Virulence and biofilms as promising targets in developing antipathogenic drugs against candidiasis

Mohd Sajjad Ahmad Khan*,1, Fatimah Alshehrei2, Saleh Bakheet Al-Ghamdi3, Majid Abdullah Bamaga4, Abdullah Safar Al-Thubiani2 & Mohammad Zubair Alam5

1Department of Basic Sciences, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia
2Department of Biology, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia
3Department of Biology, Faculty of Science, Al-Baha University, Baha, Saudi Arabia
4Department of Medical Laboratory Sciences, Fakeeh College for Medical Sciences, Jeddah, Saudi Arabia
5King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

*Candidate for correspondence: khanmsa@hotmail.com

Candida albicans has remained the main etiological agent of candidiasis, challenges clinicians with high mortality and morbidity. The emergence of resistance to antifungal drugs, toxicity and lower efficacy have all contributed to an urgent need to develop alternative drugs aiming at novel targets in C. albicans. Targeting the production of virulence factors, which are essential processes for infectious agents, represents an attractive substitute for the development of newer anti-infectives. The present review highlights the recent developments made in the understanding of the pathogenicity of C. albicans. Production of hydrolytic enzymes, morphogenesis and biofilm formation, along with their molecular and metabolic regulation in Candida are discussed with regard to the development of novel antipathogenic drugs against candidiasis.

Lay abstract: Over the last decade, candidiasis has remained a major problematic disease worldwide. In spite of the existence of many antifungal drugs, the treatment of such diseases has still remained unsuccessful due to drug inefficacy. Therefore, there is a need to discover antifungals with different modes of action, such as antipathogenic drugs against Candida albicans. Here, we describe how various types of virulence factors such as proteinase, phospholipase, hemolysin, adhesion, morphogenesis and biofilm formation, could be targeted to develop novel therapeutics. We can inhibit production of these virulence factors by controlling their molecular/metabolic regulation.

Graphical abstract:
Candidiasis is an opportunistic infection that may be acute, subacute or chronic and often results in life threatening mycoses. Healthy individuals encounter superficial infections such as vulvovaginal candidiasis, candiduria, onychomycosis and oropharyngeal candidiasis. Conversely, immunocompromised patients develop invasive systemic candidiasis such as candidemia and organs infection, especially those of brain, kidneys and eyes [1,2]. The most common Candida species (spp.) that cause candidiasis are C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis and C. tropicalis [2,3]. Among these, C. albicans dominates in causing infections of oral, genital and cutaneous sites, including patients in intensive care units with indwelling devices, as well as those who have undergone bone marrow transplantation and individuals treated improperly with broad spectrum antibiotics or corticosteroids [3,4]. A significant number of women witness vulvovaginal candidiasis at least once in their lifetime [5–7]. \textit{Candida} spp. are the commonly isolated pathogens in nosocomial infections and are ranked as the fourth major causative agents of systemic infections, with a mortality rate of 50% [6,8,9].

Antifungal drugs used for invasive fungal infections are categorized as polyenes, azoles and echinocandins [10]. The careful use of these available antifungal agents and the management of underlying diseases have led to success in the treatment of invasive fungal diseases [11]. However, the emergence of drug-resistant strains and drug toxicity have indicated the need for a continuous search for novel antifungal drugs. In a blatant contrast with antibacterial drugs, the existing armaments of antifungal drugs are extremely diminutive. Moreover, the advancements in antifungal drug discovery programmes are slower than those for antibacterial drug discovery [12,13]. The currently available antifungal drugs target fungal growth. The drug that targets cell growth enforces a higher level of selective pressure, which results in the emergence of antibiotic-resistant strains [14]. Moreover, both host cells and fungi are eukaryotic and therefore share common physiological processes. This is also one of the main reasons for the noticeable host-toxicity of some of the existing antifungals. Hence, it is difficult to identify a drug with pathogen-specific targets during drug discovery and development programmes [12,15].

An alternative approach to antifungal drug development is to target pathogen-specific virulence factors. It is a quite effective strategy, as it maintains the host microflora with reduced cellular toxicity [14]. Also, considering the immunological aspects, the treatment of hosts with an antivirulence compound would result in a scenario similar to the use of live attenuated vaccines [12]. Therefore, understanding the infection biology of a pathogen is mandatory in recognizing new drug targets. In this review, we have highlighted some of the recent developments made in understanding how virulence traits including biofilm formation regulated at metabolic and molecular levels and, how this could be exploited as promising anticaldindal drug targets.

Current antifungal drug therapy: targeting cell growth & its challenges

Antifungal agents currently in use belong to seven classes of drugs: polyenes, azoles, allylamines, candins, morpholines, thiocarbamates and pyrimidine analogues [16]. All of these agents target cell growth and their mechanism of action are represented by inhibition of ergosterol biosynthesis; inhibition of DNA or RNA synthesis; and inhibition of glucan, chitin or mannan synthesis [17]. The principal targets of these antifungal drugs are varied and are depicted in Figure 1 as well as listed in Table 1. The host toxicity and the rapid emergence of resistant strains are the main problems associated with these antifungal drugs, though low potency, poor solubility and limited or inconvenient dosage forms may also be accounted [19]. Amphotericin B fungal cell toxicity is due to its higher affinity toward ergosterol, resulting in pore formation and leakage of cytoplasmic material. However, it has been considered as toxic to hosts as well because it also has shown sufficient affinity toward cholesterol in the host cell membrane, and thereby affecting permeability of renal tubules [19–21]. 5-Fluorocytosine is known to obstruct DNA synthesis and may lead to bone marrow toxicity, leukopenia and imbalance of liver enzymes [22]. Nonetheless, globally, the most commonly prescribed antifungal drug is fluconazole because it is considered as the safest. However, its fungistatic nature has led to the development of drug-resistance and higher doses are reported to be hepatotoxic [23,24]. The echinocandins and the nikkomycins target the fungal cell wall by inhibiting glucan and chitin synthesis respectively. Such targets are not present in a mammalian cell; therefore, these drugs are less toxic to the host cells. Hence, these classes of antifungals have become attractive for use in humans, but are very expensive [18,25,26]. Recent reports have established increased drug resistance against these classes of drugs as well [13].

Keywords: antifungal • antipathogenic • biofilm • \textit{Candida albicans} • candidiasis • drug discovery • hydrolytic enzymes • metabolic pathways • morphogenesis • virulence factors
### Table 1. Antifungal agents: activities, mechanism of action and resistance against fungal pathogens.

| Antifungal drugs | Activity spectrum | Mechanism of action | Mechanism of resistance | Ref. |
|------------------|-------------------|---------------------|-------------------------|------|
| **Polyenes**     |                   |                     |                         |      |
| Amphotericin B   | Broad activity against Candida spp (except C. lusitaniae), Cryptococcus neoformans and filamentous fungi (except Aspergillus spp. A. terreus and A. flavus) | Binding to ergosterol and destabilization of cell membrane functions | Decreased access of AMB to drug target in fungal membrane, altered membrane ergosterol content and reduced intercalation, increased cell wall rigidity, sequestration of fungi to lysosomes, enhanced catalase activity | [15,30] |
| Lipid formulations | Interaction with ergosterol, intercalation of fungal membrane that leads to increased permeability to univalent and divalent cations and cell death |                         |                         |      |
| Liposomal nystatin | Alternative mechanism of action through oxidation of fungal membrane | Decreased oxidative damage (a) over expression of catalases and superoxide dismutases of A. fumigatus, (b) anaerobic environment |                         | [15,16] |
| **Azoles**       |                   |                     |                         |      |
| Miconazole, Ketoconazole, Clotrimazole, Fluconazole | Active against Candida spp, Cryptococcus spp, less active against C. glabrata, no activity against C. krusei and filamentous fungi | Interaction with cytochrome P-450 and inhibition of C-14 demethylation of Lanosterol (ERG11), causes ergosterol depletion and accumulation of toxic and aberrant sterols in membrane leading to perturbation of fungal cell membrane | Enhanced efflux by upregulation of multi-drug transporter genes (CDR, MDR), over expression of target enzyme, decreased affinity to the binding site, target alterations by occurrence of mutations (ERG3), alteration of specific steps in the ergosterol biosynthetic pathway, altered drug uptake, exogenous cholesterol import | [15,16,18] |
| Itraconazole     | Like fluconazole but enhanced activity against filamentous fungi and other yeasts |                         |                         |      |
| Voriconazole, Posaconazole, Ravuconazole | Like fluconazole but enhanced activity against filamentous fungi including Aspergillus and Fusarium spp. Active against C. krusei, C. glabrata and Candida isolates with acquired azole resistance |                         |                         |      |
| **Allylamine**   |                   |                     |                         |      |
| Terbinafine      | Active against most of dermatophytes, but poorly active against Candida spp | Inhibition of squalene epoxidase (ERG1), with subsequent ergosterol depletion and accumulation of toxic sterol intermediates | Increased drug efflux (CDR1, CDR2), over expression of target site (ERG1), over expression of salicylate mono-oxygenase (drug degradation) | [18] |
| **Morpholine**   |                   |                     |                         |      |
| Amorolfin        | Active against most of dermatophytes, but poorly active against Candida spp | Inhibition of sterol Δ14 reductase and Δ7,8 isomerase | Over expression of ERG24, ERG4 genes | [18,30] |
| **Nucleoside analogue** |                   |                     |                         |      |
| 5-Fluorocytosine (SFC) | Active against Candida spp and Cryptococcus spp. | Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine antimetabolites | Decreased uptake of 5-FC, decreased formation of toxic antimetabolites, defect in cytosine permease | [15,16,18] |
| **Echinocandins** |                   |                     |                         |      |
| Caspofungin, Micafungin, Anidulafungin | Active against Candida spp., moderately active against Aspergillus spp, poorly active against C. neoformans | Inhibition of the cell wall synthesis enzyme β-1,3-glucan synthase, leading to susceptibility of fungal cell to osmotic lysis | Up regulation of homeostatic stress-response pathways (HSP90; calcineurin), over expression of target site, up regulation of genes encoding for β-1,3-glucan synthase (FK51 genes), over expression of genes related to transport of cell wall components | [15,16,18,30] |

AMB: Amphotericin B.
The evolution of antifungal drug resistance and its epidemiological spread is a key issue in the management of fungal diseases [27]. Molecular mechanisms of drug resistance in fungi are summarized in Table 1. The formation of biofilms by many pathogenic Candida spp. are reported to confer drug resistance up to 1000-fold higher than the majority of antifungal drugs, particularly azoles and polyenes [16,28]. The mechanism of drug resistance shown by biofilms involves restricted infiltration of drugs inside the exopolymer matrix; limitations with the availability of nutrients, resulting in a decreased growth rate; overexpression of resistance genes, especially efflux pumps; and the existence of persister cells [29,30].

