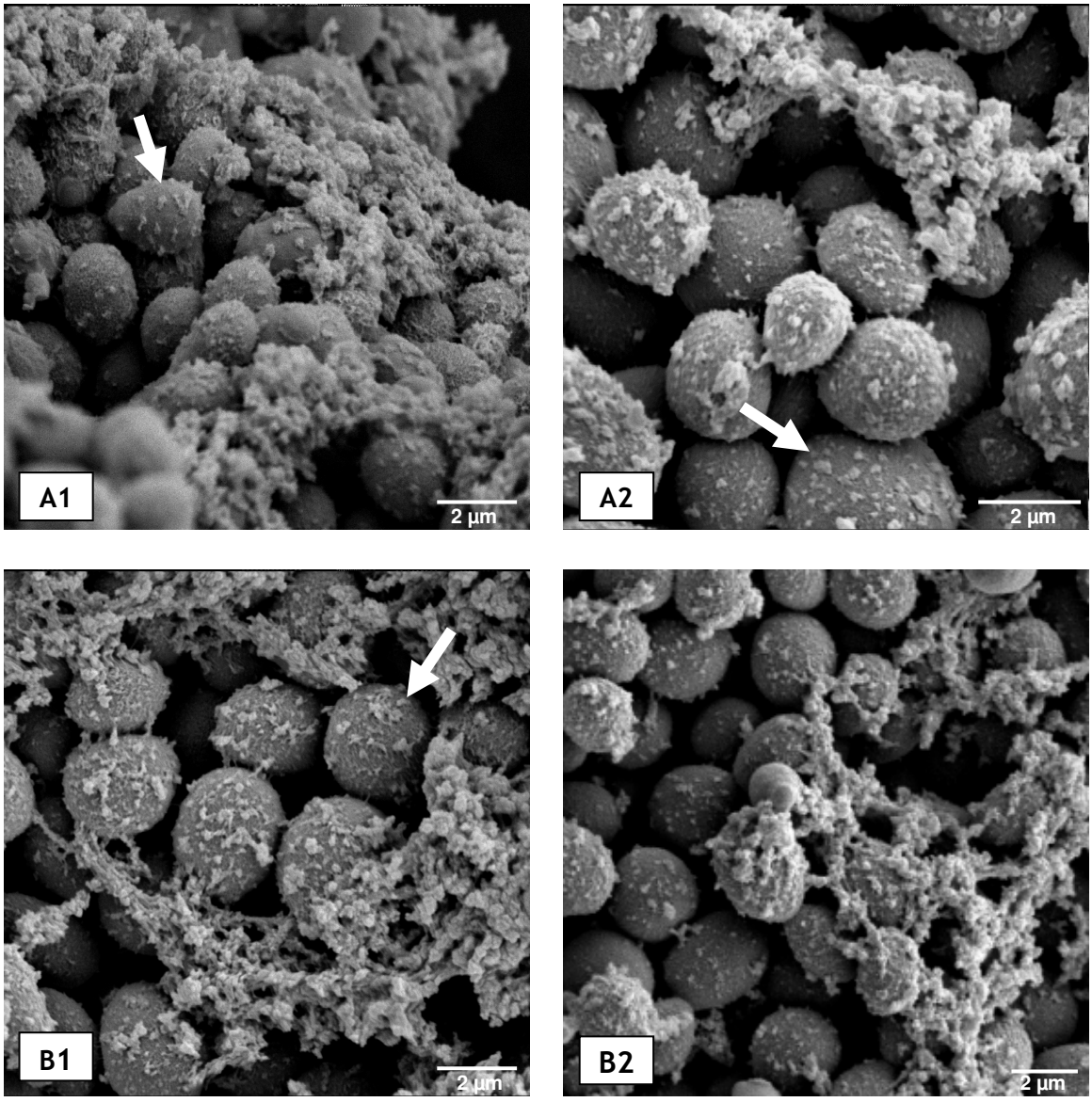


Figure 34. Scanning electron micrographs of the bottom layers of biofilms of *Candida* species exposed to amphotericin B

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with amphotericin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of amphotericin B (78 µg/ml) (2). Arrows indicate globule-like matrix particle.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

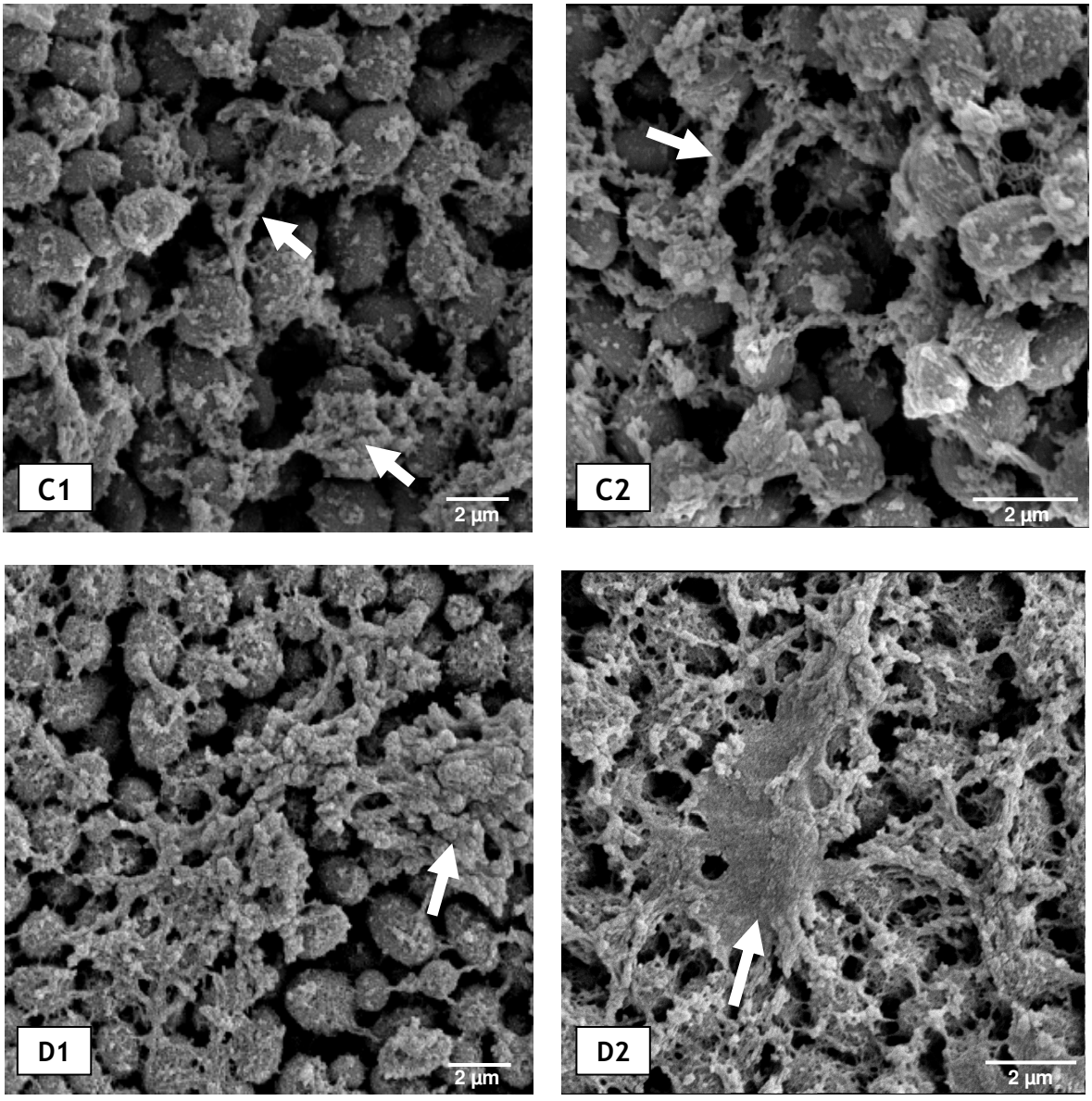


Figure 34

(**continued**)

Arrows in C indicate matrix material; arrows in D indicate slime-like matrix material.

C. *C. glabrata*

D. *C. parapsilosis*

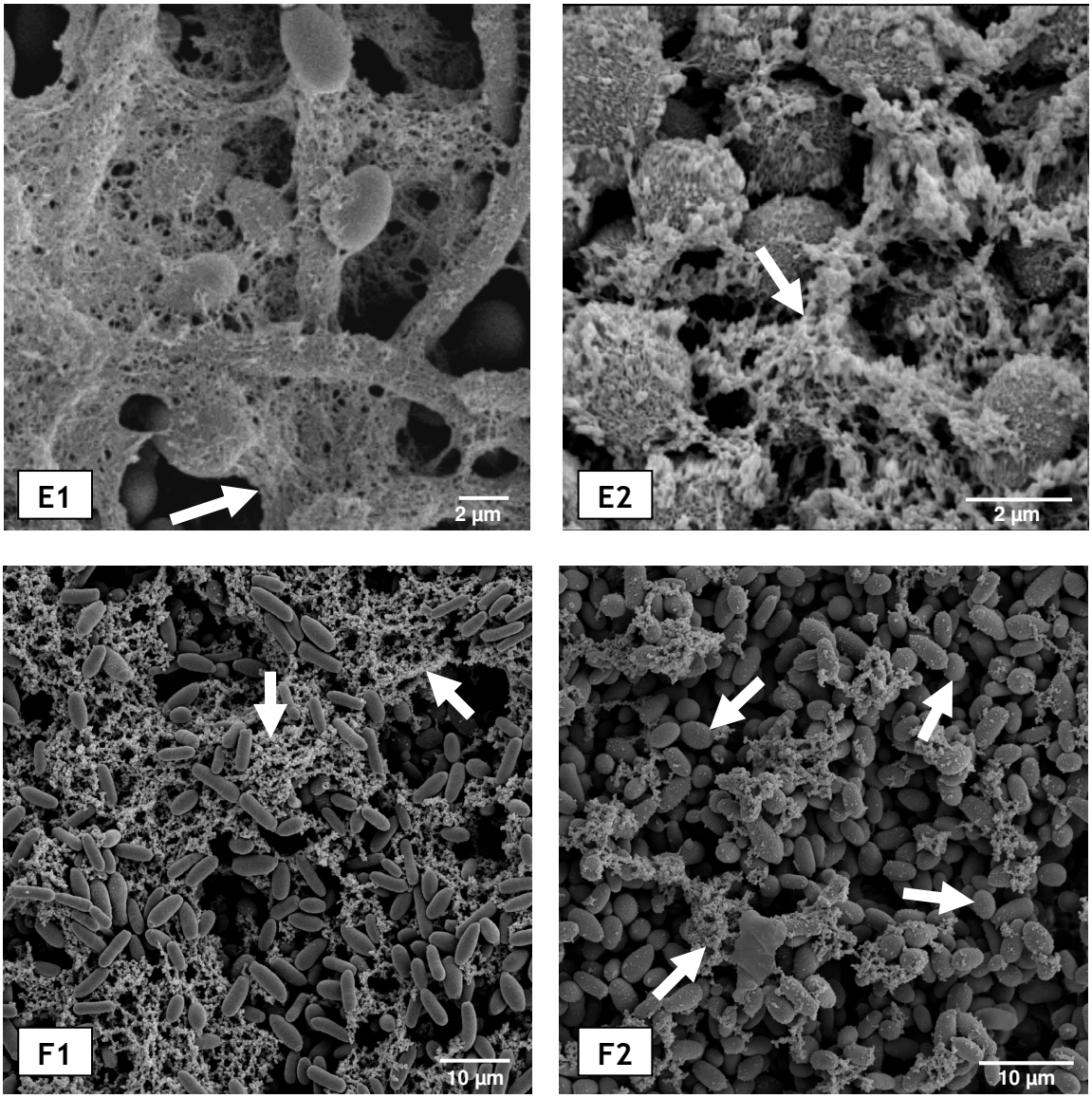


Figure 34

(**continued**)

Arrows in E and F1 indicate matrix material; arrows in F2 indicate remaining matrix material and spherical yeast cells.

E. *C. tropicalis*

F. *C. krusei*

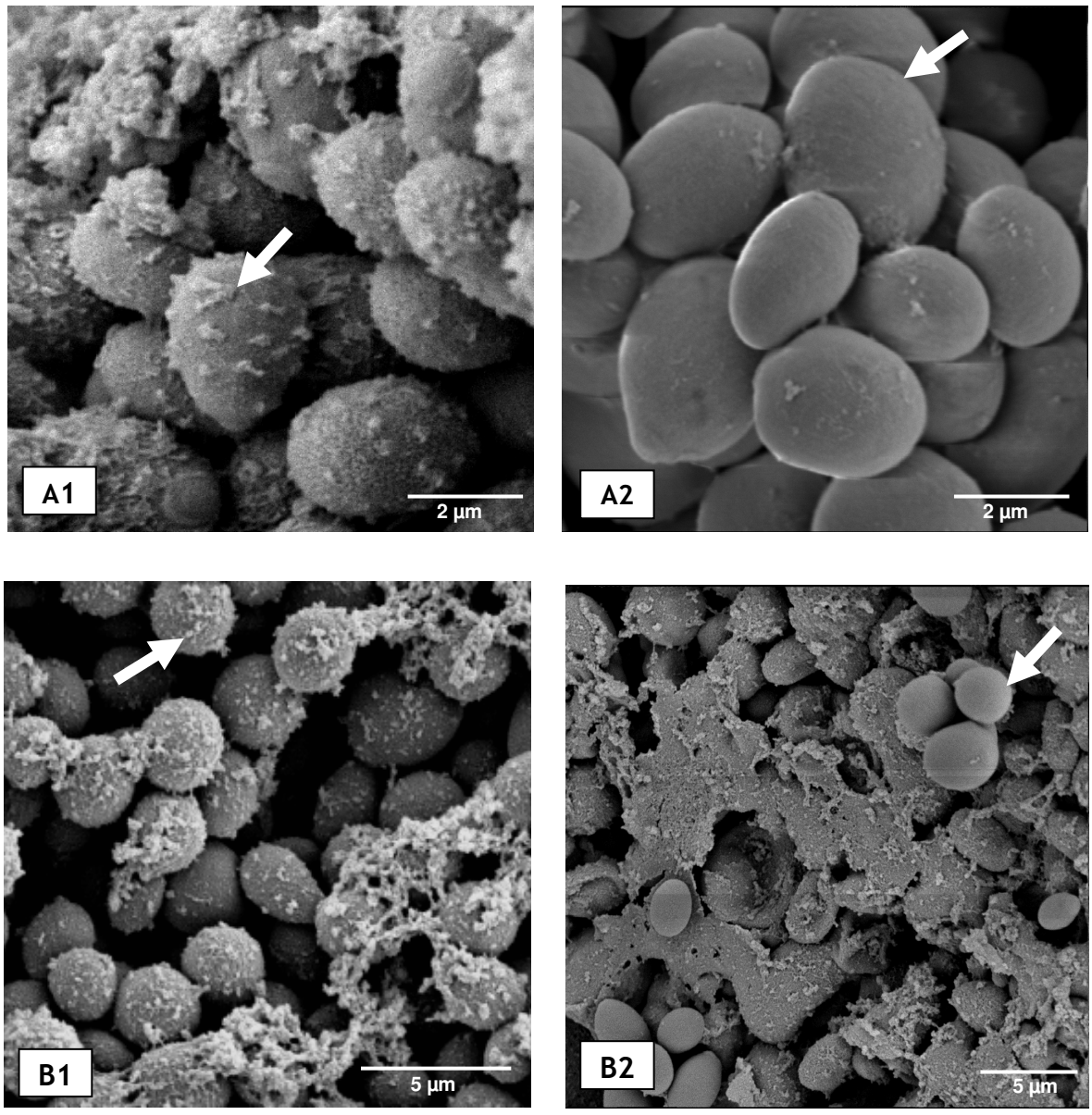


Figure 35. Scanning electron micrographs of the bottom layers of biofilms of *Candida* species exposed to caspofungin

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with caspofungin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of or caspofungin (24 µg/ml) (2). Arrows in A1 and B1 indicate globule-like matrix; arrows in A2 and B2 indicate yeast cells without surface matrix material.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

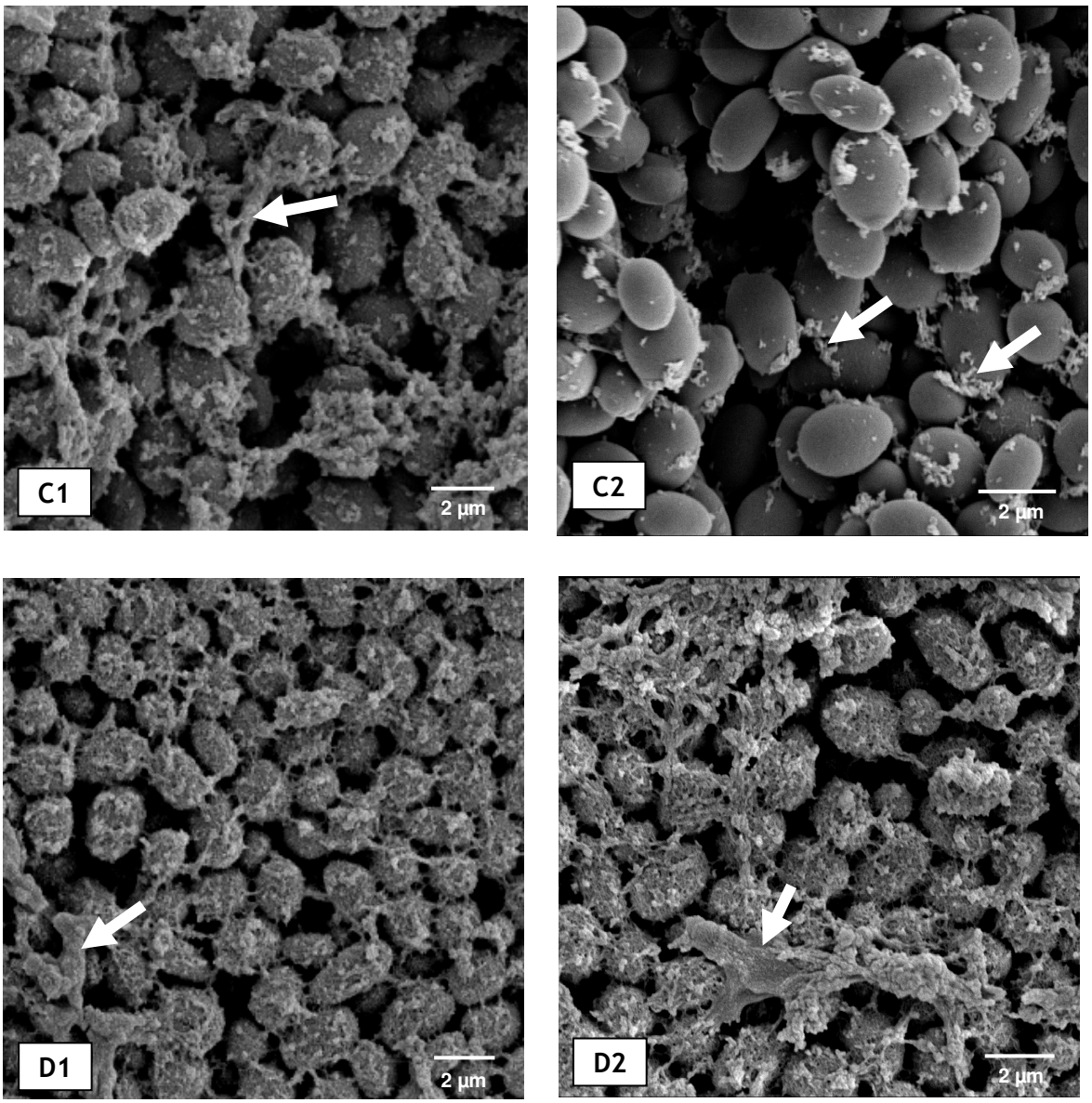


Figure 35

(**continued**)

Arrows in C2 indicate severe destruction of matrix material; arrows in C1 and D indicate matrix material.

C. *C. glabrata*

D. *C. parapsilosis*

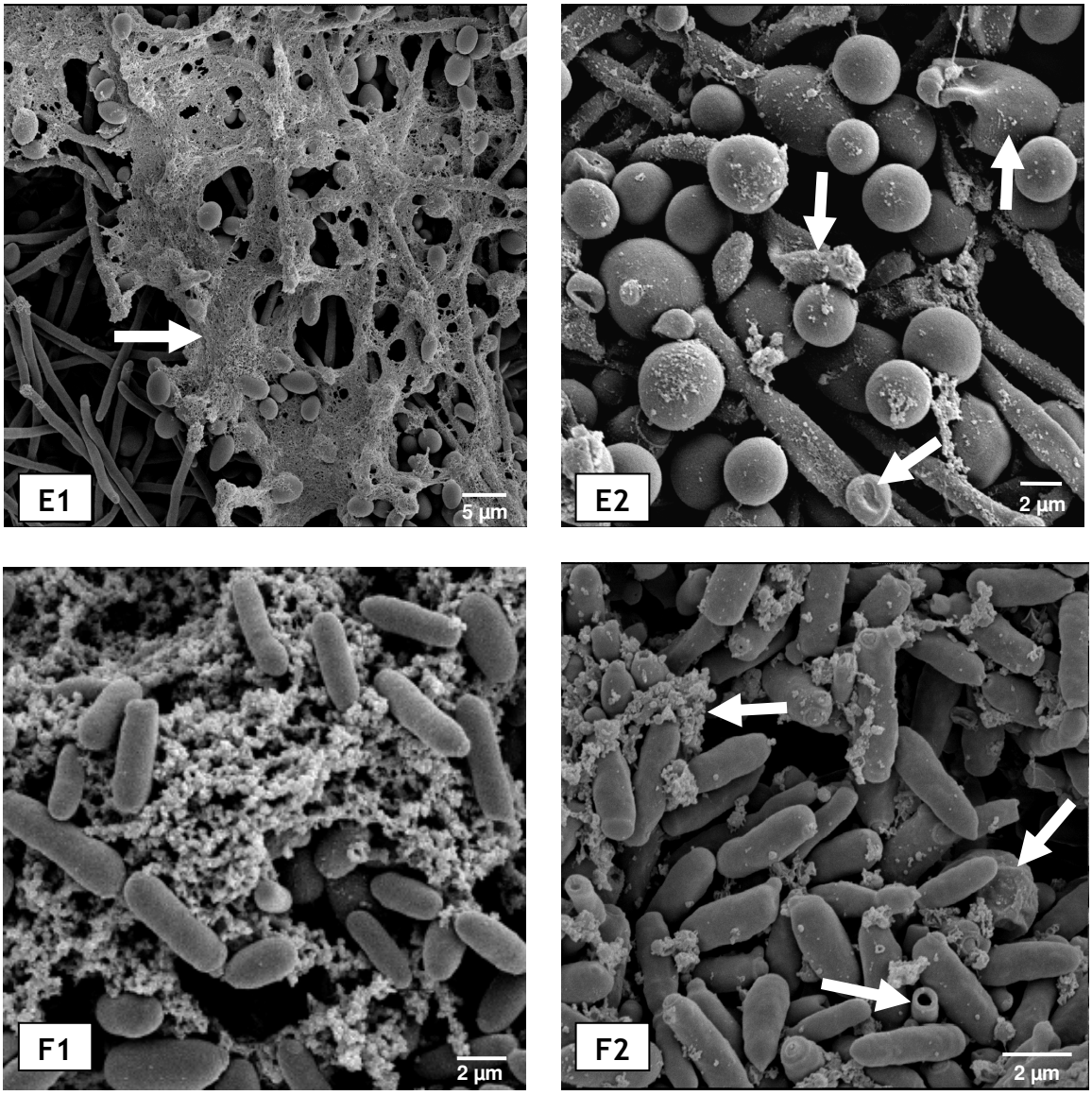


Figure 35

(**continued**)

Arrow in E1 indicates slime-like matrix material; arrows in E2 indicate damaged yeast cells; arrows in F2 indicate severe destruction of the meshwork structure and damaged yeast cells.

E. *C. tropicalis*

F. *C. krusei*

4.5.1.3 The membrane-attached basal region of the biofilm

SEM observations of the membrane-attached basal region of control biofilms showed, in most cases, yeast cells submerged in a dense slime-like material. This was clearly visualized in *C. albicans* GDH2346 (Figs. 36 and 37, A1), *C. albicans* SC5314 (Figs. 36 and 37, B1), *C. glabrata* AAHB12 (Fig. 36 and 37, C1), *C. parapsilosis* (Fig. 36, D1), and *C. krusei* (Figs. 36 and 37, F1). On the other hand, *C. tropicalis* showed a thick meshwork of matrix material connecting cells and attaching them to the surface (Figs. 36 and 37, E1) in addition to the slime-like material (Fig. 36, E1)

After treatment with amphotericin B, the slime-like material which was covering the basal region disappeared in most *Candida* biofilms. The removal of this type of material in *C. albicans* GDH2346 biofilms revealed a thick network of strands that seemed to anchor the cells to the substrate (Fig. 36, A2). The slimy material was also absent in amphotericin-treated biofilms of *C. glabrata* and *C. parapsilosis* but here no strands were apparent (Figs. 36, C2 and D2). On the other hand, *C. albicans* SC5314 biofilms retained a little slimy matrix material (Fig. 36, B2), whereas *C. krusei* biofilms appeared to retain as much intact slimy material as control biofilms (Fig. 36, F2). The basal region of amphotericin-treated *C. tropicalis* biofilms had a thick meshwork of strands connecting cells together and to the substrate (Fig. 36, E2). In some cases, yeast cells at the basal region were elongated, as in *C. glabrata* (Fig.36, C2) or shortened and ballooned as *C. krusei* (Fig. 36, F2).

Caspofungin caused a high degree of damage to biofilm cells as well as damage to the biofilm structure of all six *Candida* isolates. The basal slime-like material was completely removed in all those *Candida* species that appeared to have it (Fig. 37, A2, B2, C2, and F2). The removal of this slimy material revealed a thick network of matrix strands as in *C. albicans* SC5314 (Fig. 37, B2) or short fibrils of matrix material surrounding single yeast cells as in *C. glabrata* (Fig. 37, C2), *C. parapsilosis* (Fig. 37, D2), and *C. krusei* (Fig. 37, F2). The meshwork structure of *C. tropicalis* was highly damaged (Fig. 37, E2). Caspofungin had also caused some damage to the yeast cells at the basal region and cell 'footprints' were found which are presumably due to lysed cells (Fig. 37, C2, D2, F2).

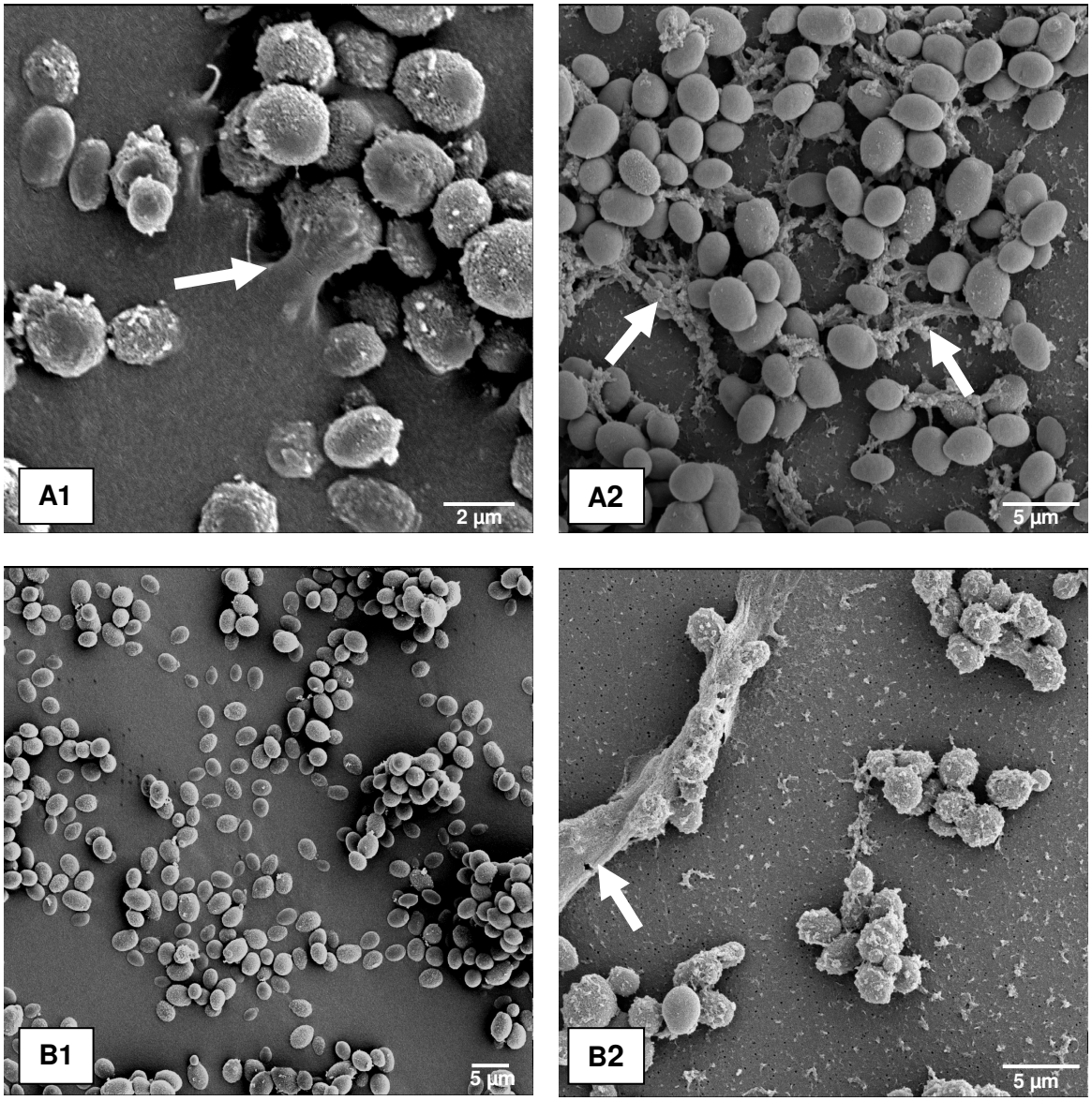


Figure 36. Scanning electron micrographs of the membrane-attached basal region of biofilms of *Candida* species exposed to amphotericin B

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with amphotericin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of amphotericin B (78 µg/ml) (2). Arrow in A1 indicates slime-like matrix material; arrows in A2 indicate thick strands of matrix material; arrow in B2 indicates remaining slime-like material.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

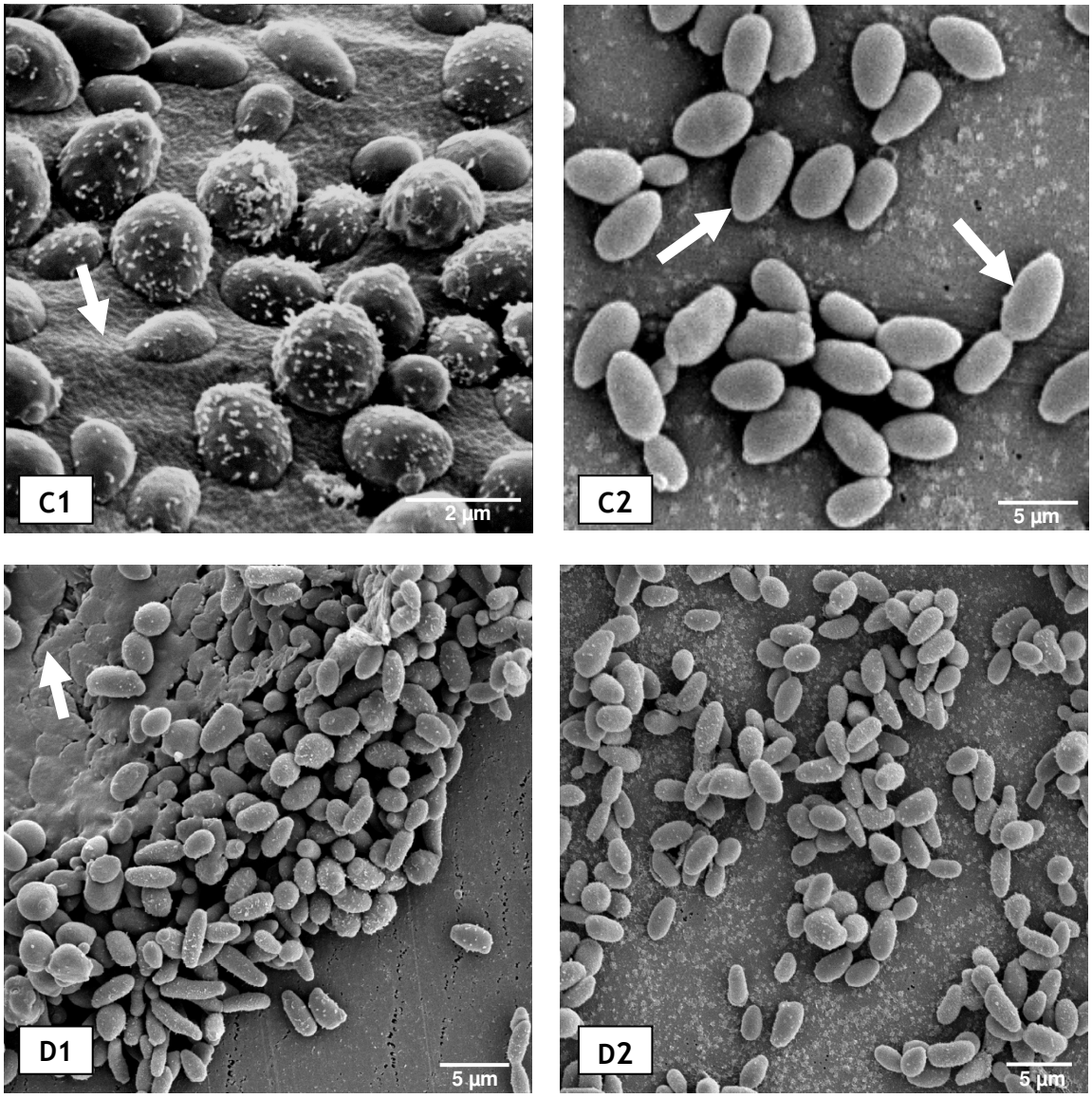


Figure 36

(**continued**)

Arrows in C1 and D1 indicate slime-like material; arrows in C2 indicate elongated yeast cells.

