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PATHOGENICITY AND DRUG RESISTANCE IN *CANDIDA ALBICANS* AND OTHER YEAST SPECIES

A REVIEW

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Pathogenic yeasts from the genus *Candida* can cause serious infection in humans particularly, in immunocompromised patients and are now recognized as major agents of hospital acquired (nosocomial) infections. In the recent years, there has been a marked increase in the incidence of treatment failures in candidiasis patients receiving long-term antifungal therapy, which has posed a serious problem in its successful use in chemotherapy. *Candida* cells acquire drug resistance (MDR) during the course of the treatment. The mechanisms of resistance to azole antifungal agents have been elucidated in *Candida* species and can be mainly categorized as (i) changes in the cell wall or plasma membrane, which lead to impaired drug (azole) uptake; (ii) alterations in the affinity of the drug target Erg11p (lanosterol 14∝-demethylase) especially to azoles or in the cellular content of Erg11p due to target site mutation or overexpression of the *ERG11* gene; and (iii) the efflux of drugs mediated by membrane transport proteins belonging to the ATP-binding cassette (ABC) transporters, namely *CDR1* and *CDR2* or to the major facilitator superfamily (MFS) transporter, *CaMDR1*. Many such manifestations are associated with the formation of *Candida* biofilms including those occurring on devices like indwelling intravascular catheters. Biofilm-associated *Candida* show uniform resistance to a wide spectrum of antifungal drugs. A combination of different resistance mechanisms is responsible for drug resistance in clinical isolates of *Candida* species.

Keywords: drug resistance, efflux pump, ABC transporters, biofilm, *Candida,* efflux pumps

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Introduction

The apparent exaltation of fungal infections may be explained by the increasing number of immunocompromised patients [1]. Having emerged as the major causes of morbidity and mortality in immunocompromised hosts, *Candida* spp., mainly *Candida albicans* and to a smaller extent, also other non-*albicans* species (*C. dubliniensis, C. glabarata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, C. tropicalis,* etc.) represent the major group of yeast species recovered from infected individuals [2]. Incidence of *C. albicans* cells acquiring resistance to antifungals like azoles has increased considerably, causing serious problems in successful chemotherapy. Both *C. albicans* as well as non-*albicans* species have developed a variety of mechanisms to combat resistance to antifungal drugs [3].

Pathogenesis and candidiasis

In the last decades, the incidence of fungal infections by *C. albicans* and other related human opportunistic yeast species has increased dramatically due to the rise in the number of immunocompromised patients. Several *Candida* spp., especially *C. albicans,* normally inhabit the oral cavity, respiratory, intestinal tracts and vaginal cavity of humans and animals. Colonization with *C. albicans* can lead to systemic infection when the host presents risk factors. Predisposing factors for candidiasis (Table I) include immunosuppressive and cytotoxic therapies, treatment with broad spectrum antibiotics, AIDS, diabetes, drug abuse, use of catheters and indwelling devices, etc. Depending on the underlying host defects, the microorganism may cause a wide variety of infections ranging from mucosal to life threatening disseminated candidiasis [2]. Therefore, development of candidiasis depends on a delicate balance between the fungi and the host's immune status which determines the commensal or parasitic relationship.

Interactions between *C. albicans* cells and mammalian host tissues are highly complex. *Candida* infections arise as a sequence of time-scaled steps. First, adhesion to an epithelial surface is required to initiate the colonization of the actual surface. Penetration into the epithelial surfaces is the limit of the infectious process in most cases, leading to the establishment of a superficial candidiasis; normally the fungi are incapable of further invasion into the immunologically intact host. When fungi reach the bloodstream, they must face the blood-borne cellular defense system. Finally to cause invasive infections *Candida* cells should be

Table I

Incidence of candidiasis, locations, symptoms and predisposing factors

Type	Location	Symptoms	Predisposing factors
Superficial			
Oral thrush	Oropharynx	White lesions resembling milk curd on the surface of throat, tongue, gum linings	Old age, infancy
Stomatitis	Palate	Erythema, oedema of palate	Old age
Leukoplakia	Inner cheek surface	Chronic lesions	Tobacco smoking
Vulvo-vaginal	Vagina, perinal area	White discharge, intense erythema	Pregnancy, diabetes
Candida onychia	Brown, greenish discoloration of nails	Swollen, painful inflammation of nail and nail folds	Occupational hazards (fruit canners)
Systemic			
Oesophageal	Oesophagus	Dysphagia, retrosternal pain	Fatally ill patients, AIDS patients
Gastric	Stomach	Lesions, vomiting	Pre-existing lesions, parenteral nutrition
Candidiasis of lower respiratory tract	Bronchi, lungs	Lesions	Aspiration
Candiduria	Kidney, urinary tract	Microabscesses dissemination in urinary tract	Diabetes, old age, drug abuse
Candida endocarditis	Heart, aortic and mitral valve	Intracardiac vegetation blocking blood vessels	Cardio pulmonary bypass, open heart surgery
Candida meningitis	Granulomas of ventricle, lesions	Microabscess, headaches, lesions	No particular factor
Candida arthritis	Necrosis of cartilages, abscess formation	Swelling, painful joints	Intra-articular steroid injection, trauma, cancer patients
Disseminated	Eye, skin, blood		Leukaemic patients, burn victims, latrogenic factors

able to penetrate the endothelial surfaces and invade the tissues. Different factors of host and fungal cells may have a major role at each step of the infectious pathway, although no component has yet been found to be absolutely essential [4]. From clinical data, it appears that both antibody- and cell-mediated immune response contributes to host protection against candidiasis [5, 6].

C. albicans pathogenicity depends, in addition to the immune status of the host, on a complex set of microorganism-related putative virulence factors. These

include the secretion of hydrolytic enzymes, antigenic variability, adhesion to host cells and tissues as well as to inert substrates, dimorphic transition (yeast-to-hypha), phenotypic switching or the ability to switch among different cell phenotypes and modulation of the host's immune response [7–12]. Expression of most virulence factors is regulated *in vitro* by environmental parameters and *in vivo* it depends on the stage of infection and varies also according to host species or tissues.

It is generally accepted that dimorphism (the ability to grow either as budding yeast or as a filamentous form or hypha) is a virulence trait *per se,* and is coregulated with other virulence factors associated with the cellular morphology, too [13, 14]. In addition to the yeast-hypha transition, *C. albicans* may undergo also another type of morphological change called phenotypic switching, involving the spontaneous and reversible generation of different morphological and physiological states expressing different patterns of pathogenicity-related traits (virulence factors, antigenicity and resistance to antifungal drugs) readily observed by the morphology of the colonies [11, 15]. Both processes, dimorphic transition and switching, confer on *C. albicans* the ability to generate variants allowing a better selective adaptation to changing environmental conditions, and particularly to evade the host's immune system.