Antifungal drug discovery: processes & new approaches
Antifungal drug development relies on the use of Saccharomyces cerevisiae as a successful model of a eukaryotic organism because it shares many molecular and metabolic processes similar to humans. This model is also being used to predict the potential efficacy or toxicity of drug candidates such as small molecule compounds [10,31]. The most common tactic to discover active small molecules as antifungal agents, is to screen libraries of compounds
of natural or synthetic origin, which inhibit the growth of selected fungi [10]. Many researchers have attempted to develop newer chemotherapeutic drugs against fungi. Babazadeh-Qazijahani et al. [32] synthesized a series of imidazolylchromanone oximes containing phenoxyethyl ether moiety similar to omoconazole. These compounds exhibited potent activity against Cryptococcus gattii. In search of newer kinds of azole drugs, Hashemi et al. [33] designed a series of triazole alcohols in which one of the 1,2,4-triazol-1-yl group in fluconazole structure has been replaced with 4-amino-5-aryl-3-mercapto-1,2,4-triazole motif. Furthermore, they removed the amino group from the structure to obtain 5-aryl-3-mercapto-1,2,4-triazole derivatives. In their study, the majority of the compounds were shown to be potential antifungal candidates even greater than fluconazole against Candida spp.

Targeting virulence factors to discover novel antifungal drugs

In the past few years, virulence factors of fungi and their inhibitors have been considerably investigated and characterized, which has led to the development of new alternatives for antifungal therapeutics [35]. Many different types of determinants such as genes (e.g., adhesins) and gene products (e.g., secreted enzymes) are considered as virulence factors that are involved in the host–microbe relationship, leading to superficial as well as invasive infections in humans [36]. Virulence factors facilitate adherence, infiltration and spread in host tissues, even at elevated temperatures. These factors also help pathogens to tolerate the hosts adaptive immunity for example, the evasion from phagocytes and the complement-mediated pathway. Additionally, nutritional, metabolic and necrotic factors or even morphology variations, including phenotype switching and biofilm formation, could be considered as virulence factors [37]. Virulent strains of albicans and non-albicans Candida specifically, C. albicans, C. glabrata, C. auris and C. haemulonii, are reported to potentially cause invasive fungal infections even in immunocompetent individuals [38].

Targeting virulence in developing antifungals has the edge over other strategies since it enumerates the number of potential targets that are needed for discovery of newer drug candidates; it maintains natural host microflora, which is of utmost importance especially for C. albicans; it exerts a low level of selective pressure, therefore it minimizes the development of antibiotic resistance [12,14]. Here, we have summarized some of the potential virulence factors in C. albicans and how they are being targeted by some of the compounds exhibiting antivirulence activity that could be a lead in drug discovery for antifungal drug development. Table 2 describes some of the compounds discovered as antivirulence and antibiofilm agents, which are expected to be drug leads.

Extracellular hydrolytic enzymes

Extracellular hydrolytic enzymes assist fungi to rupture and enter host tissues [39]. Therefore, they are expected to be potential virulence factors. Some of these important enzymes are lipases, phospholipases and proteinases. Several studies have reported a reduction in virulence of Candida spp. due to the absence or reduced expression of these hydrolytic enzymes. These enzymes also help Candida cells to undergo morphological transitions, colonization and penetration of host tissues [39–41].

Phospholipases facilitate Candida cells in the invasion of host tissues by hydrolyzing ester linkages of glycolipids. It is evident from a study conducted by Ibrahim et al., that invasive strains of C. albicans produce a higher amount of phospholipases compared with noninvasive strains [42]. In C. albicans, four types of phospholipases are categorized, phospholipase A, B, C and D, depending upon the ability of the enzyme to cleave a specific ester bond [43]. Many researchers have observed that the invasiveness of Candida cells toward the epithelial tissues is facilitated by the higher production of phospholipases [43–46].

The secreted aspartyl proteinases (SAPs) play central role in Candida pathogenicity. A family of ten SAPs (Sap proteins) fulfill a number of specialized functions during the infective process. SAPs digest hemoglobin to acquire nutrition for Candida cells. They destroy the host cell membrane by hydrolyzing many tissue proteins such as albumin, collagen, cystatin A, keratin, laminin and fibronectin, to facilitate adhesion and tissue invasion. SAPs also digest cells and molecules of the host immune system such as IL-1β, immunoglobulin A and mucinoid salivary lactoferrin, to avoid or resist antimicrobial attack by the host [47]. Several workers have reported that the production
Table 2. Occurrence of various virulence factors in fungi, their role and known inhibitors.

| Virulence factors | Organisms | Role | Inhibitors | Ref. |
|-------------------|-----------|------|------------|------|
| **Proteinases**   |           |      |            |      |
|                   | *C. albicans* | Hydrolytic enzyme | Pepstatin A, saquinavir, indinavir Human domain antibodies | [175,176] |
| **Phospholipases**|           |      |            |      |
|                   | *C. albicans, C. neoformans, Aspergillus flavus* | Hydrolytic enzyme | Alexidine dihydrochloride, 1,12 bis-(tributylphosphonium)-dodecane dibromide | [177] |
| **Haemolysin**    | *C. albicans* | Hydrolytic enzyme | Cationic lipo-benzamide compound 9M | [178] |
| **Candidalysin**  | *C. albicans* | Hydrolytic enzyme | Cis-2-dodecenoic acid | [179] |
| **Elastase**      | *Trichophyton mentagrophytes, Candida spp* | Hydrolytic enzyme | Aliphatic aldehydes | [65] |
| **Glyoxilate cycle** | *C. albicans* | Metabolic pathways | Caffeic acid, rosmarinic acid and apigenin | [142] |
| **Inositol phosphoryl ceramide synthase (IPC1)** | *C. neoformans, Candida spp, Aspergillus spp* | Metabolic pathways | Aureobasidin A, khafrefungin | [180] |
| **Isocitratelyase (ICL)** | *C. albicans* | Metabolic pathways | 3-nitropropionate, 3-bromopyruvate, mycenon, mohangamide A and mohangamide B | [153,154] |
| **Target of Rapamycin (TOR) signaling pathway** | *Saccharomyces cerevisae* | Metabolic pathways | Small molecule CID 3528206 | [181] |
| **Calcineurin**   | *C. albicans, C. neoformans* | Metabolic pathways | Tacrolimus, cyclosporin A | [150] |
| **Hyphal formation** | *Candida spp, C. neoformans* | Morphogenesis | Saponins | [88] |
| **Adhesion, morphogenesis, biofilm** | *C. albicans* | Ras1-CAMP-Efg1 pathway | Magnolol and honokiol | [182] |
| **Biofilm**       | *Candida spp* | Drug-resistance | Farnesol, Diazaspiro-decane structural analogs, cationic lipo-benzamide compound 9M | [101,113,179] |

of SAPs is accompanied by other factors such as adherence, hyphal formation and phenotype switching, which enhances pathogenicity [47–49].

Hemolysin, a mannoprotein attached to the cell surface, enables *C. albicans* to exploit iron from host protein [50]. Iron is an indispensable cofactor for several proteins and is a prerequisite for various metabolic processes such as, cellular respiration and DNA synthesis. Hence, it is considered as a crucial virulence factor in *C. albicans*. [51]. It has been reported that colonization and the spread of fungal cells are more pronounced if iron was easily available in an ample amount to the fungus [52]. *C. albicans* can also acquire iron from host ferritin via hyphal-associated adhesin and invasin Als3 [53].

A newly identified peptide toxin, candidalysin, which is derived from the product of the ECE1 gene, is also categorized as an important virulence factor of *C. albicans* because it damages the host cell plasma membrane [54]. Candidalysin is found to stimulate the transcription factor c-Fos of activating protein 1 (AP-1) (via p38–mitogen-activated protein kinase [MAPK]) and the MAPK phosphatase MKP1 (via extracellular signal-regulated kinases 1 and 2 [ERK1/2]–MAPK), which trigger and regulate proinflammatory cytokine responses, respectively [55]. During pathogenesis, candidalysin triggers NLRP3 inflammasome-dependent caspase-1 activation via potassium efflux and acts as the main facilitator of inflammasome-independent cytolysis of macrophages and dendritic cells in the host [56].

Anti-secreted hydrolytic enzymes

A very important group of antipathogenic drugs are inhibitors of protease in fungi. It has been found that under *in vitro* conditions, some of the potential HIV protease inhibitors such as saquinavir and indinavir have dose-dependent inhibitory effects on SAPs [57,58]. SAPs of *C. albicans*, as well as HIV proteinases, belong to the group of aspartic proteinases and are inhibited by pepstatin A. The inhibitory effects of saquinavir and indinavir on SAPs *in vitro* are similar to pepstatin A. The study by Ollert *et al.* reported that pepstatin A retards adhesion of *Candida* to the host cells [59]. Therefore, the development of saquinavir and indinavir as potential antipathogenic drugs against *Candida* could be justified [35].

Another virulence factor that significantly contributes to the invasiveness of *C. albicans* during infection is phospholipase. Ganendren *et al.* [60] reported that commercially available compounds, like alexidine dihydrochloride...
and 1,12-bis-(tributylphosphonium)-dodecane dibromide with structural similarities to phospholipid substrates, had relatively broad antifungal activities under in vitro conditions against *C. albicans*, *Cryptococcus neoformans* and *Aspergillus flavus*. Orlistat is a saturated derivative of lipstatin, a powerful natural inhibitor of pancreatic lipases, first isolated from the bacterium *Streptomyces toxytricini*. It was prepared to treat obesity [61]. It has been shown to possess ten binding possibilities against the active site of *Candida rugose* lipase as observed in a docking study [62]. Other lipase inhibitors such as quinine and ebelactone B have been reported to retard the growth of *Candida* spp. [63,64].

Some aliphatic aldehyde compounds obtained from olive fruit extracts such as hexanal, nonanal, (E)-2-hexenal, (E)-2-heptenal, (E)-2-octenal and (E)-2-nonenal, have been reported to possess anti-elastase activities against dermatophytes and *C. albicans* [65]. Some studies have highlighted the efficacy of natural products such as oils of *Carum coticum*, *Cinnamomum verum*, *Syzygium aromaticum*, *Thymus vulgaris* and their active compounds, cinnamaldehyde and eugenol, at subinhibitory concentration, in inhibiting the production of proteinaes, hemolysin and biofilm formation in multi-drug resistant strains of *C. albicans* [66,67]. El Zawawy et al. [68] evaluated the efficacy of *Pluchea dioscoridis* leaf extract on growth, morphogenesis and virulence gene expression of *Candida albicans* and, observed greater than 70% decrease in expression of phospholipase, proteinase and hemolysin genes. The compounds inhibiting extracellular hydrolytic enzymes could be attractive molecules for anticandidal drug discovery.

**Morphogenesis**

Morphogenesis in *C. albicans* is defined as a switch from yeast form to hyphal form. These morphological transitions are reversible and occur during growth. These kinds of physical plasticity have been considered to ease pathogenicity [31,69]. Morphogenetic transitions in fungi occur in reaction to an external stimulus, which may be encountered in a human host such as body temperature, pH, serum, nutrient and oxygen supply and certain hormones [69]. Yeast cells are capable of disseminating more efficiently than filamentous forms, which are rather well adapted for penetration and damaging the tissues [31,70]. Morphological transitions in *Candida* cells lead to the successful establishment of disease and further progression in the form of biofilms. Therefore, these factors are well accepted as contributors to pathogenicity in *C. albicans* [12,31].