C. *C. glabrata*

D. *C. parapsilois*

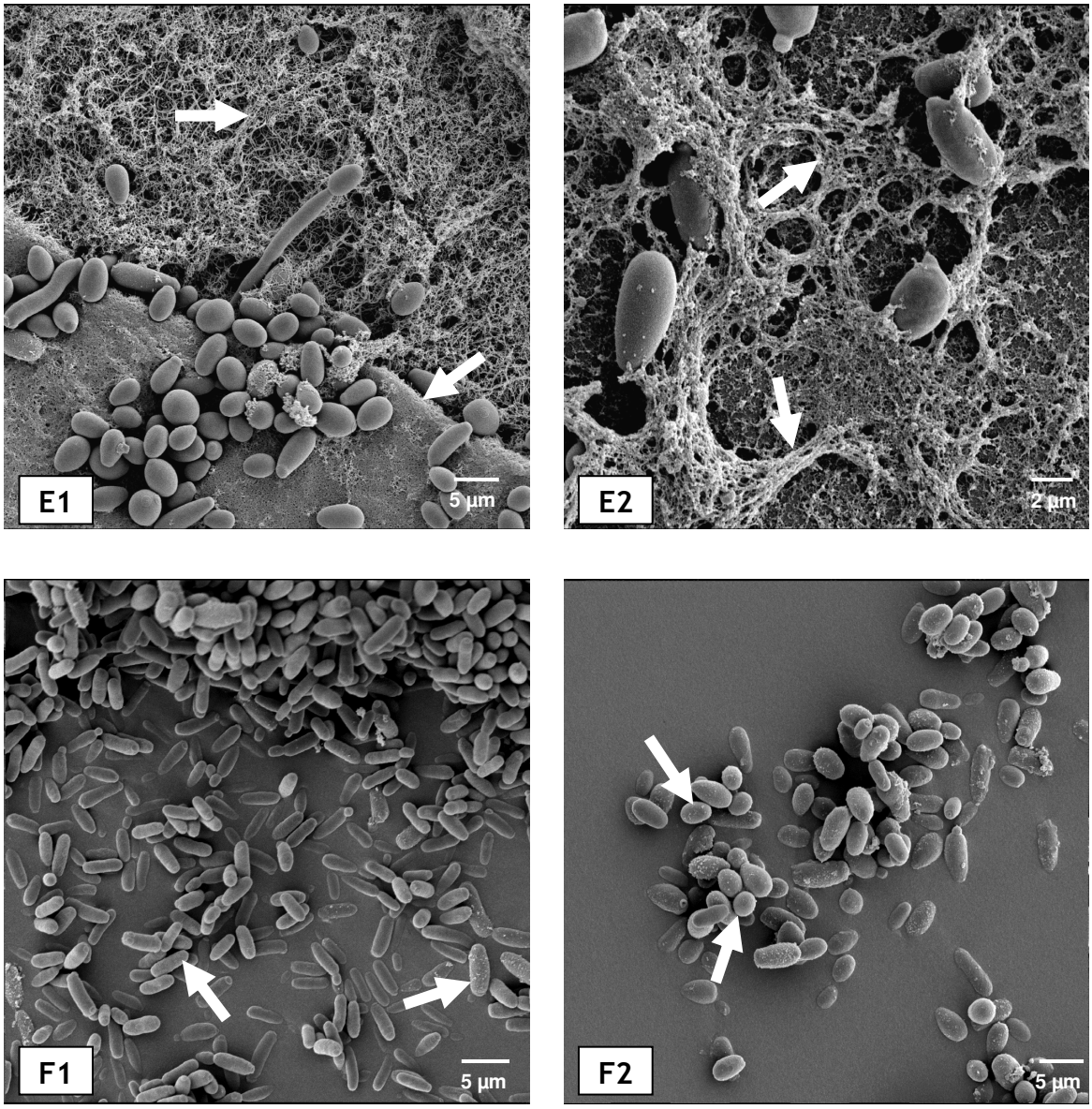


Figure 36

(**continued**)

Arrows in E1 indicate thick meshwork of strands as well as slime-like material; arrows in E2 indicate thick meshwork of strands attached to the surface; arrows in F1 indicate control elongated yeast cell; arrows in F2 indicate shortened, ballooned cells.

E. *C. tropicalis*

F. *C. krusei*

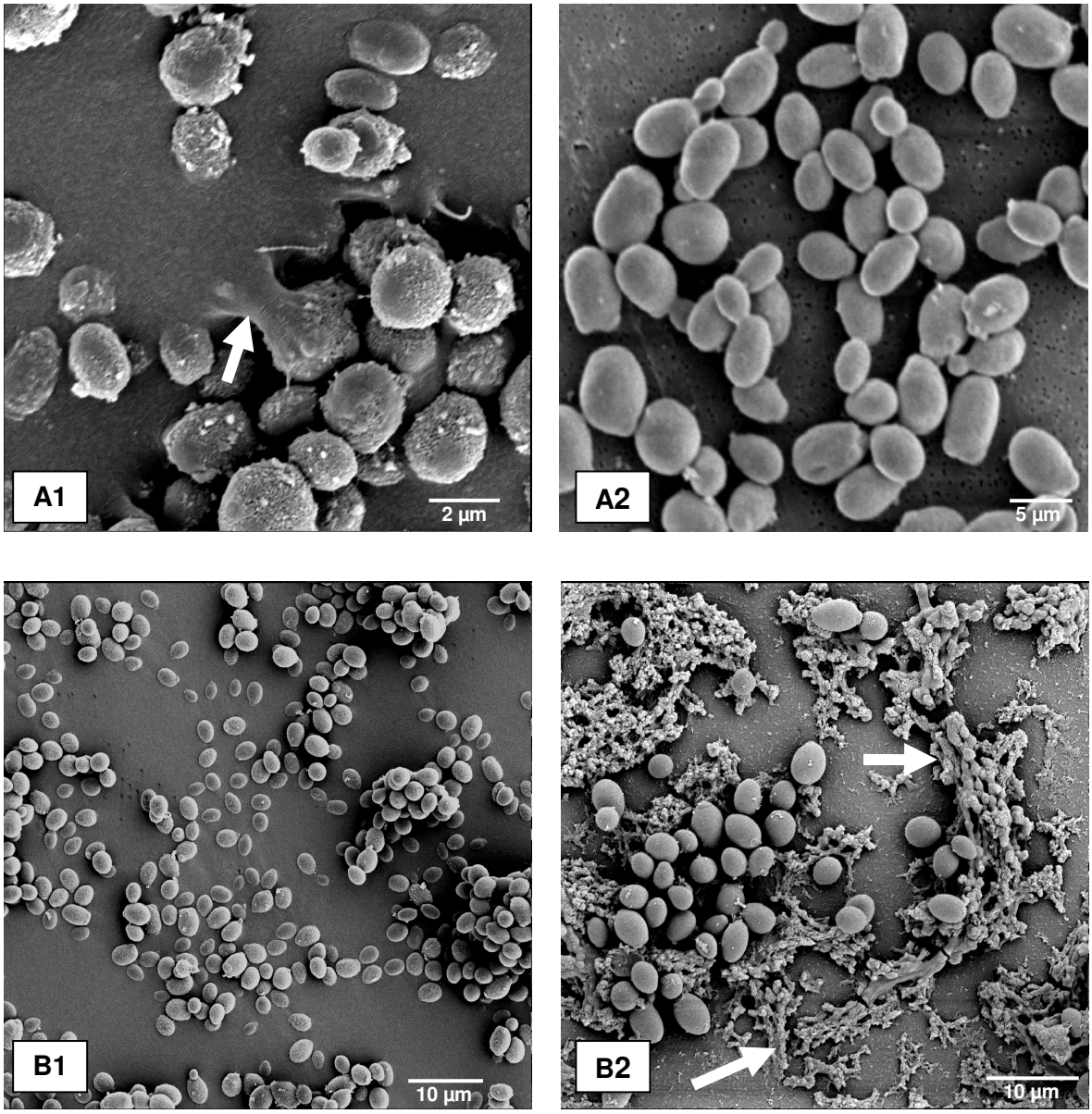


Figure 37. Scanning electron micrographs of the membrane-attached basal region of biofilms of *Candida* species exposed to caspofungin

Biofilms were grown on polycarbonate membrane filters for 48 h, and then treated with caspofungin for 24 h. The figure shows control biofilms (1) and biofilms grown in the presence of or caspofungin (24 µg/ml) (2). Arrow in A1 indicates slime-like matrix material; arrows in B2 indicate a thick network of strands attached to the surface.

A. *C. albicans* GDH2346

B. *C. albicans* SC5314

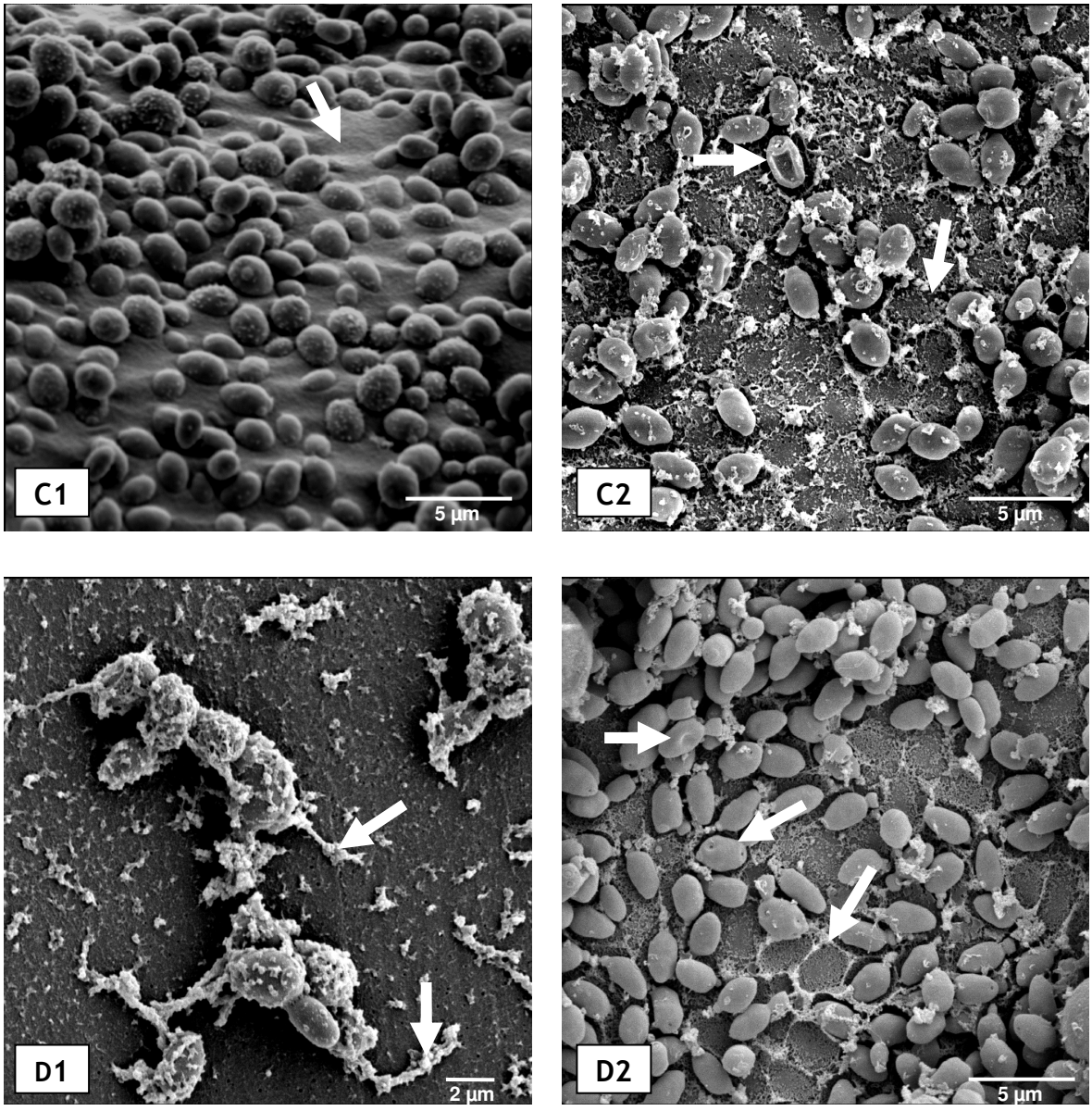


Figure 37

(**continued**)

Arrow in C1 indicates slime-like material; arrows in C2 and D2 indicate damaged yeast cells and 'cell footprints'; arrows in D1 indicate strands of matrix material attached to the surface.

C. *C. glabrata*

D. *C. parapsilosis*

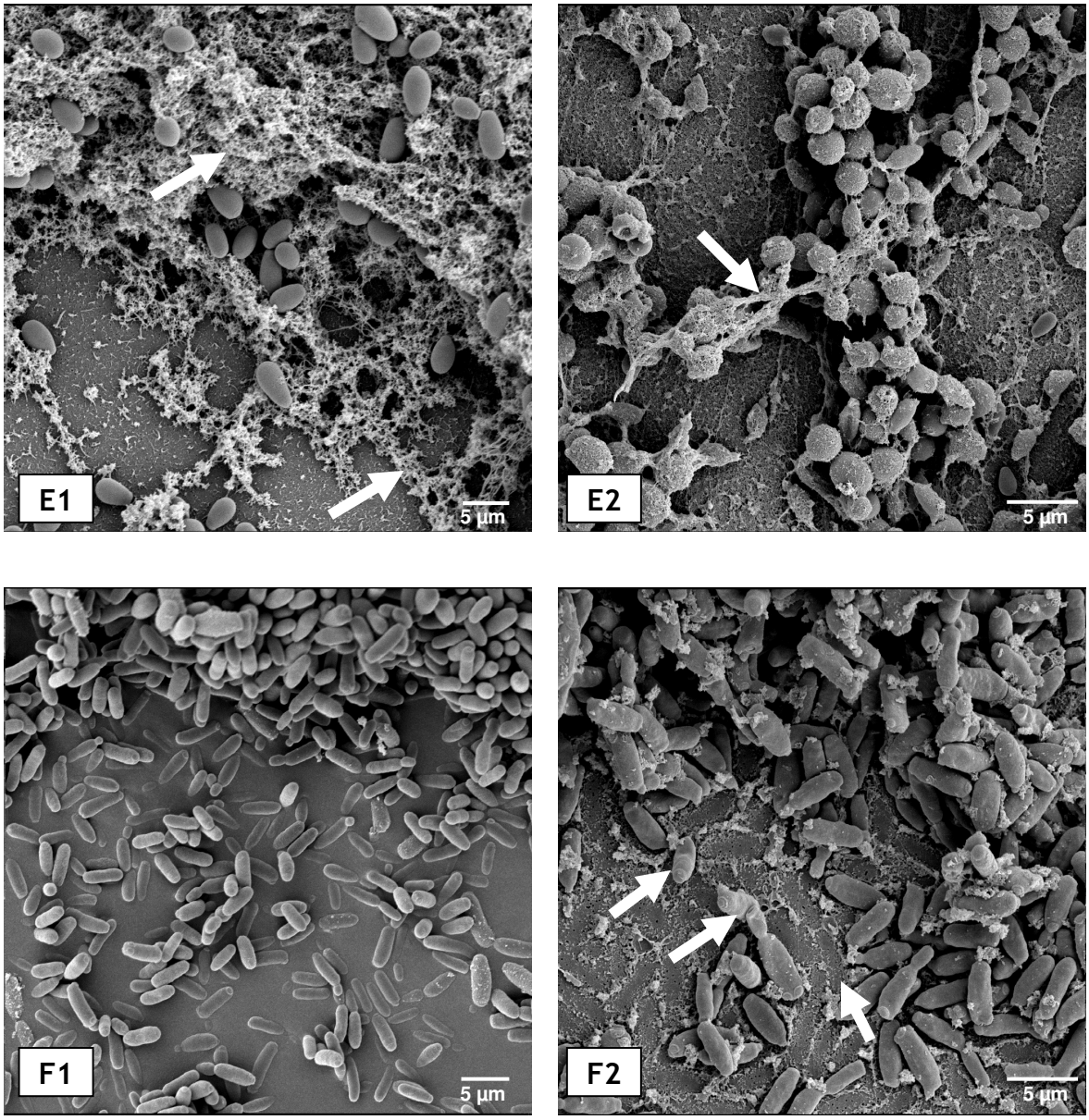


Figure 37

(**continued**)

Arrows in E1 and E2 indicate thick meshwork of strands of matrix material; arrows in F2 indicate damaged yeast cells and 'cell footprints'.

E. *C. tropicalis*

F. *C. krusei*

In summary, cells in both the membrane-attached basal region and the bottom surface of the biofilms (close to the drug-containing agar surface) were more affected by antifungal drugs than those at the top surface, which were more likely to be found intact. Caspofungin caused more damage to the biofilm structure and biofilm cell morphology than did amphotericin B. Moreover, the highest degree of damage overall was caused by caspofungin, and was observed in biofilms of *C. krusei* and *C. glabrata*.

5 Persister cells in planktonic cultures and biofilms of different *Candida* species

Planktonic cultures (exponential and stationary-phase) and biofilms (grown on catheter discs for 48 h) of six isolates (i.e. *C. albicans* GDH2346, *C. albicans* SC5314, *C. glabrata* AAHB12, *C. parapsilosis* AAHB4479, *C. krusei* Glasgow and *C. tropicalis* AAHB73) were exposed to different concentrations of antifungals (amphotericin B and caspofungin; 5 μg – 100 μg/ml) for 24 h and examined for the presence of persister cells using standard viable cell counts. Caspofungin was tested against both strains of *C. albicans* but showed limited effects on stationary-phase cells and biofilms, producing at most only a 10-fold decrease in viability (Figs. 38 and 39). Planktonic cells in exponential growth phase were rather more susceptible to caspofungin (Figs. 38 and 39). On the basis of these preliminary studies it was decided that caspofungin was an unsuitable antifungal agent for use in investigations on persister cells, and further experiments were carried out with amphotericin B only.

5.1 Persister cells in planktonic cultures of *Candida* species

5.1.1 Persister cells in planktonic cultures of *C. albicans* strains

Planktonic cells of strain GDH2346, in both exponential and stationary growth phases were effectively killed by a low concentration of amphotericin B (5 μg/ml) with no detectable survivors (Fig. 40); the MIC for this strain is 1.3 μg/ml. Planktonic cells of strain SC5314 in exponential and stationary growth

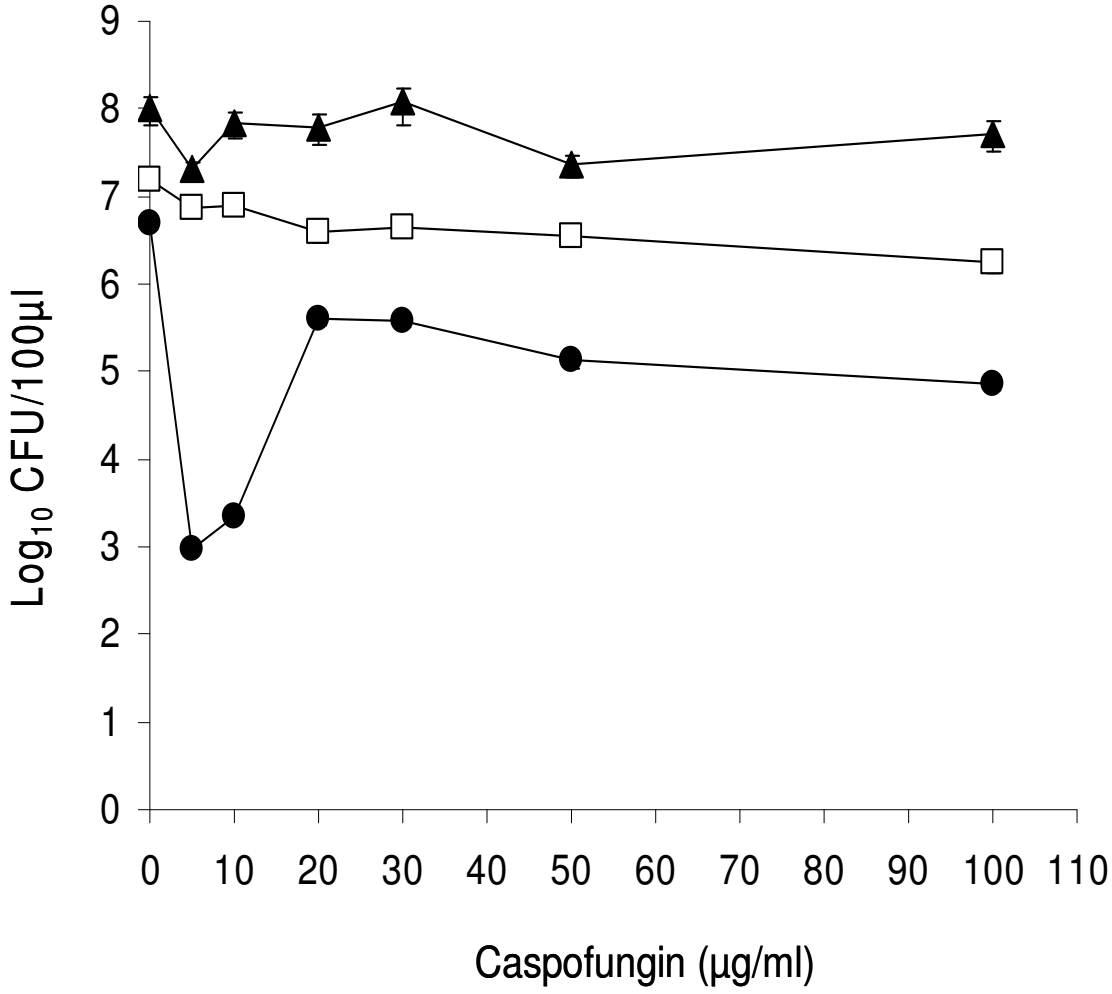


Figure 38. Survival of biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) of *C. albicans* GDH2346 exposed to different concentrations of caspofungin

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and caspofungin at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase planktonic cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

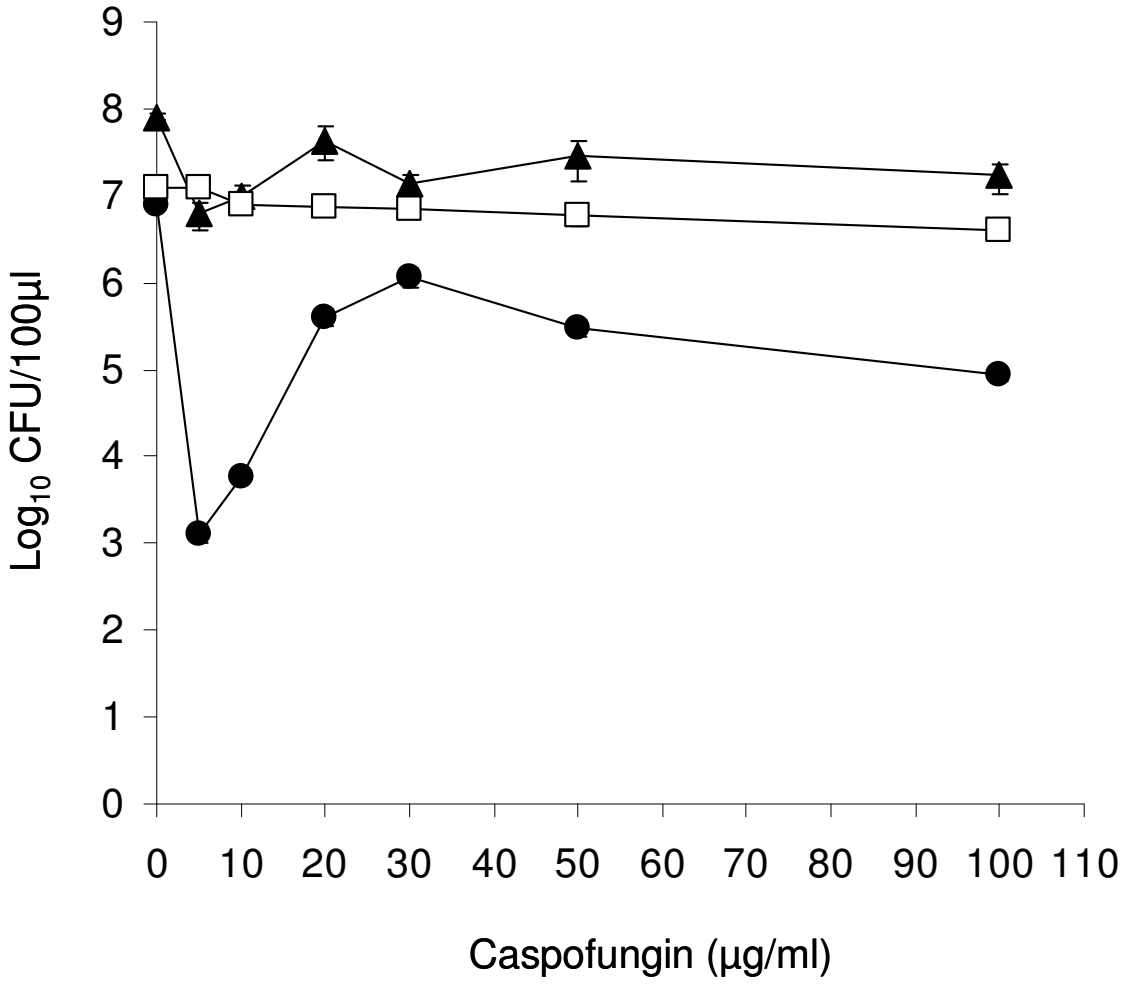


Figure 39. Survival of biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) of *C. albicans* SC5314 exposed to different concentrations of caspofungin

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and caspofungin at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase planktonic cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

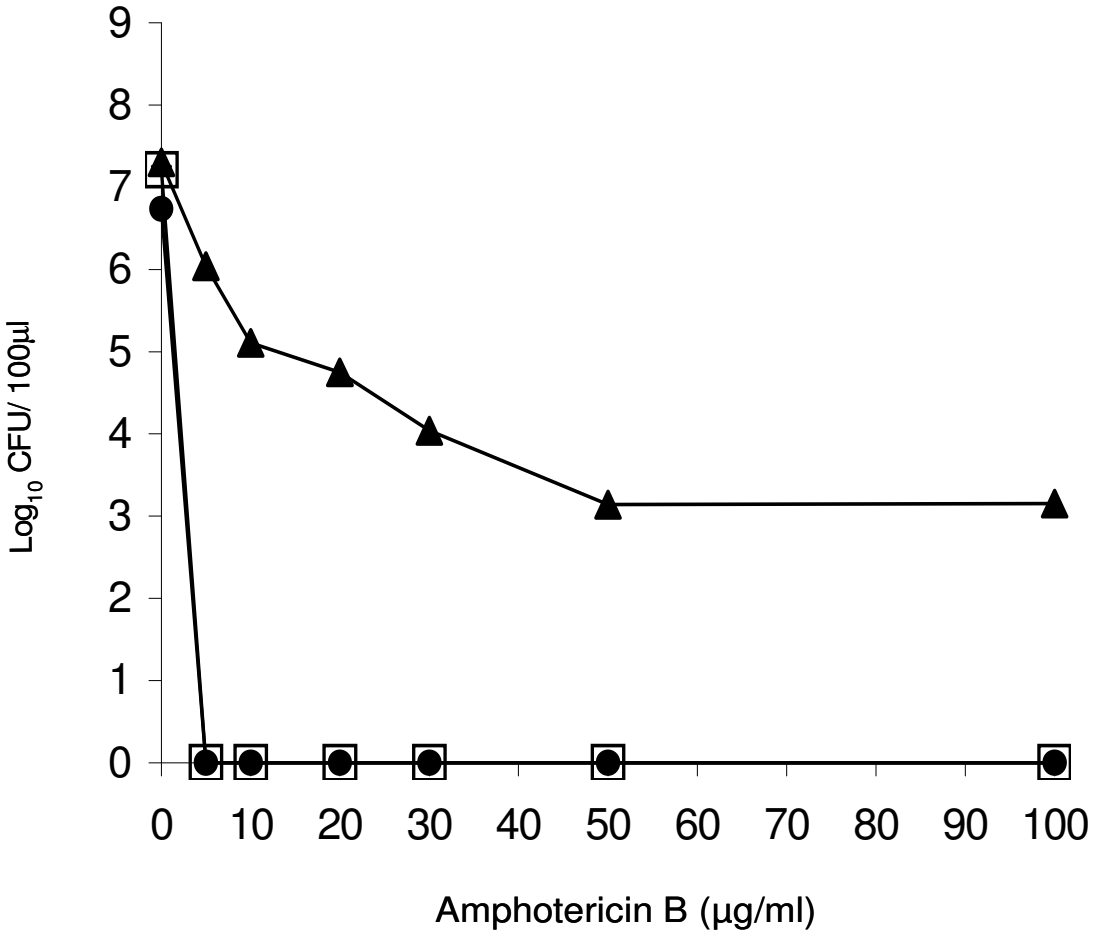


Figure 40. Survival of biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) of *C. albicans* GDH2346 exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

phases were also completely eliminated at this low concentration of amphotericin B (Fig. 41). The MIC for strain SC5314 is 0.8 μg/ml.

5.1.2 Persister cells in planktonic cultures of non-*C. albicans* species

Planktonic cells of non-*C. albicans* species were also susceptible to amphotericin B. Exponentially growing cells of *C. glabrata, C. tropicalis* and *C. krusei* were killed by amphotericin B at a concentration of 5 μg/ml (Figs. 42-44) whereas those of *C. parapsilosis* were relatively more resistant (Fig. 45). Stationary-phase cells of *C. tropicalis*, *C. krusei* and *C. parapsilosis* were less susceptible than exponentially growing cells (Figs. 43-45), while stationary-phase cells of *C. krusei* showed a distinct tolerance to amphotericin B (Fig. 44). Overall, there was no indication of persisters among planktonic cells of any *Candida* species tested since all of these cells were eliminated completely at an amphotericin B concentration of 100 μg/ml.

5.2 Persister cells in biofilms of *Candida* species

5.2.1 Persister cells in biofilms of *C. albicans* strains

Unlike planktonic cells, biofilms of *C. albicans* GDH2346 seemed to contain a small fraction (0.01 %) of cells tolerant to amphotericin B at concentration of 100 μg/ml (Fig. 40), i.e. drug-tolerant persister cells. Unexpectedly, biofilms of *C. albicans* SC5314 appeared to lack such tolerant cells as no cells survived exposure to 100 μg/ml amphotericin B (Fig. 41). Nevertheless, in this strain, as in strain GDH2346, biofilm cells were more drug resistant than planktonic cells.

5.2.2 Persister cells in biofilms of non-*C. albicans* species

Although biofilms of all four non-*C. albicans* species were considerably more resistant to amphotericin B than planktonic cells, only biofilms of *C. krusei* and *C. parapsilosis* gave biphasic killing curves indicative of the presence of persisters (Figs. 44 and 45). These biofilms, unlike those of *C. glabrata* and *C. tropicalis*, still showed some viability even after exposure to a drug concentration of 100 μg/ml. However, for both species the persister population

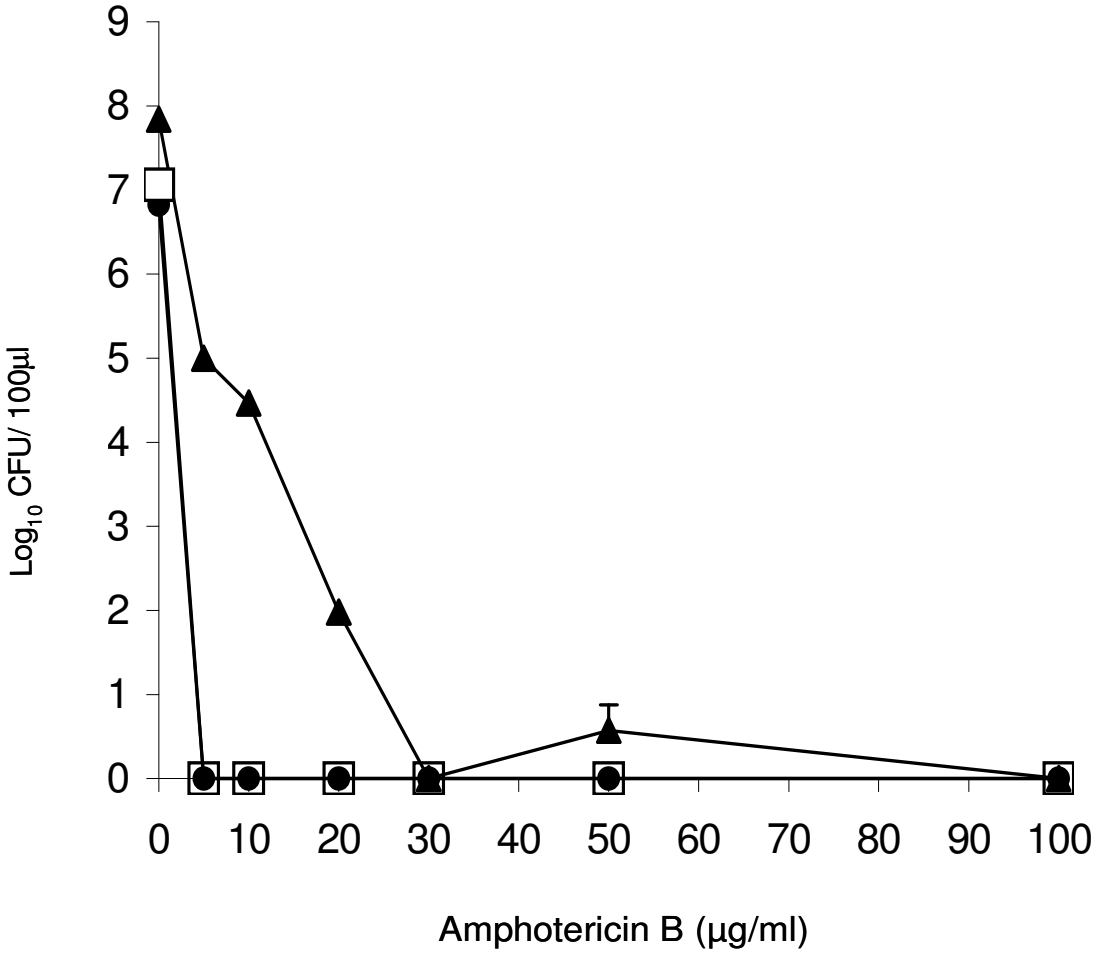


Figure 41. Survival of biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) of *C. albicans* SC5314 exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

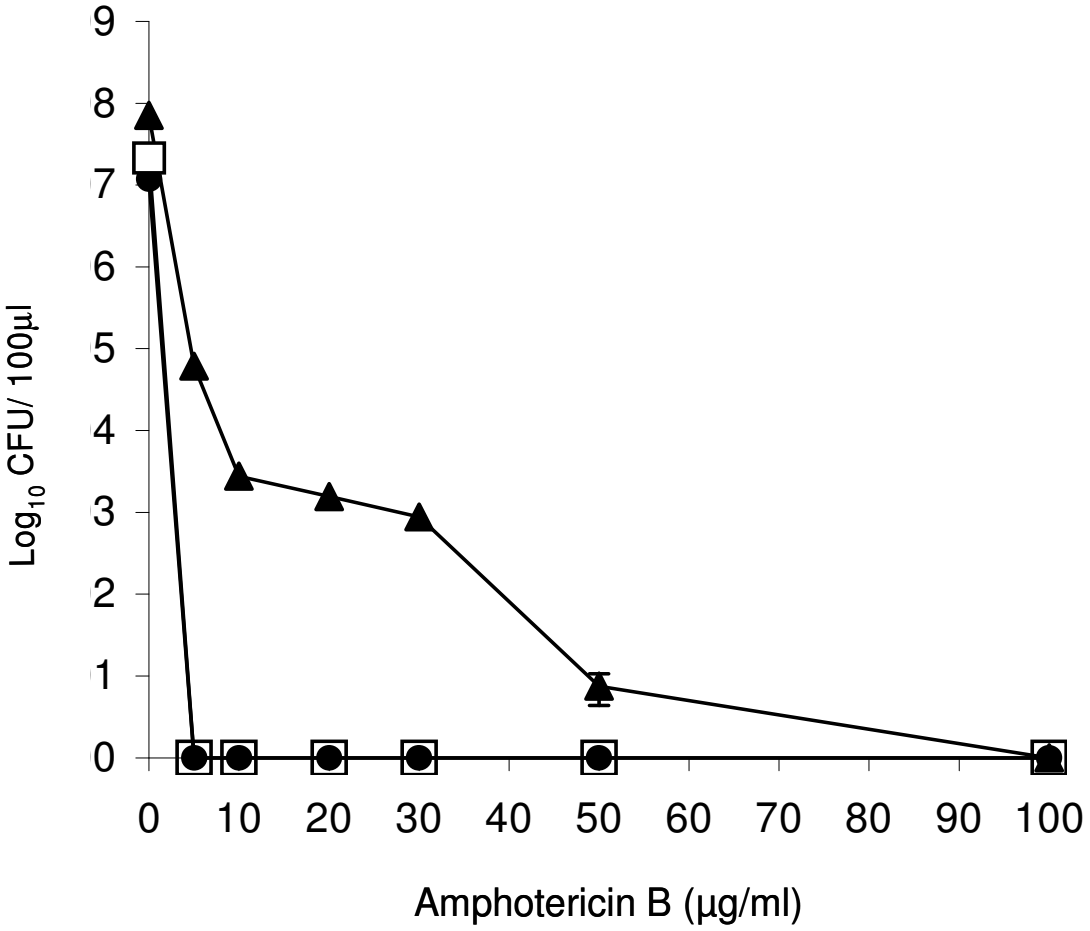


Figure 42. Survival of *C. glabrata* biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