As with many pathogenic microorganisms, adhesion of *C. albicans* to the host surface is a crucial step in the pathogenic process and a prerequisite for colonization of the host's tissues. *C. albicans* adhesion to host (epithelial and endothelial cells, soluble factors, extracellular matrix) and inert materials implanted in the body of the host involves multiple adherence mechanisms mediated by cell wall components designated as adhesins [9].

A complex assortment of hydrolytic enzymes is found in culture filtrates of *C. albicans* cells. Although some of the proteins present in these filtrates represent products specifically secreted, some others may be components shed from the cell wall, or even released from cytoplasm by spontaneous cell lysis. Several of the secreted proteins may be considered as virulence factors since the hydrolytic action of these extracellular proteins affects the function and viability of the host, thus contributing to the establishment and progress of infection [9].

Role of cell wall associated components in pathogenicity

This section includes (i) secreted proteins, mainly hydrolytic enzymes that act on host substrates, (ii) proteins involved in adhesion to host ligands and tissues

and (iii) the role of cell wall components as inducers or mediators of the host immune response.

Secreted protein

Secreted hydrolytic enzymes may be considered as virulence factors contributing to the establishment and progress of infection. Expression and intracellular distribution depend on environmental conditions.

Secreted acid proteinase

The extracellular proteolytic activity described for *C. albicans* is due to aspartyl proteinase enzymes constituting a family of enzymes (*SAP:* secreted aspartyl proteinases) whose expression depends on both the yeast strain and the environment [12, 16]. Several observations suggest a major role for Sap proteins in the pathogenesis of candidiasis: (i) *SAP* are secreted by pathogenic *Candida* species *in vivo* during infection and there is a correlation between virulence and the level of proteinase production in both the clinical isolates and laboratory strains of *C. albicans;* (ii) the enzymes are able to degrade a number of important defensive host proteins such as immunoglobulins and complement and (iii) genetically engineered strains harboring disruptions in *SAP* genes showed a reduced virulence in an animal model of disseminated candidiasis. Therefore, proteinase production appears to enhance the ability of the fungal cells to colonize and penetrate host tissues and to evade the host immune system.

Phospholipase

C. albicans cells exhibit phospholipase A, B and C as well as lysophospholipase and lysophospholipasetransacetylase activities [16, 17]. Phospholipase activities are considered as putative virulence factors since they are associated with host cell membrane damage, adherence and penetration. Molecular organization of *C. albicans* cell wall reveals that a scaffold of branched β-1,3-glucan serves for the covalent attachment of other cell wall components; covalent linkages (as disulfide bridges) between different cell wall mannoproteins (CWP) also exist and some mannoproteins are linked to chitin [18]. Phospholipase production by *C. albicans* strains therefore result in adhesion to epithelial cells and virulence in a mouse model.

Hemolytic factor

C. albicans exhibits hemolytic activity when cultured on glucose-enriched blood agar; this activity is detected in intact cells, particularly in hyphae, and secreted also into the culture medium. This hemolytic activity, is associated to a mannoprotein residing in its mannan moiety, and may be considered as a putative virulence factor as it can allow iron acquisition from hemoglobin released after lysis of host erythrocytes, thus favoring the ability to grow in the host's serum [9].

Immunosuppressive and B-cell mitogenic protein

An immunosuppressive, B-cell mitogenic protein (ISM p43) is secreted into the culture medium by *C. albicans* [10, 19]. This protein belongs to the ISM proteins produced by other pathogens, that are virulence factors producing immunosuppression, as a consequence of B-cell overstimulation, and is crucial for the survival of the pathogen within the host. The immunosuppressive and B-cell mitogenic properties of p43 are quantitatively associated with the host susceptibility to *C. albicans* infections.

Other secreted proteins

C. albicans cells are able to secrete into the medium, under proper inducing conditions, also other enzymes like acid phosphatase, trehalase, glucoamylase, esterase, lipase, hyaluronidase, chondroitin sulfatase and metallopeptidase [9, 10, 16, 20, 21]. Some of these catalytic mannoproteins are thought to be virulence factors contributing to candidal infection, particularly by the combined action with acid proteinases and phospholipases. In addition, a variety of proteins whose primary location is the cytosol were also found incorporated into the cell wall, such as several glycolytic proteins (GAPDH, PGK, ADH, enolase) and members of the *HSP* family (*HSP70*, *HSP90* and others *HSP*s). Some of these proteins may have relevant roles in pathogenesis, either as putative adhesion factors or as modulators of the host immune response [9, 10].

Binding proteins for host ligands

Physical interactions between *C. albicans* cells and the host are mediated by active radicals on the cell wall as the cell wall is the outermost surface of the microorganism. Potential interactions may involve host soluble proteins, extracellular matrix proteins as well as phagocytic cells and any host cell or organ

that can be infected by the fungus. The role of these interactions in pathogenesis is complex, as there is a wide variety of adhesion factors and their participation in virulence may depend on the fungal strain, type of infected host tissue and host's immune status.

Although cell wall components, such as chitin, glucans or lipid molecules play some role in adhesion, protein and mannoproteins are the major mediators in adhesion of *C. albicans* to the host. Adhesion at molecular level may involve distinct types of interactions between microbial and host molecules. Also, adhesion to plastic surfaces and fungal cell surface hydrophobicity (CSH), which may play an important role in pathogenesis, are mediated by cell wall-associated mannoproteins. In this section we summarize the most relevant features of the candidal adhesion [9, 10, 22–26].

Lectin-like molecules

Proteinaceous candidal adhesin might interact with glycoside receptors (glycoprotein or glycolipid) in a lectin-like manner, as described in many bacterial adhesion mechanisms [9, 27, 28]. This type of interaction seems to be a major mechanism of adhesion to epithelial cells. Several lectin-like molecules have been described in *C. albicans*. Binding proteins may recognize *L*-fucose-, *N*-acetylgalactosamine- or *N*-acetylglucosamine-containing glycosides, possibly in the form of blood group antigens, which can serve as epithelial cell receptors for the microorganism.

Binding of fungal cells to erythrocytes mediated by sugar ligands on erythrocyte surface may contribute to a fungal system for iron acquisition. Fimbriae bind directly to epithelial buccal cells and the protein moiety of the 66 kDa, a major structural fimbrial glycoprotein appears to mediate binding to glycosphingolipids displayed on these cells. In addition, among several adhesins that *C. albicans* utilizes for interacting with type 4 collagen, there is some lectin that recognizes specific oligosaccharide residues at the receptor level [29]. *Candida* lectin-like receptors also recognize salivary proteins, such as mucins, as well as bacterial complex polysaccharides mediating adhesion to *Streptococcal* species; such interactions may contribute to oral colonization [9, 30].

Proteins recognizing RGD-containing ligands

Candidal cell surface proteins displaying RGD (argininеglycine-aspartic acid motif)-binding activity can be considered as integrin analogues [26, 27], RGD motifs are present in extracellular matrix proteins, complement C3 frag-

ments and fibrinogen, which bind to β_1 and β_2 mammalian integrins, heterodimeric transmembrane proteins that play a major role in adhesion of mammalian cells to the extracellular matrix (ECM).