**Antimorphogenesis**

To understand the molecular mechanisms involved in morphogenetic conversions in fungi, many researchers have investigated activities of signaling pathways, along with key transcriptional regulators [31]. Here, we have briefly summarized studies conducted to explore antimorphogenetic approaches to discover antifungal compounds.

**Targeting filamentation in *C. albicans***

Filamentation is regulated in coordination with other virulence factors associated with cellular morphology [71]. Studies have evidenced that filamentation plays an active role in the progression of infection and could be targeted to obtain newer antifungal agents [12,70,72]. Additionally, immune cells respond differently to yeast and hyphal cells. In general, a protective host response is elicited against the exposure to yeast forms, whereas a nonprotective host response is elicited against filamentous forms [73,74]. Therefore, one very fascinating possibility is that anti-filamentation drug compounds could also benefit the human host by tempering immune responses [12].

**Controlling multiple signaling pathways of filamentation in *C. albicans***

The process of filamentation in *C. albicans* is tightly regulated either positively or negatively, through manifold signaling pathways congregated on either the same, or different transcription factors [31]. Tup1 in combination with Nrg1 or Rfg1, or Rbf1 alone carries out negative regulation whereas Efg1, Cph1, Tec1, Czf1, Hgc1, Ume6, Brg1 and Rim101 are involved in positive regulation [75,76]. Efg1 is a chief filamentation regulator under most environmental conditions since it is accountable for the induction of filamentation in response to pathways stimulated by N-Acetyl Glucosamine (GlcNac), pH and CO2 [75,77]. A MAPK pathway is required for inducing hyphal growth through Cph1. On the other hand, the signaling pathway involving Dck1 is responsible for the stimulation of filamentation by Czf1, which is triggered by hypoxic conditions [78]. Hgc1 forms a complex with Cdc28 and induces hyphal growth by phosphorylating Efg1 resulting in suppression of cell separation machinery [79]. It is believed that Ume6 drives filamentation through this Hgc1 pathway [80]. The GATA-family transcription factor Brg1 plays a key role in regulating filamentation in *C. albicans* [76].
The hunt for small molecules against \textit{C. albicans} filamentation

Researchers have recognized a large number of small molecules tempering with morphogenetic conversions, thereby inhibiting filamentation. Examples include farnesol, phenazine and other autoregulatory alcohols such as retigeric acid and bisbibenzyls, which act as quorum sensing (QS) molecules that target regulators of yeast-to-hyphae transition in \textit{C. albicans} \cite{77–84}. Johnson \textit{et al.} recognized up to 21 different inhibitors of \textit{C. albicans} filamentation using small molecule screening and, consequently, verified that some of these inhibitors were acting through diverse signaling pathways \cite{85,86}. In search of anti-adhesion compounds, Fazly \textit{et al.} screened a series of compounds from the University of Massachusetts Medical School (MA, USA) Small Molecule Facility DIVERset Library (Chembridge), using a high-throughput phenotypic assay, and obtained several bioactive molecules. One of the lead compounds of this study named filastatin, inhibited \textit{Candida} adhesion to human epithelial cells, yeast to hypha transition, biofilm formation and pathogenesis in a nematode infection model. It also inhibited the induction of hyphal specific HWP1 promoter, which is an early and essential event in the process of hyphal development. Zhang \textit{et al.} demonstrated that two steroid saponins isolated from \textit{Tribulus terrestris} L., as tigogenin-3-O-\beta-D-xylopyranosyl (1–2)-\[\beta-D-xylopyranosyl (1–3)]-\beta-D-glucopyranosyl (1–4)-[alpha-L-rhamnopyranosyl (1–2)]-\beta-D-galactopyranoside ( = TTS-12) and igogenin-3-O-\beta-D-glucopyranosyl (1–2)-[\beta-D-xylopyranosyl (1–3)]-\beta-D-glucopyranosyl (1–4)-\beta-D-galactopyranoside ( = TTS 15), inhibited hyphal formation in \textit{C. albicans} \cite{88}.

To obtain inhibitors of \textit{C. albicans} filamentation, large-scale phenotypic screening of 30,000 drug-like small molecules within ChemBridge’s DIVERSet chemical library was performed by Romo \textit{et al.} \cite{89,90}. They obtained several novel bioactive compounds, out of which one main compound with a common biaryl amide core structure was identified as N-[3-(allyloxy)-phenyl]-4-methoxybenzamide. This compound exhibited its antivirulence properties by inhibiting filamentation and biofilm formation in \textit{C. albicans} under \textit{in vitro} and \textit{in vivo} conditions. Pierce \textit{et al.} \cite{91} performed a cell-based phenotypic screening of 2,293 compounds, using three different chemical libraries from the National Cancer Institute’s Open Chemical Repository Collection (Naturalset, Structural Diversity set, and Challenge set). They identified 17 confirmed compounds as inhibitors of filamentation in \textit{C. albicans}.

Adhesion

Adherence of \textit{Candida} to the host tissues or medical devices is a prerequisite for colonization and biofilm formation. Adherence of \textit{Candida} to the host tissues exploit several adhesins, which are expressed on its surface. The hydrophobic proteins entrenched in a matrix of \textit{Candida} cell walls, underneath the fibrillar layer, deliver the hydrophobic interactions required to convert initial interaction between the fungus and the host surface into a strong bond \cite{92}. Adhesins are agglutinin-like sequences and are member of a family of seven glycosylated proteins. The enhanced tissue invasiveness of hyphal cells is attributed to increased adhesiveness due to the expression of agglutinin-like sequence adhesins \cite{31}. The adhesins Als1p, Als3p and Als5p (Ala1p) are located on the cell surface of hyphae and help in adhering to the host’s buccal epithelial cells, collagen, endothelial cells, fibronectin and laminin \cite{93}. Als4p, Als6p and Als9p bind to endothelial cells, collagen and laminin, respectively. Als5p is also required for cell aggregation, whereas the role of Als7p is uncertain \cite{31,94}. These adhesins, along with HWP1 and EAP1, are known to mediate biofilm formation of \textit{C. albicans} into the abiotic surfaces or epithelial cells \cite{95,96}.

Biofilms

Biofilms are controlled assemblies of microbial communities onto biotic or abiotic planes, in which the phenotypic behavior of cells, including growth rate and gene expression, is changed compared with the planktonic cells \cite{97}. In \textit{Candida} biofilms, the basal layer is formed by the adherent yeast cells and invasive hyphal forms construct an upper layer above it. The layers are surrounded by a self-produced extracellular polymer matrix of chitins, eDNA, polysaccharides and proteins, which forms a 3D structure with water channels \cite{95,98,99}. A wide array of medical implants such as catheters, endotracheal tubes, pacemakers and other prosthetic devices are easily colonized by biofilm forming fungi, resulting in persistent fungal infections. \textit{C. albicans} remains the most commonly allied species in this context and exhibits predominant prevalence in nosocomial infections. The majority of clinical manifestations of candidiasis are linked to biofilm formation on such devices. These devices, in turn, not only provide a platform for candidal cells to form a biofilm, but also promote dissemination through the host defense \cite{29,30,100}. Many mucosal-associated diseases such as oropharyngeal candidiasis, denture stomatitis and vaginal candidiasis, are also linked to the ability of \textit{C. albicans} to form biofilms \cite{100}. Similar to filamentation, biofilm development is also QS controlled and is regulated at the molecular level by a complex network. The two molecules – farnesol and tyrosol – regulate the development of biofilms via
QS regulated gene expression [99,101–103]. This cell to cell communication regulates overpopulation, nutritional competition and facilitates dissemination of old biofilm cells to establish infection at a distal site [104]. Therefore, biofilm cells are more resistant to eradication, and results in long lasting persistent infections [105].

**Transcription factors regulating biofilm formation**

There are six transcriptional factors regulating biofilm development in *C. albicans* of which Efg1 and Bcr1 are the most studied [103]. The biofilm structure in *C. albicans* is stabilized by hyphal development, which is controlled by a regulatory network of gene *BCR1* [106]. In addition, as *efg1* is the main regulator of morphogenesis and metabolism, it regulates biofilm formation and pathogenesis [107]. Many hypha-specific genes, such as *als1, als3* and *hwp1* are controlled by Bcr1 and Efg1 [108,109]. Therefore, *ber1, efg1* and genes under their control may provide new drug targets for developing antipathogenic drugs. One of the *ber1*-dependent genes is *eel1*, which is responsible for hypha induction in *C. albicans* [110]. Researchers have found that *eel1* regulates adhesion as it is indicated by the fact that overexpression of *eel1* restores biofilm formation in *ber1/ber1* mutant strains [108,109]. Therefore, it has become a perfect target for antifungal drug discovery.

**Antibiofilm**

Biofilm-associated infections of *Candida* cells can be treated in two ways; by inhibition of biofilm formation and eradication of preformed biofilms. To combat device-related biofilm infections, a strategy is required to restrict the dispersion of biofilm cells. As cells dispersed from the biofilms are responsible for dissemination, extravasation and establishment of invasive candidiasis [105]. The biofilm matrix can also be a target for development of antipathogenic drugs. Extracellular DNA is a key component of the *C. albicans* biofilm matrix and it has been discovered that a combination of DNase with some antifungal drugs improves their efficacies [111]. Since filamentation and biofilm development are coregulated in *C. albicans*, anti-morphogenetic drugs could also potentially retard the development of biofilms. Similar justification stands for the use of QS modulators [12]. However, the strategies may differ for the development of biomaterials resisting *C. albicans* biofilm growth, such as catheter coatings and lock solutions [112,113].

**Targeting biofilm development in *C. albicans***

Preformed biofilms are usually targeted through various approaches, depending on the nature of the host-candida interaction. However, in many cases, a strategy is adapted to inhibit the formation of biofilm by *Candida* cells [100]. Currently, to overcome the problem of biofilm-associated drug resistance, researchers have exploited calcineurin inhibitors such as cyclosporine A (CsA), tacrolimus (FK506) and heat shock protein 90 (Hsp90) inhibitors for example, geldanamycin [114,115].

Calcineurin is a Ca$^{2+}$-calmodulin-activated phosphatase that regulates intracellular calcium homeostasis; cell cycle progression; morphogenesis; mating and cytokinesis; recovery from pheromone arrest; cell wall biosynthesis; antifungal drug resistance; and pathogenesis [115,116]. Combinations of calcium and calcineurin inhibitors with known antifungal compounds have been shown to inhibit the growth of drug-resistant fungal strains [116–118]. Therefore, inhibition of calcineurin signaling is a novel antifungal strategy, that both attenuates fungal virulence and increases the efficacy of the existing antifungals with concomitant suppression of antifungal resistance [119]. Under *in vitro* and *in vivo* conditions, the planktonic and biofilm cells of *Candida* have been reported to be sensitive to the combination of fluconazole with FK506 or with CsA [115]. A study by Chen et al. [120] reported both *in vitro* and *in vivo* synergistic interaction of posaconazole with FK506, against drug-susceptible or resistant *C. albicans* strains. Moreover, Hsp90 inhibitors have exhibited potential synergistic antifungal activity in combination with azoles and echinocandins against the *C. albicans* [121].