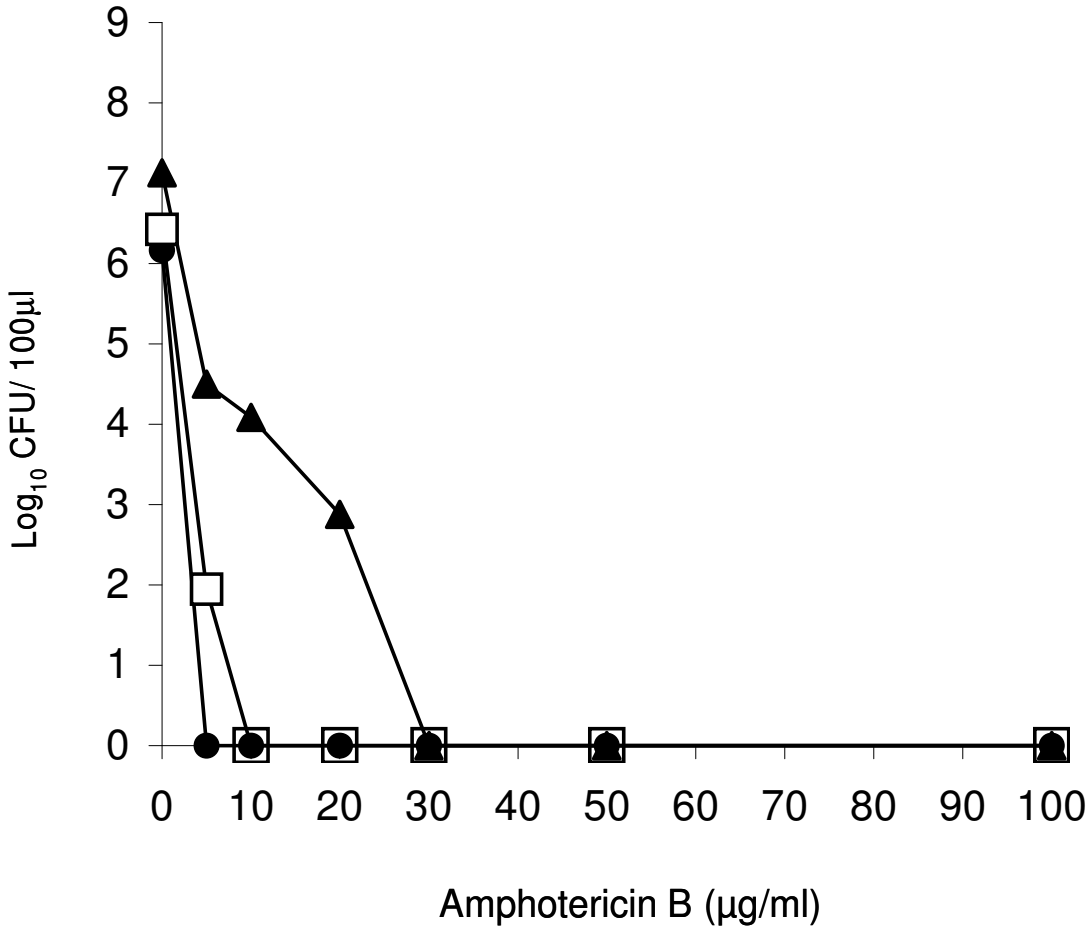


Figure 43. Survival of *C. tropicalis* biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

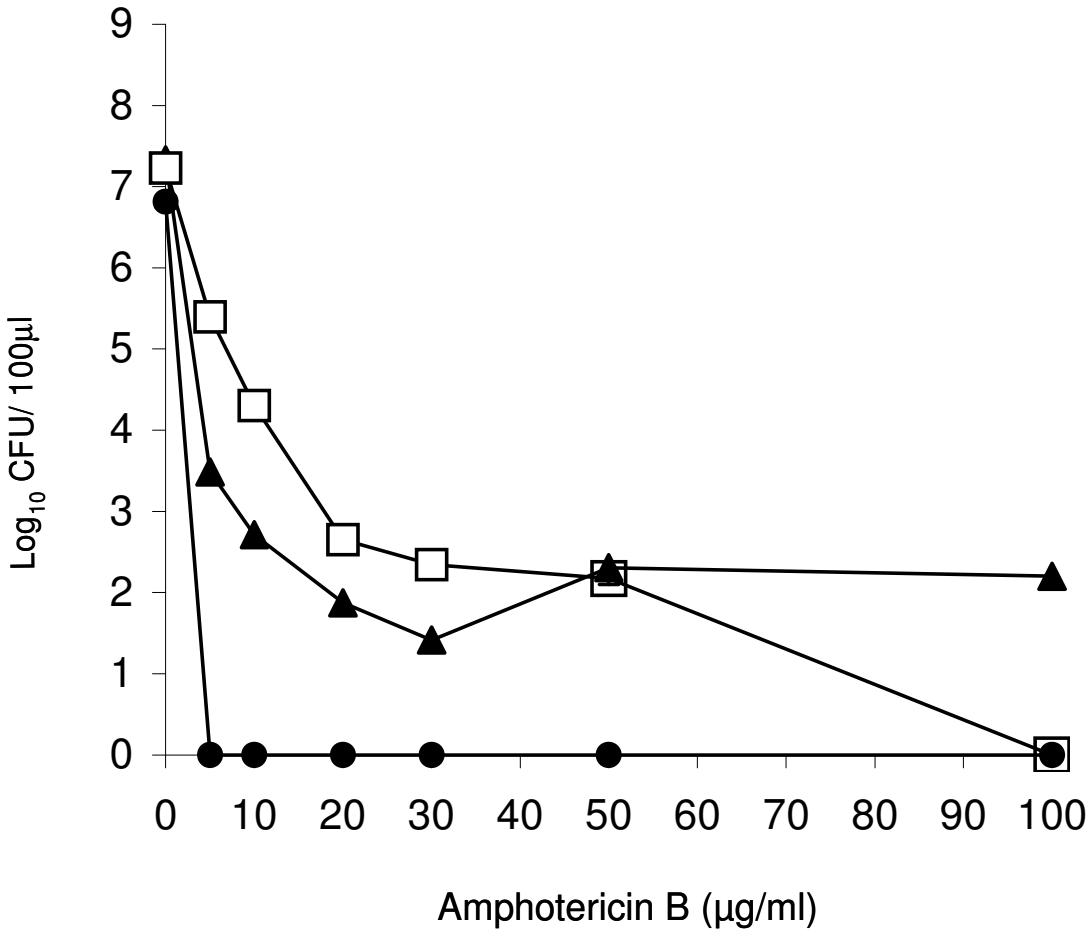


Figure 44. Survival of *C. krusei* biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

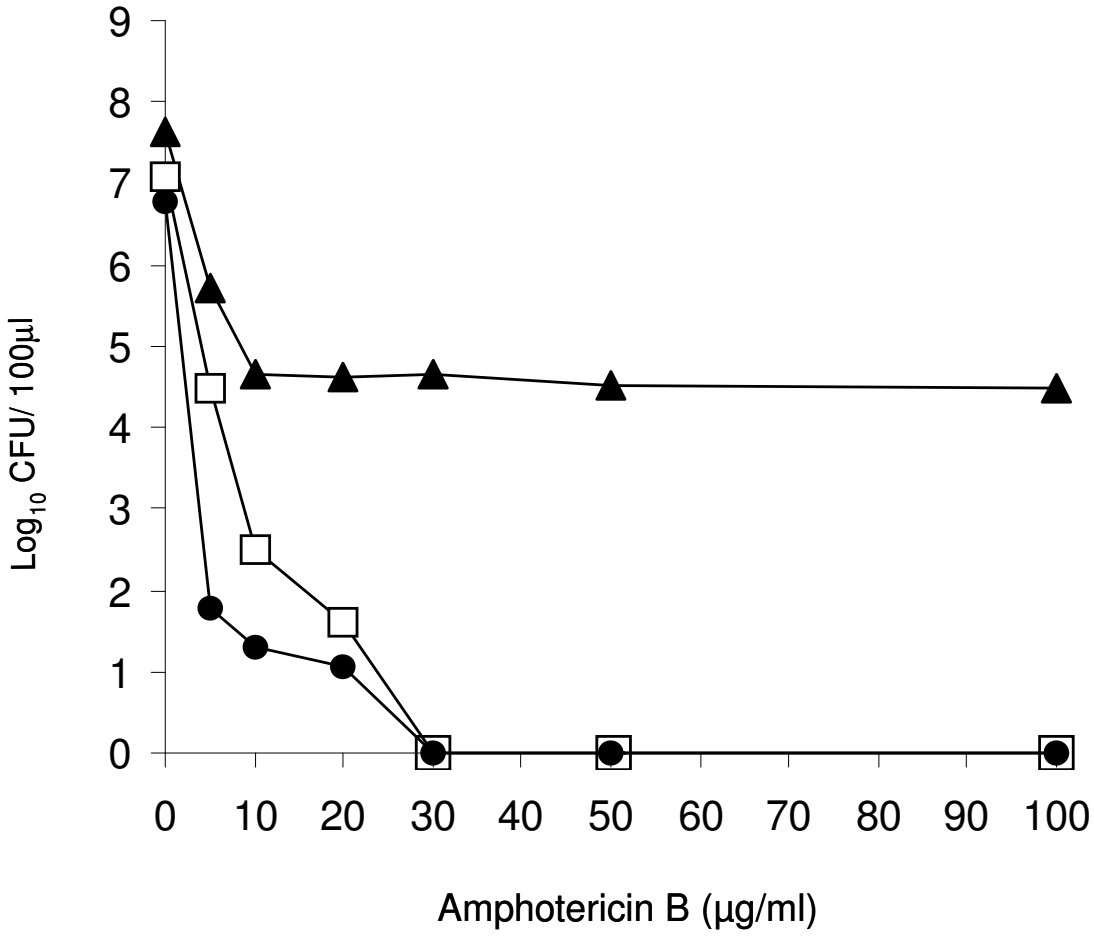


Figure 45. Survival of *C. parapsilosis* biofilm cells (▲), planktonic exponential-phase cells (●), and planktonic stationary-phase cells (□) exposed to different concentrations of amphotericin B

Biofilms were grown on PVC catheter discs for 48 h in YNB medium containing 50 mM glucose and amphotericin B at the concentration indicated, and then processed for viable cell counts. Exponential- and stationary-phase cultures were grown in the same medium. Results are means ± standard errors of two independent experiments carried out in duplicate.

was small, representing approximately 0.001% and 0.07% of the total cell count of biofilms of *C. krusei* and *C. parapsilosis*, respectively. *C. tropicalis* and *C. glabrata* biofilm cells were eliminated at amphotericin concentrations of 30 μg/ml and 100 μg/ml, respectively (Figs. 42 and 43).

5.3 Live-dead staining of persister cells

To confirm the existence of persister cells in some *Candida* isolates but not in others, a live-dead staining procedure with fluorescein diacetate was used (LaFleur *et al.*, 2006).

5.3.1 Staining with fluorescein diacetate

Fluorescein diacetate was used to discriminate between live and dead yeast cells; this dye specifically stains dead yeast cells with a bright green fluorescence. Mature (48-h) *Candida* biofilms (*C. albicans* GDH2346 and SC5314, *C. krusei* Glasgow, and *C. parapsilosis* AAHB4479) were treated with a high concentration of amphotericin B (100 μg/ml) for 24 h at 37°C and stained with fluorescein diacetate. A small number of unstained cells (live) were detected in three of the isolates. These cells appeared to have normal morphology similar to that of untreated control cells and their numbers varied among the different *Candida* species. *C. parapsilosis* biofilms (Fig. 46) contained more of the live cells than did biofilms of *C. albicans* GDH2346 or *C. krusei* (Figs. 47 and 48, respectively). In contrast, *C. albicans* SC5314 failed to show live cells (Fig. 49). With all four isolates, fluorescence staining was rarely observed in untreated control cells (i.e. 72-h biofilm cells). These findings further confirm the earlier viability measurements which showed that persisters were present in biofilms of *C. albicans* GDH2346, *C. krusei*, and *C. parapsilosis* but absent from biofilms of *C. albicans* SC5314.

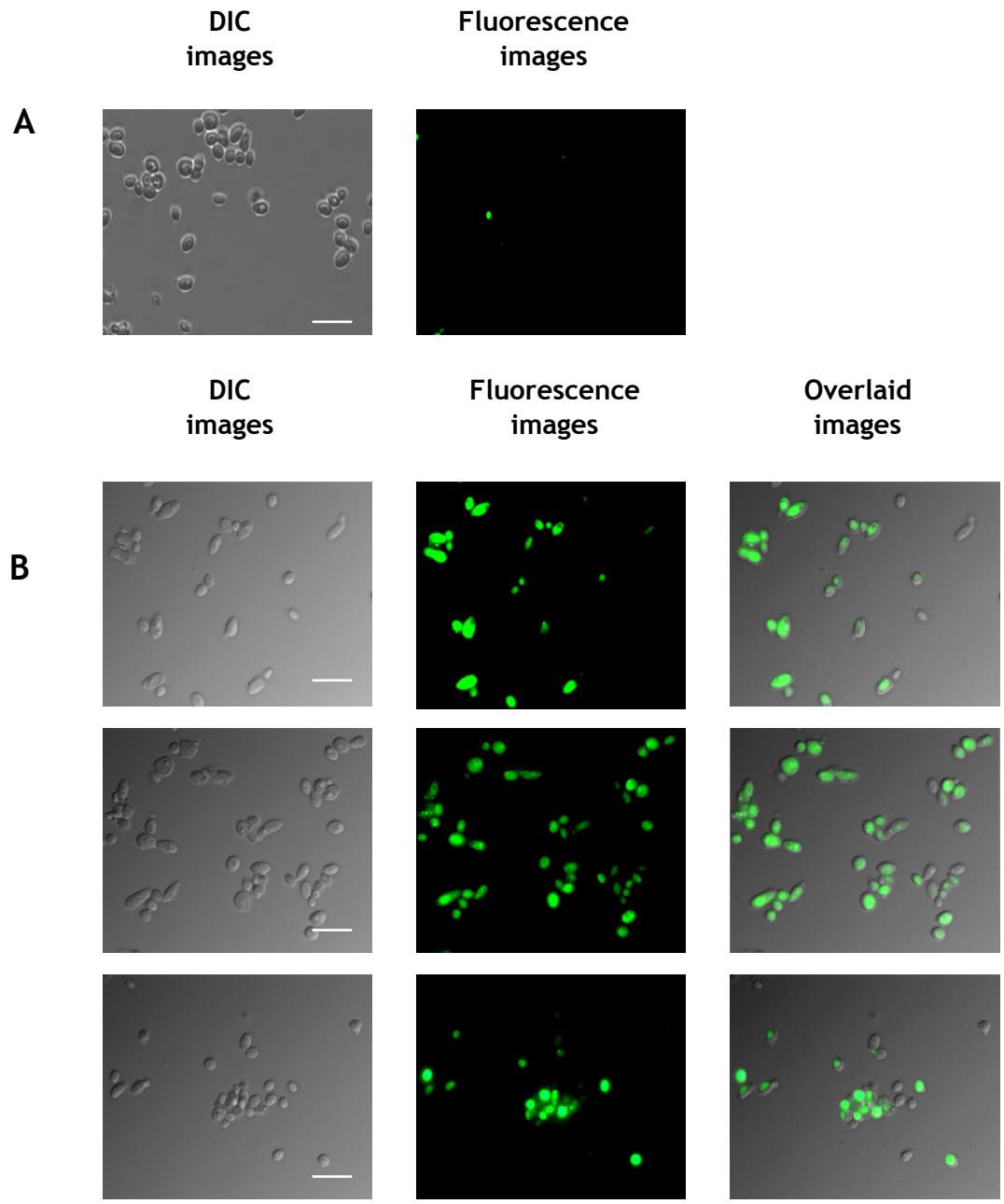


Figure 46. Live-dead staining of *C. parapsilosis* biofilm cells with fluorescein diacetate

Biofilms (48-h) were incubated with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) for 24 h at 37ºC. Washed, resuspended biofilm cells were then examined by fluorescence microscopy. Differential interference contrast (DIC), fluorescence, and overlaid images are shown of untreated biofilm cells (A) and biofilm cells treated with amphotericin B (B). Dead cells fluoresce green. Bar, 13 µm

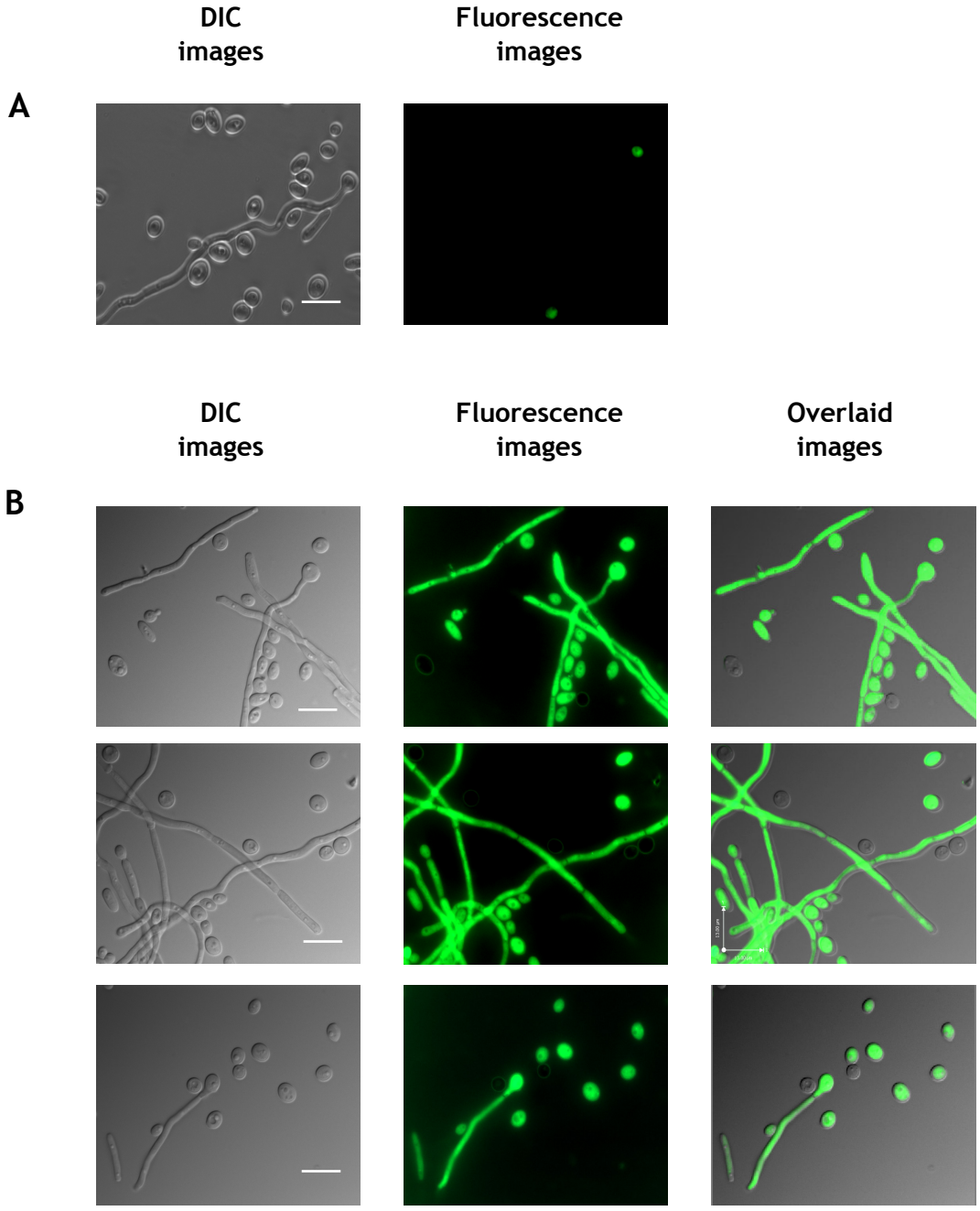


Figure 47. Live-dead staining of *C. albicans* GDH2346 biofilm cells with fluorescein diacetate

Biofilms (48-h) were incubated with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) for 24 h at 37ºC. Washed, resuspended biofilm cells were then examined by fluorescence microscopy. Differential interference contrast (DIC), fluorescence, and overlaid images are shown of untreated biofilm cells (A) and biofilm cells treated with amphotericin B (B). Dead cells fluoresce green. Bar, 13 µm

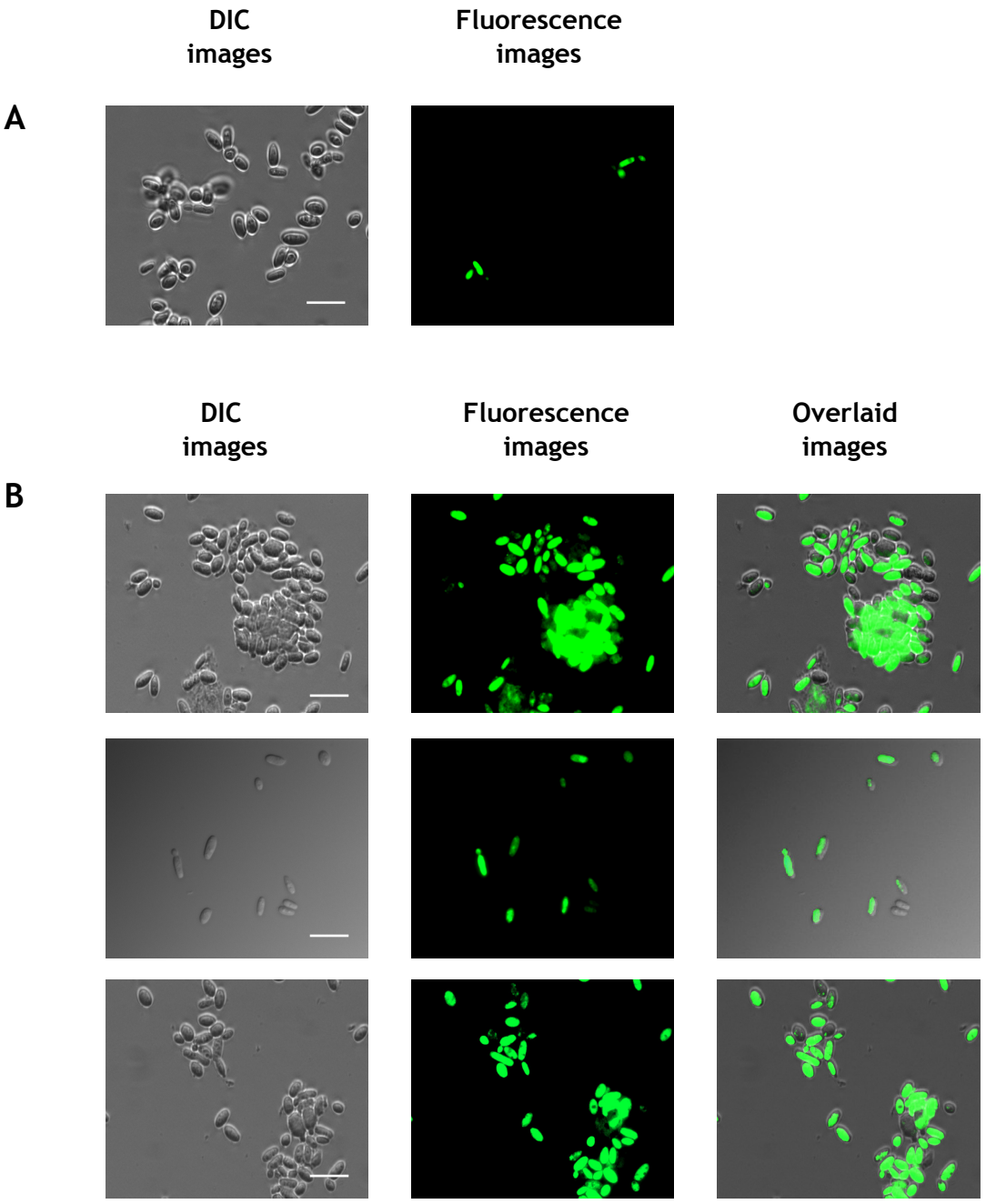


Figure 48. Live-dead staining of *C. krusei* biofilm cells with fluorescein diacetate

Biofilms (48-h) were incubated with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) for 24 h at 37ºC. Washed, resuspended biofilm cells were then examined by fluorescence microscopy. Differential interference contrast (DIC), fluorescence, and overlaid images are shown of untreated biofilm cells (A) and biofilm cells treated with amphotericin B (B). Dead cells fluoresce green. Bar, 13 µm

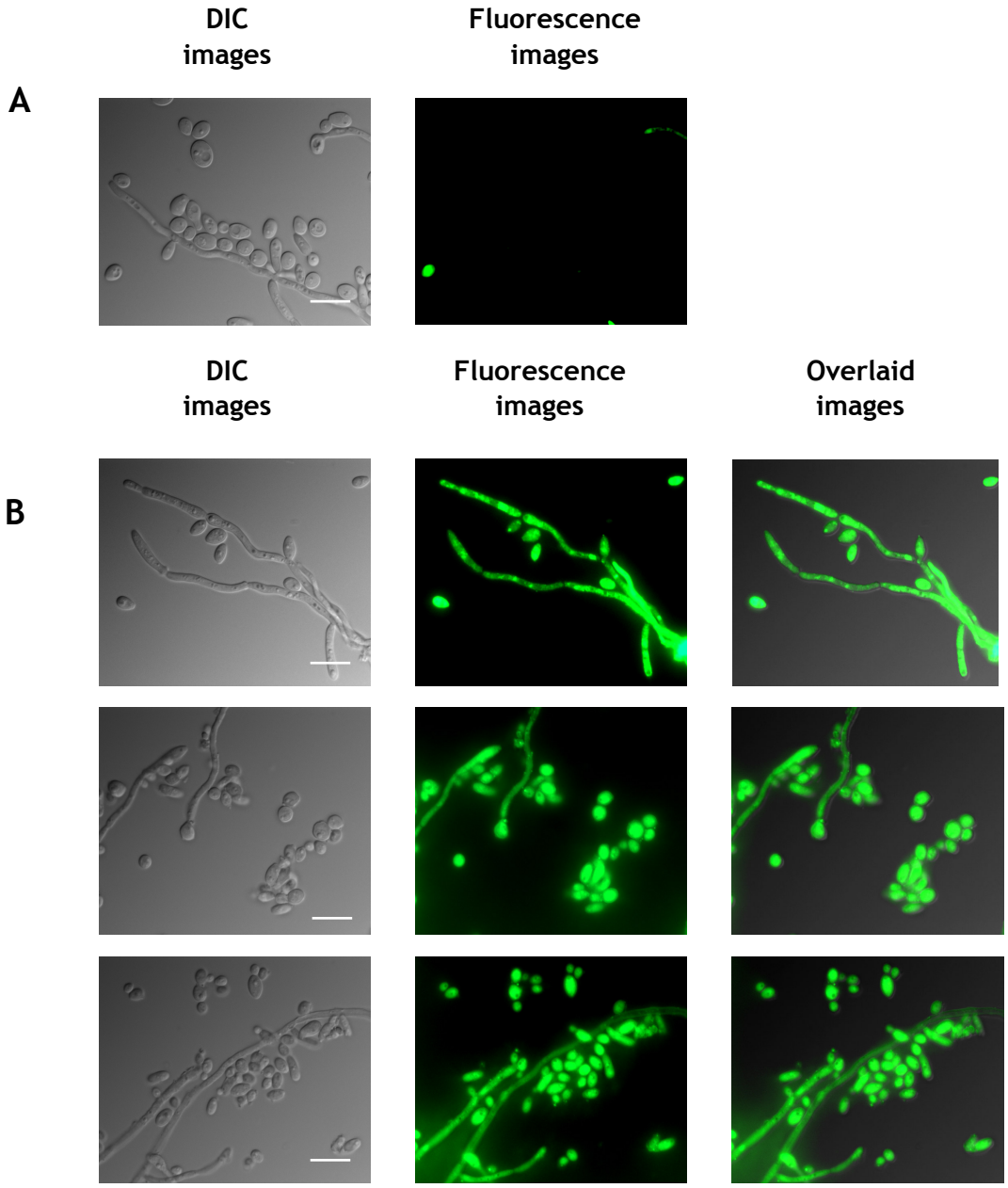


Figure 49. Live-dead staining of *C. albicans* SC5314 biofilm cells with fluorescein diacetate

Biofilms (48-h) were incubated with amphotericin B (100 µg/ml) and fluorescein diacetate (100 µg/ml) for 24 h at 37ºC. Washed, resuspended biofilm cells were then examined by fluorescence microscopy. Differential interference contrast (DIC), fluorescence, and overlaid images are shown of untreated biofilm cells (A) and biofilm cells treated with amphotericin B (B). Dead cells fluoresce green. Bar, 13 µm

6 Apoptosis in *Candida* biofilms

6.1 Detection of apoptotic cells in *Candida* biofilms

6.1.1 Caspase detection using a polycaspase SR-FLICA reagent

The FLICA reagent (for fluorochrome-labelled inhibitor of caspase) was used to detect the presence of caspases in *Candida* biofilm cells. When the reagent enters a cell, any active caspases present will bind covalently to the peptide inhibitor sequence of FLICA (VAD). As a result, the fluorescent label, sulforhodamine (SR) is retained within the cell which fluoresces red. Here, 48-h biofilms of *C. krusei*, *C. parapsilosis* and both strains of *C. albicans* were treated with amphotericin B (10 or 50 µg/ml) for 5 h or 24 h. The cells were then labelled with the reagent and viewed under a fluorescence microscope.

Exposure of biofilms of *C. albicans* SC5314 to amphotericin B at a concentration of 10 µg/ml for 5 h resulted in relatively few cells that were stained with FLICA reagent (Fig. 50, A2) while biofilms exposed to a higher concentration of AMB (i.e. 50 µg/ml) showed slightly more stained cells, indicating a higher level of caspase activity (Fig. 50, A3). Considerably more stained cells resulted after 24 h of exposure (Fig. 50, B2 and 3). Similarly, exposing biofilms of *C. albicans* GDH2346 to amphotericin B at a concentration of 50 µg/ml for 5 h resulted in relatively few cells that were stained with the FLICA reagent (Fig. 51, A2 and 3). Again, considerably more stained cells (93%) were seen in biofilms that had been exposed to the drug for 24 h (Fig. 51, B2 and 3), suggesting an ongoing process of apoptosis induction. After such treatment, higher numbers of apoptotic cells appeared to be present in biofilms of *C. albicans* (both strains) and *C. krusei* (Fig. 52, A2 and 3) than in biofilms of *C. parapsilosis* (Fig. 52, B2 and 3). Some cells were faint red which may indicate a lower concentration of active caspase; these cells were probably just beginning to enter apoptosis at the time the reagent was added. Unstained, non-apoptotic cells appeared to be intact and had a morphology similar to that of untreated control cells.

Pre-treatment of biofilm cells with unlabelled caspase inhibitor Z-VAD-FMK for 1h before the addition of FLICA failed to block the caspase-like protease

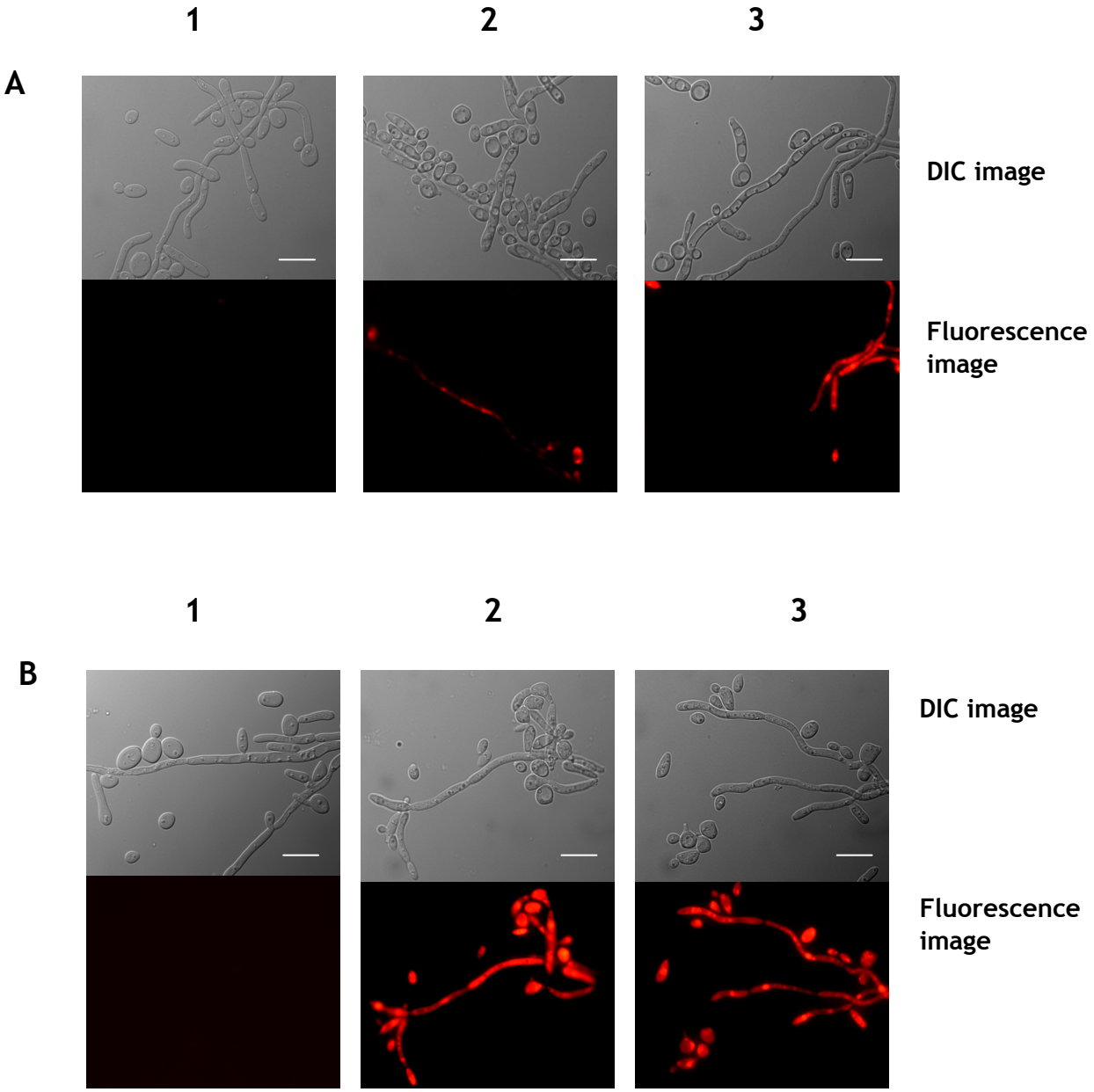


Figure 50. Caspase detection using a polycaspase SR-FLICA reagent: *C. albicans* SC5314

Biofilms (48-h) of *C. albicans* SC5314 were treated with AMB (10 or 50 µg/ml) for 5 h or 24 h. Biofilm cells were then labelled with the SR-VAD-FMK reagent for 60 min at 37°C and examined by fluorescence microscopy. DIC and fluorescence images of *C. albicans* SC5314 exposed to AMB for 5 h (A) or 24 h (B) are shown: (1) untreated control biofilm cells; (2) biofilm cells treated with AMB (10 µg/ml); and (3) biofilm cells treated with AMB (50 µg/ml). Orange/red fluorescence indicates caspase activity. Bar, 13 µm.