C. albicans expresses cell surface receptors for complement C3 conversion products: C3d-binding (complement receptor 2, *CR2*) and iC3b-binding (complement receptor 3, CR3) proteins [9, 26, 27]. Deposition of complement fragments on any surface (epithelial, endothelial or plastic) would provide a potential bridge for adhesion of *C. albicans*. Clumping between opsonized and non-opsonized *C. albicans* cells by using CR may lead to the formation of aggregates that might protect the fungal cell from phagocytosis. Furthermore, the *C. albicans* cell surface can bind complement regulator factors H and FHL-1 and is able to regulate alternative complement activation at its surface and to inactivate toxic complement activation products [31].

It is well known that *C. albicans* exhibits an affinity for laminin, fibronectin, and other ECM proteins mediated by fungal integrin-like receptors facilitating colonization of endothelial and epithelial surfaces [9, 26, 27]. Laminin, a major component of the basement membrane, is recognized by several surface receptors of *C. albicans*. Studies of adhesion to endothelium indicated a role for fibronectin as the most important host ligand for candidal adhesion.

Different receptors have been identified at the cell wall of both yeast and hyphal forms. Adhesion of *C. albicans* to type I and type IV collagen, a component of the basement membrane, is almost completely inhibited by fibronectin, indicating that common receptors interact with both ligands and similar molecular species have been described to bind collagen and fibronectin. Binding among fungal receptors and collagen molecules may involve different types of interactions, as RGD peptides only inhibited adhesion to type IV collagen, and a collagen lectin-like candidal adhesion has been described [9, 29]. Several moieties from yeast and hyphal forms were found to bind entactin, and this interaction is partially mediated by RGD-binding sites. Besides their role in adhesion, candidal integrin-like molecules may have additional roles in *C. albicans* physiology, as described for Intp, an integrin-like receptor involved in adhesion, dimorphic yeast-to-hyphal transition and therefore in virulence [26, 32].

Plastic-binding proteins

Adherence of *C. albicans* to plastic medical devices (medical implants, prostheses and catheters) allows the organism to propagate and establish biofilms; release of microorganisms from these biofilms contributes to or initiates acute dis-

seminated nosocomial infections [9, 33]. Several components of the microfibrillar surface layer of *C. albicans* were implicated in plastic binding and plastic adherence appears to be mediated by CSH through hydrophobic bonds between plastic surfaces and the peptide moieties of the mannoproteins; these mannoproteins may play other significant roles in cell physiology rather than specifically bind to plastic surfaces. Salivary proteins, adsorbed to plastic material of dental or voice prostheses may also help their colonization by fungal cells [30, 34, 35].

Mannan adhesins and other polysaccharides

The mannan portion of mannoproteins may be involved in the fungus – host interaction. There is evidence indicating that distinct mannan epitopes are components of candidal adhesin activity involved in adhesion to epithelial and endothelial surfaces [9, 27, 28]. Mannan also binds to human red blood cells and causes hemolysis, an activity that may be associated with the ability of the organism to utilize hemoglobin and iron [36]. Mannan adhesin activity also participates in adherence to salivary components, and interaction with bacteria other than *Streptococcal* species may involve lectin-like molecules interacting with candidal surface carbohydrate [9, 34]. O-linked mannose residues of the 58 kDa fibrinogen binding protein mediates binding to its ligand [29] and, in addition, differential mannosylation of cell wall proteins appears to control CSH [30]. Lectin-like receptors were found on macrophage membranes. A similar mannose receptor and mannose-binding lectins and a glucan receptor, dectin-1, are involved in phagocytosis and cytokine production [31]. PLM also serves as a ligand for Toll-like receptor (TLR) 4 on the surface of phagocytic cells [32]. Further, several observations indicate that chitin may play a role in adherence to epithelial surfaces, and although chitin can be exposed at the cell surface at early stages of growth, the role of chitin in natural infections is uncertain [27].

Other binding proteins

Several *C. albicans* cell surface mannoproteins interact with fibrinogen, as well as with other ligands (laminin and C3d), suggesting their relationship to mammalian integrins [9, 26, 27]. The best characterized fibrinogen receptor is a 58 kDa mannoprotein that appears to interact with fibrinogen through its O-linked oligosaccharide domain. The ability of *C. albicans* to bind to fibrinogen may represent a virulence factor, as coagulation proteins such as fibrin appear to be the receptor for fungal cell adhesion to blood clots *in vivo,* and disseminated intravascular coagulation may lead to severe *Candida* septicemia. Besides, bind-

ing of *C. albicans* cells to platelets appears to be mediated by fibrinogen. *C. albicans* cells also bind to plasminogen, although no plasminogen activators have been detected in *C. albicans;* several plasminogen-binding proteins have been isolated from fungal cell walls and binding is not mediated by mannan moieties but appears to involve predominantly C-terminal lysine residues [33]. Activation of fungal-bound plasminogen by mammalian activators may potentially increase the capacity of the fungus for tissue invasion and necrosis.

The *ALS* (agglutinin-like sequence) gene family of *C. albicans* encodes cell surface glycoproteins (Als) implicated in adhesion to host surfaces [34]. *ALS* proteins and the structurally related adhesin *HWP1* are members of the GPI-dependent cell wall proteins [35], containing N-terminal signal peptides and C-terminal features mediating GPI membrane anchor addition and other domains leading to attachment to cell wall glucan. *ALS* proteins adhere to several host ligands (laminin, fibronectin, collagen and others) probably by recognizing patches of certain amino acids (threonine, serine or alanine) [36]. *HWP1* is a substrate for epithelial transglutaminase, a mammalian activator that contributes to the attachment of fungal cells to epithelial surfaces through covalent linkages [37]. Some of these adhesins, in addition to their role in virulence, also play significant roles in *C. albicans* physiology such as dimorphic transition and mating [38, 39].

Immunologic response to cell wall components

Fungal cell wall components play an essential role in triggering and modulating the natural and adaptive anti-*Candida* host immune responses [10, 31, 40]. Some of the most relevant aspects concerning host immune response and cell wall components are summarized below. Cell wall *PLM* has been identified as the candidal component that stimulates cytokine production by acting as a ligand for *TLR4* [32], a member of the *TLR* family of evolutionarily conserved transmembrane proteins that are the main pattern recognition receptors on the surface of phagocytic cells and function as sensors of infection that induce innate and adaptive immune responses [31, 41, 42]. Phagocytosis of *C. albicans* cells also involves recognition of fungal cells by host receptors, such as the mannose receptor and lectin-binding proteins and dectin-1, which recognize oligomannosides and β-glucan as their ligands, respectively [31]. *C. albicans* cell wall-associated components or secreted proteins are fungal antigens that include all potential elicitors of immunoprotective antibody responses [10].