Hsp90 is an essential and highly conserved molecular chaperone that facilitates folding, assembly and maturation of proteins in eukaryotes. It has demonstrated its role in biofilm formation and the evolution of drug resistance in *C. albicans* [114,122]. In *C. albicans*, Hsp90 regulatesazole resistance through its key downstream effectors, calcineurin and the Mkc1 kinase [123]. Hsp90 stabilizes calcineurin by direct interactions, therefore, the inhibition of Hsp90 is expected to result in depletion or inactivity of the client protein calcineurin [124]. Moreover, Hsp90 inhibitors such as geldanamycin and its derivatives, nongeldanamycin, have exhibited a potential synergistic antifungal activity in combination with azoles or echinocandins against the *C. albicans* [121,125]. It has been observed that compromising Hsp90 function in *C. albicans* resulted in the abrogation of resistance of biofilms to the azoles. The reduction of Hsp90 levels led to a marked decrease in matrix glucan levels, providing a compelling mechanism through
which, Hsp90 might regulate biofilm azole resistance. In a rat venous catheter infection model, the weakening of Hsp90 function either genetically or pharmacologically could transform ineffective fluconazole into highly effective drugs in eradicating biofilms [114]. Cowen et al. [126] demonstrated the benefit of combining the fluconazole with clinically relevant Hsp90 inhibitors that are structurally related to the natural product geldanamycin (GdA). Using the Galleria mellonella model for pathogenesis, they demonstrated synergistic effects between GdA and echinocandin against Aspergillus fumigatus. Whereas in a murine model of C. albicans disseminated candidiasis, genetical compromise with Hsp90 expression resulted in enhanced activity of fluconazole. Li et al. [125] explored the effect of non-Geldanamycin Hsp90 inhibitor molecule HSP90 on the activity of fluconazole against C. albicans. They observed that the efficacy of fluconazole against biofilm formation in vitro and in a murine model of disseminated candidiasis, is significantly enhanced when used in combination with HSP90. These combination therapies could provide less toxic treatment to the patient and more effective killing of pathogens in a wider range of Candida spp.

The high-throughput screening of small molecule compounds from different chemical libraries [127], along with the evaluation of a variety of natural products for anti-biofilm activity [128] could lead to the development of newer alternative therapeutics. To identify compounds inhibiting C. albicans biofilm, Pierce et al. [129] conducted a large-scale whole-cell assay screen of 20,000 small molecules from the research-intensive and medicinally relevant NOVACore chemical library (Chembright, CA, USA). They recognized a novel hit series of diazaspiro-decane structural analogs. Compound 61894700 was extensively characterized and displayed potent inhibitory activity against filamentation as well as biofilm in C. albicans.

In a study, a high-throughput microarray-based technology was utilized to investigate the biofilm formation in C. albicans. They used CaBChip (Candida albicans Biofilm Chip), comprised of 768 spatially discrete and equivalent nano-biofilms on a standard microscope glass slide. Although nanoscale biofilms were miniaturized as 2000-fold, CaBChip displayed phenotypic properties such as morphological, architectural and increased drug resistance analogous to biofilm cells formed using conventional 96-well microtiter plate. This automated nanobiofilm chip is easy to handle and is fully compatible with standard microarray technology and equipment [130]. Thus, the use of small-molecule compounds alone or in combination with existing antifungal drugs may provide a potential therapeutic strategy for fungal infectious disease. The use of such technology will encourage the antifungal drug discovery program since it will allow an inexpensive approach that is convenient and rapid in the screening of hundreds-to-thousands of compounds concurrently [12].

Transcription factors as unique antifungal drug targets

Transcription factors (TFs) are attractive as novel antifungal drug targets since they are evolutionarily divergent between fungi and humans and can be exploited as selective drug targets. Several natural or synthetic or peptidomimetic compounds have been identified that interfere with TFs which regulate many vital gene expressions. These compounds can inhibit hetero- or homodimerization of TFs, TF-binding DNA elements, DNA-binding domains of TFs, or the interaction between a TF and its essential modulating proteins [131]. The functional characterization of fungal TFs and their role in pathogenicity has become a demanding area of research work. Fungal TFs have been analyzed using functional genomic analyses at large-scale in C. albicans and C. neoformans. In this regard, Nobile and Mitchell [132] generated 83 TF mutants of C. albicans and addressed their roles in biofilm formation. Homann et al. have characterized in vitro functions of 166 TFs under 50 different growth conditions [133]. Furthermore, the comparative functional analysis of TFs in C. albicans and C. neoformans has provided an insight into the kinds of TFs being exploited as drug targets of broad or narrow spectrum action. The TFs Crz1, Nrg1, Rim101, Bcr1/Usv101, Zap1/Zap104 and Brg1/Gat201, are involved in virulence processes of both of these pathogens. Crz1 directs down-stream target that modulates ion homeostasis, pH response, thermo-tolerance, cell wall integrity, developmental processes and many other different virulence factors in C. albicans, C. neoformans and A. fumigatus [134].

Considering the industrial perspectives, broad-spectrum antifungals are commercially more rewarding. However, these drugs have associated potential hindrance to be developed as a successful candidate because such drugs disturb normal commensal microflora of the host and cause secondary infections of undesirable pathogens. In fact, C. albicans inhabit the gastrointestinal tract of humans as a normal microflora. Therefore, the advantage of this approach is that if the disease-causing specific fungal pathogen is determined in an early stage of infection, the pathogen-specific narrow-spectrum targets could be developed. Any drugs targeting such TFs would facilitate drug discovery to obtain more optimal drugs, reducing the toxic effects. In C. albicans, out of many narrow-spectrum
Virulence & biofilms as promising targets in developing antipathogenic drugs against candidiasis

TFs targeting agents. Efg1 is best characterized. Targeting of Efg1 also enhances the susceptibility of Candida to azole drugs [135,136].

**Metabolic pathways controlling virulence factors**

**Glyoxylate cycle**

The glyoxylate cycle is a modification of the tricarboxylic acid cycle, in which the steps for the production of CO2 are bypassed, resulting in carbons to be reserved as substrates for gluconeogenesis. In microorganisms this cycle is essential for the uptake and utilization of nonfermentable carbon sources, such as ethanol, acetate and fatty acids [137]. Since this metabolic pathway enables C. albicans to survive in a nutrient-restricted environment of the host, it is an essential virulence factor of C. albicans [137,138]. Isocitrate lyase (ICL) and maltate synthase are the key enzymes involved in the Glyoxylate cycle. During host infection, pathogenic microorganisms such as Aspergillus fumigatus, Magnaporthe grisea, Burkholderia pseudomallei, Mycobacterium tuberculosis and C. albicans cause upregulation of the glyoxylate cycle [139,140]. In particular, the icl gene of C. albicans is strongly activated when cells are exposed to macrophages [137]. Research has shown that disruption of icl gene rendered C. albicans unable to utilise acetate, ethanol or oleic acid. It has also been found that ICL is crucial for the survival of C. albicans in response to macrophage engulfment [137–139].

As this cycle does not exist in mammalian cells, ICL appears to be a prospective target for the development of antifungal drugs [127,138–140]. Several inhibitors of ICL, including 3-nitropropionate, 3-bromopyruvate, 3-phosphoglycerate, mycenon, oxalate and itaconate, have been identified [141]. However, most of these inhibitors are not pharmacologically suitable for use in vivo due to their toxicity and nonspecificity. It is expected that inhibitors of specific ICL show lower toxicity [140]. Thus, natural specific inhibitors of ICL derived from organisms have been sought as they may have many suitable pharmacological properties [142]. Bae et al. [143] isolated two compounds, mohangamide A and mohangamide B, from a marine actinomycete Streptomyces spp. that have shown specific inhibitory activity against the ICL of C. albicans. Further development of selective ICL inhibitors with suitable pharmacological properties would require more tests in animal models to establish both the importance of the glyoxylate cycle in C. albicans and the evidence for the therapeutic potential of ICL inhibitors in fungal infections [140].

**High osmolarity glycerol pathway**

Responding and adapting to different microenvironments within a host is advantageous for pathogens. The MAPK pathway is considered an important signal network in eukaryotes as it allows adaptation to changes in the environment. Four MAPK signalling pathways in C. albicans have been recognized as Mkc1, Cek1, Cek2 and the high osmolarity glycerol (HOG) pathway [144]. These pathways in C. albicans are exploited in the biogenesis of cell wall, morphogenesis and stress response [145,146]. Out of these four, the HOG pathway is the choice of the researcher to target in C. albicans [147]. It is comprised of a two-component system having a phosphorelay system and Hog1-type MAPK cascade. Most importantly, this two-component-system is conserved in organisms ranging from bacteria to higher plants, but not in mammals [144,147]. Therefore, it can be considered as a promising molecular target for developing new antifungal agents with lesser or no toxicity to the host [140].

**Target of Rapamycin signaling pathway**

The target of rapamycin (TOR) is a member of the phosphoinositide 3-kinase-related protein kinase family and regulates normal physiology and growth in eukaryotes [148,149]. Many studies have shown its contribution in regulating virulence in C. albicans [150,151]. The Tco89 sequence homolog, as a member of the TORC1 complex in some fungi and does not exist in mammals. Therefore, it could be a perfect antifungal target [152].

**Cell calcium homeostasis**

Calcium homeostasis in the fungal cell is required for survival, pathogenicity and is responsible for maintaining many closely associated physiological processes in C. albicans such as adhesion, hyphal development and stress response [153]. The calcium cell survival pathway in C. albicans facilitates the survival of cells in stress conditions of the environment. It elicits Ca2+ influx through the Cch1-Mid1 channel and triggers calcineurin and its downstream transcription factor Crz1p [154]. Calcineurin is a calcium-regulated signaling enzyme and needed by C. albicans to survive in the serum, therefore, it is also a promising virulence target [155]. Consequently, transcription factor Crz1p is now considered as a newly discovered calcineurin target in C. albicans [119].
Calmodulin is a small and universal Ca\(^{2+}\) binding protein that is highly conserved in all eukaryotes. It plays an important role in mediating calcium cell survival and response to other stressors in *C. albicans*. Thus, all these transcription factors including Cch1-Mid1 channel, calmodulin, calcineurin, Crz1p and the other components, which are involved in calcium homeostasis, could serve as potential drug targets for developing newer antifungals against *C. albicans* [140,119].

**QS molecules**

Farnesol, a lipid signaling molecule, regulates the invasiveness of hyphae in *C. albicans* by affecting the expression of genes involved in hyphal development [82]. Research has shown that farnesol produced *in situ* by planktonic *C. albicans* cultures prevented biofilm formation. Also, it has been found that the accumulation of farnesol blocks the morphological shift from yeast to hyphal form at high cell densities [101]. A study conducted by Decanis *et al.* [156] suggest that farnesol may reduce *Candida* pathogenesis through a downregulation of yeast-to-hypha morphogenesis, which may involve a modulation of SAPS gene expression. The study by Navarathna *et al.* [157] also supports the view of using farnesol as a new antifungal agent. Since *C. albicans* synthesizes farnesol from farnesyl pyrophosphate (FPP), and FPP is the biosynthetic precursor of both farnesol and ergosterol [158]. They hypothesized that drugs blocking the sterol biosynthetic pathway after FPP might lead to the accumulation of FPP, which, in turn, could lead to enhanced farnesol production. This hypothesis proved to be correct as they found that the treatment of mouse model of disseminated candidiasis with subinhibitory concentrations of fluconazole has resulted in a several-fold increase in production of farnesol by treated *Candida* cells compared with untreated cells. The increased production of farnesol inhibits hyphal morphogenesis in *Candida* cells and, it explains the mechanism of antimicrobial action of farnesol [101]. Therefore, farnesol could be considered as a potential candidate to develop antipathogenic antifungal drug against candidiasis.