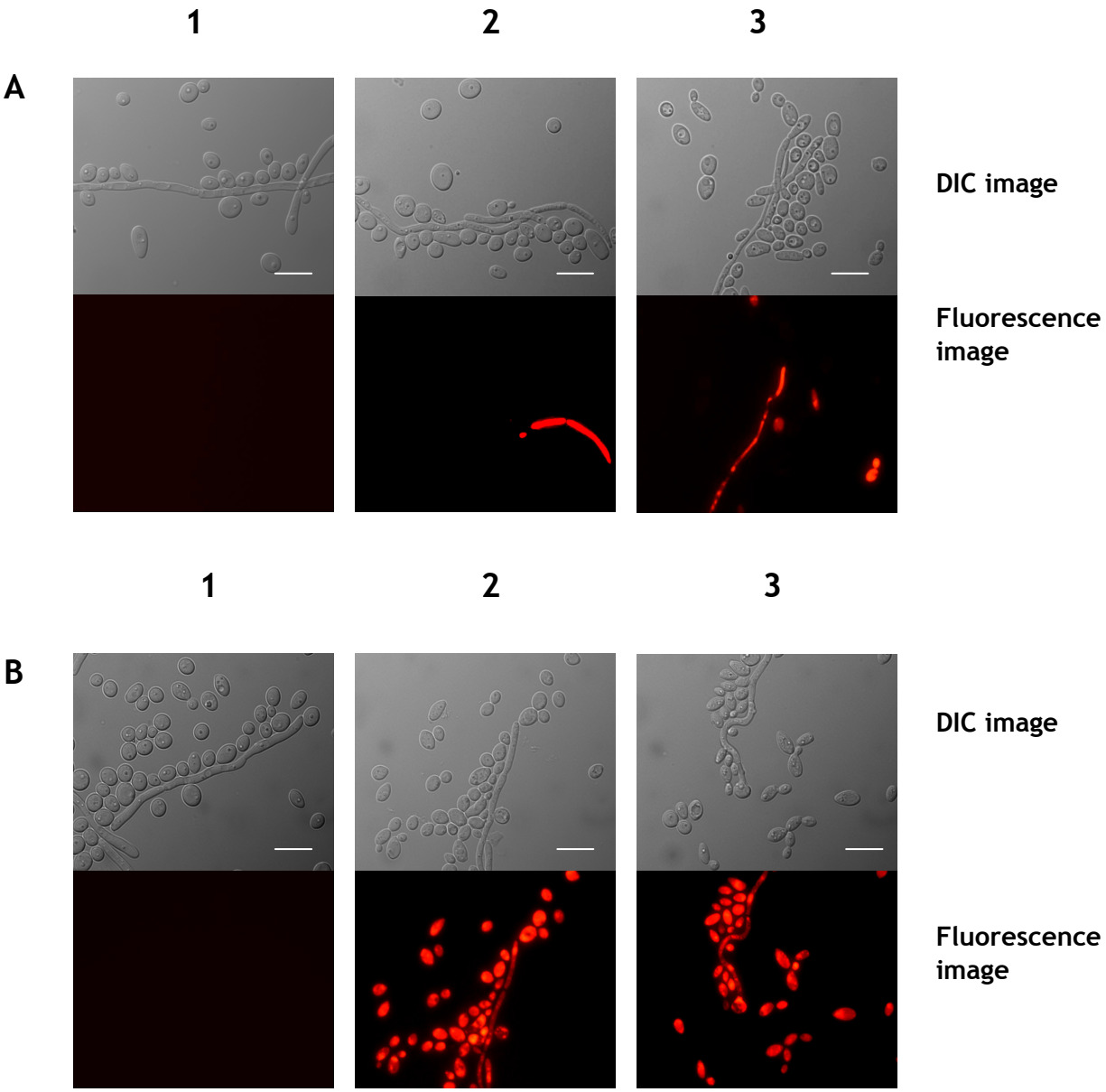


Figure 51. Caspase detection using a polycaspase SR-FLICA reagent: *C. albicans* GDH2346

Biofilms (48-h) of *C. albicans* GDH2346 were treated with AMB (50 µg/ml) for 5 h or 24 h. Biofilm cells were then labelled with the SR-VAD-FMK reagent for 60 min at 37°C and examined by fluorescence microscopy. DIC and fluorescence images of *C. albicans* GDH2346 exposed to AMB for 5 h (A) or for 24 h (B) are shown: (1) untreated control biofilm cells; (2 and 3) treated biofilm cells. Orange/red fluorescence indicates caspase activity. Bar, 13 µm.

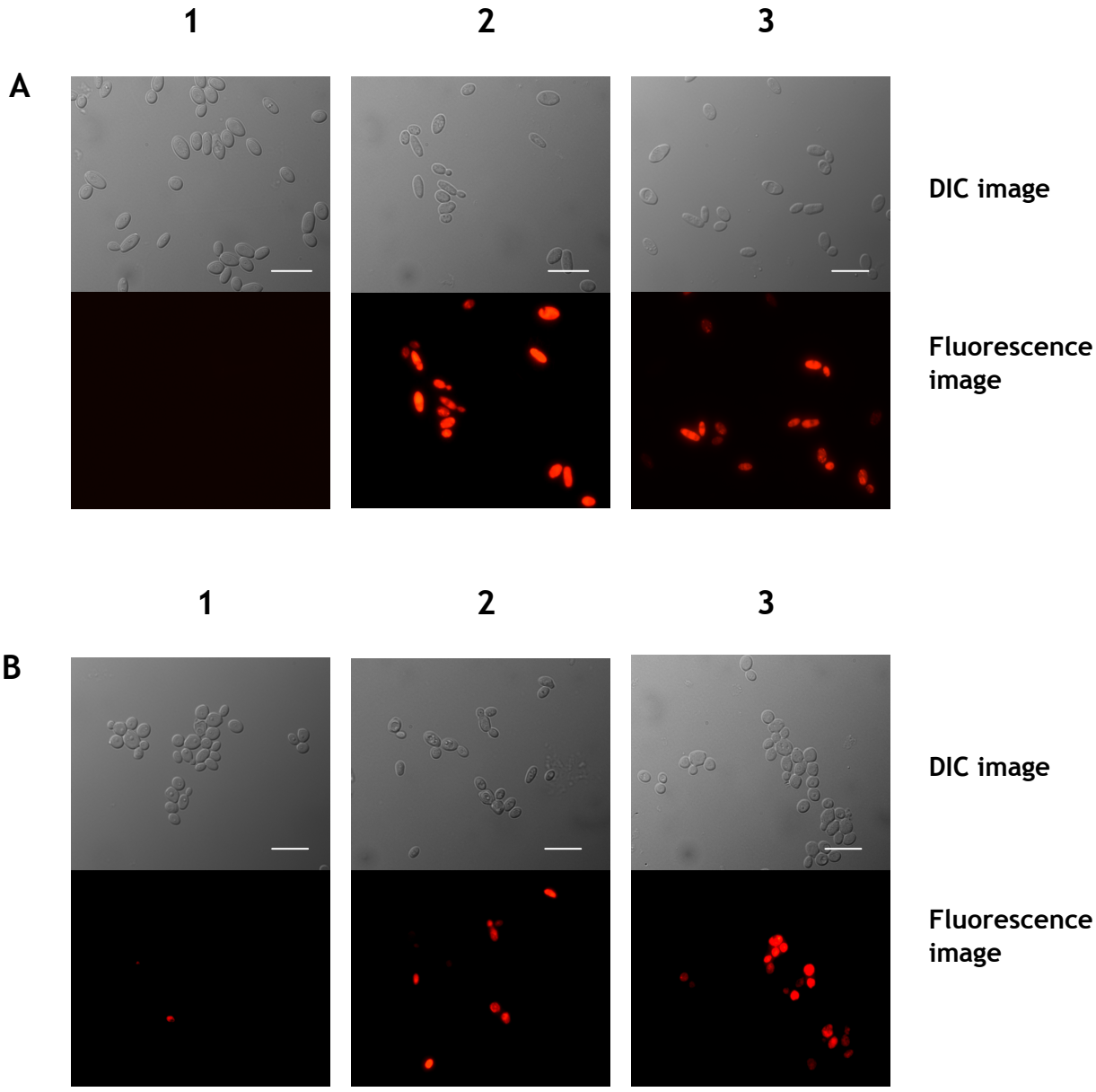


Figure 52. Caspase detection using a polycaspase SR-FLICA reagent: *C. krusei* and *C. parapsilosis*

Biofilms (48-h) of *C. krusei* and *C. parapsilosis* were treated with AMB (50 µg/ml) for 24 h. Biofilm cells were then labelled with the SR-VAD-FMK reagent for 60 min at 37C and examined by fluorescence microscopy. DIC and fluorescence images of *C. krusei* (A) and *C. parapsilosis* (B) are shown: (1) untreated control biofilm cells; (2 and 3) treated biofilm cells. Orange/red fluorescence indicates caspase activity. Bar, 13 µm.

binding sites. Furthermore, subsequent binding of FLICA was not completely prevented by addition of the unlabelled caspase inhibitor at the time of apoptosis induction; the number of stained cells did not significantly decrease (94 % of 1300 cells counted, *P*<0.37).

6.1.2 Caspase detection using a D_2R reagent

To confirm the results with the FLICA reagent, the presence of active caspase-like proteases was investigated using another method. This assay is based on the cleavage of $(aspartyl)_2$-Rhodamine 110 (D_2R) which is a substrate for caspases. D_2R is non-fluorescent but when it enters the cell it can be cleaved by active caspases to green fluorescent monosubstituted rhodamine 110 and free rhodamine (Hug *et al.*, 1999). With biofilms exposed to amphotericin B (50 µg/ml) for 24 h, the number of cells stained by D_2R was high (96%; Figs. 53 and 54); cells from untreated biofilms, however, were rarely stained.

Pre-treating biofilm cells of *C. albicans* GDH2346 with unlabelled Z-VAD-FMK for 1h prior to the addition of D_2R reagent did not prevent subsequent binding of D_2R. Furthermore, addition of the unlabelled caspase inhibitor along with the inducer of apoptosis (AMB) also did not completely prevent the subsequent binding of D_2R. Nevertheless, the number of stained cells was significantly lower (81% of 600 cells counted; *P*<0.035) than the number of stained cells in biofilms exposed to the apoptosis inducer only (97 % of 600 cells counted).

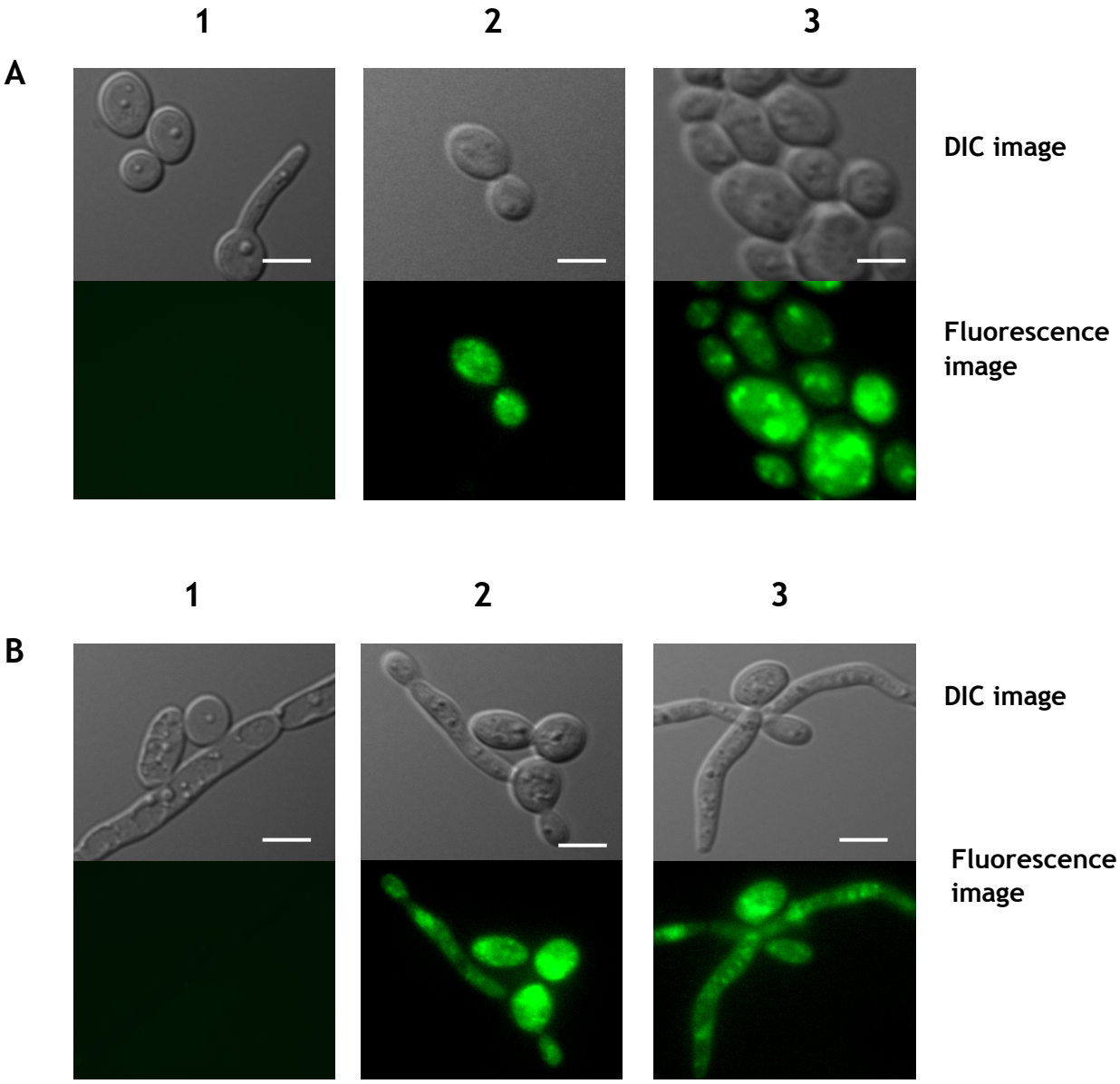


Figure 53. Caspase detection using a D_2R reagent: *C. albicans* strains GDH2346 and SC5314

Candida biofilms (48-h) were treated with AMB (50 µg/ml) for 24 h at 37 °C. Biofilm cells were then labelled with D_2R for 45 min at 37 °C and examined by fluorescence microscopy. DIC and fluorescence images of *C. albicans* GDH2346 (A) and *C. albicans* SC5314 (B) are shown: (1) untreated control biofilm cells; (2 and 3) treated biofilm cells. Green fluorescence indicates caspase activity. Bar, 5 µm.

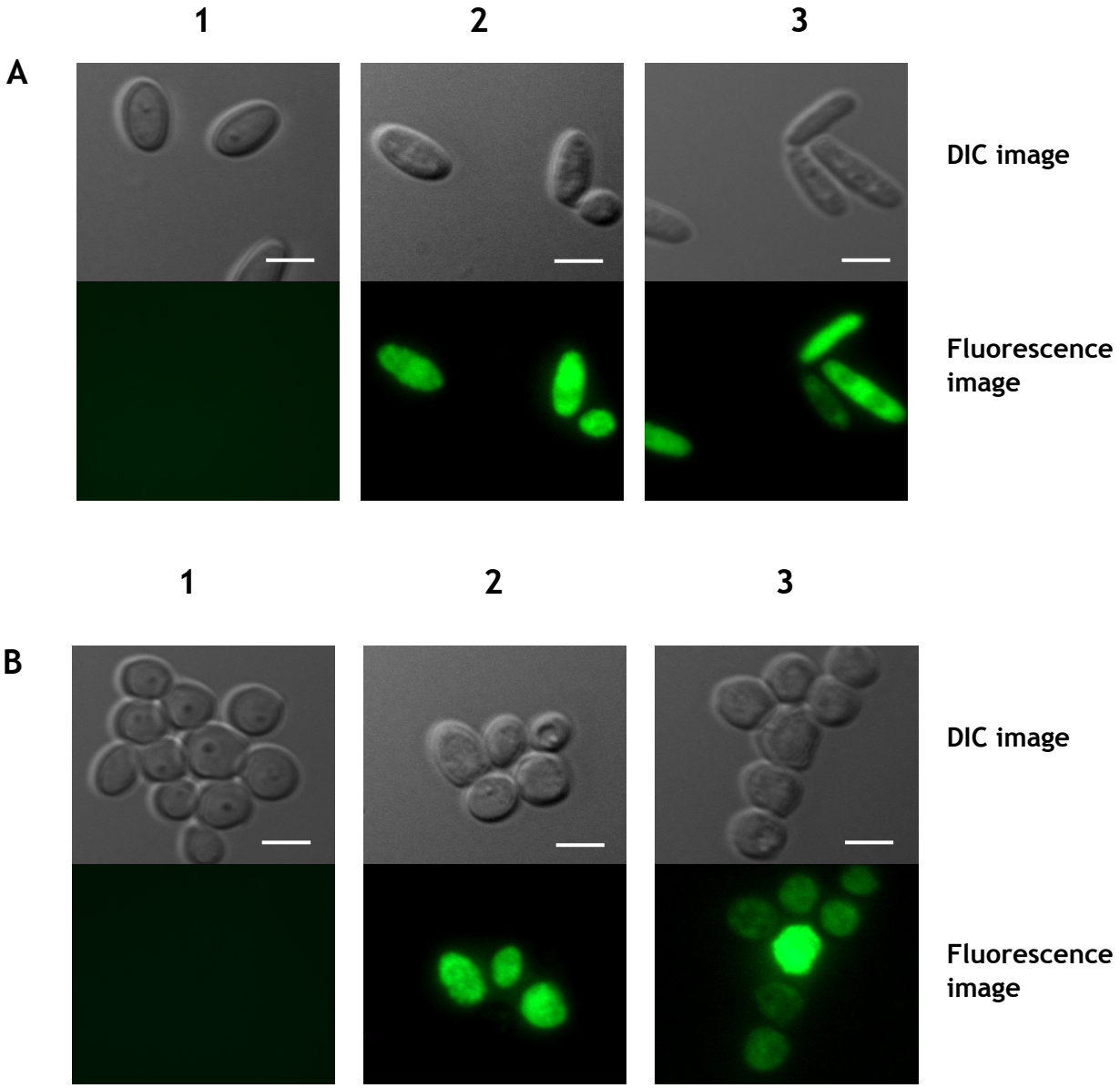


Figure 54. Caspase detection using a D_2R reagent: *C. krusei* and *C. parapsilosis*

Candida biofilms (48-h) were treated with AMB (50 µg/ml) for 24 h at 37 °C. Biofilm cells were then labelled with D_2R for 45 min at 37 °C and examined by fluorescence microscopy. DIC and fluorescence images of *C. krusei* (A) and *C. parapsilosis* (B) are shown: (1) untreated control biofilm cells; (2 and 3) treated biofilm cells. Green fluorescence indicates caspase activity. Bar, 5 µm.

6.2 Effects of caspase inhibitors on *Candida* biofilms

The effects of caspase inhibitors on amphotericin B activity against *Candida* biofilms were investigated using a broad spectrum caspase inhibitor (Z-VAD-FMK) and a variety of specific caspase inhibitors. Drug action was determined by viable cell count assays. The concentration of the solvent DMSO was maintained as low as possible ($\leq$ 0.26 %) since higher concentrations were found to interfere with the inhibitors and to increase the number of dead cells in the presence of amphotericin B; examples of this effect are shown in Figs. 55 and 56. Furthermore, according to the manufacturer of the inhibitors (Calbiochem), 'a final DMSO concentration should not exceed 0.2% as higher levels may increase the risk of cellular toxicity which may mask the effect of the caspase inhibitor'.

6.2.1 Effect of a general caspase inhibitor (Z-VAD-FMK)

Mature (48-h) biofilms of *C. albicans* GDH2346, *C. krusei* Glasgow, and *C. parapsilosis* AAHB4479 were incubated for 24 h at 37ºC in fresh growth medium in the presence of different concentrations of the caspase inhibitor Z-VAD-FMK (2.5, 5, 10, and 20 µM) alone or together with amphotericin B (50 µg/ml).

6.2.1.1 Effect of Z-VAD-FMK on viability of *Candida* biofilms

Addition of the caspase inhibitor, Z-VAD-FMK, had no significant effect on biofilm viability ($P>0.05$) for any of the three *Candida* species at any concentration tested (Fig. 57).

6.2.1.2 Effect of Z-VAD-FMK on amphotericin B activity against *Candida* biofilms

When Z-VAD-FMK was added at a concentration as low as 2.5 µM together with amphotericin B (50 µg/ml), it significantly reduced the effect of the drug on *Candida* biofilms. Biofilm viability was increased by 11.5-fold ($P<0.001$) and 1.6-fold ($P<0.05$) for *C. albicans* GDH2346 and *C. parapsilosis*, respectively. However, Z-VAD-FMK (2.5 µM) produced no significant increase in biofilm viability with *C. krusei* (Fig. 58). In contrast, Z-VAD-FMK at high concentrations (10 µM to 20 µM) was ineffective in preventing cell death; a dramatic increase in the number of dead cells was observed instead. The proportion of dead cells was

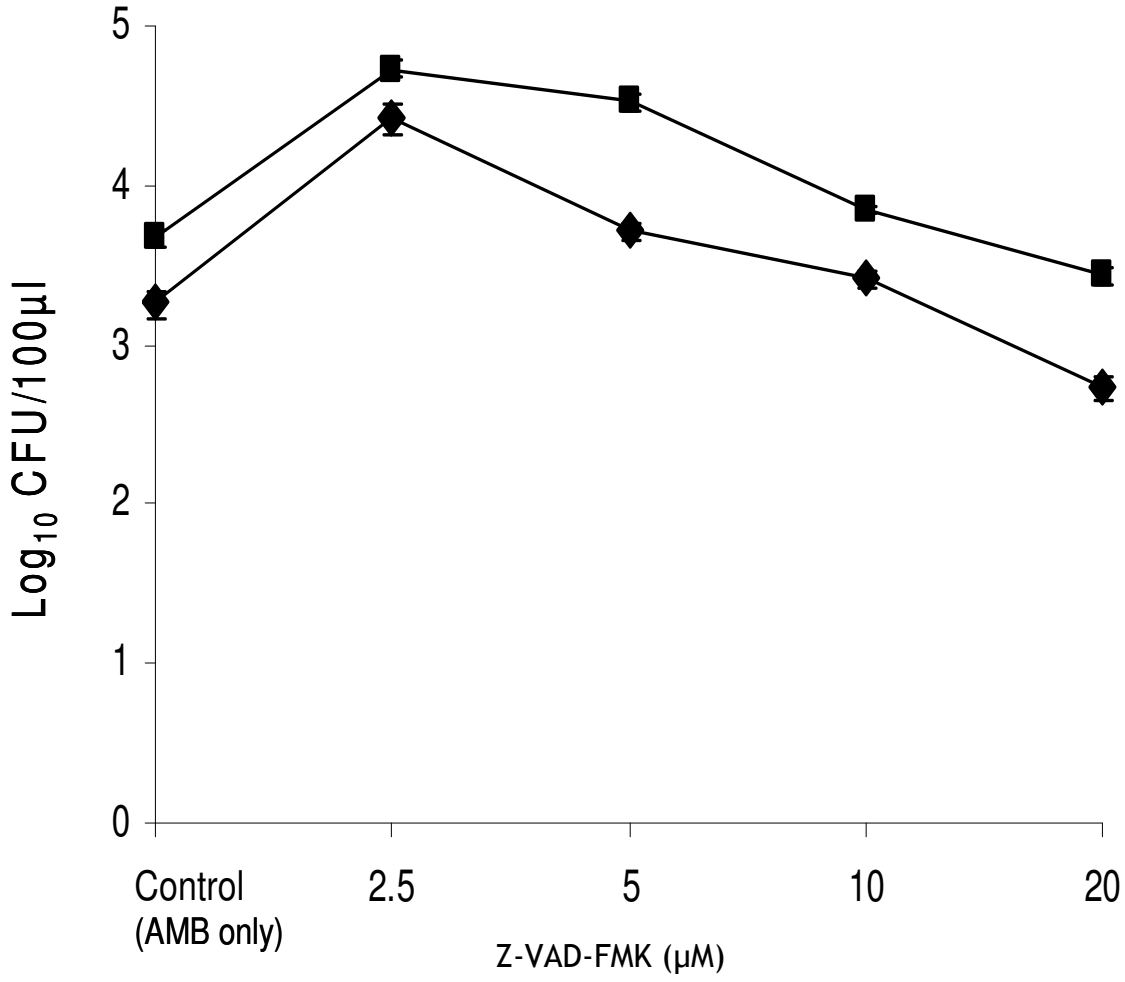


Figure 55. Effect of Z-VAD-FMK on amphotericin B (50µg/ml) activity against *C. albicans* GDH2346 in the presence of DMSO at 0.5-1.4% (♦) or 0.12-0.26% (■)

Data represent the means ± standard errors of two independent experiments carried out in duplicate.

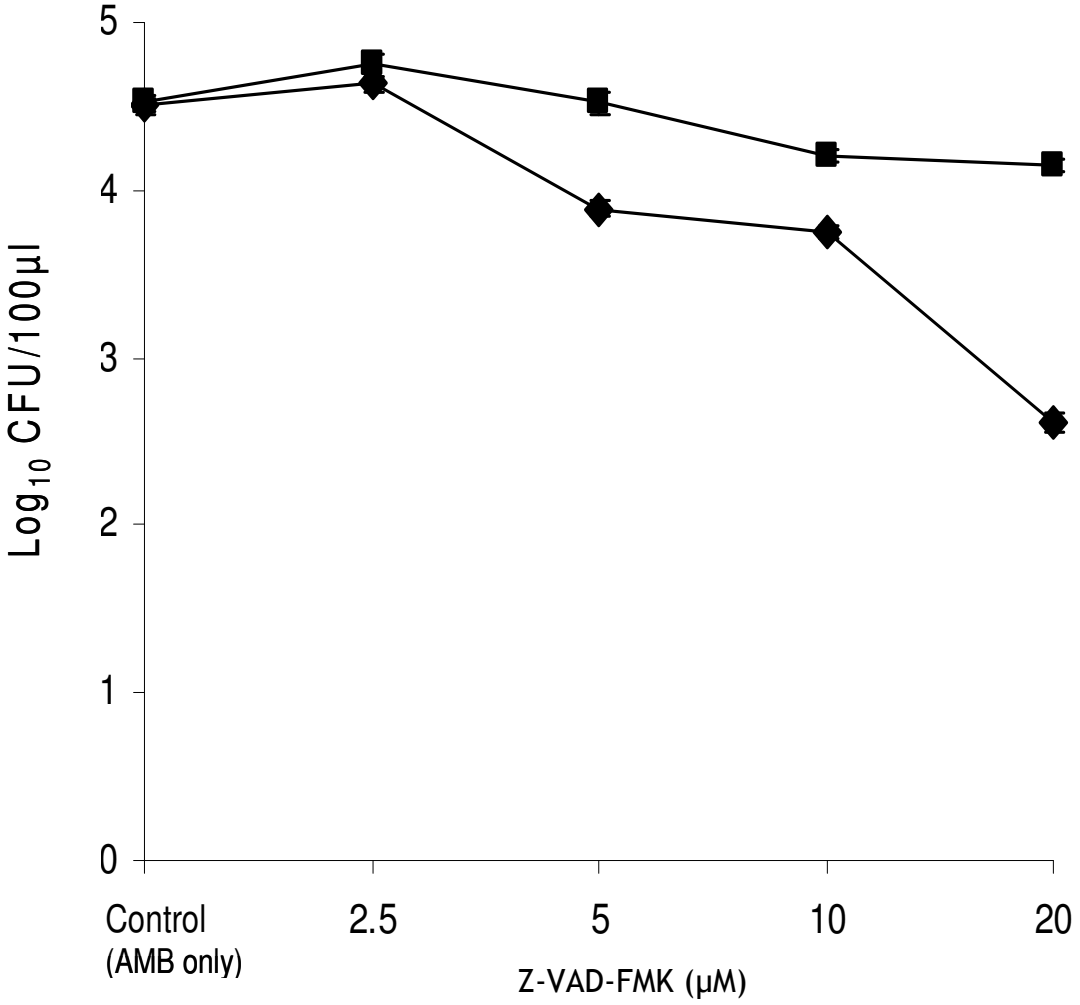


Figure 56. Effect of Z-VAD-FMK on amphotericin B (50µg/ml) activity against *C. parapsilosis* in the presence of DMSO at 0.5-1.4% (♦) or 0.12-0.26% (■)

Data represent the means ± standard errors of two independent experiments carried out in duplicate.

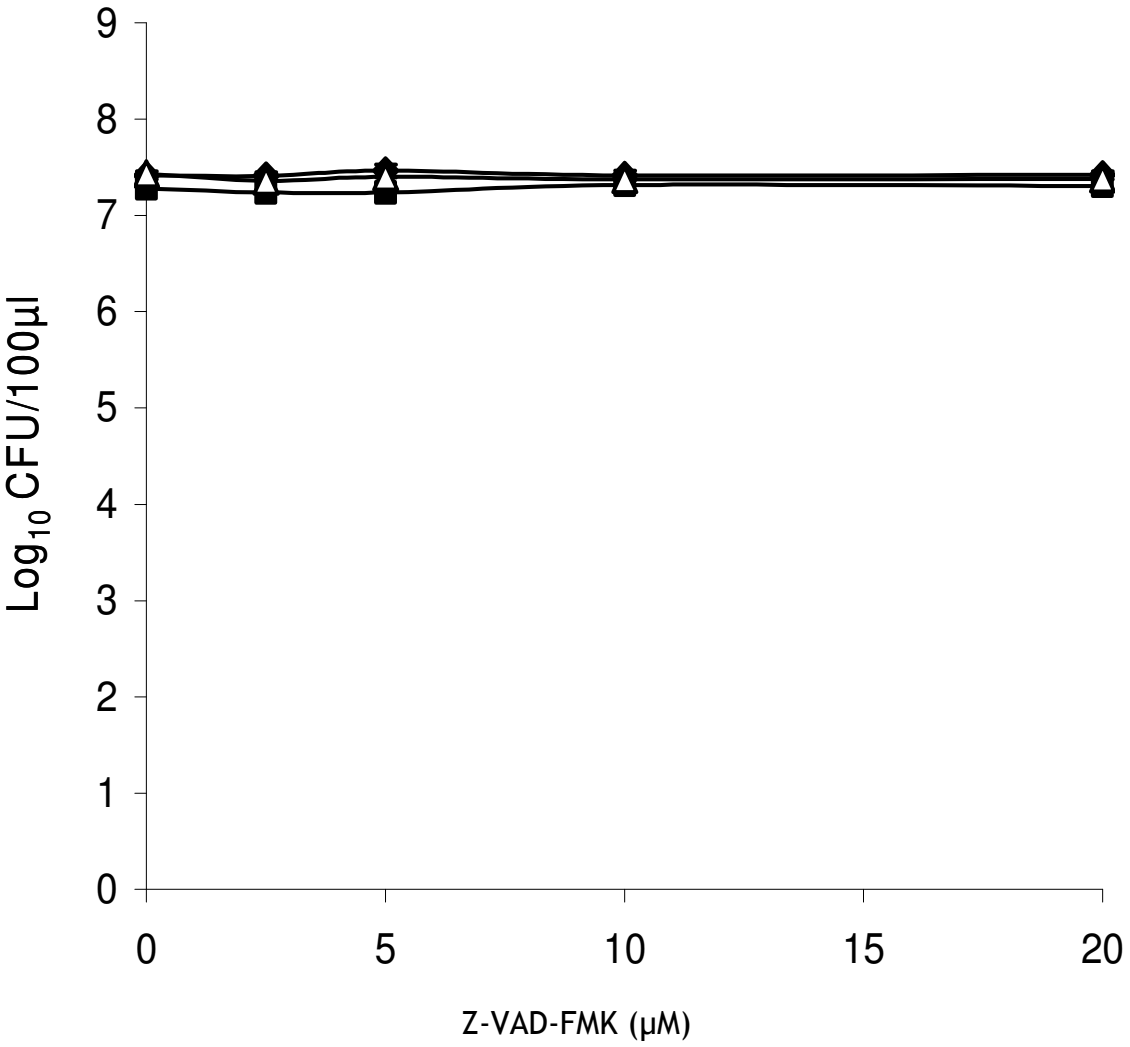


Figure 57. Effect of general caspase inhibitor Z-VAD-FMK on the viability of biofilms of *C. albicans* GDH2346 (♦), *C. krusei* (■), and *C. parapsilosis* (Δ)

Biofilms (48-h) were incubated with ZVAD-FMK for 24 h at 37°C and cell survival was determined by viable counts. Results are the means ± standard errors of two independent experiments carried out in duplicate. Final DMSO concentration, ≤ 0.014%.

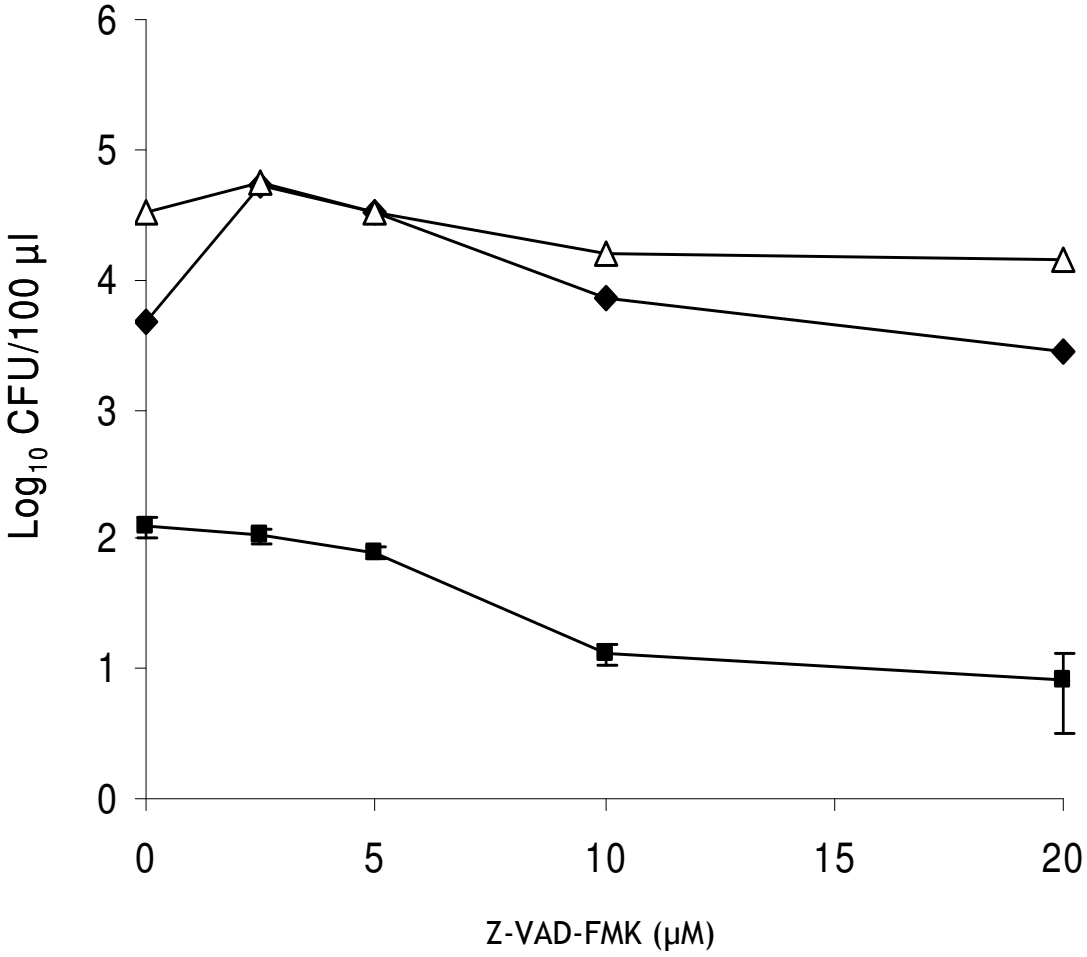


Figure 58. Effect of general caspase inhibitor Z-VAD-FMK on amphotericin B activity against biofilms of *C. albicans* GDH2346 (♦), *C. parapsilosis* (Δ), and *C. krusei* (■)

Biofilms (48-h) were incubated with Z-VAD-FMK and amphotericin B (50 µg/ml), together with Z-VAD-FMK at the concentration indicated, for 24 h at 37°C and cell survival was determined by viable counts. Results are the means ± standard errors of two independent experiments carried out in duplicate. Final DMSO concentration, 0.12-0.26%.

highest at a concentration of 20 μM: 41 %, 57 %, and 94 % for *C. albicans* GDH2346, *C. parapsilosis* and *C. krusei*, respectively (Fig. 58).