Among the immunodominant components there are glycolytic enzymes (enolase, GAPDH, PGK, ADH) and heat shock proteins (*HSP90* and its 47 kDa heat stable breakdown product, and members of the Hsp70 family), as well as other mannoproteins considered as virulence factors (*SAP*, ISM p43). Also non-protein components show antigenic and immunomodulatory properties, such as mannan, β-1,2-linked oligomannosides and glucan [10, 43]. Inspite of the increasing awareness of the crucial role of cell-mediated immunity in the defense against *C. albicans* infections, only a few antigen targets for it have been so far been identified [44–46]. These include members of the *HSP* family, enolase and a number of mannoproteins, including some with adhesin properties. One of the best characterized antigen target of the anti-*Candida* human T-cell response is Mp65p, a 65 kDa highly glycoslylated protein, which is an immunodominant antigen present in both the cell wall and secreted mannoprotein material. Mp65p is recognized by T-cells of healthy individuals, and represents a major target of anti-candidal cell-mediated immunity in humans [46–47]. The amino acid motif sequence of Mp65p representing the minimal epitope recognized by T-cell clones has been identified, although the mannan moiety is also probably involved in T-cell recognition by *C. albicans*.

Biofilm and candidiasis

Most manifestations of candidiasis are in fact associated with the formation of *Candida* biofilms on surfaces, and this phenotype is associated with infection at both the mucosal and systemic sites [48]. Biofilms are universal, complex, interdependent communities of surface-associated microorganisms. The organisms are enclosed in an exopolysaccharide occurring on any surface, particularly aquatic and industrial water systems as well as medical devices. Most microorganisms grow in structured biofilms rather than individually in suspension and while in this environment they may display altered phenotypes [49]. Biofilms can be composed of a population developed from a single species or of a community derived from multiple microbial species [50, 51]. Speculations about the ecologic advantages of forming a biofilm include protection from the environment, nutrient availability, metabolic cooperation and acquisition of new genetic traits [51, 52]. Biofilms are notoriously difficult to eliminate and are a source of many recalcitrant infections [53, 54]. A variety of microbial infections is caused by biofilms ranging from urinary tract infections, catheter infections, child middle-ear infections and dental plaque to more threatening infections such as endocarditis and infections of heart

valves [54, 55]. Immunocompromised patients such as those with cancer or HIV infection are often the most susceptible ones.

The mechanisms of biofilm resistance to antimicrobial agents are not yet fully understood. Bacterial biofilms and their role in disease have been investigated in detail over a number of years, still much less is, however, known about fungal biofilms. *Candida* biofilms share several properties with the bacterial ones. The possible mechanisms include:

Restricted penetration of drugs through the biofilm matrix

It has long been supposed that the matrix of extracellular polymeric material might exclude or limit the access of drugs to organisms residing deep in a biofilm. Most studies with bacterial biofilms indicate that the matrix does not form a major barrier to drug diffusion, although for certain compounds penetration may be delayed [56]. To investigate whether the matrix plays a role in the resistance of *C. albicans* biofilms to antifungal agents, susceptibility profiles of biofilms incubated statically (which have relatively little matrix) were compared to those incubated under gentle shaking (which produce much more matrix material). Biofilms grown with or without shaking failed to exhibit significant differences in susceptibility to any of the drugs tested, indicating that drug resistance is unrelated to the extent of matrix formation [57]. However, separate studies with biofilms produced under flow conditions showed that resuspended cells (which presumably had lost most of their matrix) were some 20% less resistant to amphotericin B than intact biofilms, suggesting that the matrix might play just a minor role in drug resistance [58, 59].

Slow growth rate or nutrient limitation

Biofilm cells are thought to grow slowly because of the limited availability of nutrients, particularly at the base of the biofilm. A slow growth rate is often accompanied by changes in cell surface composition that could, in turn, affect the susceptibility of the microorganisms to antimicrobial agents. Growth rate could therefore be an important modulator of drug activity in biofilms [56, 60]. To investigate this possibility with *C. albicans,* a perfused biofilm fermenter was used to generate biofilms at different growth rates and the susceptibility of the biofilm

cells to amphotericin B was compared to that of planktonic organisms grown at the same growth rate in a chemostat. The results showed that biofilms were resistant to the drug at all growth rates tested, whereas planktonic cells were resistant only at low growth rates [59]. Biofilm resistance is therefore not only the result of a low growth rate but depends also on some other factors of growth of the biofilm. A separate study [58] using the cylindrical cellulose filter model system demonstrated that glucose-limited and iron-limited biofilms grown at the same low rate were equally resistant to amphotericin B. Iron-limited biofilms probably resemble more closely those growing *in vivo,* as although there is an abundance of iron in the human body, most of it is located intracellularly or tightly complexed to iron-binding glycoproteins and therefore it is relatively inaccessible to microorganisms. In the same study, daughter cells from iron limited biofilms were significantly more susceptible to the drug [58]. An acute disseminated infection produced by the release of such cells from an implanted biofilm might therefore respond rapidly to amphotericin B while the biofilm itself would remain unaffected.

Surface-induced expression of resistance genes

When microorganisms attach to a surface and form a biofilm they express an altered phenotype. There have been efforts to identify genes that are activated or repressed in *Candida* biofilms compared with planktonic cells, and genes that might contribute to drug resistance are of particular interest. For example, upregulation of genes coding for multidrug efflux pumps would result in a multidrug-resistant phenotype. To date, evidence for this as a resistance mechanism in bacterial biofilms is equivocal [61, 62]. *C. albicans* possesses two different types of efflux pumps: ATP-binding cassette (ABC) transporters and major facilitators, which are encoded by *CDR* and *MDR* genes, respectively. A recent study has demonstrated that genes encoding both types of efflux pumps are upregulated during biofilm formation and development. However, mutants carrying single or double deletion mutations in some of these genes were found to be highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth [63]. These results strongly suggest that drug resistance in *C. albicans* biofilms, similar to bacterial biofilms, is a complex process that cannot be explained by a single molecular mechanism.

Mechanism of drug resistance

Antifungal drug resistance

Many different types of mechanisms are known to contribute to a drug-resistant phenotype in eukaryotic cells. The antifungals which are commonly used to treat *Candida* infections and their mechanism of action are listed in Table II. The different sites of action for the antifungals is cited in Figure 1. The most frequent resistance mechanisms include reduction in the import of the drug into the cell; modification or degradation of the drug once it is inside the cell; changes in the interaction of the drug with the target enzyme (binding, activity); changes in other enzymes of the same enzymatic pathway and an increased efflux of the drug from the cell [64].