**Antimetabolic pathways**

A lot of research has been conducted so far to target metabolic enzymes to develop anti-candidal agents. A study has revealed that tacrolimus (FK 506) and cyclosporin A isolated from a marine actinomycete also supports the view of using farnesol as a new antifungal agent. Since *C. albicans* synthesizes farnesol from farnesyl pyrophosphate (FPP), and FPP is the biosynthetic precursor of both farnesol and ergosterol [158]. They hypothesized that drugs blocking the sterol biosynthetic pathway after FPP might lead to the accumulation of FPP, which, in turn, could lead to enhanced farnesol production. This hypothesis proved to be correct as they found that the treatment of mouse model of disseminated candidiasis with subinhibitory concentrations of fluconazole has resulted in a several-fold increase in production of farnesol by treated *Candida* cells compared with untreated cells. The increased production of farnesol inhibits hyphal morphogenesis in *Candida* cells and, it explains the mechanism of antimicrobial action of farnesol [101]. Therefore, farnesol could be considered as a potential candidate to develop antipathogenic antifungal drug against candidiasis.

**Use of molecular approaches to identify new targets**

With the introduction of microbial genome sequencing of relevant pathogens, greater insights of virulence can be examined. It has allowed the conventional whole-cell-based approach to be replaced by novel target-based drug discovery [35]. Indeed, the availability of genome sequence data for *Candida* spp. has assisted researchers in recognizing novel and potential antifungal targets using bioinformatics approaches including mutant collection, transcript profiling and proteomics. However, the newly explored drug targets using bioinformatic approaches have not yielded any clinically valuable antifungal drugs [162]. Genomics has been proven beneficial in developing novel antifungal agents and new diagnostic tools in various ways, as depicted in Figure 2. The functional genomics is quite useful in serving as a functional tool to explain the emergence of drug-resistance and molecular mechanisms of drugs. The strategic point regarding genomic screens is that it delivers comparatively unbiased visions of the drugs affecting the fungal cell. Bioinformatics approaches have played a successful role in combination therapies; as an example, by predicting the use of one drug targeting a particular process in the fungal cell to be combined synergistically with the second drug inhibiting mechanisms of resistance to the first drug [162,163]. To identify and validate the antifungal drug targets, researchers mainly employ three genetics based molecular tools; gene expression profiling [164–166]; RNA mediated gene silencing [167–169]; and insertional mutagenesis [170–172].
An important approach is to analyze the gene expression profile at the level of transcriptome and proteome to assess the effects of the drugs. In *C. albicans*, transcript profiling has revealed the effects of ketoconazole interfering with the expression of genes involved in lipid, fatty acid and sterol metabolism and, caspofungin, influencing the expression of genes involved in cell wall biosynthesis [173,174]. A variety of RNA-mediated gene silencing or knockout methods have been identified such as the introduction of antisense RNA, double-stranded RNA (dsRNA) (also known as RNA interference or RNAi) and sense transgenes (also known as cosuppression in plants or quelling in fungi). These processes are generally termed as post-transcriptional gene silencing, and have proven their applications in the clinically important fungal human pathogen *C. albicans* [167]. In a study conducted by Disney et al. [169], it was found that an antisense DNA oligonucleotide mimicking rRNA was easily taken up by the cell and it could inhibit the growth of a *C. albicans* strain below pH 4.0. Therefore, it is expected that the use of oligonucleotide silencing methods could be a promising approach in antifungal drug discovery. On the other hand, for the creation of homozygous insertion mutants in the diploid *C. albicans*, a new gene disruption cassette, UAU1 marker cassette has been described [170]. The UAU1 marker cassette can be incorporated into a Tn7 transposon that allows the generation of random homozygous insertion mutants in *C. albicans* by *in vitro* transposition [171]. In general, the Tn7-UAU1 approach facilitates a large-scale first-pass assessment of the essentiality of genes and fast phenotypic (e.g., filamentous growth) screening for gene insertions of interest. Whereas, Haselbeck et al. [172] reported on a gene replacement and conditional expression method for genome-wide gene identification in *C. albicans*. This approach could also lead to the construction of fungal strains carrying gene fusions or promoter replacements. Additionally, nucleotide biosynthesis could be considered as a suitable antifungal drug target. Flucytosine as such is not active as an antifungal but once metabolized by the cell; it rapidly converts into 5-fluorouracil and blocks the synthesis of DNA and proteins by inhibiting cytosine deaminase, which is absent in human hosts [28]. Overall, the experimental power of genomics has significantly improved the diagnosis and therapeutics in antifungal drug discovery. More could be expected through the wider use of genome-wide profiling and mutant collections and in combination with well-targeted, clinically relevant molecular and cellular approaches.
Conclusion

So far, several virulence factors in Candida have been identified, but many more are yet to be discovered with the advancements of molecular approaches. Interestingly, these determinants are unique to fungi. In the antifungal drug discovery programme, the options of novel potential targets will be widened as more newer virulence factors will be discovered. As hydrolytic enzymes are responsible for increasing the pathogenicity in Candida spp., a greater effort should be made in understanding their roles in fungal pathogens to develop antipathogenic drugs. Moreover, because of its prime role during disease progression, the morphogenetic conversion in C. albicans has been most studied by researchers. However, the knowledge of filamentation and biofilm formation in C. albicans at the molecular level has been quite augmented, but until now it has not been possible to harness these targets for the development of new drugs for candidiasis. In search of filamentation and biofilms specific inhibitors, the accumulation of information along with the implementation of high-throughput screenings might provide much needed newer compounds to the antifungal armamentarium. Studies have yielded unique insights into the regulatory metabolic circuits and transcription factors that control different virulence functions associated with these processes and have led to the identification of small-molecule inhibitors. Indeed, an increasing number of small molecules are being discovered that can modulate morphogenetic conversions and prevent hyphal or biofilm development in C. albicans. Future investigations into precise targets of virulence processes as mentioned in this article are needed to understand the fundamental mechanisms of pathogenicity in C. albicans and to facilitate the antifungal drug discovery.

Future perspective

The viability of the antipathogenic drug development approach is due to the recent advancements in infection biology and bioinformatics. In the search for future antifungals, investigations of antivirulence compounds, target validation and preliminary screenings are being performed by various researchers to identify potential drug candidates. However, none of these compounds with the proposed newer mechanism of action have yet been introduced in clinical settings. It is still undecided if these approaches are or will be valuable to our expectations or not. We keenly await novel research in the next few years that could shed light on the performance of such antifungals, their ability to reduce resistance development and whether they could potentiate the efficacy of current antifungals in combination or would have the ability to make existing antifungals obsolete.

Executive summary

Currently available antifungal drugs are ineffective in controlling candidiasis:

- Despite the existing armamentarium of antifungal drugs such as azoles and amphotericin B, the rate of morbidity and mortality associated with candidiasis has been increasing.
- The emergence of drug resistance and host toxicity is a major hindrance to the success of these drugs.
- There is a need for alternative strategies to employ to discover newer antifungal agents:
  - Targeting virulence and biofilm formation in Candida could be a fruitful strategy to develop newer antifungal drugs.
  - Various virulence factors have been recognized in fungi, especially in C. albicans, such as the production of extracellular hydrolytic enzymes such as proteinase, phospholipase, hemolysin, adhesion, morphogenesis and biofilm formation.
- These virulence factors could be controlled by regulating their production at a molecular or metabolic level:
  - Several research efforts have envisaged the antipathogenic drugs that inhibit the production of these virulence factors.
  - Screening of small molecules using high-throughput sequencing has further led to the discovery of many compounds that target one or more virulence factors in fungi.
- The successful use of antipathogenic drugs has not yet been reported in clinical practice:
  - Ongoing research equipped with advancements of genomics and proteomics in studying host–pathogen relationships could reveal the success or failure of this strategy in the near future.

Acknowledgments

The authors acknowledge the support and co-operation received from department of Scientific Research at Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia.
Author contributions
The first author, MSA Khan, designed, developed and drafted the manuscript. All other authors critically reviewed the manuscript including the text, figures and tables, and provided significant comments.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Open access
This work is licensed under the Creative Commons Attribution 4.0 License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

References
Papers of special note have been highlighted as: ● of interest; ●● of considerable interest
1. Tong Y, Tang J. Candida albicans infection and intestinal immunity. Microbiol. Res. 198, 27–35 (2017).
2. Haynes KA, Westerneng TJ. Rapid identification of Candida albicans, C. glabrata, C. parapsilosis and C. krusei by species-specific PCR of large subunit ribosomal DNA. J. Med. Microbiol. 44(5), 390–396 (1996).
3. Pfaffer MA, Pappas PG, Wingard JR. Invasive fungal pathogens: current epidemiological trends. Clin. Infec. Dis. 43(Suppl. 1), S3–S14 (2006).
4. Lopez-Martinez R. Candidosis, a new challenge. Clin. Dermatol. 28(2), 178–184 (2010).
5. Sobel J. Vulvovaginal candidosis. Lancet 369(9577), 1961–1971 (2007).
6. Sardi J, Pitanguy NS, Gallo FP, Giannini A. A mini review of Candida species in hospital infection: epidemiology, virulence factor and drugs resistance and prophylaxis. Trop. Med. Surg. 1(5), 1–7 (2013).
7. Donders GGG, Sobel JD. Candida vulvovaginitis: a store with a buttery and a show window. Mycoses 60(2), 70–72 (2017).
8. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20(1), 133–163 (2007).
9. Bandara HMHN, Matsubara VH, Samaranayake LP. Future therapies targeted towards eliminating Candida biofilms and associated infections. Exp. Rev. Anti-Infect. Ther. 15(3), 299–318 (2017).
10. Roemer T, Krysan DJ. Antifungal drug development: challenges, unmet clinical needs, and new approaches. Cold Spr. Harb. Perspectives Med. 4(5), a019703 (2014).
11. Perfect JR. Is there an emerging need for new antifungals? Exp. Opin. Emerg. Drugs 21(2), 129–131 (2016).
12. Pierce CG, Lopez-Ribot JL. Candidiasis drug discovery and development: new approaches targeting virulence for discovering and identifying new drugs. Exp. Opin. Drug Disc. 8(9), 1117–1126 (2013).
13. Cui J, Ren B, Tong Y, Dai H, Zhang L. Synergistic combinations of antifungals and anti-virulence agents to fight against Candida albicans. Virulence 6(4), 362–371 (2015).
14. Clatworthy AE, Pierson E, Hung DT. Targeting virulence: a new paradigm for antimicrobial therapy. Nat. Chem. Biol. 3(9), 541–548 (2007).
15. Cowen LE. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. Nat. Rev. Microbiol. 6(3), 187–198 (2008).
16. Sanglard D, Coste A, Ferrari S. Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. FEMS Yeast Res. 9(7), 1029–1050 (2009).
17. Eliopoulos GM, Perea S, Patterson TF. Antifungal resistance in pathogenic fungi. Clin. Infect. Dis. 35(9), 1073–1080 (2002).
18. Odds FC, Brown AJ, Gow NA. Antifungal agents: mechanisms of action. Trends Microbiol. 11(6), 272–279 (2003).
19. Balkis MM, Leidich SD, Mukherjee PK, Ghannoum MA. Mechanisms of fungal resistance: an overview. Drug 62(7), 1025–1040 (2002).
20. Onyewu C, Heitman J. Unique applications of novel antifungal drug combinations. Anti-Infect. Agents Med. Chem. 6(1), 3–15 (2007).
21. Laniado-Labori R, Cabrales-Vargas M. Amphotericin B: side effects and toxicity. Rev. Iberoam. Micol. 26(4), 225–227 (2009).
References

22. Vermes A, Guchelaar HJ, Dankert J. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J. Antimicrob. Chemother. 46(2), 171–179 (2000).