The effect of combined treatment with Z-VAD-FMK and amphotericin B was also investigated with biofilms of *C. albicans* SC5314. This strain seems to lack persisters, and biofilms lose all viability after exposure to amphotericin B at a concentration of 30 μg/ml (Section 5.2.1). When Z-VAD-FMK (2.5 μM) was added to 48-h biofilms along with amphotericin B (50 μg/ml), after incubation for 24 h at 37ºC there was a complete loss of viability like that observed with control biofilms exposed only to the antifungal agent. On the other hand, treating biofilms with Z-VAD-FMK and a lower concentration of amphotericin B (10 μg/ml) resulted in an 11.9-fold increase in viability ($P<0.001$).

The specificity of the effect noted with the general caspase inhibitor was investigated using pepstatin A, an inhibitor of aspartic proteinases which are known to be produced by *Candida* species. Mature (48-h) biofilms of *C. albicans* GDH2346 and *C. albicans* SC5314 were exposed to pepstatin A (2.5 μM) plus amphotericin B (50 μg/ml) for 24 h at 37ºC. Pepstatin A, with either *C. albicans* strain, had no significant effect in improving cell survival (Fig. 59). As noted above, *C. albicans* SC5314 biofilms produce no persisters and are completely eliminated at concentrations of amphotericin B $\geq$30 μg/ml (Section 5.2.1).

6.2.2 Effect of some specific caspase inhibitors

A set of specific caspase inhibitors, active individually against caspases-1, -2, -3, -5, -6, -8, and -9, was also tested against *C. albicans* GDH2346 biofilms, at a concentration of 2.5 μM.

6.2.2.1 Effect of specific caspase inhibitors on viability of *Candida* biofilms

Mature (48-h) biofilms of *C. albicans* were incubated for 24 h at 37ºC in fresh growth medium in the presence of specific caspase inhibitors (2.5 μM). None of the inhibitors showed a significant effect ($P>0.05$) on biofilm viability asdetermined by viable counts (Fig. 60).

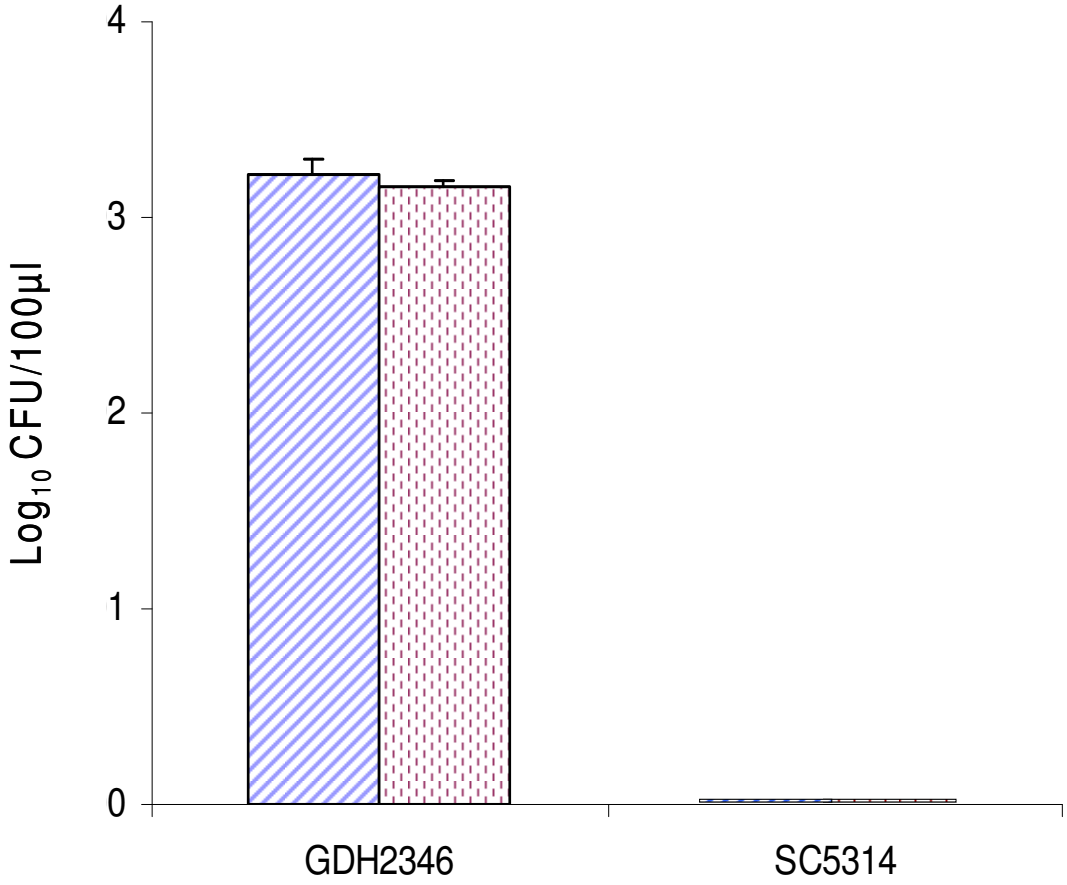


Figure 59. Effects of pepstatin A on amphotericin B activity against biofilms of *C. albicans* GDH2346 and *C albicans* SC5314

Biofilms (48-h) were incubated with pepstatin A (2.5 µM) and amphotericin B (50µg/ml) for 24 h at 37°C. Cell survival was determined by viable counts. Control (AMB only) (▨); pepstatin A + AMB (▦). Results are means ± standard errors of two independent experiments carried out in duplicate.

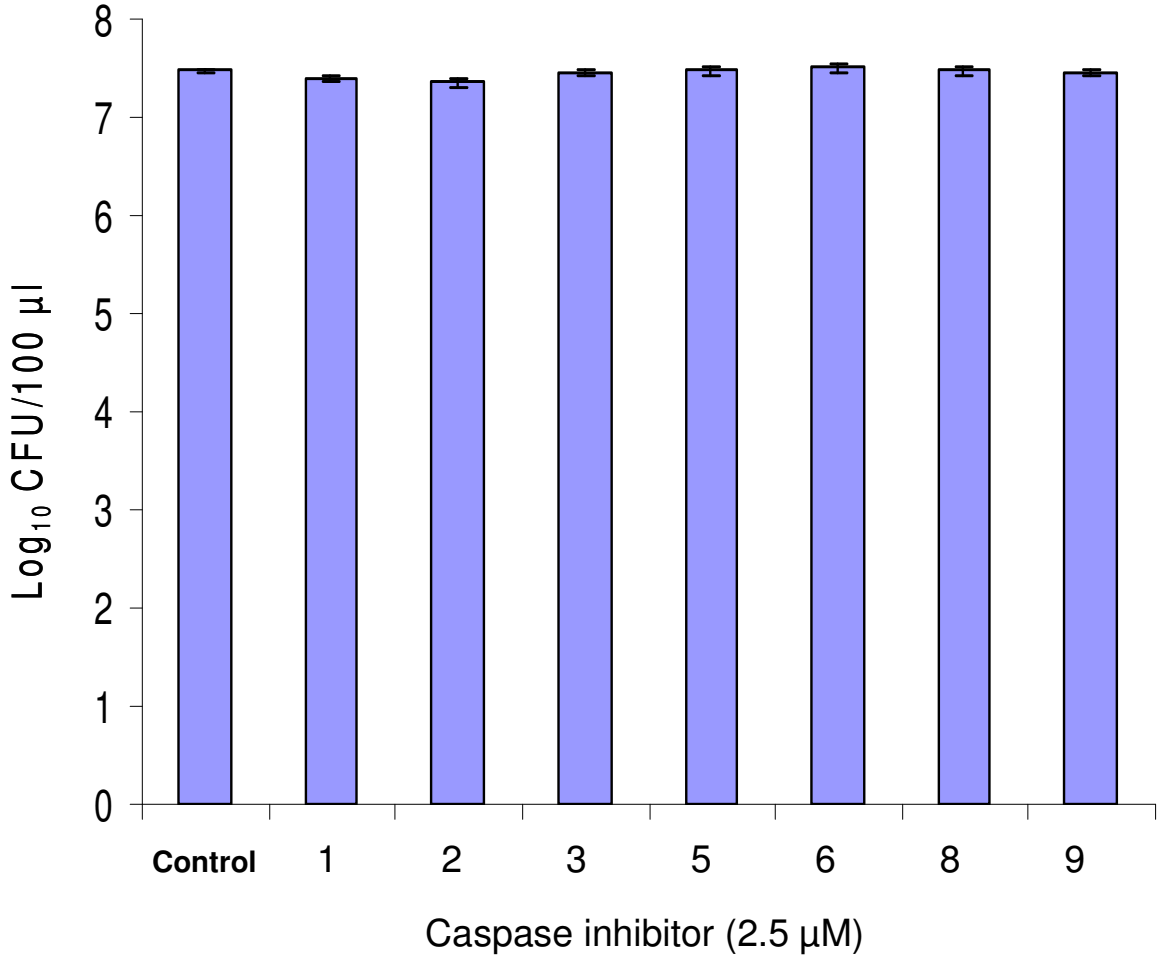


Figure 60. Effects of specific inhibitors of caspases-1, -2, -3, -5, -6, -8, and -9 on viability of *C. albicans* GDH2346 biofilms

Biofilms (48-h) were incubated with inhibitor (2.5 µM) for 24 h at 37°C. Cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate. Final DMSO concentration, 0.125%.

6.2.2.2 Effect of specific caspase inhibitors on amphotericin B activity against *Candida* biofilms

When specific caspase inhibitors (2.5 μM) were added along with amphotericin B (50 μg/ml) to 48-h *C. albicans* biofilms, some of the inhibitors significantly enhanced the survival of biofilm cells. Caspase-1 inhibitor VI produced in a 40-fold increase in biofilm cell survival ($P<0.001$) compared with amphotericin B-treated controls. Similarly, inhibitors of caspases -9, -5, -3, and -2 increased cell survival 8-fold ($P<0.001$), 3.5-fold ($P<0.001$), 1.9-fold ($P<0.001$), and 1.7-fold ($P<0.01$), respectively. In contrast, caspase-6 and caspase-8 inhibitors decreased biofilm cell survival as compared with the amphotericin-treated controls (Fig. 61).

6.3 Effects of histone deacetylase (HDA) inhibitors on *Candida* biofilms

The effects of four HDA inhibitors, namely sodium butyrate, sodium valproate, apicidin, and trichostatin A, on *Candida* biofilms were investigated using two methods. Initial experiments were carried out using a tetrazolium salt reduction (XTT) assay. However, very low XTT readings were obtained with one *Candida* species (*C. parapsilosis*). Additional experiments were therefore carried out using a viable cell count assay. In preliminary experiments, HDA inhibitors were added at time zero of biofilm formation but this procedure produced biofilms that were fragile, loosely adherent to PVC discs and difficult to handle (especially with trichostatin A and apicidin). In all subsequent experiments, the effects of HDA inhibitors were tested on mature (48-h) *Candida* biofilms instead.

6.3.1 Effects of HDA inhibitors on growth and viability of *Candida* biofilms

6.3.1.1 Tetrazolium salt reduction (XTT) assays

HDA inhibitors (sodium butyrate, sodium valproate, apicidin, and trichostatin A) at different concentrations (2 to 32 μg/ml, or 2 to 32 mM for sodium butyrate) were added to incubation mixtures at time zero of biofilm production. Incubation was continued for 48 h at 37 °C. Sodium valproate had no significant effect on the growth of *C. albicans* GDH2346 biofilms, whereas sodium butyrate and apicidin reduced growth by 8 to 15 % ($P<0.05$). On the other

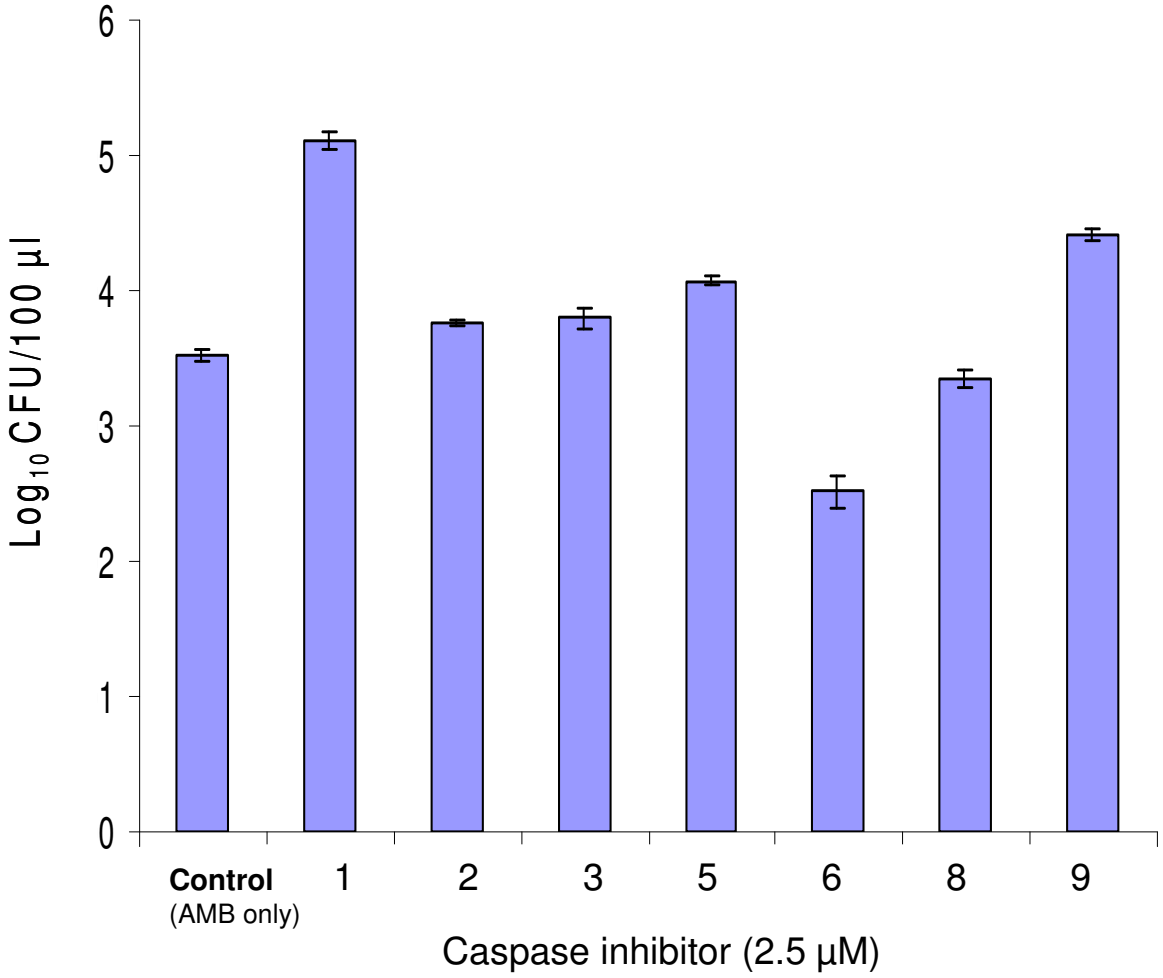


Figure 61. Effects of specific inhibitors of caspases-1, -2, -3, -5, -6, -8, and -9 on amphotericin B activity against biofilms of *C. albicans* GDH2346

Biofilms (48-h) were incubated with inhibitor (2.5 µM) and amphotericin B (50µg/ml) for 24 h at 37°C. Cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate. Final DMSO concentration, 0.25%.

hand, trichostatin A seemed to enhance growth slightly at low concentrations (Fig. 62).

Growth of *C. krusei* and *C. parapsilosis* biofilms was reduced by all four HDA inhibitors by 10 to 36% (Fig. 63 and 64). Using the XTT assay, low metabolic activity was consistently observed with *C. parapsilosis* biofilms. The reason for this is not clear; similar observations have been made by a colleague working in this laboratory (**Filip Ruzicka,** personal communication). Kuhn and co-workers have reported that XTT readings in this assay can vary in accordance with the sensitivity of different strains and species of *Candida* to tetrazolium salts. They have also demonstrated decreased XTT activity with *C. parapsilosis* (strains P/A71 and P92) compared to *C. albicans* strains (Kuhn *et al.*, 2002a; Kuhn *et al.*, 2002b; Kuhn *et al.*, 2003).

6.3.1.2 Viable cell counts

Mature (48-h) biofilms of *C. albicans* GDH2346, *C. parapsilosis* and *C. krusei* were incubated for 24 h at 37ºC in fresh growth medium in the presence of different concentrations of the HDA inhibitors. None of the inhibitors affected the viability of any of the *Candida* species (Figs. 65, 66, and 67).

6.3.2 Effects of HDA inhibitors on fluconazole activity against *Candida* biofilms

The effects of HDA inhibitors on fluconazole activity against *C. albicans* biofilms were evaluated by the XTT metabolic assay. The addition of HDA inhibitors (2 to 32 µg/ml, or 2 to 32 mM for sodium butyrate) at time zero of biofilm formation increased the action of fluconazole, at either 10 or 50 µg/ml, against biofilms of *C. albicans* GDH2346. The greatest effect on viability was observed in the presence of sodium butyrate (32 mM); biofilm viability was reduced by 36 % and 26 % ($P< 0.001$) at fluconazole concentrations of 10 and 50 µg/ml, respectively (Fig. 68 and 69). Valproate, apicidin, and trichostatin A at their highest concentration (32 µg/ml) also produced a significant effect on biofilm viability at a fluconazole concentration of 10 µg/ml; viability decreased by 28 %, 28 %, and 23.8 %, respectively ($P< 0.001$) (Fig. 68). Interestingly, valproate and apicidin at the higher concentration of fluconazole (50 µg/ml)

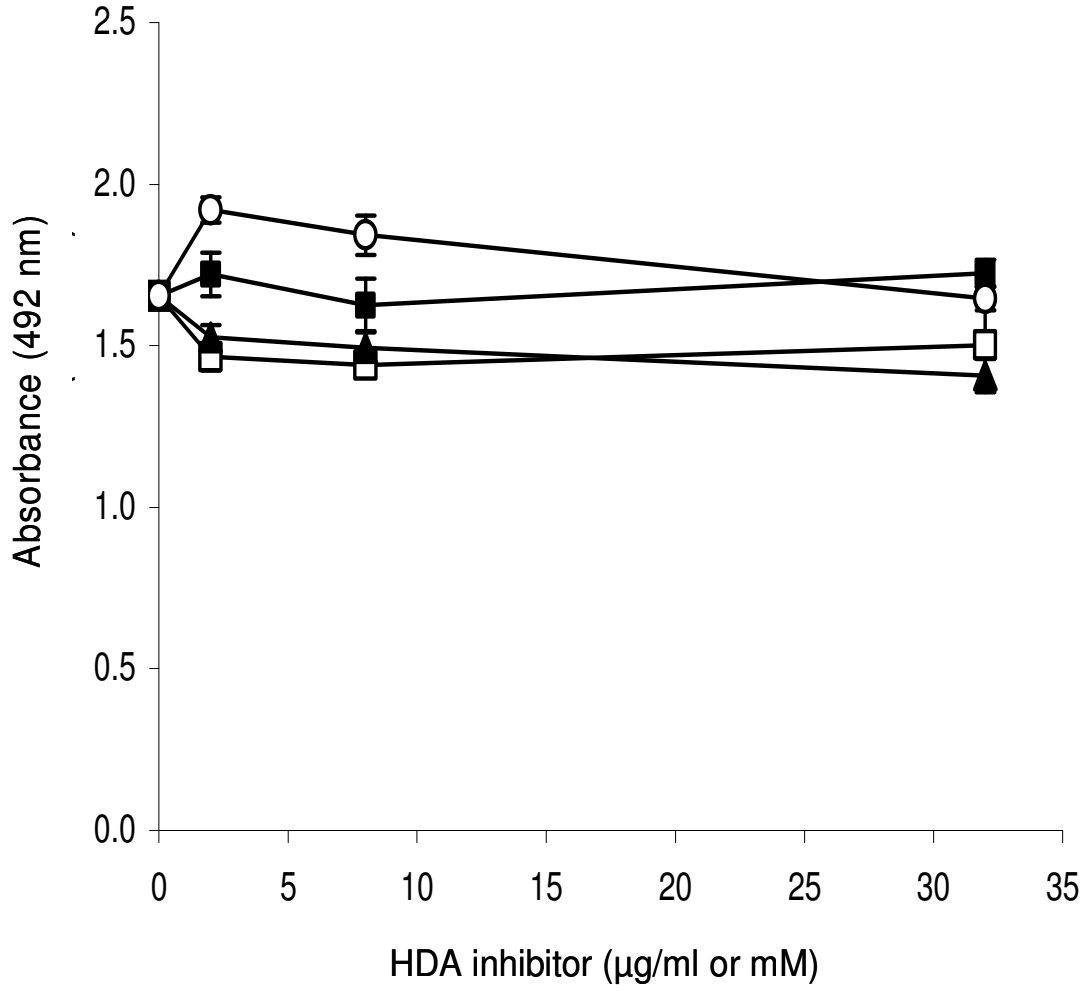


Figure 62. Effects of HDA inhibitors on the growth of biofilms of *C. albicans* GDH2346 as determined by XTT assays

HDA inhibitors (2, 8, 32 µg/ml or mM) were added at time zero of biofilm formation. Incubation was for 48 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

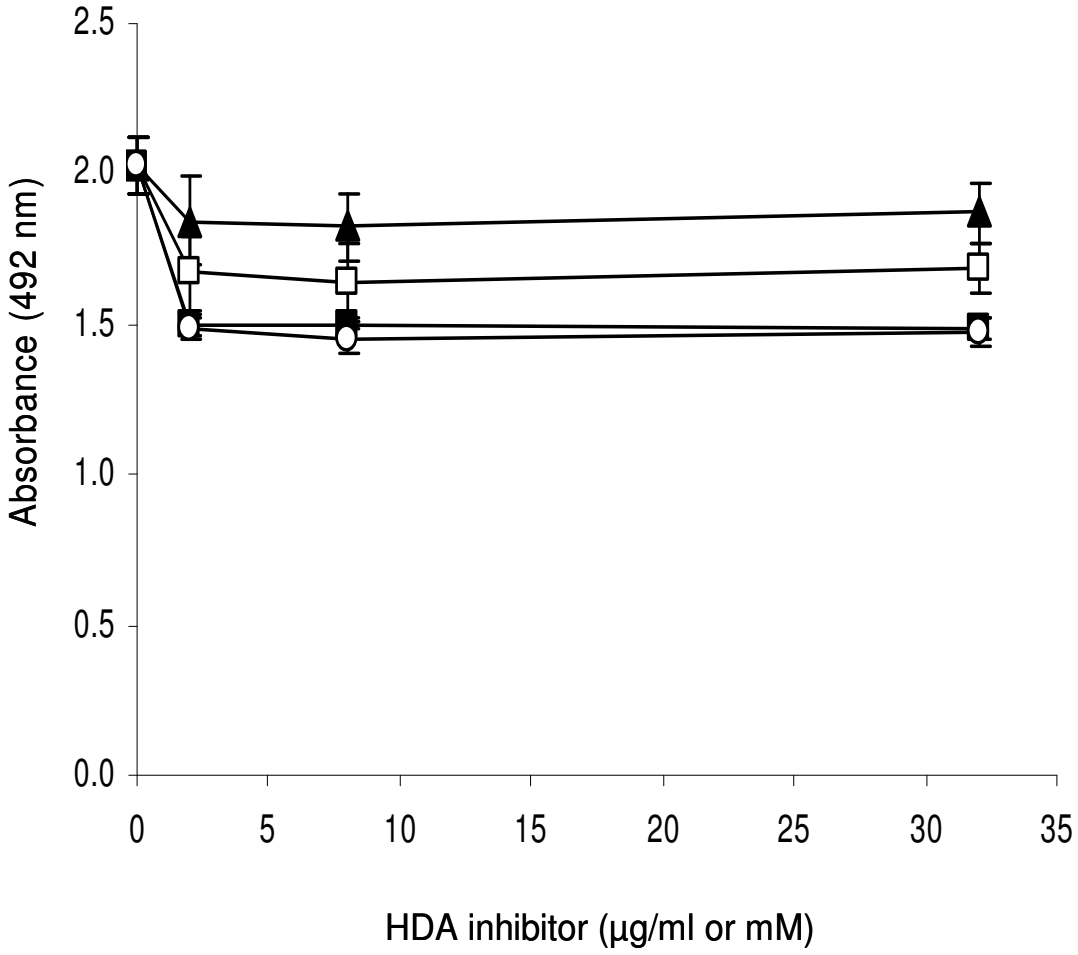


Figure 63. Effects of HDA inhibitors on the growth of *C. krusei* biofilms as determined by XTT assays

HDA inhibitors (2, 8, 32 µg/ml or mM) were added at time zero of biofilm formation. Incubation was for 48 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

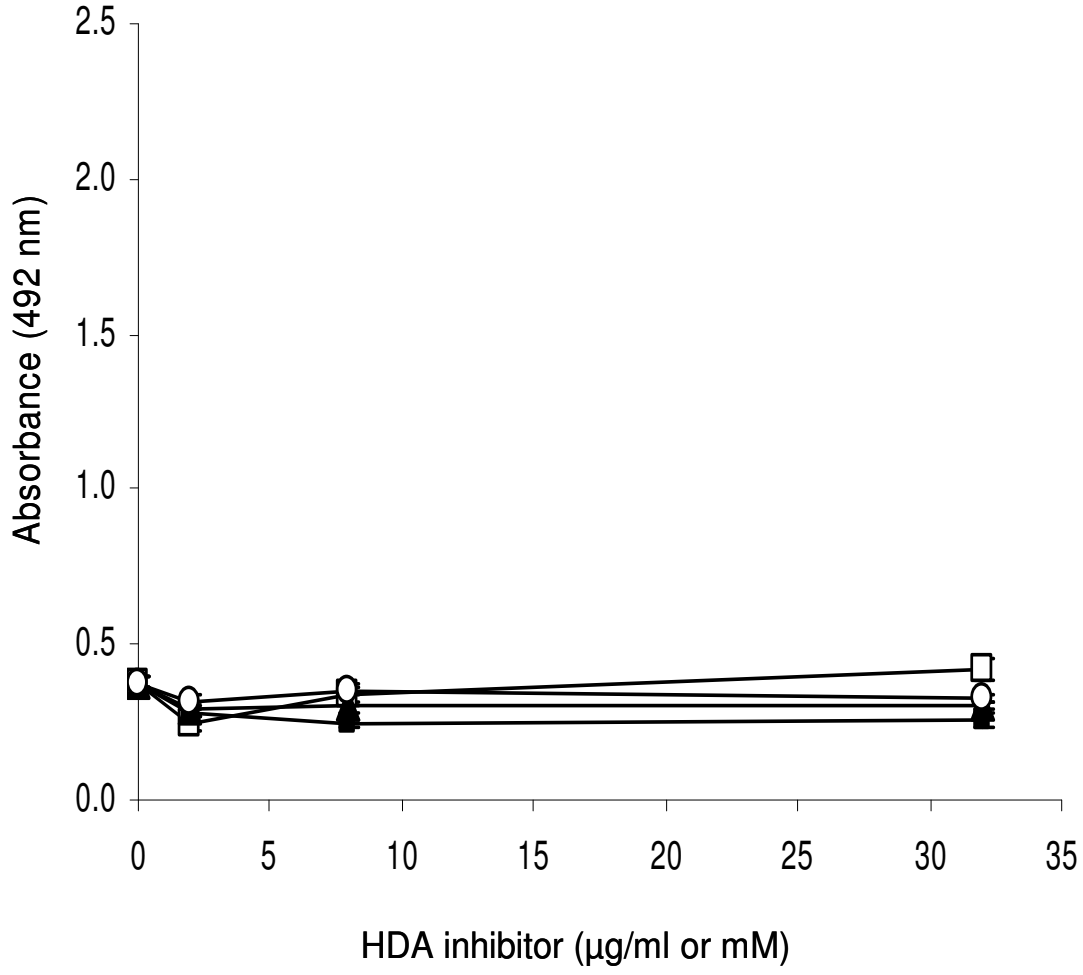


Figure 64. Effects of HDA inhibitors on the growth of *C. parapsilosis* biofilms as determined by XTT assays

HDA inhibitors (2, 8, 32 µg/ml or mM) were added at time zero of biofilm formation. Incubation was for 48 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

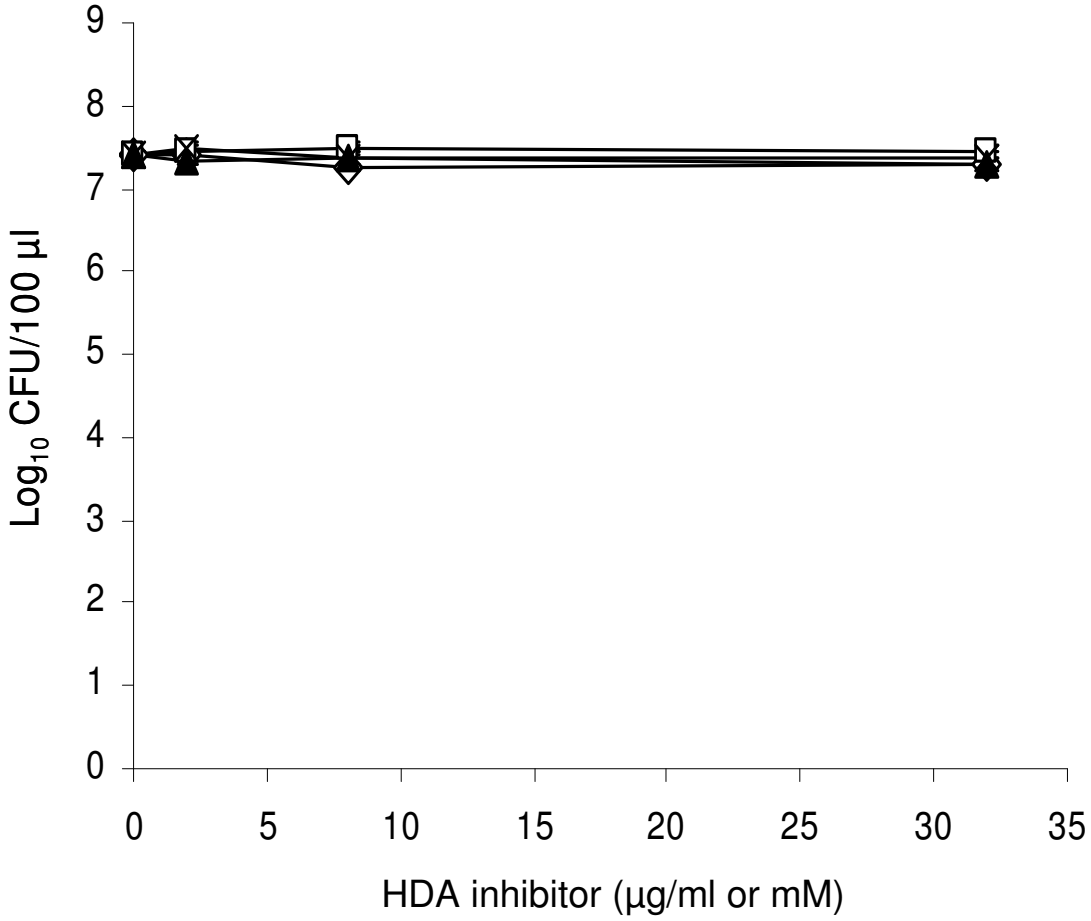


Figure 65. Effect of HDA inhibitors on the viability of *C. albicans* GDH2346 biofilms

Biofilms (48-h) were incubated with HDA inhibitors for 24 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

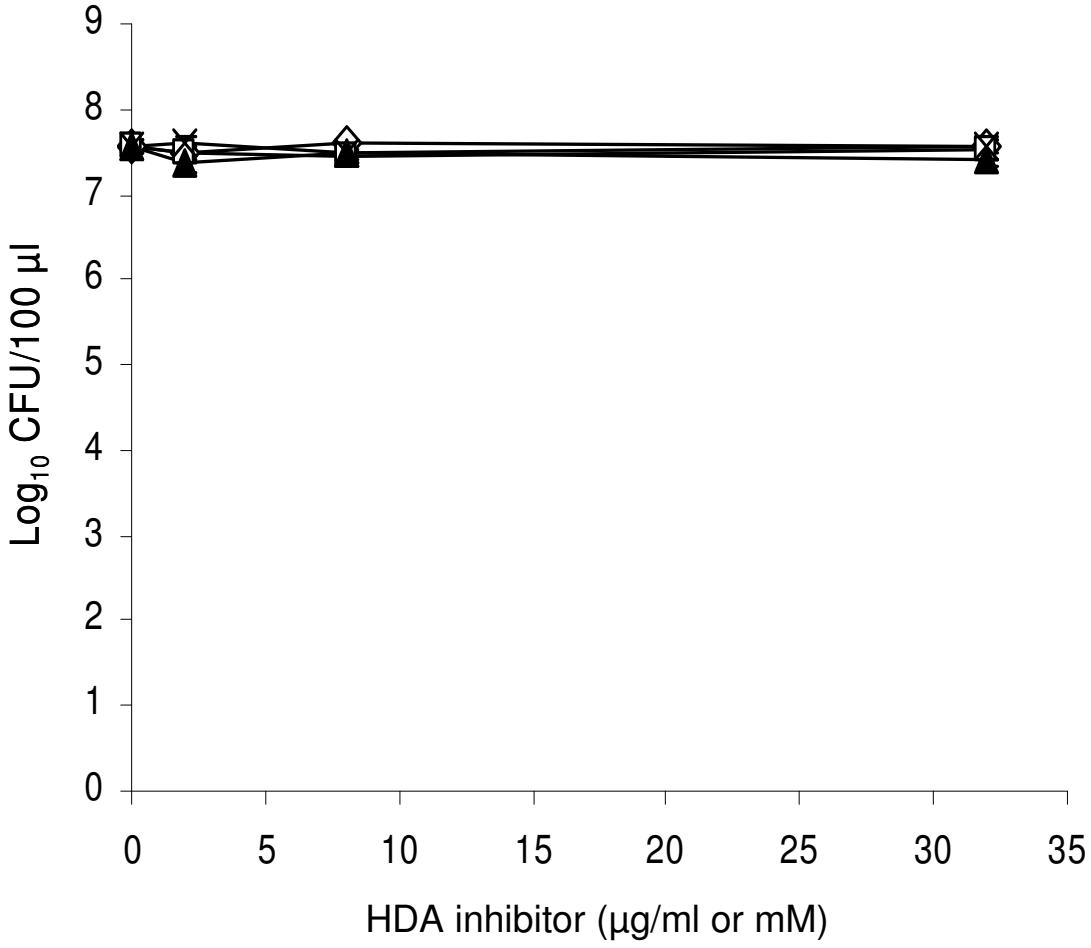


Figure 66. Effect of HDA inhibitors on the viability of *C. krusei* biofilms

Biofilms (48-h) were incubated with HDA inhibitors for 24 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

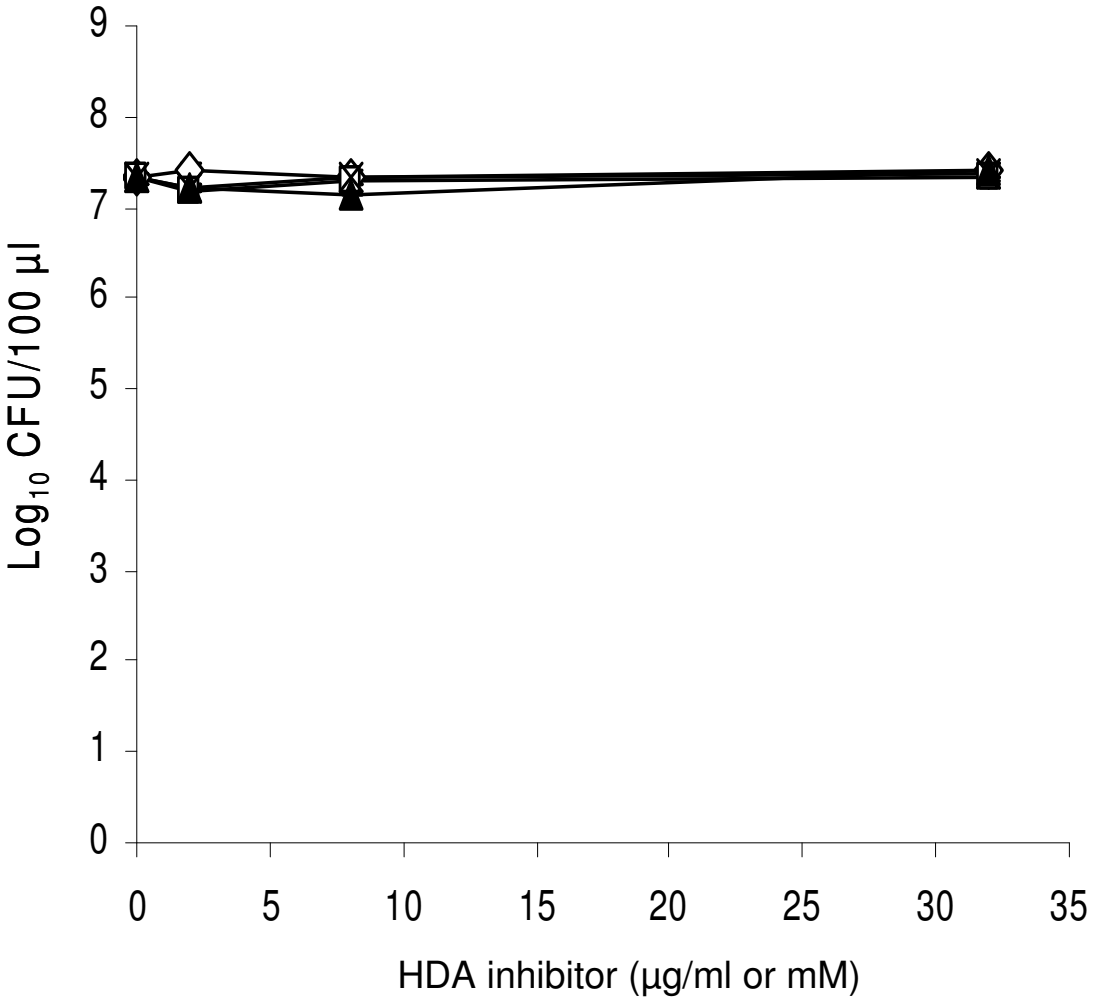


Figure 67. Effect of HDA inhibitors on the viability of *C. parapsilosis* biofilms

Biofilms (48-h) were incubated with HDA inhibitors for 24 h at 37°C. Results are means ± standard errors of two independent experiments carried out in triplicate. Sodium butyrate (mM; □), sodium valproate (µg/ml; ■), apicidin (µg/ml; ▲), and trichostatin A (µg/ml; ○).