It is important to note that alterations in drug processing (modification or degradation) are important drug resistance mechanisms in a variety of bacterial and eukaryotic systems [65]. To date, little analysis of drug modification or degradation within a resistant cell has been performed for the medically important fungi. It has been mentioned that azoles are inert to metabolism in *C. albicans*

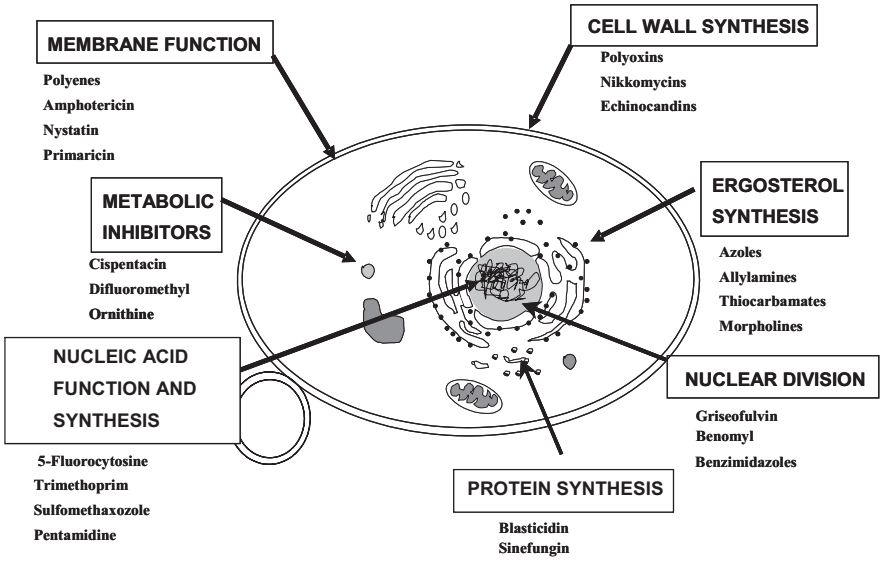

Figure 1. Different sites of action of antifungal agents

Table II

Drugs used against *Candida* and their mechanism of action

Drugs	Mechanism of action
INHIBITORS OF ERGOSTEROL BIOSYNTHESIS	
ALLYLAMINES	
e.g. Naftitine and terbinafine	Both allylamines and thiocarbamates inhibit the conversion of squalene to 2,3-oxidosqualene by the enzyme squalene expoxidase (which is a product of *ERGI* gene) leading to accumulation of squalene in the cells.
THIOCARBAMATES	
e.g. Tolnaftate and tolcitate	
AZOLES	
e.g. Imidazole (ketoconazole and miconazole) e.g. Triazoles (fluconazole, intraconazole and voriconazole)	Azoles inhibit cytochrome P_{450} dependent 14α-lanosterol demethylase (encoded by *ERG11*) resulting in the accumulation of 14α-methylated sterols, which are toxic to the cells.
PYRIDINES	
e.g. Buthiobate and pyrifenox	Pyridines and pyrimidines also inhibit lanosterol demethylase and are used in agriculture but not in medicine.
PYRIMIDINES	
e.g. Triarimol and fenarimol	
MORPHOLINES	
e.g. Fenopropimoph and amorolfine	Inhibit D_{14}-reductase and D_8–D_{-7} isomers.
OTHER ANTIFUNGAL DRUGS	
POLYENES	
e.g. Nystatin, amphotericin B (AmB) and primaricin	These polyenes intercalate into plasma membranes containing ergosterol and forms the channel through which cellular components like potassium ions, leak out and destroy the proton gradient within the membrane.
5-FLUCYTOSINE (5-FC)	Inside the cell, flucytosine is deaminated to 5-fluorouracil (5-FU). 5-FU is converted into 5-fluorouridine monophosphate (5-FUMP), and 5-fluoro-deoxyuridine monophosphate (5-FdUMP). 5-FUMP is incorporated in RNA, thus disrupting protein synthesis of fungus.

[66], although no studies yet have analyzed the sensitivity of azole drugs to metabolism in resistant strains or non-*albicans* species.

Studies on fungi have usually used labeled drugs to monitor the amount of drug that accumulates within the cell over several minutes. These accumulation studies have led to the identification of several fungal drug efflux mechanisms.

One study with *C. albicans* suggested that accumulation of [3H] ketoconazole required glycolytically derived energy and was controlled by cell viability, environmental pH and temperature [67]. This study also showed that ketoconazole accumulation at low extracellular concentrations was saturable, implying a specific facilitator of import, while accumulation at high concentrations appeared to be by passive diffusion. Curiously, this study found no evidence for export, since the addition of unlabeled drug did not lower the concentration of labeled drug in the cells. Finally, data in this study suggested that other azole drugs and amphotericin B increased the accumulation of labeled ketoconazole in the cell [67]. The energy requirement for ketoconazole accumulation described in this study is inconsistent with the energy-requiring efflux mechanisms. Reduction of energy levels would inhibit energy-dependent efflux pumps, resulting in increased drug accumulation, the opposite is seen with ketoconazole. These results suggest that active transport may be involved in ketoconazole import. It is possible that the ketoconazole and fluconazole are imported by two different mechanisms.

Another common mechanism of drug resistance is modification of the target enzyme and/or other enzymes in the same biochemical pathway. The ergosterol biosynthetic pathway is one such important target for azole drugs. Analysis of the sterols in a yeast cell can provide a wealth of information concerning the alterations that might have occurred in a resistant strain (Table I). Modifications in the ergosterol pathway are likely to generate resistance not only to the drug to which the cells are exposed but also to other related drugs.

A defective lanosterol demethylase (the predominant azole target enzyme) will result in the accumulation of 14α-methyl sterols, especially 14α-methyl fecosterol and the diol 14-methyl-ergosta-8, 24(28)-dien-3b, 6a-diol [68, 69]. In azole-treated cells, the presence of 14α-methyl sterols can modify the function and fluidity of the plasma membrane. In addition, it appears that cells with 14α-methyl sterols have an increased sensitivity to oxygen-dependent microbicidal systems of the host [70]. The accumulating toxic diol is known to cause growth arrest in *Saccharomyces cerevisiae* [69, 71] but is thought to be tolerated in *C. albicans* [72]. The toxic effects of the diol in *S. cerevisiae* are eliminated by a mutation in *ERG3,* encoding C-5 sterol desaturase [69, 72, 73].

Plasmid complementation of the ERG3 mutation in *S. cerevisiae* suggests that the *ERG3* mutation alone can cause azole resistance [69, 74]. Biochemical analysis suggests that *ERG3* mutations are responsible for resistance in at least two clinical isolates of *C. albicans* [75]. Also the analysis of sterol composition of *Cryptococcus neoformans* revealed that when cells were exposed to itraconazole

[76], there was an altered sterol composition since the drug affects both lanosterol demethylase and the C-4 sterol demethylase enzyme, 3-ketosteroid reductase.

The analysis of cell extracts from fungi has been important in monitoring overall sterol synthesis and the level of inhibition of lanosterol demethylase by azole drugs [77–79]. For example, cell extracts from a polyene- and azole-resistant strain of *C. albicans* (D10) did not contain any detectable lanosterol demethylase activity whereas the revertant strain, D10R, has lanosterol demethylase activity [80]. In the absence of the drug, strain D10 accumulates 14α-methyl sterols similar to an azole-treated wild-type cell. Strain D10 is also defective in the formation of hyphae, while the revertant forms hyphae at normal rates. This finding suggests that hyphal formation, which is an important virulence factor [81] can be affected by changes in the ergosterol pathway. This has also been found true for *A. fumigatus* [82].