23. Proia LA. New antifungal therapies. Clin. Microbiol. News 28(22), 169–173 (2006).

24. Groll AH, Kolve H. Antifungal agents: in vitro susceptibility testing, pharmacodynamics, and prospects for combination therapy. Eur. J. Clin. Microbiol. Infect. Dis. 23(4), 256–270 (2004).

25. Denning DW. Echinocandins: a new class of antifungal. J. Antimicrob. Chemother. 49(6), 889–891 (2002).

26. Sable CA, Strohmaier KM, Chodakewitz JA. Advances in antifungal therapy. Ann. Rev. Med. 59, 361–379 (2008).

27. Kanafani ZA, Perfect JR. Resistance to antifungal agents: mechanisms and clinical impact. Clin. Infec. Dis. 46(1), 120–128 (2008).

28. Lamping E, Monk BC, Niumi K et al. Characterization of three classes of membrane proteins involved in fungal aspartate resistance by functional hyperexpression in Saccharomyces cerevisiae. Eukaryot. Cell 6(7), 1150–1165 (2007).

29. Douglas LJ. Candida biofilms and their role in infection. Trends Microbiol. 11(1), 30–36 (2003).

30. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. Candida biofilms: an update. Eukaryot. Cell. 4(4), 633–638 (2005).

31. Vila T, Romo JA, Pierce CG, McHardy SF, Saville SP, Lopez-Ribot JL. Targeting Candida albicans filamentation for antifungal drug development. Virulence 8(2), 150–158 (2017).

32. Babazadeh-Qazijahani M, Badali H, Irannejad H, Afsarian MH, Emami S. Imidazolylchromanones containing non-benzylic oxime ethers: synthesis and molecular modeling study of new azole antifungals selective against Cryptococcus gattii. Eur. J. Med. Chem. 76, 264–273 (2014).

33. Hashemi SM, Badali H, Irannejad H, Shokrzadeh M, Emami S. Synthesis and biological evaluation of fluconazole analogs with triazole-modified scaffold as potent antifungal agents. Bioorg. Med. Chem. 23(7), 1481–1491 (2015).

34. Sangamwar A, Deshpande U, Pekamwar S. Antifungals: need to search for a new molecular target. Ind. J. Pharm. Sci. 70(4), 423–430 (2018).

35. Gauwerky K, Borelli C, Korting HC. Targeting virulence: a new paradigm for antifungals. Drug. Disc. Today 14(3), 214–222 (2009).

36. Casadevall A. Determinants of virulence in the pathogenic fungi. Fungal Biol. Rev. 21(4), 130–132 (2007).

37. Khan M, Ahmad I, Aqil F, Owais M, Shahid M, Musarrat J. Virulence and pathogenicity of fungal pathogens with special reference to Candida albicans. In: Combating Fungal Infections. Ahmas I, Owais M, Shahid M, Aqil F (Ed.), Springer, Heidelberg, Germany, 21–45 (2010).

38. Fakhim H, Vaezi A, Dannaoui E et al. Comparative virulence of Candida auris with Candida haemulonii, Candida glabrata and Candida albicans in a murine model. Mycoses 61(6), 377–382 (2018).

39. Park MDE, Jung WH. Lipolytic enzymes involved in the virulence of human pathogenic fungi. Mycobiology 41(2), 67–72 (2013).

40. Fotedar R, Al-Hedaihty SSA. Comparison of phospholipase and proteinase activity in Candida albicans and C. dubliniensis. Mycenes 48(1), 62–67 (2005).

41. Park M, Do E, Jung WH. Lipolytic enzymes involved in the virulence of human pathogenic fungi. Mycobiology 41(2), 67–72 (2013).

42. Ibrahim AS, Mirbod F, Filler SG et al. Evidence implicating phospholipase as a virulence factor of Candida albicans. Infect. Immun. 63(5), 1993–1998 (1995).

43. Ghamnoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13(1), 122–143 (2000).

44. YangYL. Virulence factors of Candida species. J. Microbiol. Immunol. Infect. 36(4), 223–228 (2003).

45. Theiss S, Ishdorj G, Brenot A et al. Inactivation of the phospholipase B gene PLB5 in wild-type Candida albicans reduces cell-associated phospholipase A2 activity and attenuates virulence. Int. J. Med. Microbiol. 296(6), 405–420 (2006).

46. Mattei AS, Alves SH, Severo CB, Guazzelli Lda S, Oliveira Fde M, Severo LC. Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of Candida albicans. Rev. Bras. Micr. Med. Trop. 46(3), 340–342 (2013).

47. Naglik JR, Challacombe SJ, Hube B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. 67(3), 400–428 (2003).

48. Monod M, Zepelin MB. Secreted proteinases and other virulence mechanisms of Candida albicans. Chem. Immunol. 81, 114–128 (2002).

49. Buu LM, Chen YC. Sap6, a secreted aspartyl proteinase, participates in maintenance of the cell surface integrity of Candida albicans. J. Biomed. Sci. 20(1), 101 (2013).

50. Watanabe T, Takan M, Murakami M et al. Characterization of a haemolytic factor from Candida albicans. Microbiology 145(3), 689–694 (1999).

51. Welch KD, Van Eden ME, Aust SD. Modification of ferritin during iron loading. Free Radic. Biol. Med. 31(8), 999–1006 (2001).
52. Surak R, Lesuisse E, Tachezy J, Richardson DR. Crusade for iron: iron uptake in unicellular eukaryotes and its significance for virulence. *Trends Microbiol.* 16(6), 261–268 (2008).
53. Almeida RS, Brunke S, Albrecht A *et al.* The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* 4(11), e1000217 (2008).
54. Wu HJ, Wang AH, Jennings MP. Discovery of virulence factors of pathogenic bacteria. *Curr. Opin. Chem. Biol.* 12(1), 93–101 (2008).
55. Richardson JP, Mogavero S, Moyes DI *et al.* Processing of *Candida albicans* Eec1p is critical for candidalysin maturation and fungal virulence. *mBio* 9, e02178–17 (2018).
56. Kasper L, Konig A, Koenig PA *et al.* The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes. *Nat. Commun.* 9(1), 4260 (2018).
57. Hoeg L, Thoma-Greber E, Rocken M, Korting HC. HIV protease inhibitors influence the prevalence of oral candidosis in HIV-infected patients: a 2-year study. *My coses* 41(7–8), 321–325 (1998).
58. Cauda R, Tacconelli E, Tumarello M *et al.* Role of protease inhibitors in preventing recurrent oral candidosis in patients with HIV infection: a prospective case–control study. *J. Acquir. Immune Defic. Syndr.* 21(1), 20–25 (1999).
59. Ollert MW, Wende C, Gerlich M *et al.* Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. *J. Clin. Micro biol.* 33(10), 2543–2549 (1995).
60. Ganendren R, Widmer F, Singhal V, Wilson C, Sorrell T, Wright L. *In vitro* antifungal activities of inhibitors of phospholipases from the fungal pathogen *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* 48(5), 1561–1569 (2004).
61. Barbier P, Schneider F. Syntheses of tetrahydrolipstatin and absolute configuration of tetrahydrolipstatin and lipstatin. *Helv. Chim. Acta* 70(1), 196–202 (1987).
62. Khedidja B, Abderrahman L. Selection of orlistat as a potential inhibitor for lipase from *Candida* species. *Bioinformatics* 7(3), 125–129 (2011).
63. Trofa D, Agovino M, Stehr F *et al.* Acetylsalicylic acid (aspirin) reduces damage to reconstituted human tissues infected with *Candida* species by inhibiting extracellular fungal lipases. *Microb. Infect.* 11(14), 1131–1139 (2009).
64. Gacser A, Schafer W, Nosanchuk JS, Salomon S, Nosanchuk JD. Virulence of *Candida parapsilosis*, *Candida orthobipolaris*, and *Candida metapsilosis* in reconstituted human tissue models. *Fungal Genet. Biol.* 44(12), 1336–1341 (2007).
65. Batinelli L, Daniele C, Cristiani M, Bisignano G, Saija A, Mazzanti G. *In vitro* antifungal and anti-elastase activity of some aliphatic aldehydes from *Olea europaea* L. fruit. *Phyto medicines* 13(8), 558–563 (2006).
66. Khan MSA, Ahmad I. *In vitro* influence of certain essential oils on germ tube formation, cell surface hydrophobicity, and production of protease and hemolysin in *Candida albicans*. *J. Nat. Pharm.* 3, 110–117 (2012).
67. Khan MSA, Ahmad I, Camoetra SS, Botha F. Sub-MICs of *Carum copticum* and *Thymus vulgaris* influence virulence factors and biofilm formation in *Candida* spp. *BMC Complement. Altern. Med.* 14, 337 (2014).
68. El Zawawy NA, Hafez SS. Efficacy of *Pluchea dioscoridis* leaf extract against pathogenic *Candida albicans*. *J. Infect. Dev. Ctries.* 11(4), 334–342 (2017).
69. Braun BR, Kadoch D, Johnson AD. NRG1, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *The EMBO J.* 20(17), 4753–4761 (2001).
70. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. Engineered control of cell morphology in *Candida albicans* during infection. *Fungal Genet. Biol.* 38(1), 1053–1060 (2003).
71. Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. *Trends Microbiol.* 9(7), 327–335 (2001).
72. Saville SP, Lazzell AL, Bryant AP *et al.* Inhibition of filamentation can be used to treat disseminated candidiasis. *Antimicrob. Agents Chemother.* 50(10), 3312–3316 (2006).
73. Cheng SC, Joosten LA, Kullberg BJ, Netea MG. Interplay between *Candida albicans* and the mammalian innate host defense. *Infect. Immun.* 80(4), 1304–1313 (2012).
74. Netea MG, Brown GD, Kullberg BJ *et al.* An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol.* 6(1), 67–78 (2008).
75. Sudbery PE. Growth of *Candida albicans* hyphae. *Nat. Rev. Microbiol.* 9(10), 737–748 (2011).
76. Cleary IA, Lazzell AL, Monteagudo C, Thomas DP, Saville SP, BRG1 and NRG1 form a novel feedback circuit regulating Candida *albicans* hypha formation and virulence. *Mol. Microbiol.* 85(3), 557–573 (2012).
77. Roche CR, Schroppel K, Harsuc D *et al.* Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell.* 12(11), 3631–3643 (2001).
78. Brown Jr DH, Giusani AD, Chen X, Kumamoto CA. Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique CZF1 gene. *Mol. Microbiol.* 34(4), 651–662 (1999).
79. Wang A, Raniga PP, Lane S, Lu Y, Liu H. Hyphal chain formation in *Candida albicans*; Cdc28-Hgc1 phosphorylation of Efg1 represses cell separation genes. *Mol. Cell Biol.* 29(16), 4406–4416 (2009).
80. Carlisle PL, Banerjee M, Lazzell A, Monteagudo C, Lopez-Ribot JL, Kadosh D. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. Proc. Natl Acad. Sci. USA 106(2), 599–604 (2009).