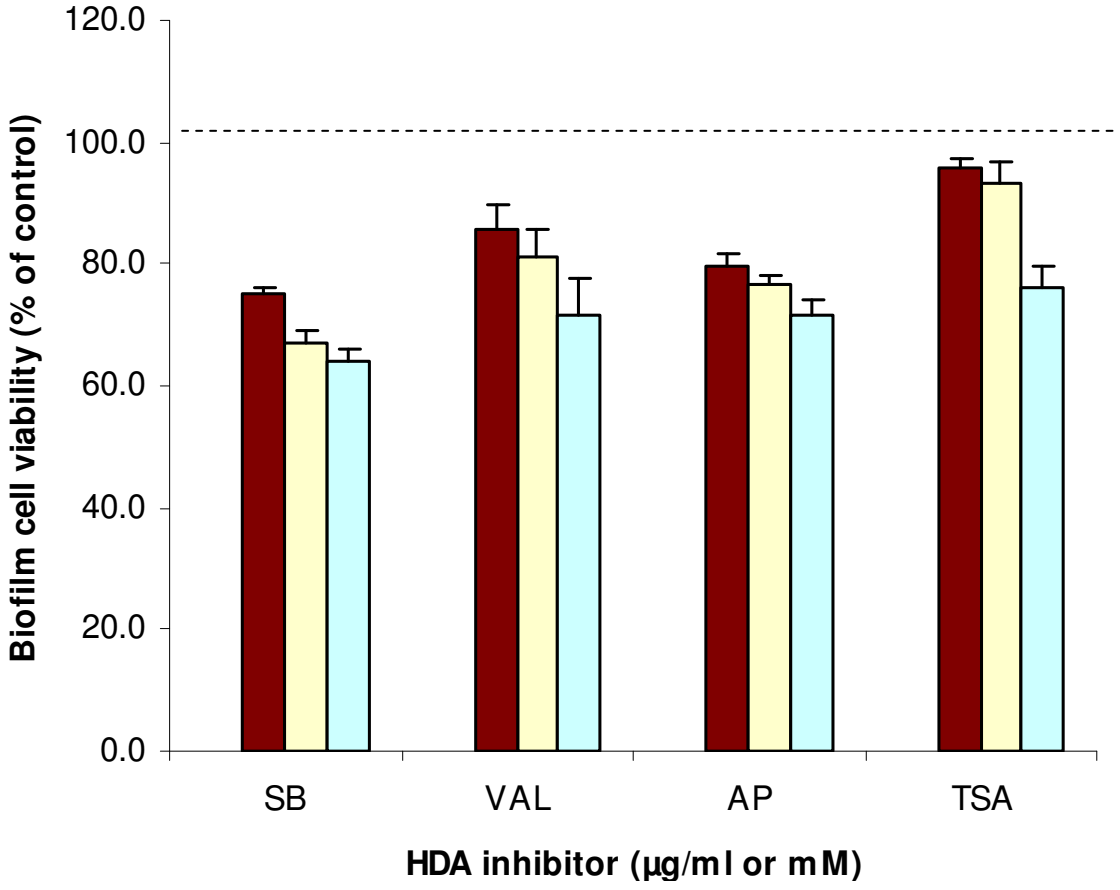


Figure 68. Effects of HDA inhibitors on fluconazole (10 µg/ml) activity against biofilms of *C. albicans* GDH2346

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to fluconazole (10 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

were less effective (10.5 % and 19 %, respectively), whereas trichostatin A appeared to enhance growth (Fig. 69).

A similar experiment was performed in which the HDA inhibitors were added to mature (48-h) biofilms of *C. albicans* GDH2346 rather than at time zero of biofilm formation. All four HDA inhibitors at their highest concentration (32 µg/ml or mM) had a marked effect on biofilm viability at both fluconazole concentrations (10 or 50 µg/ml); however, sodium butyrate (32 mM) had the greatest effect and reduced biofilm viability by 40 % and 24 %, at fluconazole concentrations of 10 and 50 µg/ml, respectively (P< 0.01) (Figs. 70 and 71).

6.3.3 Effect of HDA inhibitors on amphotericin B activity against *Candida* biofilms

Similar experiments were carried out to investigate the effects of HDA inhibitors (sodium butyrate, sodium valproate, apicidin, and trichostatin A) at different concentrations on the activity of amphotericin B against *Candida* biofilms. Tetrazolium reduction (XTT) and viable cell count assays were again used to assess the effects.

6.3.3.1 Tetrazolium salt reduction (XTT) assays

The addition of HDA inhibitors at time zero of biofilm formation produced varied effects on amphotericin B activity. Sodium butyrate at all concentrations (2 to 32 mM) significantly enhanced the activity of amphotericin B against *C. albicans* GDH2346 biofilms. Sodium butyrate at concentrations of 2, 8, and 32 mM with amphotericin B (10 µg/ml) reduced biofilm viability by 34 %, 56 %, and 68 %, respectively (P<0.001; Fig. 72), and in the presence of a higher concentration of amphotericin B (50 µg/ml) by 47%, 59.7%, and 76%, respectively (P<0.001; Fig. 73). In contrast, valproate and apicidin at all concentrations (2 to 32 mM) with amphotericin B (50 µg/ml) had no significant effect on *C. albicans* biofilm viability (P>0.05; Fig. 73). However, significant effects on viability (decreases of 37% and 31%, respectively) were observed when these inhibitors were used at a concentration of 32 µg/ml with amphotericin B at 10 µg/ml (P<0.001; Fig. 72). Results with trichostatin A were not reliable in this assay; it was noticed that this inhibitor in the presence of amphotericin B causes detachment of *C. albicans* biofilms from the PVC discs. Surprisingly,

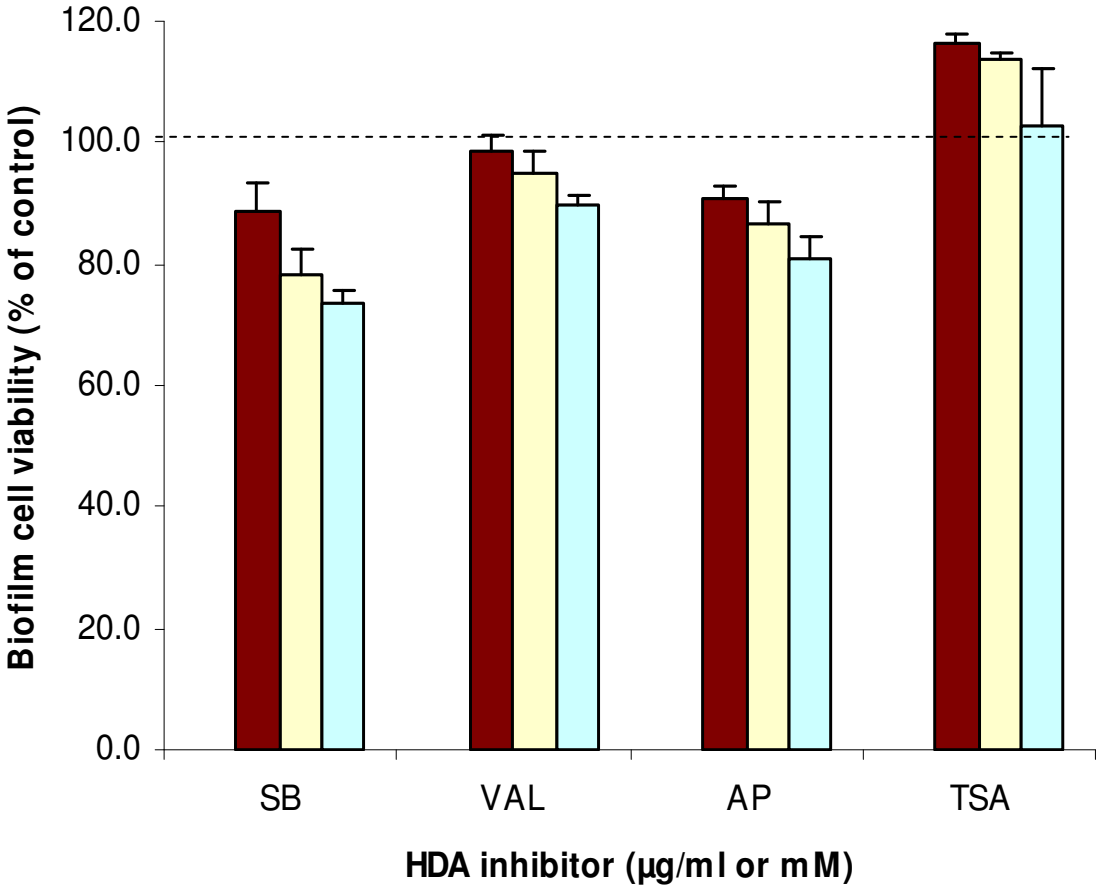


Figure 69. Effects of HDA inhibitors on fluconazole (50 µg/ml) activity against biofilms of *C. albicans* GDH2346

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to fluconazole (50 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

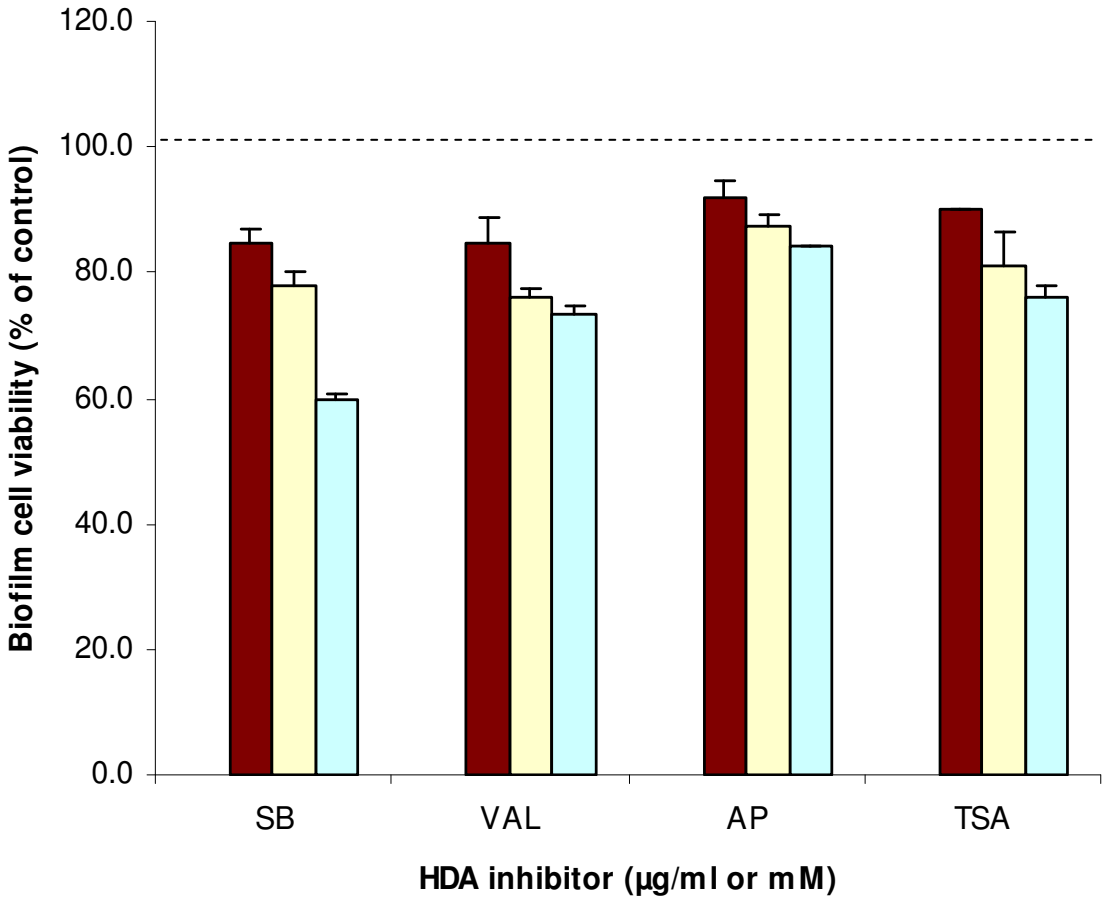


Figure 70. Effects of HDA inhibitors on fluconazole (10 µg/ml) activity against biofilms of *C. albicans* GDH2346: inhibitors added to mature biofilms

Biofilms (48-h) were incubated with HDA inhibitors and fluconazole (10 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 mM or µg/ml. Results are means ± standard errors of two independent experiments carried out in triplicate.

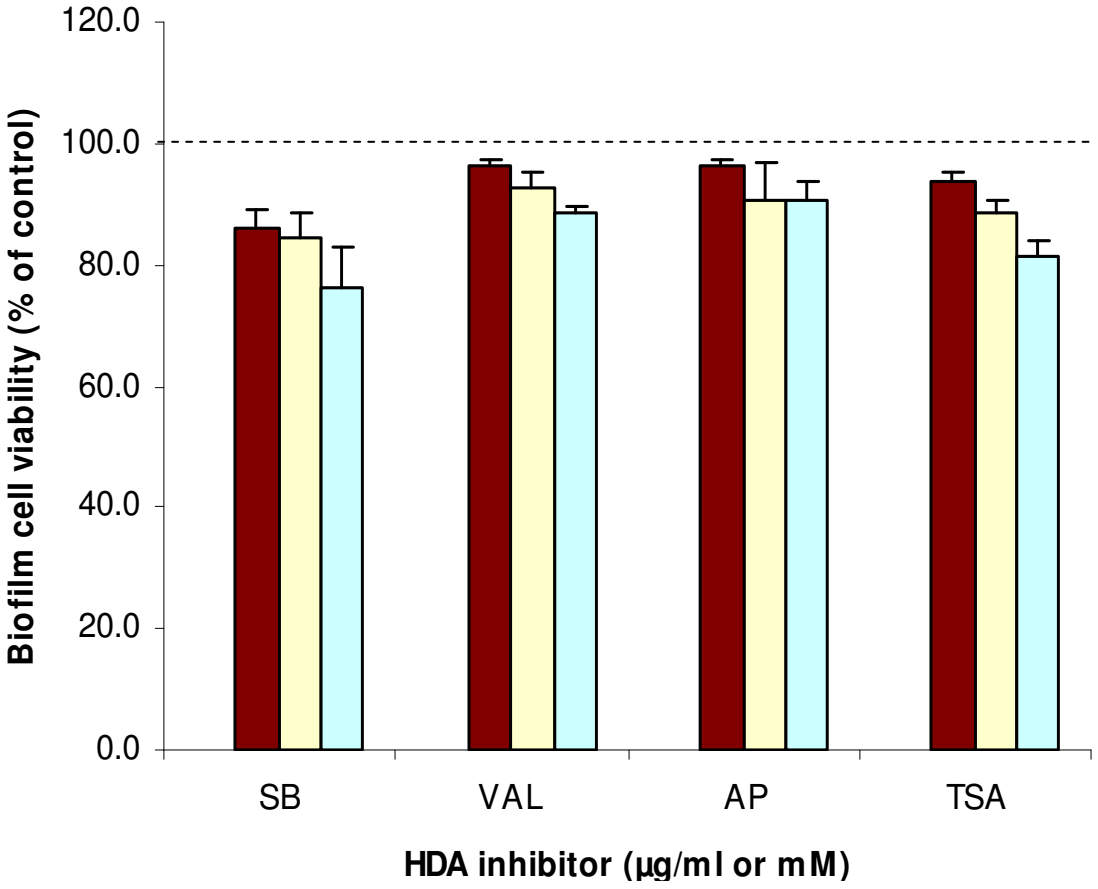


Figure 71. Effects of HDA inhibitors on fluconazole (50 µg/ml) activity against biofilms of *C. albicans* GDH2346: inhibitors added to mature biofilms

Biofilms (48-h) were incubated with HDA inhibitors and fluconazole (50 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 mM or µg/ml. Results are means ± standard errors of two independent experiments carried out in triplicate.

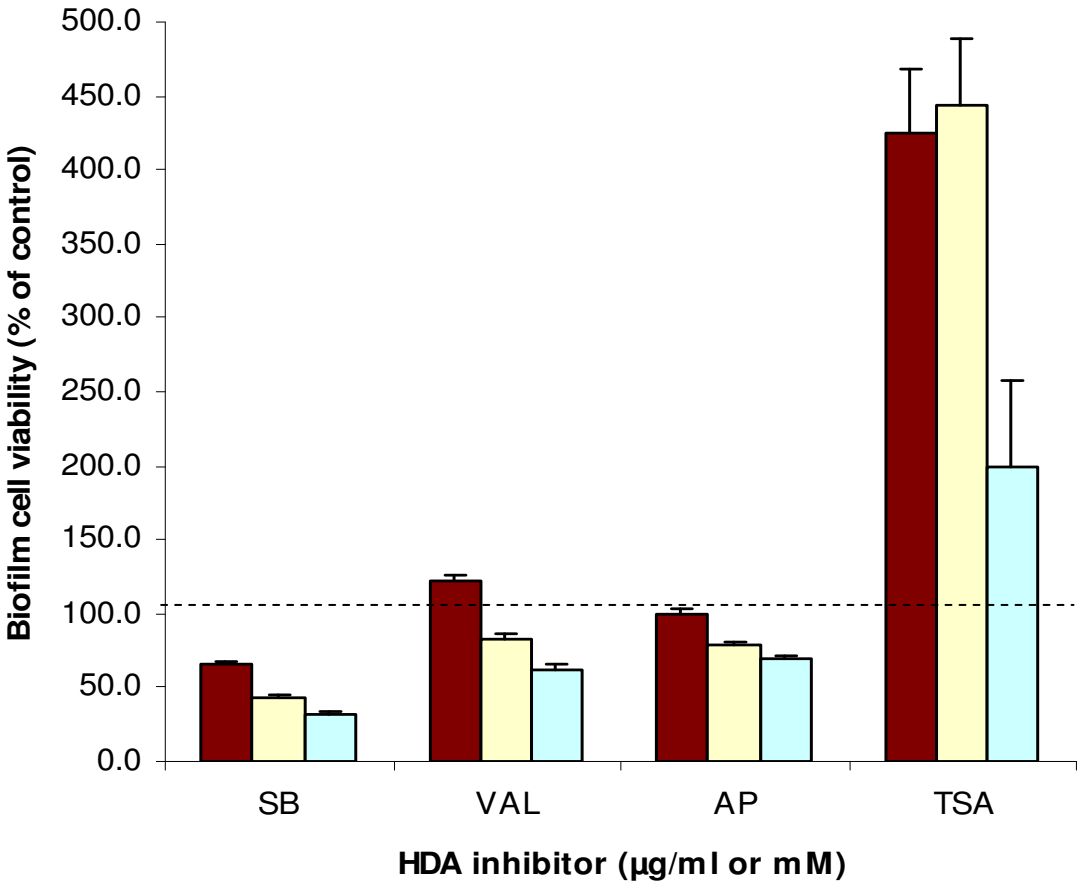


Figure 72. Effects of HDA inhibitors on amphotericin B (10 µg/ml) activity against biofilms of *C. albicans* GDH2346

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (10 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

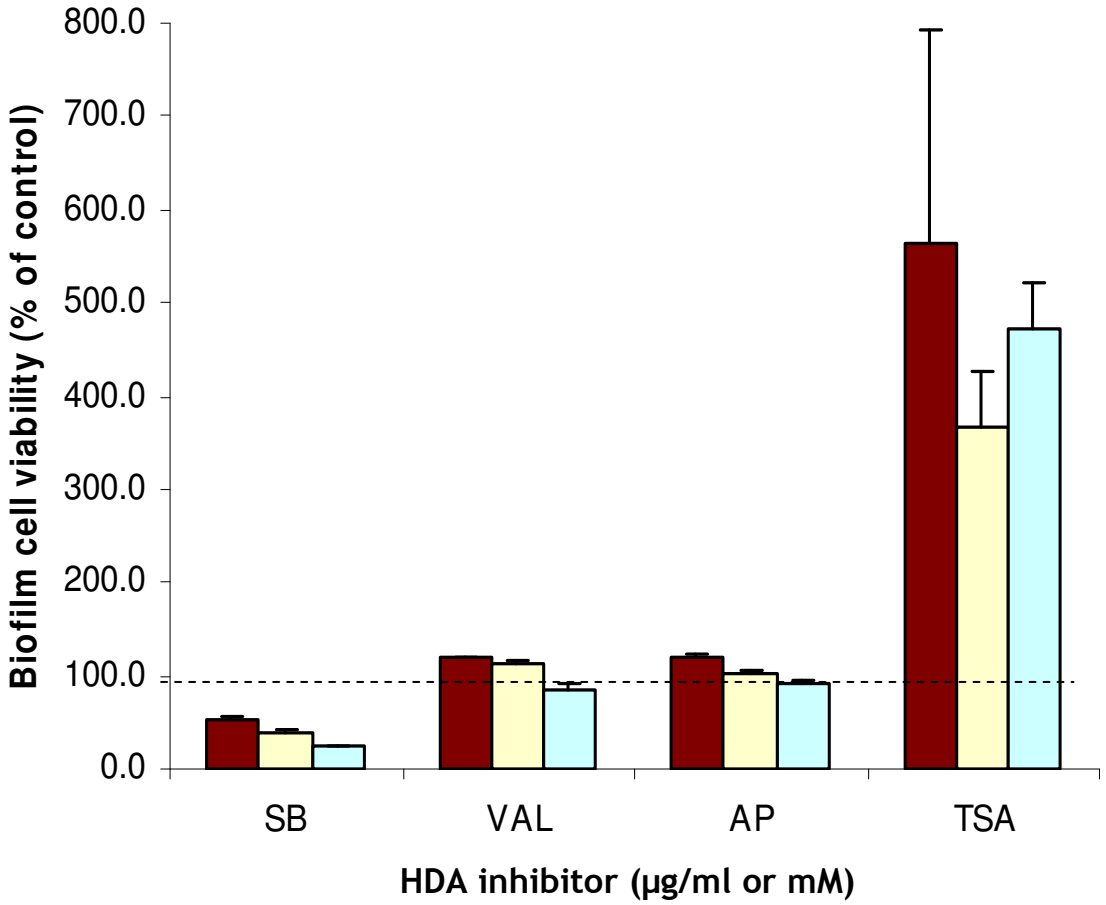


Figure 73. Effects of HDA inhibitors on amphotericin B (50 µg/ml) activity against biofilms of *C. albicans* GDH2346

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (50 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

the XTT readings for trichostatin A were significantly higher than that of the control (P<0.001); the reason for this result is unclear. However, since biofilm cells are enclosed in an extracellular polymeric matrix, detachment of the biofilm could expose cells directly to the XTT reagent which in turn might result in higher readings.

Biofilms of *C. krusei* and *C. parapsilosis* were also examined for the effects of HDA inhibitors on amphotericin B activity. With biofilms of *C. krusei*, sodium butyrate (32 mM) together with amphotericin B (10 or 50 µg/ml) decreased viability by 50% and 66%, respectively (P< 0.001; Figs. 74 and 75). Valproate and apicidin had their greatest effects at a concentration of 32 µg/ml with amphotericin B at 10 µg/ml (i.e. decreases of 37% and 45%, respectively; P<0.001). In contrast, trichostatin A, produced no significant effect at any concentration tested. With biofilms of *C. parapsilosis*, on the other hand, results were less striking overall. Biofilms treated with a high concentration of HDA inhibitors (32 µg/ml or mM) and amphotericin B (10 µg/ml) showed decreases in viability of 23%, 18%, and 10% with valproate, apicidin, and sodium butyrate, respectively (P<0.05; Fig. 76). At the higher concentration of amphotericin B (50 µg/ml), there was no significant effect with any of the inhibitors used (Fig. 77).

6.3.3.2 Viable cell counts

Mature (48-h) biofilms of *C. albicans* GDH2346, *C. parapsilosis* and *C. krusei* were incubated for 24 h at 37ºC in fresh growth medium in the presence of different concentrations of the HDA inhibitors plus amphotericin B (10 or 50 µg/ml). HDA inhibitors had a marked effect on biofilm viability in the presence of the drug. Biofilm populations of *C. albicans* were completely eliminated by sodium butyrate (8 or 32 mM) at low concentrations of amphotericin B (10 µg/ml; Fig. 78), and by even lower concentrations of butyrate (2 mM) at higher concentrations (50 µg/ml) of the drug (Fig. 79). In the absence of butyrate, biofilms of this strain of *C. albicans* produce persisters which remain viable at amphotericin B concentrations of up to 100 µg/ml (Section 5.2.1). Biofilms of *C. krusei* and *C. parapsilosis*, which also produce persisters, were rather less susceptible; combined treatment with butyrate (32 mM) and amphotericin B (50 µg/ml) reduced biofilm viability by 66% and 75%, respectively (Fig. 80 and 81).

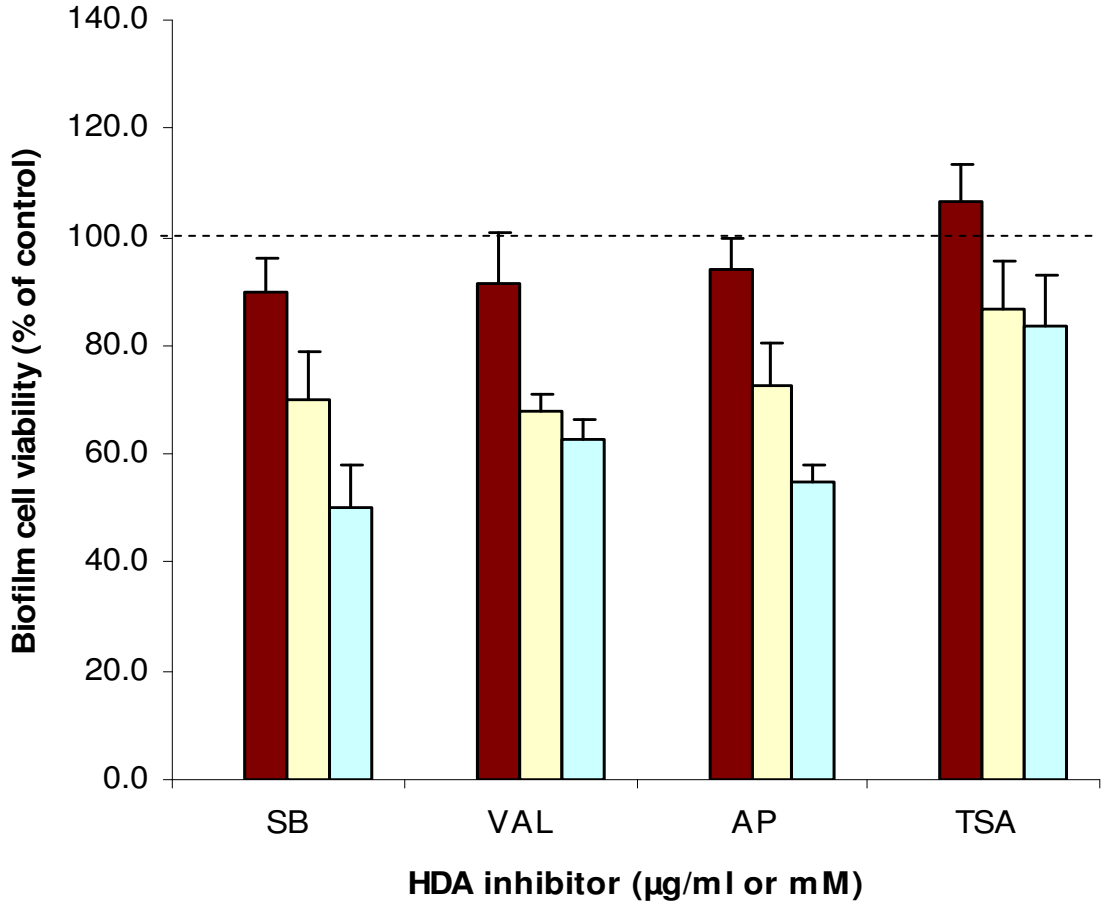


Figure 74. Effects of HDA inhibitors on amphotericin B (10 µg/ml) activity against biofilms of *C. krusei*

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (10 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

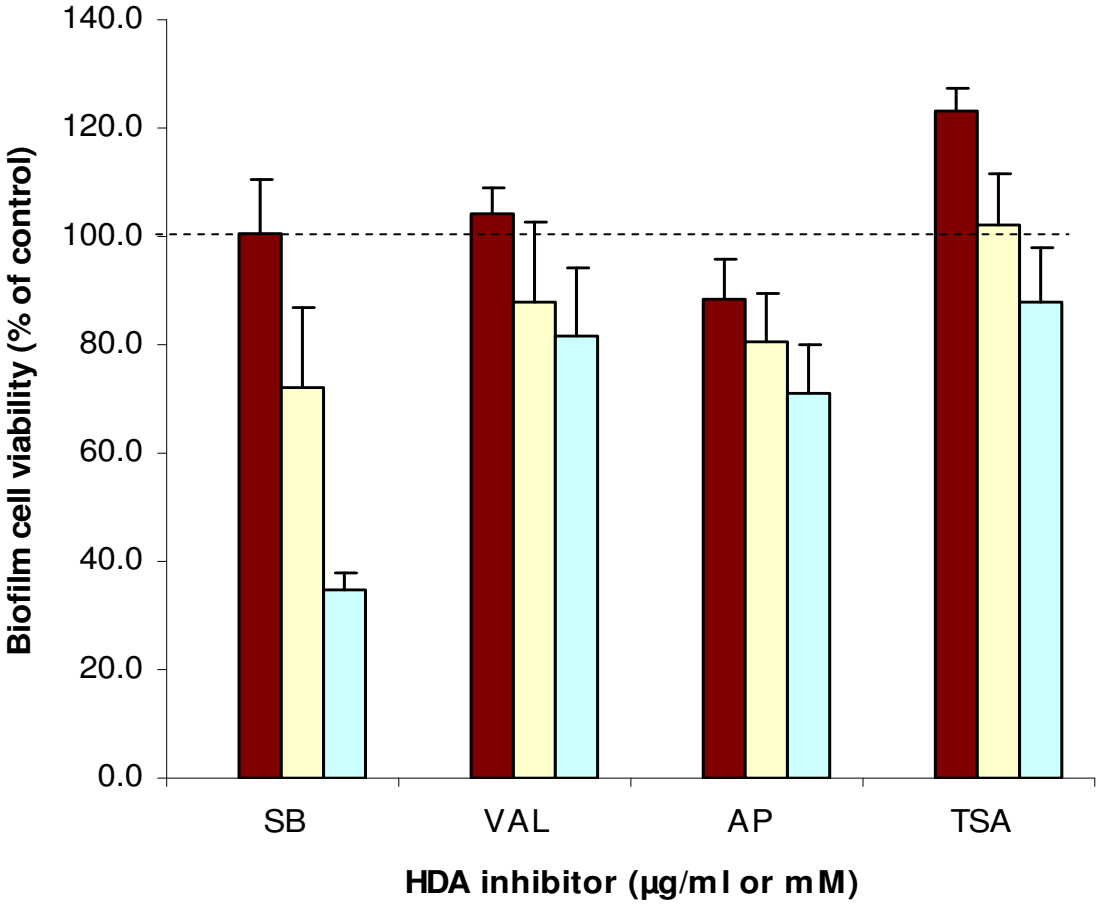


Figure 75. Effects of HDA inhibitors on amphotericin B (50 µg/ml) activity against biofilms of *C. krusei*

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (50 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

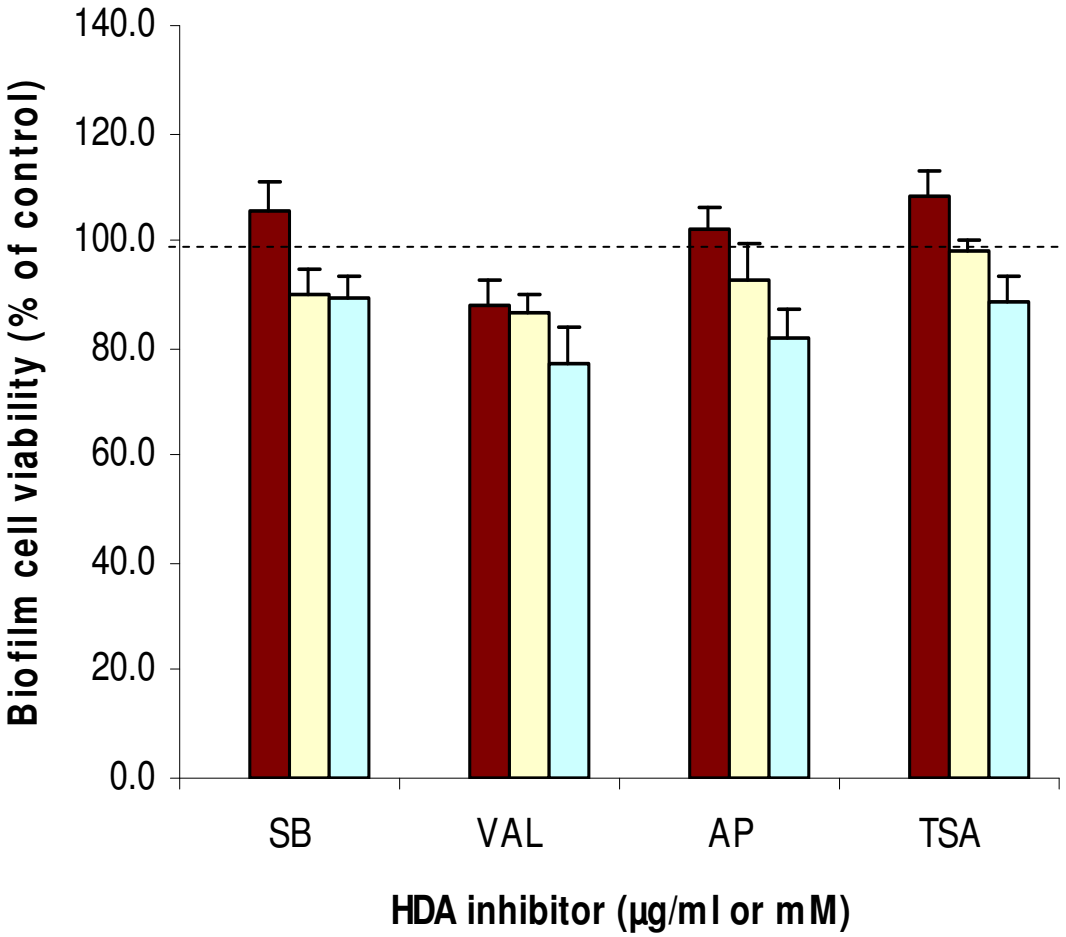


Figure 76. Effects of HDA inhibitors on amphotericin B (10 µg/ml) activity against biofilms of *C. parapsilosis*

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (10 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

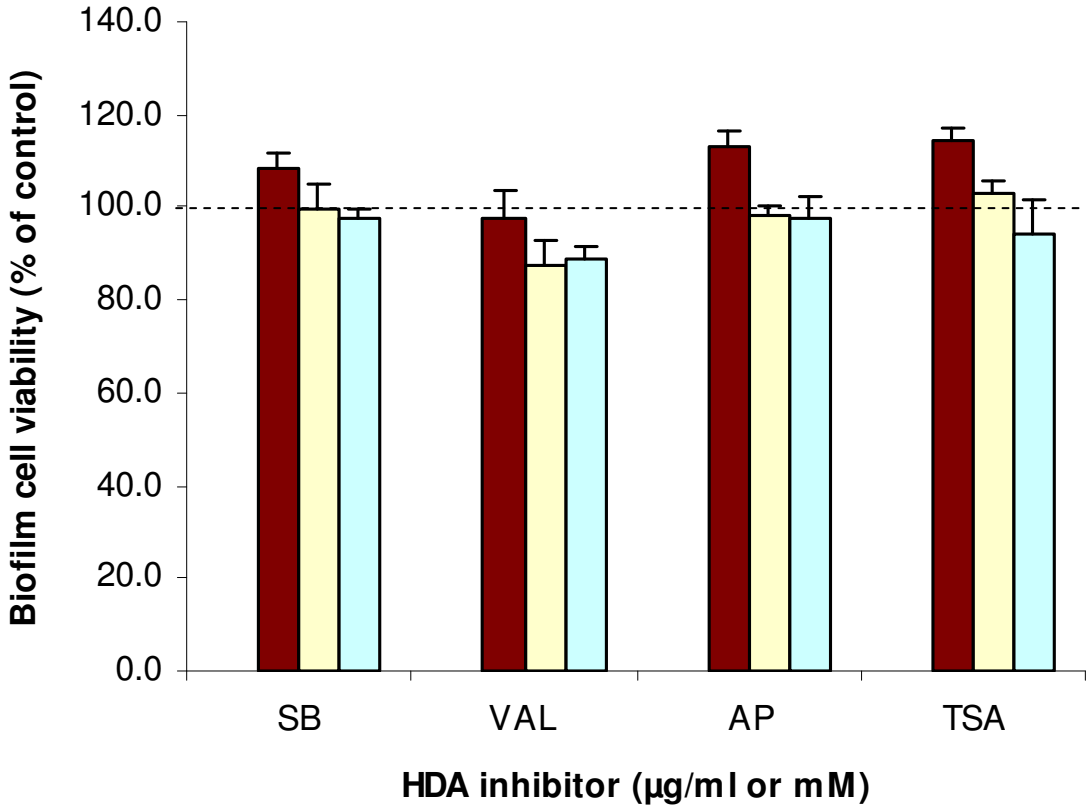


Figure 77. Effects of HDA inhibitors on amphotericin B (50 µg/ml) activity against biofilms of *C. parapsilosis*

HDA inhibitors were added at time zero of biofilm formation. After incubation for 48 h, biofilms were exposed to amphotericin B (50 µg/ml) for 24 h at 37°C. Cell viability was measured by the XTT assay. Sodium butyrate (SB; mM), sodium valproate (VAL; µg/ml), apicidin (AP; µg/ml), and trichostatin A (TSA; µg/ml) were used at concentrations of ■ 2; □ 8; □ 32 µg/ml or mM. Results are means ± standard errors of two independent experiments carried out in triplicate.