The predominant target enzyme of the azole drugs is lanosterol demethylase. The gene encoding this protein is currently designated *ERG11* in all fungal species, although it has previously been referred to as *ERG16* and *CYP51A1* in *C. albicans*. Several genetic alterations were identified associated with the *ERG11* gene of *C. albicans,* including point mutations in the coding region, overexpression of the gene, gene amplification (which leads to overexpression) and gene conversion or mitotic recombination.

Point mutations in *ERG11* were developed in laboratory strains that result in azole resistance. The point mutation T315A (the replacement of threonine [T] with alanine [A] at position 315) was constructed in the *C. albicans ERG11* gene [78] based on the current understanding of the active site of the enzyme [83]. The active site represents a pocket positioned on the top of the heme cofactor. Substrates or inhibitors enter the active site through a channel accessible only with a shift in α-helix of the apoprotein.

Mutations in the active-site pocket, in the channel and/or in the mobile helix affect the function of the enzyme. The T315A mutation, situated in the active-site pocket, was studied in *S. cerevisiae,* which is more amenable to genetic manipulation. T315A causes a reduction in the enzymatic activity and a reduction in azole binding to the active site, resulting in fluconazole resistance. Another point mutation was identified in the *S. cerevisiae ERG11* gene from a laboratory strain resistant to azole drugs [84, 85]. This mutation, D310G (replacement of aspartic acid [D] with glycine [G] at position 310), is located in the active site of the enzyme in close proximity to the T315A mutation and renders the enzyme inactive. However, the azole resistance of this strain is likely due to an *ERG3* suppressor rather than to an inactive *ERG11* gene product [69].

A survey of resistant and sensitive clinical isolates has identified seven different point mutations associated with azole-resistant isolates [86]. However, the matched sensitive isolate was not available from these resistant isolates, which could be used to determine if the point mutations were associated with resistance or just the result of allelic variation. *ERG11* gene from a variety of fungal sources was expressed and manipulated in *S. cerevisiae* [78, 87, 88] and in *Escherichia coli* [89]. A recent study used functional expression in *S. cerevisiae* to identify and characterize five *ERG11* point mutations from matched sets of sensitive and resistant isolates of *C. albicans* [90]. Overexpression of *ERG11* was described in several different clinical isolates [91–93). In each case, the level of overexpression is not substantial (less than a factor of 5). It is difficult to assess the contribution of *ERG11* overexpression to a resistant phenotype, since these limited cases of overexpression were always accompanied by other alterations associated with resistance, including the R467K mutation, and overexpression of genes regulating efflux pumps. Overexpression was not extensively evaluated, so it is difficult to assess its importance. It should be noted that low-level overexpression was also documented in strains of *S. cerevisiae* [94–97]. In each case, the effect of overexpression on antifungal susceptibility was minimal. It remains to be seen if overexpression of *ERG11* alone can result in azole resistance.

Another genetic alteration associated with *ERG11* was described in the same clinical isolates in which the R467K mutation was described [98]. Most, if not all, strains of *C. albicans* are diploid, having two alleles of each gene [99]. Clinical isolates are usually clonal [100] and contain several sequence differences between the two copies of a gene (allelic differences). While analyzing the R467K mutation, it was observed that all of the allelic differences present in the sensitive isolate were eliminated in the resistant isolates containing R467K. These allelic differences were eliminated from the region of the *ERG11* gene including the promoter, coding region, and terminator region and into the *THR1* gene immediately downstream of the *ERG11* gene [98]. The simplest explanation is that the allelic differences were eliminated by a gene conversion or mitotic recombination involving the *ERG11* gene, although other possibilities (gene or chromosome deletion or mating) could not be completely excluded. This gene conversion or mitotic recombination resulted in a cell in which both copies of the *ERG11* gene contain the R467K mutation.

In addition to alterations in the lanosterol demethylase, a common mechanism of resistance is an alteration in other enzymes in the same biosynthetic pathway. Most of the genes of the ergosterol biosynthetic pathway shown in Table III were cloned in *S. cerevisiae* [101, 102, 103]. To date, *ERG1, ERG2, ERG3, ERG4,*

ERG7, and *ERG11* were cloned from *C. albicans* [104], ERG3 and ERG11 were cloned from *C. glabrata* [105, 106], and *ERG11* was cloned from *C. krusei* [105] and *C. tropicalis* [107]. In addition, *PRD1,* the NADPH-cytochrome P-450 reductase important for the function of *ERG11,* was cloned from *C. albicans* [104].

Table III

Ergosterol biosynthetic pathway from squalene to ergosterol

Enzyme	Gene	Farnesyl pyrophosphate	Antifungal inhibitors
Squalene synthase	*ERG9*	↓ SQUALENE	
Squalene epoxidase	*ERG1*	↓ 2,3-OXIDOSQUALENE	← Allylamines/ Thiocarbamates
Lanosterol synthase	*ERG7*		
Lanosterol 14α- demethylase		↓ 4,4-DIMETHYL-CHOLESTA -8,14,24-TRIENOL	← Azoles
C-14 sterol reductase	*ERG24*	↓ 14-DIMETHYL LANOSTEROL	← Morpholines
C-4 methyl oxidase	*ERG25*	↓ 4-METHYL ZYMOSTEROL	
C-4 decarboxylase	*ERG26*	↓ ZYMOSTEROL	
C-24 sterol methyltransferase	*ERG6*	↓ FECOSTEROL	← Tomatidine
C-8 sterol isomerase	*ERG2*	↓ EPISTEROL	← Morpholines
C-5 sterol desaturase	*ERG3*	↓ ERGOSTA-5,7,24(28)-TRIENOL	← Azoles
C-22 sterol desaturase	*ERG5*	↓ ERGOSTA-5,7,22,24(29) -TETRAENOL	
C-24 sterol desaturase	*ERG4*	↓ ERGOSTEROL	

The antifungal resistance patterns are not similar, suggesting that the pattern of sterol control and antifungal resistance differs between the closely related yeast species [106]. This suggests that analysis of the ergosterol biosynthetic pathway in one species may not be applicable to other yeasts. Sterol analysis of *C. albicans* clinical isolates suggested that alterations in *ERG3* may be a major cause of azole resistance [108, 109]. *ERG3* defects prevent the production of the diol that would cause growth arrest. *ERG3* defects have been suggested to be a major source of azole resistance in a number of fungi, including the plant pathogen *U. maydis* [110]. In *Cryptococcus neoformans,* the azoles appear to block directly or indirectly the C-4 sterol demethylase enzyme complex, preventing the production of the diol without an *ERG3* defect [111]. Sterol analysis of fluconazole resistance in *C. neoformans* suggested that resistant strains may have defects in *ERG2* or in *ERG3* [112]; these strains appear to be crossresistant to amphotericin B. In addition to its interactions with lanosterol demethylase, itraconazole interacts with *ERG24* in *C. neoformans* and *H. capsulatum,* which explains the increased potency of itraconazole against these species [113].