81. Joyner PM, Liu J, Zhang Z, Merritt J, Qi F, Cichewicz RH. Mutanobactin A from the human oral pathogen Streptococcus mutans is a cross-kingdom regulator of the yeast-mycelium transition. Org. Biomol. Chem. 8(24), 5486–5489 (2010).

82. Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA, Oliveira R. Morphogenesis control in Candida albicans and Candida dubliniensis through signaling molecules produced by planktonic and biofilm cells. Eukaryot. Cell. 6(12), 2429–2436 (2007).

83. Murzyn A, Krasowska A, Stefanowicz P, Działkowiak D, Łukaszewicz M. Capric acid secreted by S. bouardii inhibits C. albicans filamentous growth, adhesion and biofilm formation. PLoS ONE 5(8), e12050 (2010).

84. Zhang L, Chang W, Sun B, Groh M, Speicher A, Lou H. Bisbibenzyls, a new type of antifungal agent, inhibit morphogenesis switch and biofilm formation through upregulation of DPP3 in Candida albicans. PLoS ONE 6(12), e28953 (2011).

85. Nobile CJ, Fox EP, Nett JE. Small molecule inhibitors of the pathogenic yeast Candida albicans. J. Med. Microbiol. 58(6), 779–790 (2009).

86. Midkiff J, Borochoff-Porte N, White D, Johnson DI. Small molecule inhibitors of the Candida albicans budding-to-hyphal transition act through multiple signaling pathways. PLoS ONE 6(9), doi: 10.1371/journal.pone.0025395 (2011).

87. Fazly A, Jain C, Dehner AC et al. Chemical screening identifies flastatin, a small molecule inhibitor of Candida albicans adhesion, morphogenesis, and pathogenesis. Proc. Natl Acad. Sci. USA 110(33), 13594–13599 (2013).

88. Zhang JD, Xu Z, Cao YB et al. Antifungal activities and action mechanisms of compounds from Tribulusterrestris L. J. Ethnopharmacol. 103(1), 76–84 (2006).

89. Romo JA, Pierce CG, Chaturvedi AK et al. Development of anti-virulence approaches for candidiasis via a novel series of small-molecule inhibitors of Candida albicans filamentation. MBio. 8(6), doi: 10.1128/mBio.01991-17 (2017).

**Describe** and **characterizes a series of novel small-molecule compounds inhibiting C. albicans filamentation to identify candidate for the antipathogenic drug development against candidiasis.**

90. Romo JA, Pierce CG, Esqueda M, Hung CY, Saville SP, Lopez-Ribot JL. In vitro characterization of a biaryl amide anti-virulence compound targeting Candida albicans filamentation and biofilm formation. Front. Cell. Infect. Microbiol. 8, 227 (2018).

91. Pierce CG, Saville SP, Lopez-Ribot JL. High-content phenotypic screenings to identify inhibitors of Candida albicans biofilm formation and filamentation. Path. Dis. 70(3), 423–431 (2014).

92. Masuoka J, Hazen KC. Cell wall mannann and cell surface hydrophobicity in Candida albicans serotype A and B strains. Infect. Immun. 72(11), 6230–6236 (2004).

93. Hoyer LL. The ALS gene family of Candida albicans. Trends Microbiol. 9(4), 176–180 (2001).

94. Karkowska-Kuleta J, Rapala-Kozik M, Kozik A. Fungi pathogenic to humans: molecular bases of virulence of Candida albicans, Cryptococcus neoformans and Aspergillus fumigatus. Acta Biochim. Polon. 56(2), 211–224 (2009).

95. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. Curr. Opin. Microbiol. 9(6), 588–594 (2006).

96. Cavalcheiro M, Teixeira MC. Candida biofilms: threats, challenges, and promising strategies. Front. Med. (Lausanne). 5, 28 (2018).

97. Sandai D, Tabana YM, El Ouweini A, Ayodeji IO. Resistance of Candida albicans biofilms to drugs and the host immune system. Jundishapur J. Microbiol. 9(11), e37385 (2016).

98. Kopiec EM, Darouiche RO. Biofilm-related infectious diseases. Clin. Microbiol. Rev. 17(2), 255–267 (2004).

99. Hirot A, Kuroda H, Sapaar B, Matsuo T, Ichikawa T, Miyake Y. Pathogenic factors in Candida albicans infections of medical devices. Future Sci. OA 6(3), e1000828 (2010).

100. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. Crit. Rev. Microbiol. 35(4), 340–355 (2009).

101. Ramage G, Saville SP, Wickers BL, Lopez-Ribot JL. Inhibition of Candida albicans biofilm formation by farnesol, a quorum-sensing molecule. Appl. Environ. Microbiol. 68(11), 5459–5463 (2002).

102. Lopez-Ribot JL. Candida albicans biofilms: more than filamentation. Curr. Biol. 15(12), 1150–1155 (2005).

103. Nobile CJ, Fox EP, Nett JE et al. A recently evolved transcriptional network controls biofilm development in Candida albicans. Cell 148(1–2), 126–138 (2012).

104. Alem MA, Oteef MD, Flowers TH, Douglas LJ. Production of tyrosol by Candida albicans biofilms and its role in quorum sensing and biofilm development. Eukaryot. Cell 5(10), 1770–1779 (2006).

105. Uppuluri P, Chaturvedi AK, Srinivasan A et al. Dispersion as an important step in the Candida albicans biofilm developmental cycle. PLoS Path. 6(3), e1000828 (2010).

106. Fanning S, Xu W, Solis N, Woolford CA, Filler SG, Mitchell AP. Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. Eukaryot. Cell 11(7), 896–904 (2012).

107. Lassak T, Schneider E, Bussmann M et al. Target specificity of the Candida albicans Efg1 regulator. Mol. Microbiol. 82(3), 602–618 (2011).
108. Nobile CJ, Andes DR, Nett JE et al. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. *PLoS Path.* 2(7), e63 (2006).

109. Zavrel M, Majer O, Kuchler K, Rupp S. Transcription factor Efg1 shows a haplo insufficiency phenotype in modulating the cell wall architecture and immunogenicity of *Candida albicans*. *Eukaryot. Cell* 11(2), 129–140 (2012).

110. Birse CE, Irwin MY, Fonzi WA, Sypherd PS. Cloning and characterization of ECE1, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. *Infect. Immun.* 61(9), 3648–3655 (1993).

111. Martins M, Henriques M, Lopez-Ribot JL, Oliveira R. Addition of DNase improves the in vitro activity of antifungal drugs against *Candida albicans* biofilms. *Mycoses* 55(1), 80–85 (2012).

112. Cateau E, Rodier MH, Imbert C. *In vitro* efficacies of caspofungin or micafungin catheter lock solutions on *Candida albicans* biofilm growth. *J. Antimicrob. Chemother.* 62(1), 153–155 (2008).

113. Redding S, Bhattacharyya R, Siegel G, Scott K, Lopez-Ribot J. Inhibition of *Candida albicans* biofilm formation on denture material. *Oral Surg. Oral Med. Oral Path. Oral Radiol. Endod.* 107(5), 669–672 (2009).

114. Robbins N, Uppuluri P, Nett JE et al. Hsp90 governs dispersion and drug resistance of fungal biofilms. *PLoS Path.* 7(9), e1002257 (2011).

115. Uppuluri P, Nett J, Heitman J, Andes DR. Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* 52(3), 1127–1132 (2008).

116. Liu S, Yue L, Gu W, Li X, Zhang L, Sun S. Synergistic effect of fluconazole and calcium channel blockers against resistant *Candida albicans*. *PLoS ONE* 11(3), e0150859 (2016).

117. Lamoth F, Alexander BD, Juvvadi PR, Steinbach WJ. Antifungal activity of compounds targeting the Hsp90-calcineurin pathway against various mould species. *J. Antimicrob. Chemother.* 70(5), 1408–1411 (2015).

118. Liu S, Yue L, Gu W, Li X, Zhang L, Sun S. Synergistic effect of fluconazole and calcium channel blockers against resistant *Candida albicans*. *PLoS ONE* 11(3), e0150859 (2016).

119. Liu S, Hou Y, Liu W, Lu C, Wang W, Sun S. Components of the calcium–calcineurin signaling pathway in fungal cells and their potential as antifungal targets. *Eukaryot. Cell* 14(4), 324–334 (2015).

120. Chen YL, Lehman VN, Averette AF, Perfect JR, Heitman J. Posaconazole exhibits inhibitory of biofilm and filamentation. *Am. J. Transl. Res.* 7(12), 2589–2602 (2015).

121. Cowen LE, Singh SD, Kohler JR et al. Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proc. Natl. Acad. Sci.* 106(8), 2818–2823 (2009).

122. Sardia JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* 62(1), 10–24 (2013).

123. Khan MSA, Ahmad I. Antibiofilm activity of certain phytocompounds and their synergy with fluconazole against *C. albicans* biofilms. *PLoS ONE* 10(9), e0137947 (2015).

124. LaFayette SL, Collins C, Zaas AK et al. PKC signaling regulates drug resistance of the fungal pathogen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. *PLoS Path.* 6(8), e1001069 (2016).

125. Cowen LE. The fungal Achilles’ heel: targeting Hsp90 to cripple fungal pathogens. *Curr. Opin. Microbiol.* 16(4), 377–384 (2013).

126. Li L, An M, Shen H. The non-Geldanamycin Hsp90 inhibitors enhanced the antifungal activity of fluconazole. *Am. J. Transl. Res.* 7(12), 2589–2602 (2015).

127. Birse CE, Irwin MY, Fonzi WA, Sypherd PS. Cloning and characterization of a small molecule inhibitor of biofilm and filamentation. *npj Biofilms Microbiomes* 1(2015), doi: 10.1038/npjbiofilms.2015.12 (2015).

128. Juvvadi PR, Lee SC, Heitman J, Steinbach WJ. Antifungal activity of compounds targeting the Hsp90-calcineurin pathway comprised of Mkc1, calcineurin, and Hsp90. *PLoS Path.* 6(8), e1001069 (2016).

129. LaFayette SL, Collins C, Zaas AK et al. PKC signaling regulates drug resistance of the fungal pathogen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. *PLoS Path.* 6(8), e1001069 (2016).

130. Cowen LE, Singh SD, Kohler JR et al.Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *Proc. Natl. Acad. Sci.* 106(8), 2818–2823 (2009).