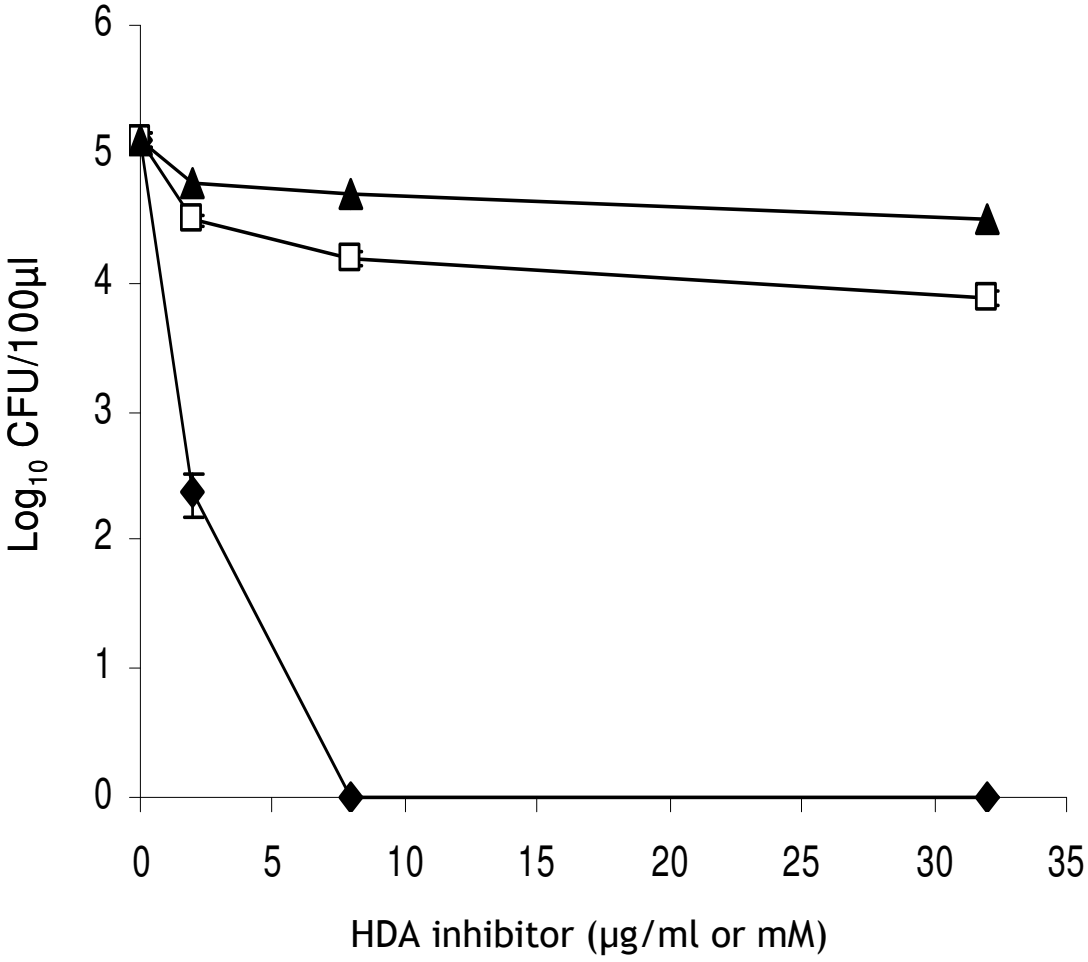


Figure 78. Effects of HDA inhibitors on amphotericin B (10 µg/ml) activity against biofilms of *C. albicans* GDH2346

Biofilms (48-h) were incubated with amphotericin B (10 µg/ml) and sodium butyrate (mM; ♦) or valproate (µg/ml; □) or apicidin (µg/ml; ▲) for 24 h at 37°C and cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate.

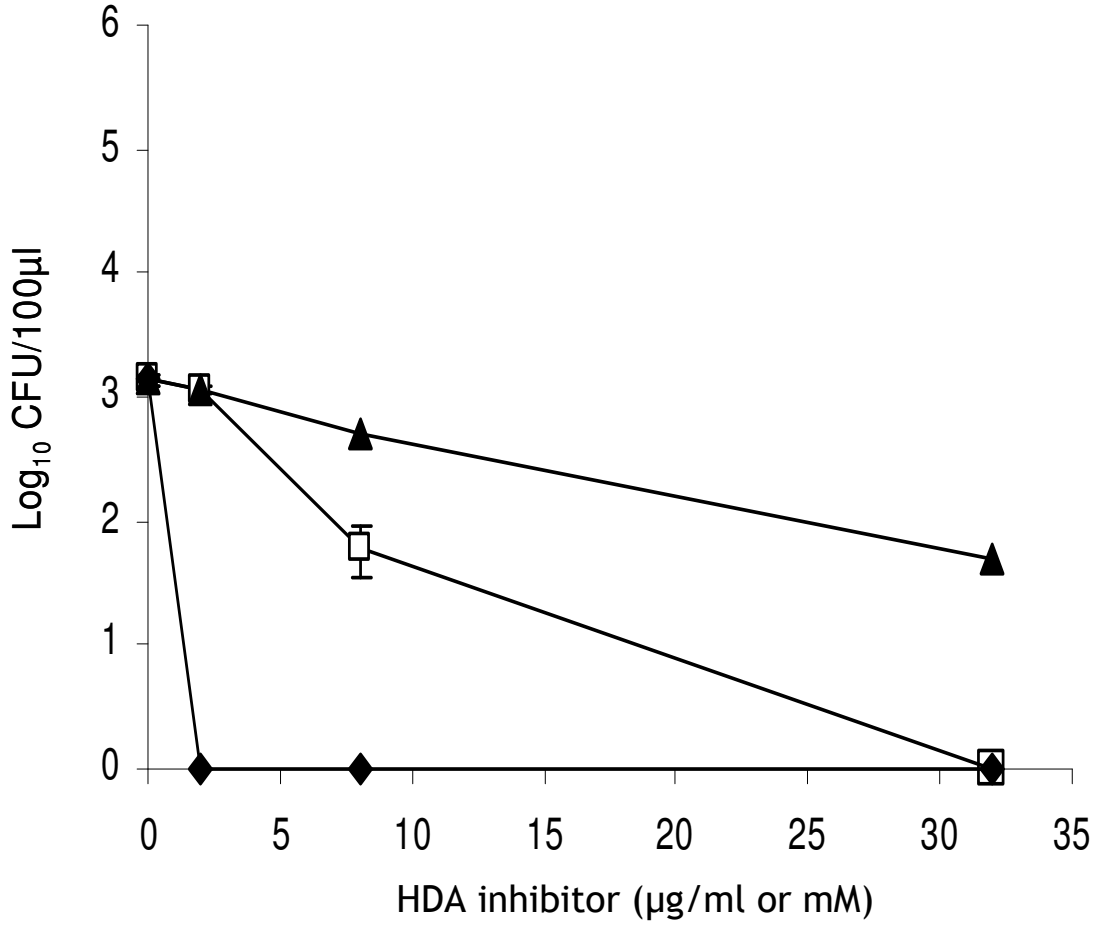


Figure 79. Effects of HDA inhibitors on amphotericin B (50 µg/ml) activity against biofilms of *C. albicans* GDH2346

Biofilms (48-h) were incubated with amphotericin B (50 µg/ml) and sodium butyrate (mM; ♦) or valproate (µg/ml; □) or apicidin (µg/ml; ▲) for 24 h at 37°C and cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate.

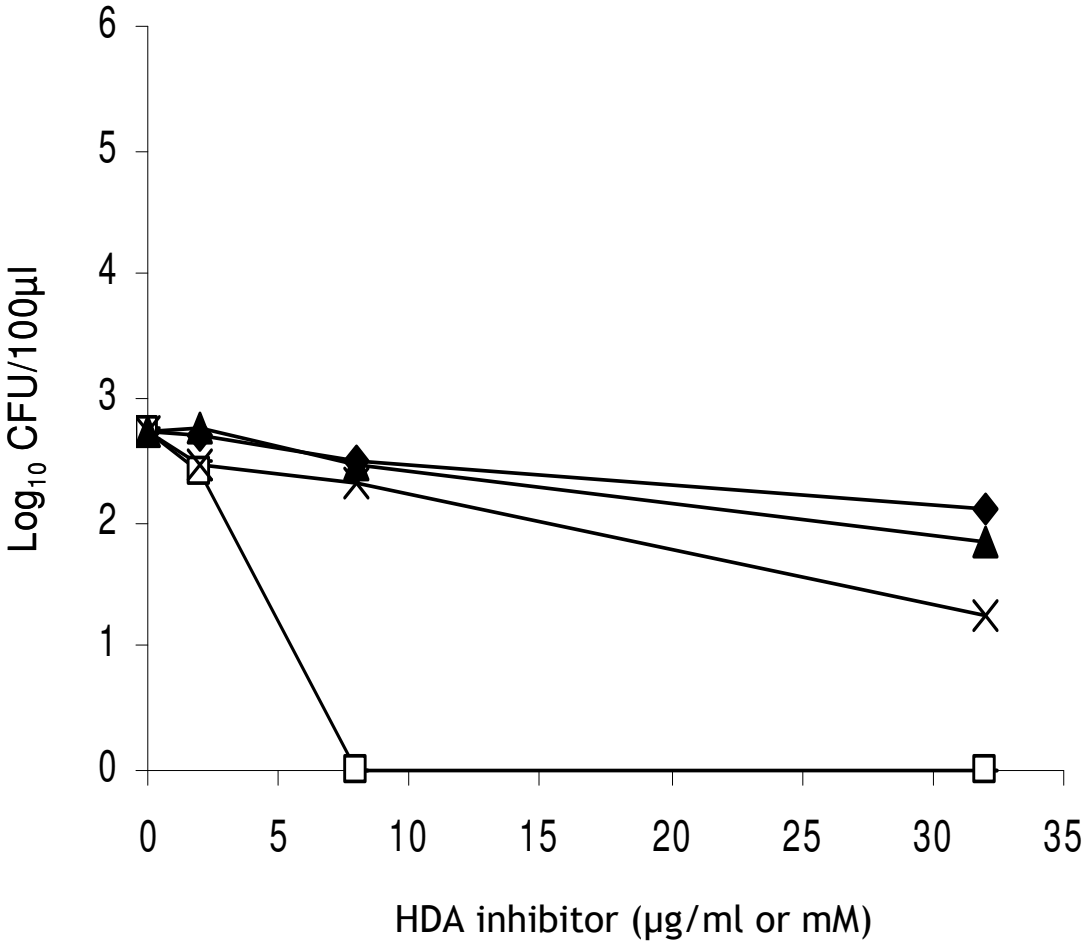


Figure 80. Effects of HDA inhibitors on amphotericin B activity against biofilms of *C. krusei*

Biofilms (48-h) were incubated with amphotericin B (50 µg/ml) and sodium butyrate (mM; ♦) or valproate (µg/ml; □) or apicidin (µg/ml; ▲) or trichostatin A (µg/ml; x) for 24 h at 37°C and cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate.

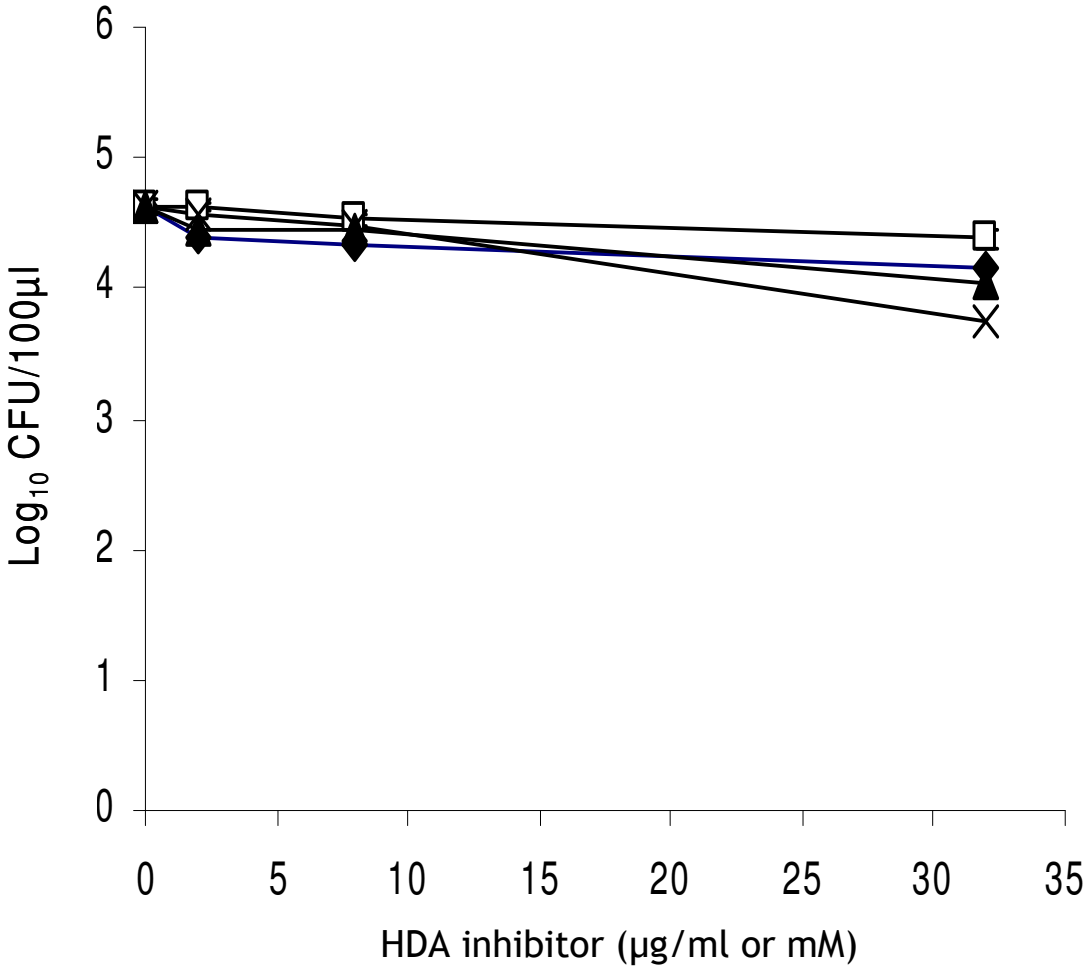


Figure 81. Effects of HDA inhibitors on amphotericin B activity against biofilms of *C. parapsilosis*

Biofilms (48-h) were incubated with amphotericin B (50 µg/ml) and sodium butyrate (mM; ♦) or valproate (µg/ml; □) or apicidin (µg/ml; ▲) or trichostatin A (µg/ml; x) for 24 h at 37°C and cell survival was determined by viable counts. Results are means ± standard errors of two independent experiments carried out in duplicate.

A second HDA inhibitor, sodium valproate, also effectively eliminated biofilm populations of *C. albicans* when used at a concentration of 32 µg/ml with amphotericin B at 50 µg/ml (Fig. 79). Biofilms of *C. krusei* were even more sensitive to this inhibitor and lost all viability after treatment with 8 µg/ml valproate and 50 µg/ml amphotericin B (Fig. 80). On the other hand, biofilms of *C. parapsilosis* lost only 43% of their viability when treated in an identical manner (Fig. 81).

The other HDA inhibitors, apicidin and trichostatin A, both significantly reduced biofilm viability of all species tested ($P<0.002$) when used in conjunction with amphotericin B, although neither inhibitor produced complete killing of the biofilm population. Apicidin at a concentration of 32 µg/ml and amphotericin B (50 µg/ml) effectively reduced biofilm viability of *C. albicans*, *C. krusei*, and *C. parapsilosis* by 96 %, 87 %, and 74 %, respectively (Figs. 79, 80, and 81). Biofilms of *C. krusei* and *C. parapsilosis* were similarly sensitive to trichostatin A and viability was reduced by 97 % and 87 %, respectively (Fig. 80 and 81). Trichostatin A was not tested against *C. albicans* since it was noted that this inhibitor used in combination with amphotericin B caused some detachment of *C. albicans* biofilms from PVC discs.

DISCUSSION

1 *In vitro* activity of amphotericin B and caspofungin at different developmental phases of *Candida* biofilms

Candida cells within biofilms are much more resistant to a variety of antimicrobial agents than are their planktonic counterparts (Hawser & Douglas, 1995; Kuhn *et al.*, 2002b; Mukherjee *et al.*, 2003). These agents include the clinically important antifungal drugs amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole. More recently, however, it has been reported that some of the newer antifungal agents are active against *Candida* biofilms. Thus, while biofilms of *C. albicans* and *C. parapsilosis* were clearly resistant to the new triazoles, voriconazole and ravuconazole, there appeared to be some antibiofilm activity with lipid formulations of amphotericin B and two echinocandins (caspofungin and micafungin) (Kuhn *et al.*, 2002b). Caspofungin was the first echinocandin to be licensed for clinical use. It acts on planktonic cells by inhibiting the synthesis of β-1,3-D-glucan in fungal cell walls. However, its action against *Candida* biofilms is not well characterized.

In the present study, the efficacy of amphotericin B and caspofungin against biofilms of different *Candida* species, at different stages of maturation, was compared. Biofilms of *C. albicans* (strains GDH2346 and SC5314), *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* were allowed to develop over a series of time intervals (8, 17, 24, and 35 h). After each interval, antifungal drug at two or five times the MIC was added and the biofilms were further incubated for a total of 48 h. The ability of amphotericin B and caspofungin to inhibit fungal metabolism at the different stages of biofilm maturation was then evaluated using XTT reduction assays. Under the experimental conditions of this study, the activity of amphotericin B and caspofungin was assessed according to three parameters: (i) the *Candida* species; (ii) the maturation age of the biofilm; and (iii) the concentration of the antifungal drug.

1.1 Activity of amphotericin B

Previous work by Chandra *et al.* (2001a) demonstrated that antifungal resistance of *C. albicans* biofilms increased in conjunction with the developmental phase of the biofilms; in other words, the progression of drug resistance was associated with the increase in metabolic activity of developing

biofilms. However, a recent study by Tobudic *et al.* (2009) failed to observe a correlation between the age of *Candida* biofilms and resistance to amphotericin B; the metabolic activity of biofilms grown for 24 h and 72 h was reduced significantly, but a lesser little effect was observed in 48-h biofilms.

In the present investigation, biofilms of both strains of *C. albicans* were more resistant to amphotericin B throughout development (except for the earliest stage) than the other *Candida* species. Amphotericin B at two times the MIC (ranging from 1.6 to 4.6 μg/ml) had the least effect on *Candida* biofilms. However, when the drug concentration was increased to five times the MIC (ranging from 4 to 11.5 μg/ml), the effect of amphotericin B increased. Amphotericin B concentration-dependent activity has been reported previously with *C. albicans* biofilms (Ramage *et al.*, 2002c; Tobudic *et al.*, 2009). Amphotericin B at the higher concentration showed relatively high activity against *C. tropicalis*, *C. parapsilosis* and *C. glabrata* biofilms, especially at the late development phase (35-h biofilms). Further, the least effect was observed in biofilms grown for 24 h, except with *C. parapsilosis* which showed the lowest metabolic activity in biofilms grown for 17 h.

Amphotericin B binds to the major sterol in the fungal cell membrane, ergosterol, and causes the death of the fungal cell (Slavin *et al.*, 2004; Wingard & Leather, 2004). Mukherjee and colleagues analyzed the membrane sterols of *C. albicans* and revealed that the levels of ergosterol were substantially decreased in intermediate and mature stages compared with those in early-stage biofilms (Mukherjee *et al.*, 2003). They also found that the levels of ergosterol biosynthetic intermediates varied among biofilm developmental stages. Alteration in sterol composition may result in membrane permeability changes that prevent or slow down the entry of antifungal drugs into the *Candida* cell. In addition to this mechanism, the absence of extracellular material in young (8-h) biofilms might play some role in their enhanced susceptibility to the higher concentration of amphotericin B and in the decreased susceptibility of biofilms at later stages of maturation.

1.2 Activity of caspofungin

Data regarding the effect of caspofungin on biofilms of *Candida* species *in vitro* are rather contradictory. Previous studies have reported that caspofungin displays potent antibiofilm activity *in vitro* with decreases in biofilm metabolic activity of ≥50 % at concentrations similar to the MIC (Bachmann *et al.*, 2002; Katragkou *et al.*, 2008; Kuhn *et al.*, 2002b; Tobudic *et al.*, 2009). In contrast to these results, Cocuaud *et al.* (2005) found that caspofungin used at the MIC failed to modify the metabolic activity of *C. albicans* biofilms in all development phases tested. Nevertheless, the same concentration of caspofungin significantly reduced the metabolism of *C. parapsilosis* biofilms grown for 2 h or 48 h (Cocuaud *et al.*, 2005). Related investigations have shown that when caspofungin is used at higher therapeutic concentrations (2 mg/l), it causes a significant reduction in biofilm metabolic activity of different *Candida* species, including both *C. albicans* and *C. parapsilosis* (Cateau *et al.*, 2008; Cocuaud *et al.*, 2005; Ferreira *et al.*, 2009; Katragkou *et al.*, 2008; Ramage *et al.*, 2002c). However, Choi *et al.* (2007) have reported that caspofungin, at therapeutic concentrations, is active against biofilms formed by *C. albicans* and *C. glabrata* but not against those formed by *C. tropicalis* and *C. parapsilosis*.

Although caspofungin is a fungicidal agent for planktonic cells of *Candida* species, it failed to show a fungicidal effect, defined as 95 % killing, on *C. albicans* biofilms tested in any of the developmental phases (Tobudic *et al.*, 2009). By contrast, Ramage *et al.* (2002c) found that caspofungin killed >99 % of biofilm cells of *C. albicans* at therapeutically attainable concentrations. Subsequent studies have shown that complete sterility of mature *Candida* biofilms is difficult to achieve (Bachmann *et al.*, 2002; Katragkou *et al.*, 2008). However, caspofungin has shown considerable efficacy in the treatment of *C. albicans* biofilms *in vivo* (Lazzell *et al.*, 2009; Shuford *et al.*, 2006).

In the current work, caspofungin, at two times the MIC (ranging from 0.6 to 1.6 μg/ml), had a greater effect on *Candida* biofilms than did amphotericin B, although this was not seen with *C. parapsilosis* biofilms at some stages of development (i.e. 24-h and 35-h biofilms). Caspofungin, at five times the MIC (ranging from 1.5 to 4 μg/ml), was rather less effective than at the lower concentration against *C. tropicalis* in all development phases tested and against

C. albicans SC5314 in some phases (i.e. 8-h and 35-h biofilms). The species most susceptible to caspofungin, at both concentrations and throughout biofilm development, was *C. glabrata*.

Reduced activity of caspofungin at high concentrations, above the MIC, has been described for both planktonic and biofilm cells; this phenomenon is called the paradoxical effect or paradoxical growth. It has been reported for planktonic cells of different *Candida* spp. (Ostrosky-Zeichner *et al.*, 2003; Stevens *et al.*, 2004; Stevens *et al.*, 2005) and for *C. albicans* biofilms (Ramage *et al.*, 2002c; Tobudic *et al.*, 2009). In a recent survey of five different *Candida* species (Melo *et al.*, 2007) all isolates except those of *C. tropicalis* displayed paradoxical growth more frequently when they were grown as biofilms than when they were grown as planktonic cells. Moreover, paradoxical growth was undetectable among *C. metapsilosis* isolates grown planktonically but was present in 100% of isolates grown as biofilms. In a separate investigation (Ferreira *et al.*, 2009) paradoxical growth was confirmed in biofilms of *C. albicans* and *C. tropicalis* but not in those of *C. parapsilosis*.

So far, the mechanism of the paradoxical effect of caspofungin has not been elucidated and its clinical significance is still unclear. In this study, paradoxical growth was detected in planktonic cells of some *Candida* isolates at different concentrations of the drug; these were *C. albicans* (both strains) and *C. tropicalis*. Reduced activity of caspofungin was observed at high drug concentrations: at 4 to 16 μg/ml, 8 to 16 μg/ml and 3 to 16 μg/ml for *C. albicans* GDH2346, *C. albicans* SC5314 and *C. tropicalis*, respectively. Among *Candida* biofilms tested, caspofungin at five times the MIC showed reduced activity against *C. tropicalis* at all developmental phases and against *C. albicans* SC5314 biofilms grown for 8 h and 35 h only. Recently, Tobudic *et al.* (2009) also demonstrated decreased caspofungin activity at high concentrations in *C. albicans* biofilms, particularly biofilms grown for 48 h.

Overall, this part of the study has demonstrated that amphotericin B has a concentration-dependent activity against *Candida* biofilms. Activity varied, depending on the *Candida* species and the development phase of the biofilm. Caspofungin, at two times the MIC, exhibited a greater effect on *Candida* biofilms than amphotericin B, except with *C. parapsilosis* biofilms grown for 24 h and 35 h.

The efficacy of caspofungin in inhibiting the metabolism of biofilm cells was dependent on the concentration of antifungal used, the *Candida* species, and the phase of biofilm development. It was also complicated by the paradoxical effect. Several resistance mechanisms may be activated during the course of biofilm development. These could include the time-dependent production of quorum sensing molecules, activation of efflux pumps, alterations of cell wall assembly and the presence of persister cells (Tobudic *et al.*, 2009). Further research is required to elucidate the role of these mechanisms over the various stages of biofilm maturation.

2 Penetration of antifungal agent (caspofungin) through *Candida* biofilms

Biofilm-mediated antifungal resistance has been well documented and one of the mechanisms that may contribute to biofilm resistance is restriction of drug penetration through the biofilm matrix. The extracellular polymeric matrix, which envelops biofilm cells, may affect and limit the diffusion of solutes and antimicrobials. For example, the ability of a drug to penetrate the biofilm can be severely reduced if it is reactively neutralized as it diffuses into a cell cluster; this may involve enzymatic degradation or drug binding to charged extracellular polymers (Anderl *et al.*, 2000; Stewart *et al.*, 2001). Anderl *et al.* (2000) attributed the ability of ciprofloxacin to penetrate a bacterial biofilm faster than ampicillin to its low reactivity with the biofilm. A similar finding was reported by Stewart *et al.* (2001) who observed that chlorosulfamate penetrated bacterial biofilms more rapidly than hypochlorite due to a slower reaction rate with biofilm components. These results suggest that the extent of antibiotic penetration through bacterial biofilms depends greatly upon both the nature of the antimicrobial agent and the organism involved.

Most studies on drug penetration have been conducted with bacterial biofilms. However, three recent investigations (Al-Fattani & Douglas, 2004; Samaranayake *et al.*, 2005; Subha & Gananamani, 2008) have evaluated the penetration of antifungals through *Candida* biofilms. These studies were carried out using an *in vitro* filter disc assay with commonly used antifungals such as

amphotericin B, fluconazole, itraconozole, ketoconazole, voriconazole, and flucytosine. Al-Fattani & Douglas (2004) showed that fluconazole diffused into single-species *Candida* biofilms more rapidly than flucytosine. Both drugs penetrated biofilms of *C. glabrata* and *C. krusei* faster than those of *C. parapsilosis* and *C. tropicalis*. Moreover, amphotericin B showed rapid penetration through *C. albicans* biofilms. In mixed-species biofilms containing *C. albicans* and *S. epidermidis*, the diffusion of the antifungal drugs was very slow. Subsequently, Samaranayake *et al.* (2005) reported that fluconazole and flucytosine demonstrated a similar degree of penetration through biofilms of *C. albicans*, *C. parapsilosis*, and *C. krusei*, while amphotericin B penetrated least well. Biofilms of all three *Candida* species showed a significant increase in drug penetration with increasing fluconazole and flucytosine concentrations but not with increasing concentrations of amphotericin B. In contrast to both these earlier studies, Subha & Gnanamani (2008) reported that all drugs tested (amphotericin B, fluconazole, itraconozole, ketoconazole) were unable to penetrate biofilms of *C. albicans* and *C. tropicalis* and reach the topmost disc (the concentration disc) even at concentrations as high as 2000 µg/ml. However, partial penetration by azoles was observed.

In this study, the penetration of caspofungin through biofilms of different *Candida* species was investigated using a similar model system. To date, the ability of this drug to penetrate *Candida* biofilms has not been reported. The rates of diffusion of caspofungin through biofilms of different strains of *C. albicans* were similar. Al-Fattani & Douglas (2004) described related findings with fluconazole and flucytosine; the rates of diffusion of either drug through biofilms of three strains of *C. albicans* were similar. However, different levels of penetration through biofilms of non-*C. albicans* species were observed. Caspofungin diffusion through biofilms of *C. glabrata* and *C. parapsilosis* was faster and greater than that through biofilms of *C. krusei* and *C. tropicalis*. Faster drug penetration through biofilms of *C. glabrata* and slower penetration through those of *C. tropicalis* was also reported by Al-Fattani & Douglas (2004).

The ability of antifungal agents to diffuse into *Candida* biofilms will depend on the chemical nature of the drug as well as the nature of the biofilm matrix material. In this connection, it is noteworthy that in biofilms of *C.*

albicans, *C. parapsilosis* and *C. glabrata* the major matrix component is carbohydrate, probably glucan, whereas in *C. tropicalis* biofilms, which are poorly penetrated by drugs, it is hexosamine (Al-Fattani & Douglas, 2006; Silva *et al.*, 2009). Hexosamine is also present in the matrix of *S. epidermidis* biofilms in the form of a polysaccharide of ß-1,6-linked N-acetylglucosamine (Mack *et al.*, 1996); this polymer mediates cell-cell interaction within the biofilm (Gotz, 2002) and its production has been related to *S. epidermidis* virulence in catheter-infection models in animals. However, synthesis of glucan as a matrix component of some *Candida* biofilms could be inhibited by caspofungin and this might explain the rapid penetration of the drug through such biofilms. This is discussed further in Section 2.2.

2.1 Effect of antifungal agents on the viability of biofilm cells

The effect of antimicrobial agents on biofilm cell viability following drug penetration has been investigated previously. Anderl *et al.* (2000) reported that ciprofloxacin perfused bacterial biofilms within a few hours but bacteria were not killed even after 24 h of exposure. Likewise, a biofilm formed by a ß-lactamase deficient mutant was fully perfused by ampicillin but the cells were not effectively killed. Further, Stewart *et al.* (2001) demonstrated that both chlorosulfamate and hypochlorite completely penetrated bacterial biofilms after 60 min of treatment but neither antimicrobial was able to kill biofilm cells very effectively. Similar findings were reported by Al-Fattani & Douglas (2004). After 3 to 6 h, both fluconazole and flucytosine had fully penetrated biofilms of different *Candida* species, yet neither drug was able to produce complete killing of biofilm cells even after 24 h of treatment.

In this study, the effect of caspofungin and amphotericin B on biofilm cell viability was assessed by viable counts. Biofilms 'sandwiched' between the two membranes were exposed to antifungal agent-containing agar for 6 h or 24 h. An earlier investigation in this laboratory showed rapid penetration of amphotericin B throughout *C. albicans* biofilms (Al-Fattani & Douglas, 2004); however, after 6 h of amphotericin B and caspofungin penetration, about 70% of the biofilm cells remained viable. Both drugs failed to produce complete killing of biofilm cells even after 24 h. Indeed, caspofungin showed less activity than amphotericin B. This may be associated with the paradoxical effect of caspofungin at high

concentrations. A drug concentration of 24 µg/ml was used in this assay. Low drug concentrations are unsuitable as high concentrations are required to obtain well defined zones of growth inhibition.