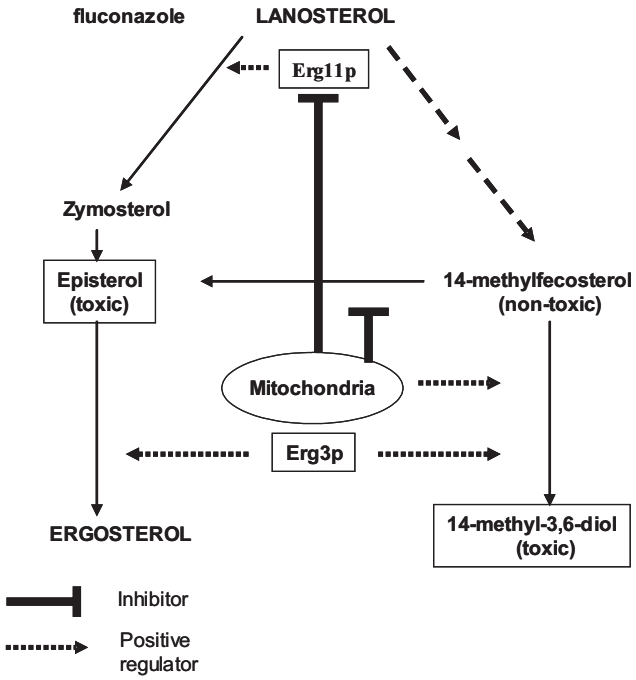

Figure 2. Erg3p mediated production of toxic intermediate

Drug efflux and uptake

ABC (ATP-binding cassette) transporters

C. albicans, clinical resistance to fluconazole as a result of reduced intracellular accumulation was reported for other pathogenic *Candida* species including *C. tropicalis, C. glabrata, C. krusei* and *C. dubliniensis*. The azole resistant isolates of *Candida* cells mainly overexpress genes encoding multidrug efflux transporter proteins belonging to two superfamilies, the ABC transporters and MFS [114].

ABC proteins are generally made up of two transmembrane domains (TMDs) and two cytoplasmically located nucleotide binding domains (NBDs), although 'half proteins' that probably dimerize for full functionality are known in some prokaryotes [115]. Typically, the TMDs comprise α-helices of 12 transmembrane segments (TMS) while the NBDs have α-helices and β-sheets arranged to form a Rossman-fold architecture [115]. While it appears that several TMSs associate together to form the substrate binding site(s), this alone is probably not sufficient for substrate transport across the membrane bilayer. Cdr1p was the first ABC transporter identified as a drug efflux pump of *C. albicans* [116], the gene encoding a *CDR1* was cloned by functional complementation of the *S. cerevisiae pdr5* mutant exhibiting hypersensitivity to cycloheximide and other drugs. Cdr1p is a close homologue of the ABC transporter human MDR1/P-gp. To date, Cdr1p and Cdr2p, which are very close homologues, represent two major extrusion pumps of *C. albicans*. These pump proteins eliminate not only azoles and its derivatives but also extrudes a variety of structurally unrelated compounds. Other homologues of *CDR1* and *CDR2,* namely *CDR3* and *CDR4,* were identified but neither overexpression nor deletion of *CDR3* or *CDR4* genes affect drug susceptibilities of *C. albicans* [117]. Cannon's group has found allelic variation in a number of genes involved in fungal drug resistance [118]. Considering the widely observed polyphormism of human P-gp/*MDR1* [119] the characterization of an allelic variation of drug extrusion proteins of *Candida* represents an interesting possibility, which may also contribute to azole resistance. This definitely needs to be examined to assess if allelic variation in MDR genes of *Candida* have any clinical relevance. The reported glucose induced phosphorylation of CgCdr1p and Pdh1p, drug efflux proteins of *C. glabrata* suggests that posttranslational modification of efflux proteins could represent yet another novel mechanism of drug resistance [120].

Another important characteristic feature of ABC drug transporters is that they utilize the energy of ATP hydrolysis to transport a variety of substrates across the plasma membrane. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. The NBDs of all ABC transporters, irrespective of their origin and the nature of the transport substrate, share extensive amino acid sequence identity and typical motifs [121]. For example, NBDs of ABC transporters have a β-sheet sub-domain containing the typical Walker A and Walker B motifs, as an essential feature of all ATP requiring enzymes [122], along with an α-helical sub-domain that possesses the conserved ABC signature sequence. NBD protein sequences possess certain conserved amino acid stretches, which are considered to be critical for the domain's functionality [123]. These include; the Walker A, with a consensus sequence GxxGxGKS/T, where 'x' represents any amino acid, the Walker B motif, i.e. hhhhD, where 'h' represents any aliphatic residue, and an ABC signature, LSGGQQ/R/KQR. Structural and biochemical analyses of NBDs show that the lysine residue of Walker A motif binds to the β- and γ-phosphates of ribonucleotides and plays a critical role in ATP hydrolysis [123]. Mutation of this lysine residue was shown to reduce or abolish the hydrolytic activity and in some cases impairs nucleotide binding [123]. Jha and coworkers found that an evolutionarily divergent Cys193 of the Walker A motif of NBD1 was critical for ATP hydrolysis. In a recent *in vivo* study, the relative contribution of both the N- and C-terminal NBDs in ATP binding, hydrolysis and transporter activity of native Cdr1p (full protein) was examined wherein the atypical Cys193 of the Walker A motif of NBD1 (C193K) and conserved Lys901 (K901C) in the Walker A motif of NBD2 were replaced [124]. The drug resistance profile of the Cdr1p mutant variant cells harboring C193K or K901C gave interesting insights into the functioning of the two NBDs.

During the ATP hydrolysis step, substrate binding site(s) formed by the TMDs of the protein favor dissociation rather than association of the drug substrate [125]. While similar information does not yet exist for fungal ABC transporters, it is possible to speculate that such a mechanism operates also in these proteins. Exactly how drug binding is transduced to the NBDs as a signal is however poorly understood as yet. It is to note that the well-documented drug stimulated ATPase activity of mammalian ABC transporters has not been found for fungal transporters [126, 127]. Nevertheless, it is certain that a very close interaction exists between the TMDs and the NBDs in ABC transporters. For example, it is observed that mutations or deletions in small discrete regions adjacent to the Walker B motif of P-gp, result in loss of this signaling and therefore of substrate transport [128].

Interestingly, NBDs from several ABC transporters supposedly undergo significant conformational changes due to ATP binding rather than to its hydrolysis [129, 130]. This has also been shown to be the case for isolated NBD1 from the fungal ABC transporter, Cdr1p. Thus catalytically deficient NBD1 variants (with C193A or D327A/N) continue to exhibit conformational changes similar to wild type NBD1 while the binding deficient mutant (with W326A) appears to be impaired in its ability to perform a similar conformational transition.