131. Sardia JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* 62(1), 10–24 (2013).

132. Khan MSA, Ahmad I. Antibiofilm activity of certain phytocompounds and their synergy with fluconazole against *C. albicans* biofilms. *PLoS ONE* 6(3), 618–621 (2012).

133. Pierce CG, Chaturvedi AK, Lazell AL et al. A novel small molecule inhibitor of biofilm formation, filamentation and virulence with low potential for the development of resistance. *NPJ Biofilms Microbiomes* 1, doi: 10.1038/npjbiofilms.2015.12 (2015). **Provides proof-of-concept for the implementation of antivirulence approaches against *C. albicans* by obtaining small molecule inhibitor of biofilm and filamentation.**

134. Srinivasan A, Uppuluri P, Lopez-Ribot J, Rama Subramanian AK. Development of a high-throughput *Candida albicans* biofilm chip. *PLoS ONE* 6(4), e19036 (2011).

135. Bahn YS. Exploiting fungal virulence-regulating transcription factors as novel antifungal drug targets. *PLoS Path.* 11(7), e1004936 (2015).

136. Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr. Biol.* 15(12), 1150–1155 (2005).

137. Homann OR, Dea J, Noble SM, Johnson AD. A phenotypic profile of the *Candida albicans* regulatory network. *PLoS Gen.* 5(12), e1000783 (2009).

138. Thewes S. Calcineurin-Crz1 signaling in lower eukaryotes. *Eukaryot. Cell* 13(6), 694–705 (2014).
135. Liu H. Co-regulation of pathogenesis with dimorphism and phenotypic switching in Candida albicans, a commensal and a pathogen. *Int. J. Med. Microbiol.* 292(5-6), 299–311 (2002).

136. Prasad T, Hameed S, Manoharal R et al. Morphogenic regulator EFG1 affects the drug susceptibilities of pathogenic Candida albicans. *FEMS Yeast Res.* 10(5), 587–596 (2010).

137. Dunn MF, Ramirez-Trujillo JA, Hernandez-Lucas I. Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* 155(10), 3166–3175 (2009).

138. Lorenz MC, Fink GR. Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot. Cell* 1(5), 657–662 (2002).

139. Kim H, Hwang JY, Shin J, Oh KB. Inhibitory effects of diketopiperazines from marine-derived *Streptomyces pumicicus* on the isocitrate lyase of *Candida albicans*. *Molecules*. 24(11), e2111 (2019).

140. Li X, Hou Y, Yue L, Liu S, Du J, Sun S. Potential targets for antifungal drug discovery based on growth and virulence in *Candida albicans*. *Antimicrob. Agents Chemother.* 59(10), 5885–5891 (2015).

141. Lee HS, Yoon KM, Han YR et al. Farnesol-type alkaloids, as Candida albicans isocitrate lyase inhibitors, from the tropical sponge *Hyrtio sp.* *Bioorg. Med. Chem. Lett.* 19(4), 1051–1053 (2009).

142. Cheah HL, Lim V, Sandai D. Inhibitors of the glyoxylate cycle enzyme ICL1 in *Candida albicans* for potential use as antifungal agents. *PLoS ONE* 9(4), e95951 (2014).

143. Bae M, Kim H, Moon K et al. Mohangamides A and B, new dilactone-tethered pseudo-dimeric peptides inhibiting *Candida albicans* isocitrate lyase. *Org. Lett.* 17(3), 712–715 (2015).

144. Roman E, Alonso-Monge R, Gong Q, Calderone R, Pla J. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen *Candida albicans*. *FEMS Yeast Res.* 9(6), 942–955 (2009).

145. Lawrence CL, Botting CH, Antrobos R, Coote PJ. Evidence of a new role for the high-osmolality glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. *Mol. Cell Biol.* 24(8), 3307–3323 (2004).

146. Sotoelo J, Rodriguez-Gabriel MA. Mitogen-activated protein kinase Hog1 is essential for the response to arsenite in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 5(10), 1826–1830 (2006).

147. Chauhan N, Calderone R. Two-component signal transduction proteins as potential drug targets in medically important fungi. *Infect. Immun.* 76(11), 4795–4803 (2008).

148. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell* 124(3), 471–484 (2006).

149. Neufeld TP, TOR regulation: sorting out the answers. *Cell Metab.* 5(1), 3–5 (2007).

150. Zacchi LF, Gomez-Raja J, Davis DA. Mds3 regulates morphogenesis in *Candida albicans* through the TOR pathway. *Mol. Cell Biol.* 30(14), 3695–3710 (2010).

151. Sullivan D, Moran GP. Differential virulence of *Candida albicans* and *C. dubliniensis*: a role for Tor1 kinase? *Virulence* 2(1), 77–81 (2011).

152. Betz C, Hall MN. Where is mTOR and what is it doing there? *J. Cell Biol.* 203(4), 563–574 (2013).

153. Yu Q, Wang H, Xu N et al. Spf1 strongly influences calcium homeostasis, hyphal development, biofilm formation and virulence in *Candida albicans*. *Microbiology* 158(9), 2272–2282 (2012).

154. Wang H, Lu G, Yang B et al. Effect of CCH1 or MIDI1 gene disruption on drug tolerance and pathogenesis of *Candida albicans*. *Sheng Wu Gong Cheng Xue Bao.* 28(6), 726–736 (2012).

155. Blankenship JR, Wormley FL, Boyce MK et al. Calcineurin is essential for *Candida albicans* survival in serum and virulence. *Eukaryot. Cell* 2(3), 422–430 (2003).

156. Decanis N, Tazi N, Correia A, Vilanova M, Rouabhia M. Farnesol, a fungal quorum-sensing molecule triggers Candida albicans morphological changes by downregulating the expression of different secreted aspartyl proteinase genes. *Open Microbiol. J.* 5, 119–126 (2011).

157. Navarathna DH, Hornby JM, Hoerrmann N et al. Enhanced pathogenicity of *Candida albicans* pre-treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. *J. Antimicrob. Chemother.* 56(6), 1156–1159 (2005).

158. Hornby JM, Kebara BW, Nickerson KW. Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. *Antimicrob. Agents Chemother.* 47(7), 2366–2369 (2003).

159. Hua Q, Kim HY, Liu Y et al. Crystal structure of calcineurin–cyclophilin–cyclosporin shows common but distinct recognition of immunophilin–drug complexes. *Proc. Natl Acad. Sci. USA* 99(19), 12037–12042 (2002).

160. Sato T, Ueno Y, Watanabe T, Mikami T, Matsumoto T. Role of Ca2+ concentrations of fluconazole in a mouse model of disseminated candidiasis. *Open Microbiol. J.* 5, 119–126 (2011).

161. Yu Q, Ding X, Xu N et al. *In vitro* activity of verapamil alone and in combination with fluconazole or tunicamycin against *Candida albicans* biofilms. *Int. J. Antimicrob. Agents.* 41(2), 179–182 (2013).

162. Weig M, Brown AJ. Genomics and the development of new diagnostics and anti-*Candida* drugs. *Trends Microbiol.* 15(7), 310–317 (2007).
163. De Backer MD, Van Dijck P. Progress in functional genomics approaches to antifungal drug target discovery. *Trends Microbiol.* 11(10), 470–478 (2003).

164. De Backer MD, Ilyina T, Ma XJ, Vandoninck S, Luyten WH, Vanden Bossche H. Genomic profiling of the response of *Candida albicans* to itraconazole treatment using a DNA microarray. *Antimicrob. Agents Chemother.* 45(6), 1660–1670 (2001a).

165. Nantel A, Dignard D, Bachewich C et al. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell.* 13(10), 3452–3465 (2002).

166. Rogers PD, Barker KS. Genome-wide expression profile analysis reveals coordinately regulated genes associated with stepwise acquisition of azole resistance in *Candida albicans* clinical isolates. *Antimicrob. Agents Chemother.* 47(4), 1220–1227 (2003).

167. De Backer MD, Nelissen B, Logghe M et al. An antisense-based functional genomics approach for identification of genes critical for growth of *Candida albicans*. *Nat. Biotechnol.* 19(3), 235–241 (2001b).

168. Cottrell TR, Doering TL. Silence of the strands: RNA interference in eukaryotic pathogens. *Trends Microbiol.* 11(1), 37–43 (2003).

169. Disney MD, Haidaris CG, Turner DH. Uptake and antifungal activity of oligonucleotides in *Candida albicans*. *Proc. Natl. Acad. Sci.* 100(4), 1530–1534 (2003).

170. Enloe B, Diamond A, Mitchell AP. A single-transformation gene function test in diploid *Candida albicans*. *J. Bacteriol.* 182(20), 5730–5736 (2000).

171. Davis DA, Bruno VM, Loza L, Filler SG, Mitchell AP. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* 162(4), 1573–1581 (2002).

172. Haselbeck R, Wall D, Jiang B et al. Comprehensive essential gene identification as a platform for novel anti-infective drug discovery. *Curr. Pharm. Des.* 8(13), 1155–1172 (2002).

173. Liu TT, Lee RE, Barker KS et al. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* 49(6), 2226–2236 (2005).

174. Prasad R, Pamwar SL, Krishnamurthy S. Drug resistance mechanisms of human pathogenic fungi. In: *Fungal pathogenesis* Marcel Dekker, Inc., NY, USA, 601–631 (2002).

175. Korting HC, Schaller M, Eder G, Hamm G, Böhmer U, Hube B. Effects of the human immunodeficiency virus (HIV) protease inhibitors saquinavir and indinavir on *in vitro* activities of secreted aspartyl proteinases of *Candida albicans* isolates from HIV-infected patients. *Antimicrob. Agents Chemother.* 43(8), 2038–2042 (1999).

176. De Bernardis F, Liu H, O’Mahony R et al. Human domain antibodies against virulence traits of *Candida albicans* inhibit fungus adherence to vaginal epithelium and protect against experimental vaginal candidiasis. *J. Infect. Dis.* 195(1), 149–157 (2007).

177. Ganendren R, Widmer F, Singhal V, Wilson C, Sorrell T, Wright L. *In vitro* antifungal activities of inhibitors of phospholipases from the fungal pathogen *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* 48(5), 1561–1569 (2004).

178. Jain T, Muktapuram PR, Sharma K et al. Biofilm inhibition and anti-*Candida* activity of a cationic lipo-benzamide molecule with twin-nonyl chain. *Bioorg. Med. Chem. Lett.* 28(10), 1776–1780 (2018).

179. Tscherner M, Giessen TW, Markey L, Kumamoto CA, Silver PA. A Synthetic system that senses *Candida albicans* and inhibits virulence factors. *ACS Synth. Biol.* 8(2), 434–444 (2019).

180. Takesako K, Kuroda H, Inoue T et al. Biological properties of aureobasidin A, a cyclic depsipeptide antifungal antibiotic. *J. Antibiot.* 46(9), 1414–1420 (1993).

181. Chen J, Young SM, Allen C et al. Identification of a small molecule yeast TORC1 inhibitor with a flow cytometry-based multiplex screen. *ACS Chem. Biol.* 7(4), 715–722 (2012).

182. Sun L, Liao K, Wang D. Effects of magnolol and honokiol on adhesion, yeast-hyphal transition, and formation of biofilm by *Candida albicans*. *PLoS ONE* 10(2), e0117095 (2015).