2.2 Scanning electron microscopy of biofilms

An improved scanning electron microscopy technique was used in this work to visualize biofilm structural changes associated with antifungal penetration. Conventional SEM methods appear to severely distort biofilm architecture, due to the fixation and dehydration steps required (Chandra *et al.*, 2008; Ferreira *et al.*, 2009; Ramage *et al.*, 2001a). It has been demonstrated recently that using cationic dyes during fixation improves preservation of biofilm exopolysaccharide matrix and protects it from collapse during the dehydration/critical point drying process. Moreover, various cationic dyes (such as alcian blue, safranin O, and ruthenium red) can dramatically increase visualization of the exopolysaccharide matrix structure, and different cationic dyes may selectively permit visualization of different components in the matrix (Di Bonaventura *et al.*, 2006; Erlandsen *et al.*, 2004b). For instance, the Gram-positive bacterium, *Enterococcus faecalis*, produces biofilms on cellulose tubing which have an extensive matrix (Erlandsen *et al.*, 2004a). Fixation of these biofilms in aldehyde alone, without cationic dyes, allowed the visualization of a few surface fibrils that appeared to attach the cocci to the substratum, whereas addition of alcian blue to the aldehyde cocktail resulted in a different matrix; this consisted of a branching network of fibrils on the cell surface and a thick mat of fibrils on the substratum. However, when another cationic dye, safranin O, was used, fewer filaments were present and some of them formed a meshwork above cells anchored to the substratum. Variations in size, shape and charge density of the dyes appeared to account for these differences in matrix appearance (Erlandsen *et al.*, 2004b).

Previous work by Samaranayake *et al.* (2005), using conventional SEM procedures, demonstrated that biofilms grown on polycarbonate membrane filters are distinct from those on polymethyl methacrylate strips or silicone elastomer discs (Chandra *et al.*, 2001a; Kuhn *et al.*, 2002a). For example, mature biofilms of *C. albicans* contained very few hyphal forms on membrane filter discs in comparison to the abundant filamentation seen on polymethyl

methacrylate strips (Chandra *et al.*, 2001a), silicone elastomer (Kuhn *et al.*, 2002a), or polyvinyl chloride discs (Hawser *et al.*, 1998).

In this study biofilms of six *Candida* isolates were examined using the cationic-dye procedure for SEM. The results revealed that *C. albicans* forms a dense biofilm consisting of mostly yeast cells on polycarbonate filters; occasional filamentous forms were embedded within the exopolymeric material, an observation similar to that of Samaranayake *et al.* (2005). On the other hand, *C. parapsilosis* and *C. glabrata* appeared to develop biofilms that were less dense than those of *C. albicans*; they contained mostly yeast cells, were devoid of hyphal forms and were covered by less matrix material. This was similar to the findings of Kuhn *et al.* (2002a). Subsequent observations of relatively sparse biofilm formation by *C. parapsilosis* on polycarbonate filters were made by Samaranayake *et al.* (2005). Recently, other research groups have reported that *C. glabrata* forms less extensive biofilms consisting of a multicellular structure packed with yeast cells but devoid of pseudohyphae and hyphae (Silva *et al.*, 2009; Thein *et al.*, 2007). Based on biofilm dry weight, Hawser and Douglas (1994) showed that isolates of *C. parapsilosis* and *C. glabrata* produced significantly less biofilm growth on PVC catheter discs compared with *C. albicans*. However, *C. tropicalis* and *C. krusei* exhibited more extensive biofilm growth than did *C. albicans* (Hawser & Douglas, 1994). Recently, SEM studies by Al-Fattani and Douglas (2004) have revealed that *C. tropicalis* biofilms consist of a dense network of yeast cells and filamentous forms encased in a very slimy matrix material (Al-Fattani & Douglas, 2004; Al-Fattani & Douglas, 2006).

In the current investigation, results similar to those in all of these earlier studies were obtained. Biofilms of *C. parapsilosis* and *C. glabrata*, formed on polycarbonate membrane filters, were less dense than those of *C. albicans* and consisted of yeast cells only. On the other hand, biofilms of *C. tropicalis* and *C. krusei* possessed a very extensive, slimy matrix material covering a dense network of yeast cells and filaments. The slowest rates and the lowest levels of caspofungin penetration were found with biofilms formed by *C. tropicalis* and *C. krusei* and it is possible that the complex structure with slimy matrix of these biofilms might retard antifungal penetration. In contrast, the biofilms showing the fastest rates, as well as the highest levels of caspofungin penetration were

those formed by *C. parapsilosis* and *C. glabrata*. One reason for this observation could be the formation of less extensive biofilms by both species.

Previous SEM observations of antifungal-treated *Candida* biofilms demonstrated some obvious effects of the drugs on biofilm structure. Samaranayake *et al.* (2005) reported that although amphotericin B was the drug least penetrant through biofilms of *C. albicans*, *C. parapsilosis*, and *C. krusei*, it caused more structural damage to the superficial cells of the biofilm than either fluconazole or flucytosine. Further, Bachmann *et al.* (2002) examined the effect of caspofungin on biofilms of *C. albicans* and their results indicated that caspofungin significantly affected both cellular morphology and overall biofilm architecture. In the present study, an evaluation of the ultrastructural effects of antifungal penetration through *Candida* biofilms revealed that different drugs caused different degrees of damage among biofilms of different *Candida* species.

SEM observations showed that caspofungin caused more damage to biofilms of all the *Candida* isolates tested than did amphotericin B. Caspofungin severely affected the matrix architecture as well as cell morphology. Caspofungin, a new lipopeptide antifungal, belongs to the echinocandin family that inhibits the synthesis of ß-1,3-glucan, a major component of the fungal cell wall (Denning, 2002; Georgopapadakou, 2001; Kauffman & Carver, 2008). ß-Glucans are the main component of the *C. albicans* cell wall, accounting for approximately 60% of its weight (Chaffin *et al.*, 1998; Klis *et al.*, 2001). Moreover, culture supernatants of *Candida* contain high amounts of ß-glucans (Odabasi *et al.*, 2006). Interestingly, cell walls of *C. albicans* biofilm cells contain significantly greater concentrations of ß-1,3-glucan than their planktonic counterparts; these glucans can also be found in the supernatants surrounding biofilms and in the matrix material (Nett *et al.*, 2007a; Nett *et al.*, 2007b). These observations would suggest that caspofungin exposure affects biofilm cell walls and matrix composition, which in turn change cell morphology and the architecture of the biofilm.

The chemical composition of matrix material could play a minor role in slowing the diffusion of antimicrobial agents. Biofilm cells of *P. aeruginosa* have been shown to contain periplasmic glucans which appear to interact physically with antibiotics and retard their diffusion (Mah *et al.*, 2003). It has also been

suggested that the nature and the amount of extracellular glucans produced by *Streptococcus mutans* from sucrose in dental plaque play a role in slowing acid diffusion (Hojo *et al.*, 1976). Previous work by Baillie and Douglas (2000) demonstrated that *C. albicans* biofilm matrix contains carbohydrate, protein, phosphorus, glucose and hexosamine. However, a considerable portion of the matrix material remains unidentified. These results were confirmed in recent work by Al-Fattani and Douglas (2006) on the chemical composition of the biofilm matrix. This study revealed that *C. tropicalis* biofilms, which are poorly penetrated by antifungal agents (Al-Fattani and Douglas, 2004; results of the present investigation), contain matrix material rich in hexosamine (27.4%), with smaller amounts of carbohydrate, protein, phosphorus, and uronic acid (3.3%, 3.3%, 0.2%, and 1.6%, respectively). In contrast, *C. albicans* biofilms, which are more readily penetrated by drugs (Al-Fattani and Douglas, 2004; results of the present investigation), possess a matrix that consists mainly of carbohydrate (39.6%, including glucose 32.2%) with small amounts of protein, hexosamine, phosphorus, and uronic acid (5.0%, 3.3%, 0.5%, and 0.1%, respectively). It is possible that the slow rate of drug penetration through biofilms of *C. tropicalis* is due to the production of a hexosamine-containing matrix polymer.

The impact of several enzymes on biofilm matrix polymers has also been investigated (Al-Fattani & Douglas, 2006). The greatest effect was observed with lyticase (which hydrolyses β-1,3-glucan), which caused an 85% and 53% detachment of *C. albicans* and *C. tropicalis* biofilms, respectively. This result indicates that some of the glucose present in the *C. albicans* matrix could be present as β-1,3-glucan, a polysaccharide which is a major structural component of the cell wall (Al-Fattani & Douglas, 2006). Another study by Nett *et al.* (2007b) showed that the cell walls from biofilm cells of *C. albicans* contained significantly more total carbohydrate and β-1,3-glucan compared with walls from planktonic cells, and that these glucans could be found in the supernatant surrounding the biofilm and in the matrix. Moreover, cell walls from both planktonic and biofilm cells bound a measurable amount of fluconazole. However, four- to five-fold more compound was bound to the biofilm cell walls. The effect of β-1,3 glucanase on the susceptibility of biofilm cells to fluconazole and amphotericin B was also investigated. The enzyme markedly enhanced the activity of both drugs against *C. albicans* biofilm cells. These results suggest that

a cell wall component, such as glucan, may interact physically with the antifungal and retard penetration to the site of action (Nett *et al.*, 2007b).

Recently, a study by Silva *et al.* (2009) attempted to characterized biofilms of non-*C. albicans Candida* species (NCAC). In this investigation, biofilm matrices of NCAC species were analyzed for carbohydrate and protein content. Consistent differences were found in the matrix composition of biofilms of the NCAC species. Matrix material from *C. tropicalis* biofilms had low amounts of both carbohydrate and protein while that extracted from *Candida parapsilosis* biofilms had high amounts of carbohydrate with low amounts of protein. In contrast, *C. glabrata* matrix material had higher quantities of both protein and carbohydrate compared to the other species; protein levels were on average five times higher than those of *C. parapsilosis* and *C. tropicalis* (Silva *et al.*, 2009). The results of this study are in accordance with previous work on *C. tropicalis* biofilms by Al-Fattani and Douglas (2006) which demonstrated that the matrix of this species contains mainly hexosamine, with smaller amounts of carbohydrate and protein. It also further supports the notion that biofilms which contain substantial amounts of matrix carbohydrate (those of *C. albicans, C. parapsilosis*, and *C. glabrata*) are more easily penetrated by antifungal agents, including caspofungin, than are hexosamine-rich biofilms such as those of *C. tropicalis*.

A non-destructive, *in situ* analysis by Raman microscopy was applied recently to multispecies biofilms (Ivleva *et al.*, 2008; Ivleva *et al.*, 2009; Wagner *et al.*, 2009). Without staining the sample, this technique can provide detailed information about the chemical composition and the distribution of extracellular polymeric substances and microorganisms within such biofilms. Based on their specific Raman scattering signals, the various constituents in the biofilm matrix can be classified (e.g. polysaccharides, proteins) and detailed chemical information about them obtained. Raman microscopy is therefore a promising future tool for further characterization of different matrix constituents in *Candida* biofilms.

3 Persister cells in planktonic cultures and biofilms of different *Candida* species

The discovery of persister cells in bacterial biofilms helped to clarify the puzzling resistance of biofilms to killing by antimicrobial agents (Keren *et al.*, 2004b). Persisters were originally described as dormant or nongrowing cells (Lewis, 2007) but are now recognized as drug-tolerant cells – usually 1% or less of the overall population – which neither grow nor die in the presence of microbicidal antibiotics. The ability to avoid killing is their key characteristic (Keren *et al.*, 2004a). The existence of such cells in biofilms of *C. albicans* (Khot *et al.*, 2006; LaFleur *et al.*, 2006; Lafleur *et al.*, 2009; Tsang & Tang, 2009) has also been reported recently . LaFleur *et al.* (2006) demonstrated that exposure of *C. albicans* biofilms to amphotericin B or chlorhexidine resulted in biphasic killing indicative of the presence of a persister subpopulation capable of surviving high concentrations of these fungicidal agents. In contrast to bacterial species tested, planktonic cultures (exponential or stationary phase) of *Candida* species seemed to be devoid of persisters (LaFleur *et al.*, 2006).

In this study, viability measurements and fluorescein staining were used to investigate the existence of persisters in planktonic cultures and biofilm cells of *C. albicans*, *C. tropicalis, C. glabrata*, *C. krusei*, and *C. parapsilosis* subjected to different concentrations of amphotericin B. Planktonic cultures of all these species appeared to lack persisters, in accordance with the earlier results of LaFleur *et al.* (2006). Similarly, using the criteria adopted previously for *C. albicans* (LaFleur *et al.*, 2006), persisters were absent from biofilms of some species. Persisters were found in biofilms of *C. albicans* GDH2346, *C. krusei,* and *C. parapsilosis* in low numbers (0.001 to 0.07%), while biofilms of *C. albicans* SC5314, *C. glabrata* and *C. tropicalis* were devoid of such cells. It is unlikely that persister cells were mutants since acquired resistance to amphotericin B is rare. Moreover, LaFleur *et al.* (2006) in their study clearly demonstrated that *C. albicans* persisters were phenotypic variants, not mutants, of the wild type.

In addition to viability measurements, fluorescein diacetate was also used to discriminate between living and dead biofilm cells; this dye specifically stains dead cells green. Biofilms (48-h) of *C. albicans* (both strains), *C. krusei* and *C. parapsilosis* were treated with a high concentration (100 µg/ml) of amphotericin

B for 24 h at 37ºC and the cells were then stained with fluorescein diacetate. With most of these biofilms, small numbers of unstained (live) cells were detected. These cells appeared to have a normal morphology and their numbers varied according to the *Candida* species under investigation. *C. parapsilosis* biofilms contained more of the live cells than did biofilms of *C. krusei* or *C. albicans* GDH 2346. By contrast, no live cells were detected in biofilms of *C. albicans* SC5314 exposed to a high concentration of amphotericin B. Fluorescence was rarely observed in untreated, control cells (ie. 72-h biofilm cells). On the assumption that the unstained cells represent drug-tolerant persisters, these results with fluorescein staining confirmed the viability measurements, showing that persisters were present in biofilms of *C. albicans* GDH 2346, *C. krusei* and *C. parapsilosis*, but absent from biofilms of *C. albicans* SC5314.

A recent investigation by Khot *et al.* (2006) also identified a small subpopulation of cells in *C. albicans* biofilms showing increased tolerance to amphotericin B. The biofilms were cultured in a tubular flow cell and exhibited typical *C. albicans* biofilm architecture (Baillie & Douglas, 1999a; Douglas, 2003), consisting of a thin basal yeast layer and an overlying thicker, partly filamentous layer. After growth, most of the biofilm was removed by draining and washing the tubing, but a monolayer of yeast cells remained on the surface. In dose-response experiments with amphotericin B, this yeast subpopulation showed greater tolerance of the drug than biofilm cells removed by washing. Metabolic activity, rather than viability, was measured after exposure to a range of amphotericin B concentrations for 1 h. The dose response curve for the basal yeast cells decreased to a plateau of approximately 50% metabolic activity between a drug concentration of 3.7 μg/ml and the highest concentration of 28 μg/ml. Whether these cells represent a population of persisters, as defined here is not clear. Conversely, it is not known whether the persister population identified in biofilms of *C. albicans* GDH2346 in the present study consisted entirely of yeast cells.

LaFleur *et al.* (2009) recently reported a study of *Candida* persister cells in the oral cavity of cancer patients undergoing treatment with chlorhexidine. Strains isolated from patients with long-term *Candida* carriage had high levels of

persisters. All of the high-persister isolates had an amphotericin B MIC that was the same as that for the wild type indicating that these strains were drug-tolerant rather than drug-resistant mutants. This study suggests that persister cells are clinically relevant and that antimicrobial therapy selects for high-persister strains *in vivo*.

Another study on the formation of *Candida* persister cells on titanium surfaces was reported recently (Tsang & Tang, 2009). Titanium discs were surface-treated by three different methods: group A, polishing; group B, sandblasting followed by acid-etching; and group C, sandblasting alone. Persister cells of two *C. albicans* strains (ATCC 90028 and HK30Aa), in planktonic and biofilm states, were measured by viable cell counts after 24 h of exposure to various concentrations of amphotericin B. No persister cells were detected in the planktonic cultures. However, persister cells were detected at a drug concentration of 64 μg/ml in all groups of both *C. albicans* strains. Nevertheless, group C of *C. albicans* ATCC 90028 appeared to provide a surface relatively unfavourable for the development of persister cells (Tsang & Tang, 2009).

The mechanisms by which *Candida* biofilms resist the action of antifungal agents are poorly understood. The biofilm matrix does not appear to form a major barrier to drug penetration since antifungal agents permeate *Candida* biofilms relatively easily (Al-Fattani & Douglas, 2004). However, under flow conditions resembling those found in catheter infections *in vivo*, increased production of matrix polymers can contribute to drug resistance (Al-Fattani & Douglas, 2006). Studies with a perfused biofilm fermenter (Baillie & Douglas, 1998a) have shown that drug resistance is not simply due to a low growth rate, and a related investigation (Baillie & Douglas, 1998b) demonstrated that iron limitation of biofilm growth is not solely responsible. It is possible that expression of resistance genes is induced by contact with a surface. For example, genes encoding multidrug efflux pumps in *C. albicans* are upregulated during biofilm formation and development. However, mutants lacking these genes are drug sensitive when growing planktonically but still drug resistant during biofilm growth (Ramage *et al.*, 2002a). The recent attractive suggestion that a small number of persister cells are responsible for resistance (LaFleur *et al.*, 2006) is not wholly supported by the present study. Although persister

populations are present in biofilms of several *C. albicans* isolates (LaFleur *et al.*, 2006; Tsang & Tang, 2009), the results described here demonstrate that persisters are absent from those of at least one well-characterized strain, *C. albicans* SC5314. Similarly, while biofilms of *C. krusei* and *C. parapsilosis* appear to harbour persister cells, biofilms of *C. glabrata* and *C. tropicalis* are devoid of such cells. Biofilm drug resistance in *Candida* species therefore remains unexplained and is most likely multifactorial in nature.

4 Apoptosis in *Candida* biofilms

Apoptosis is one type of programmed cell death that serves to eradicate defective cells and is essential for the development and maintenance of multicellular organisms. It is defined by a series of biochemical and morphological changes, one of the most important of which is the activation of caspases (Leist & Jaattela, 2001). During the past decade, evidence of apoptosis in both yeasts and filamentous fungi has been obtained (Hamann *et al.*, 2008; Madeo *et al.*, 2004; Mazzoni & Falcone, 2008; Ramsdale, 2008). In *C. albicans* growing planktonically, exposure of the organism to a variety of environmental conditions such as weak acid stress, oxidative stress, or ultraviolet irradiation can produce characteristics typical of apopotosis. These include externalization of phosphatidylserine, chromatin condensation, accumulation of reactive oxygen species, DNA degradation and caspase activation (Cao *et al.*, 2009; Phillips *et al.*, 2003; Shirtliff *et al.*, 2009; Yang *et al.*, 2010). The benefits of such a suicide process to unicellular organisms like yeasts are not immediately obvious. However, apoptosis could be highly advantageous for a biofilm community that, in many ways, resembles a multicellular organism. The self-destruction of damaged cells which consume scarce nutrients in a vain attempt to repair themselves could enhance the viability and reproductive success of healthier members of the community (Buttner *et al.*, 2006; Lewis, 2000). This study has shown via specific staining and the use of caspase inhibitors that apoptosis does indeed occur in *Candida* biofilms during exposure to the antifungal agent, amphotericin B.

4.1 Detection of apoptotic cells in *Candida* biofilms

Caspase activity was monitored initially in biofilm cells by the binding of a specific fluorochrome-labelled inhibitor of caspases (FLICA). FLICAs have become widely used for the detection of active caspases in yeasts as well as in mammalian cells, although their use has sometimes been controversial (Pozarowski *et al.*, 2003; Vachova & Palkova, 2007). Madeo et al. (2002) reported FLICA binding to active caspase in intact (propidium iodide-negative) cells of *S. cerevisiae*, and only in cells containing a functional *YCA1* gene; no binding was detected in an *yca 1* mutant. However, other studies have indicated nonspecific FLICA binding by heat-killed or ageing yeast cells (Vachova & Palkova, 2007; Wysocki & Kron, 2004). In the present investigation, fluorescent staining was rarely observed in control cells from biofilms not exposed to amphotericin B. Moreover, caspase activity, as detected by FLICA, increased with longer incubation times. However, to demonstrate unequivocally caspase activity in drug-treated biofilm cells, a second staining method was also used. This involves a non-fluorescent substrate, D_2R (aspartyl$_2$-Rhodamine 110), which enters intact yeast cells and is cleaved by the direct action of activated caspases to a green fluorescent compound. Our results with this staining method confirmed that drug-treated biofilm cells undergo apoptosis. Very recently, two separate research groups have demonstrated caspase activity in planktonic cultures of *C. albicans* by one or other of these staining protocols (Cao *et al.*, 2009; Shirtliff *et al.*, 2009).

4.2 Effects of caspase inhibitors on *Candida* biofilms

Synthetic caspase inhibitors have been developed both as research tools and with the hope that they may eventually be used clinically to prevent cell death. They act by binding to the active site of caspases either in a reversible or irreversible manner. The peptide recognition sequence of the inhibitor is attached to a functional group such as fluoromethylketone (FMK). FMK-containing peptides are irreversible inhibitors; those synthesized with an additional benzyloxycarbonyl group (also known as Z) show enhanced cell permeability. In this study, low concentrations of the general caspase inhibitor, Z-VAD-FMK, significantly increased the viability of biofilms of *C. albicans* GDH2346 exposed to amphotericin B, suggesting that caspase activity had been

partially suppressed. This inhibitor has a broad specificity and is capable of inhibiting human caspases-1 to -9 to varying extents, with greatest activity against caspases-1, -5, -8 and -9. The half-life for irreversible inhibition at 1 μM inhibitor is $\leq$ 40 min for all of these enzymes (Garcia-Calvo *et al.*, 1998). Some specific caspase inhibitors, each differing in the nature of its peptide recognition sequence, also increased the viability of biofilms of *C. albicans* GDH2346 treated with amphotericin B. The greatest effect, a 40-fold increase in viability, was observed with a caspase-1 inhibitor (Z-YVAD-FMK); there were also 8-fold and 3.5-fold increases with a caspase-9 inhibitor (Z-LEHD-FMK) and a caspase-5 inhibitor (Z-WEHD-FMK), respectively.

Partial suppression of caspase activity in *C. albicans* by mammalian caspase inhibitors is not wholly unexpected. To date, only one enzyme with caspase-like activity, CaMCA1, has been identified in *C. albicans*. This, like its homologue in *S. cerevisiae*, YCA1/MCA1, is a metacaspase (Cao *et al.*, 2009). Mammalian caspases are cysteine proteases with a stringent specificity for cleaving protein substrates containing aspartic acid. Plant, protozoan and fungal metacaspases, on the other hand, display arginine and lysine protease specificity instead of the aspartic acid specificity characteristic of caspases. Nevertheless, Madeo et al. (2002) reported that extracts of a YCA1 overproducing strain of *S. cerevisiae*, which had been treated with H_2O_2 to induce apoptosis, showed a high proteolytic activity towards several substrates for mammalian caspases. Addition of the general caspase inhibitor, Z-VAD-FMK, to the extracts completely abrogated this catalytic activity. Mammalian caspase inhibitors, including Z-VAD-FMK, are also known to be remarkably efficient at blocking programmed cell death in plants (Bonneau *et al.*, 2008). However, there is some evidence that plant metacaspases do not cleave caspase substrates and are not inhibited by caspase inhibitors (Bonneau *et al.*, 2008). It is therefore possible that other caspase-like activities are present in both plants and yeasts. In *S. cerevisiae*, for example, an MCA1/YCA1-independent caspase-like activity has been detected in an *mca1* mutant using the D_2R staining procedure (Vachova & Palkova, 2005). There are several other reports of MCA1-independent caspase-like activities in *S. cerevisiae* detected by different approaches (Vachova & Palkova, 2007). Moreover, very recently, Aerts et al. (2009) demonstrated that the antifungal plant defensin RsAFP2 induces apoptotic cell death in *C. albicans* that is

independent of CaMCA1, indicating the existence of at least one other unidentified caspase or caspase-like protease in this organism.

4.3 Effects of histone deacetylase (HDA) inhibitors on *Candida* biofilms

There was a striking effect on viability when *Candida* biofilms were incubated with amphotericin B in the presence of HDA inhibitors. The addition of either sodium valproate or sodium butyrate to the incubation mixtures completely eliminated biofilm populations of *C. albicans* GDH2346 at amphotericin concentrations of 10 to 50 µg/ml. Without an inhibitor, biofilms of this strain of *C. albicans* remain viable at drug concentrations of up to 100 µg/ml due to the presence of persisters. HDA inhibitors are known to induce apoptosis in mammalian cells (Kawagoe *et al.*, 2002; Medina *et al.*, 1997). Recently, valproate was reported to induce YCA1-dependent apoptosis in *S. cerevisiae*; a *yca1* mutant survived this treatment (Mitsui *et al.*, 2005). It was subsequently shown that valproate also stimulated the accumulation of neutral lipids, mainly triacylglycerol, in the apoptotic wild-type cells (Sun *et al.*, 2007). Valproic acid is a short-chain fatty acid widely used in humans as an anticonvulsant and has teratogenic and anti-tumour activities. Whether it induces lipid accumulation in *C. albicans* is not known. Sodium butyrate was even more effective than valproate at eradicating biofilms of *C. albicans* GDH2346 when added to incubation mixtures together with amphotericin B. Butyrate inhibits germination in *C. albicans* (Noverr & Huffnagle, 2004); at concentrations of 4 to 8 mM, it also enhances the activity of fluconazole against planktonic *C. albicans* cells (Smith & Edlind, 2002).

Drug-tolerant persisters capable of withstanding high concentrations of amphotericin B have been detected in biofilms of many but not all strains of *C. albicans* tested. Biofilms of *C. albicans* SC5314, for example, appear to lack persisters as demonstrated by viable counts and by fluorescein staining. Similarly, while biofilms of *C. krusei* and *C. parapsilosis* produce persisters, biofilms of some strains of *C. glabrata* and *C. tropicalis* do not. The reasons for these differences are not clear. Moreover, the mechanisms by which *Candida* persisters tolerate high drug concentrations are not understood. This investigation has demonstrated that persisters capable of surviving amphotericin

B concentrations of 100 µg/ml are nevertheless eradicated at lower drug concentrations when simultaneously subjected to an HDA inhibitor such as valproate or butyrate. This finding implies that histone acetylation might somehow activate apoptosis in these cells. The HDA inhibitor, trichostatin A, is known to affect colony-type phenotypic switching in *C. albicans*. It causes a dramatic increase in the frequency of switching in the white-to-opaque transition but has no effect on the frequency of switching in the opaque-to-white transition, suggesting that deacetylation through a trichostatin-sensitive deacetylase selectively suppresses switching in one direction (Klar *et al.*, 2001). Targeted deletion of *HDA1*, which encodes a deacetylase sensitive to trichostatin A, had the same selective effect. Subsequent studies showed that a second histone deacetylase gene, *RPD3*, plays a role in suppressing the basic switch events in both directions (Srikantha *et al.*, 2001). Whether these or any other histone deacetylase genes are involved in the regulation of caspase-like activity in *C. albicans* biofilms remains to be demonstrated.

Trichostatin A and other HDA inhibitors have also been shown to enhance the sensitivity of planktonic *C. albicans* to the azoles fluconazole, itraconazole and miconazole. Smith and Edlind (2002) reported that expression of *ERG* genes (encoding azole targets) and *CDR*/*MDR1* genes (encoding multidrug transporters) was induced by fluconazole, but that trichostatin A reduced this upregulation by 50 to 100%. The authors concluded that trichostatin A probably does not act directly on *ERG* and *CDR* gene promoters since decreased deacetylation (ie. increased acetylation) should enhance, not inhibit, transcriptional upregulation. Rather, azole treatment could be associated with histone deacetylation of the promoter region of a transcriptional repressor. The resulting down-regulation of this repressor would lead to upregulation of *ERG* and *CDR*. However, HDA inhibition by trichostatin A would result in constitutive expression of this repressor, blocking ERG/CDR upregulation (Smith & Edlind, 2002).

Recent evidence suggests that the Ras-cAMP-PKA signalling pathway in *C. albicans* regulates programmed cell death induced by exposure to acetic acid or hydrogen peroxide, either by inhibiting antiapoptotic functions (such as stress responses) or by activating proapoptotic functions (Phillips *et al.*, 2006). Mutations that block Ras signalling were shown to suppress or delay the

apoptotic response; in contrast, mutations that stimulate signalling accelerated the apoptotic response (Phillips *et al.*, 2006). The role of histone acetylation or deacetylation, if any, in this regulatory process is not known, nor is it clear whether the Ras pathway is involved in amphotericin-induced apoptosis either in planktonic or biofilm cultures. However, treatment of *C. albicans* with trichostatin A has been reported to produce a significant reduction in transcription of *EFG1*, a gene which codes for a key regulatory protein in this pathway (Simonetti *et al.*, 2007). Studies with biofilms of Ras mutants of *C. albicans* could therefore be instructive and might help to elucidate the mechanism by which drug-tolerant persisters resist programmed cell death.

5 Concluding remarks

Implanted medical devices are at risk of *Candida* biofilm formation. Microorganisms in the biofilm environment exhibit an altered phenotype and are difficult to remove since they are both recalcitrant to antifungals and isolated from host immune components. Clinical guidelines recommend device removal in order to avoid progression to systemic disease, which has a mortality rate of up to 40%. Biofilm resistance to antifungal drugs is complex and involves more than one mechanism. This study has demonstrated that even the most effective fungicidal agents, amphotericin B and the newly introduced caspofungin, are unlikely to cure or eradicate a biofilm infection.

The extent to which the matrix acts as a barrier to drug diffusion would depend on the chemical nature of both antifungal drugs as well as the matrix material. Caspofungin penetration through biofilms of *Candida* varied among species. However, although caspofungin was able to penetrate biofilms of different *Candida* species with different diffusion rates, it failed to do so at rates appreciably different from those of other drugs (Al-Fattani & Douglas, 2004) and it failed to result in complete killing of biofilm cells. These findings suggest that drug penetration is not a major factor in biofilm resistance. On the other hand, drug resistance of *C. albicans* biofilms is significantly enhanced by increased production of matrix material under flow conditions (Al-Fattani & Douglas, 2006). Thus, further detailed chemical analysis of biofilm matrix material is required to understand the nature of antifungal reactivity with

various biofilm components. For example, *C. tropicalis* matrix material, which contains significant amounts of hexosamine and appears to partially hinder the diffusion of several drugs, deserves further study.

The discovery of a persister cell population, which survives high concentrations of antifungal drugs, helps to explain the resistance of biofilms to killing. Surprisingly, in this study, not all *Candida* biofilms contained persisters which suggests that persisters cannot solely account for drug resistance. It would be interesting to carry out a more extensive survey of the existence of persisters in biofilms of different *Candida* species. Additional research aimed at determining the molecular mechanisms responsible for persister formation is also merited.

Amphotericin B induced apoptosis in *Candida* biofilms as detected by SR-FLICA and D_2R fluorochrome-based staining reagents. Mammalian general caspase inhibitor and some specific caspase inhibitors produced significant increases in the viability of drug-treated biofilms of *C. albicans*. On the other hand, histone deacetylase inhibitors enhanced the activity of amphotericin B against biofilms of *Candida* species and in some cases even eradicated persister subpopulations. Persisters may therefore represent cells with a defective apoptosis mechanism and histone acetylation could activate apoptosis in these cells. These results suggest that it might be possible to kill all biofilms cells, including persisters, *in vivo* with a combination of antifungals and apoptosis activators (i.e. histone deacetylase inhibitors), if appropriate concentrations are used.

This study could be extended further by testing drug-tolerant biofilm cells for other apoptosis markers such as phosphatidylserine externalization, chromatin condensation, or reactive oxygen species accumulation. Recently, the isolation of a CaMCA1 null mutant has been reported (Cao *et al.*, 2009) and biofilm experiments with this mutant would also be very informative. It now appears that *C. albicans* may contain a caspase activity that is distinct from the metacaspase (Aerts *et al.*, 2009). Further investigations with the null mutant should establish the relative importance of these activities in *Candida* biofilms exposed to amphotericin B.

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APPENDICES

Appendix 1 Medium

1.1 Yeast Nitrogen Base (YNB; Difco)

Chemical	Weight/litre
Ammonium sulfate	5.0 g
Monopotassium phosphate	1.0 g
Magnesium sulfate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g
L-Histidine monohydrochloride	10.0 mg
LD-Methionine	20.0 mg
LD-Tryptophan	20.0 mg
Biotin	2.0 µg
Inositol	2000.0 µg
Boric acid	500.0 µg
Niacin (Nicotinic acid)	400.0 µg
Manganese sulfate	400.0 µg
Pyridoxine HCl	400.0 µg
Zinc sulfate	400.0 µg
Thiamine HCl	400.0 µg
Calcium pantothenate (D-Pantothenic acid)	400.0 µg
Ferric chloride	200.0 µg
Sodium molybdate	200.0 µg
Riboflavin	200.0 µg
p-Aminobenzoic acid	200.0 µg
Potassium iodide	100.0 µg
Copper sulfate	40.0 µg
Folic acid	2.0µg

Final pH 5.4 ± 0.1 at 25 °C

1.2 Sabouraud Dextrose Agar (SDA; Oxoid)

Chemical	Weight (g/l)
Mycological peptone	10.0
Glucose	40.0
Agar	15.0

Autoclaved for 15 min at 15 lbs pressure (121˚C). Final pH 5.6 ± 0.2 at 25˚C

Appendix 2 Buffers

2.1 0.15 M Phosphate-buffered saline (PBS)

Phosphate buffered saline tablet

Chemical	Quantity
Phosphate buffered saline tablet	1.0
Distilled water	200 ml

Each tablet added to 200 ml distilled water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride. The solution was autoclaved at 121˚C for 15 min. The pH of the subsequent solution was 7.2, at 25˚C.

2.2 RPMI 1640 with HEPES (Sigma)

Chemicals	g/litre
Calcium Nitrate $4H_2O$	0.1
Magnesium Sulfate (anhydrous)	0.04884
Potassium Chloride	0.4
Sodium Chloride	6.0
Sodium Phosphate Dibasic (anhydrous)	0.8
L-Arginine (free base)	0.2
L-Asparagine (anhydrous)	0.05
L-Aspartic Acid	0.02
L-Cystine 2HCL	0.0652
L-Glutamic Acid	0.02
L-Glutamine	0.3
Glycine	0.01
L-Histidine (free base)	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine HCL	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine 2Na H_2O	0.02883
L-Valine	0.02
D-Biotin	0.0002
Choline Chloride	0.003
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Amino Benzoic Acid	0.001
D-Pantothenic Acid (hemicalcium)	0.00025

Pyridoxine HCL	0.001
Riboflavin	0.0002
Thiamine HCL	0.001
Vitamin B_{12}	0.000005
D-Glucose	2
Glutathione (reduced)	0.001
HEPES	5.958
Phenol Red (sodium)	0.0053
$NaHCO_3$	2.0

Final pH 7.0

2.3 RPMI 1640 with L- Glutamine (Cambrex)

Chemicals	**mg/L**
Inorganic Salts	
$Ca(NO_3)_2 \cdot 4H_2O$	100.00
KCl	400.00
$MgSO_4 \cdot 7H_2O$	100.00
NaCl	6000.00
$NaHCO_3$	2000.00
$Na_2HPO_4 \cdot 7H_2O$	1512.00
Other Components	
Glucose	2000.00
Glutathione (reduced)	1.00
Phenol Red•Na	5.00
Amino Acids	
L-Arginine	200.00
L-Asparagine•H_2O	50.00
L-Aspartic Acid	20.00
L-Cystine	50.00
L-Glutmaic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00

Hydroxy L•Proline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine•HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Trypotophan	5.00
L-Tyrosine	20.00
L-Valine	20.00
Vitamins	
p-Aminobenzoic Acid	1.00
d-Biotin	0.20
D-Ca Pantothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
Pyridoxine•HCl	1.00
Riboflavin	0.20
Thiamine•HCl	1.00
Vitamin B_{12}	0.01

Final pH 7.0

PUBLICATIONS