MFS efflux pump

MFS proteins are another class of major drug transporters involved in drug efflux thus, have a role in the multidrug resistance phenomenon displayed by yeasts. The MFS was originally defined as a superfamily of permeases characterized by two structural units of six TMS-α-helical segments, linked by a cytoplasmic loop. It is to note that out of several MFS proteins listed in *Candida*-database (http://genolist.pasteur.fr/CandidaDB) *CaMDR1,* its alleles and *FLU1* are shown to be the only drug transporters. *CaMDR1* was initially identified as a gene, conferring resistance to the tubulin binding agent benomyl and tetrahydrofolate reductase inhibitor methotrexate [131, 132]. *CaMDR1* expression in *S. cerevisiae* confers resistance to several unrelated drugs and its overexpression was linked to azole resistance in *C. albicans*. The expression of *CaMDR1* in *C. albicans* cells is enhanced by benomyl, methotrexate and several other unrelated drugs, and is found to be more pronounced in some of the azole resistant clinical isolates [133, 134]. Morschhauser and his group employed a proteomic approach to understand the molecular basis of drug resistance in *C. albicans* [135]. By comparing the protein expression pattern of matched pairs of fluconazole resistant and susceptible clinical isolates, they identified several proteins whose expression was upregulated specifically when only *CaMDR1* gene was overexpressed. These proteins, mostly belonging to putative aldo-keto reductase family were not upregulated in a fluconazole resistant strain that overexpressed only *CDR1/CDR2* and not *CaMDR1*. This implied that the expression of efflux pump encoding genes was controlled by different regulatory networks [135].

Recently *FLU1,* another gene encoding MFS protein of *C. albicans* was cloned by complementing strain of *S. cerevisiae,* which was hypersensitive to fluconazole. *FLU1* is, however, not involved in the development of fluconazole resistance in clinical isolates of *C. albicans.* Interestingly, studies revealed that the preferred substrate of Flu1p is mycophenolic acid rather than fluconazole. Al-

though more than two dozens of putative MFS genes are identified in the *Candida* genome, except for CaMdr1p none of the other proteins of this MFS superfamily are reported to have any direct role in clinical fluconazole resistance.

Drug uptake

Defects in drug uptake are a common mechanism of drug resistance. However, it is important to emphasize the distinction between the uptake of a drug into a cell and the gradual accumulation of the drug in the cell, which is the result of a balance between uptake into the cell and efflux of the drug from the cell. Analysis of drug uptake is difficult and requires that mechanisms of uptake be separated from efflux mechanisms. The hydrophobic nature of drugs permits their easy import by passive diffusion. However, the contribution of drug import to the overall scenario of MDR is not well established since technically it has not been possible to separate efflux of drugs from their uptake. Nonetheless, there are few studies particularly with mammalian cells in which passive diffusion drugs through a lipid bilayer was shown to be an important determinant of MDR [136]. The variations in membrane fluidity are expected to affect passive diffusion of drugs and in turn, their sensitivity to these drugs.

The enhanced fluidity has been linked to enhanced passive diffusion of drugs [137]. There are factors other than membrane fluidity, that can also influence diffusion of drugs across the membrane bilayer and thus can affect drug susceptibilities [138]. The uptake of drugs and its impact on drug resistance needs to be analyzed more carefully. It is expected that with better experimental designs, the contribution of import of drugs in MDR can be established.

Risk factors associated with drug resistance

Risk factors associated with fungaemia due to fluconazole-resistant *Candida* species have also been studied in non HIV-infected patients. Short case series of primary fluconazole resistance were reported in patients with severe neutropenia [139, 140]. Candidaemia due to *C. krusei* was associated with prior exposure to fluconazole [141–143]. In one of the largest cases reported, series of haematogenous *C. krusei* isolates obtained from a single hospital, it was found that in addition to prior exposure to fluconazole, acute leukaemia and severe neutropenia were risk factors for acquiring infections due to this yeast [144]. In-

fections caused by *C. glabrata* have also been associated with previous therapy with amphotericin B, with other antibiotics, permanent central venous catheters, haemodialysis, abdominal portal of entry and solid tumours [142, 143, 145]. Although an association between fluconazole use and an increase in bloodstream infections due to non-*albicans* species, including *C. glabrata,* was reported [143], some studies reported a reduction in prevalence of *C. glabrata* and *C. krusei* after the introduction of fluconazole prophylaxis.

In a retrospective case, series candidaemia by *C. glabrata* was seen in a wide range of patients with different medical conditions and predisposing factors. In this study, the greatest part of cases came from the intensive care unit and only very few were neutropenic [145]. A surveillance study of candidaemia in cancer patients done by the European Organization for Research and Treatment of Cancer (EORTC), found that candidaemia by *C. glabrata* was much more frequent in patients with haematological malignancies than in those with solid tumors [146]. With regard to moulds, invasive infections due to *Fusarium* species were described in patients with haematological malignancies, severe and prolonged neutropenia and prior treatment with broad-spectrum antibiotics [147, 148]. Similar risk factors were identified in deep mycosis caused by *S. prolificans* [149, 150]. Nearly all patients the Spanish series of *S. prolificans* infections had acute leukaemia [149]. Disease disseminated by *A. terreus* differed from other species of *Aspergillus* in that most of the patients infected with this fungus had a diagnosis of leukaemia as underlying disease and they were more likely to be neutropenic and to have a more prolonged neutropenia than patients infected with other *Aspergillus* species [151].

Future scope for strategies to overcome antifungal resistance

The increasing importance of antifungal resistance underlines the need for developing strategies to clear this problem before it spreads and reaches the rates of antibacterial drug resistance [152]. Potential mechanisms to overcome fungal resistance ranges from the synthesis of new drugs with better antifungal activity and pharmacokinetic profile to improve current therapeutic strategies with antifungal agents. In addition, antifungal-control programmes to avoid extensive and inappropriate use of antifungals in hospital and community settings may be needed [153]. As is the case with antibacterial drug resistance, appropriate use of antifungals may be critical to delay and in many cases prevent the emergence of antifungal drug resistance. Given the association between antifungal exposure and

the development of resistance, prophylaxis should ideally be restricted to carefully selected high-risk patients. There should be agreed protocols available for the use of antifungals as empiric or pre-emptive therapy, to make a more critical selection of candidates for reducing unnecessary antimicrobial pressure on fungal pathogens. Another factor that could influence the emergence of resistance is the dose of drug used for antifungal therapy. Theoretically, treatments with sufficient drug doses might avoid the development of resistance in less-susceptible fungi. Thus, the use of high rather than low doses of antifungals may be another potential measure to avoid and/or to fight against antifungal resistance. In fact, higher than usual doses of amphotericin B have already been recommended by the Infectious Diseases Society of America for treating candidaemia caused by *C. glabrata* and *C. krusei* [154]. Nevertheless, the possibility of emergence of highly resistant organisms under the increased selective pressure should also be considered. Potential strategies to fight against antifungal resistance include the use of several pharmacological and non-pharmacological measures.

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