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REVIEW

Targeting *Candida albicans* filamentation for antifungal drug development

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ABSTRACT

Candida albicans remains the main etiological agent of candidiasis, as this otherwise normal commensal of humans is capable of causing active infection in immune- and medically-compromised patients. The high morbidity and mortality rates associated with candidiasis, coupled with the emergence of drug resistance demand the development of novel therapeutic strategies. However, there is a paucity of selective targets that can be exploited in the development of new antifungals. Contrary to conventional antibiotics that kill or curtail growth, specifically targeting virulence mechanisms represents an attractive option for antifungal drug development. In *C. albicans*, a growing body of research over the last few decades has provided important insights into its virulence factors and their contribution to the pathogenesis of candidiasis. Of these, filamentation is the one that has received the most attention and perhaps shows the most promise as a target for new anti-virulence strategies to combat *C. albicans* infections.

Infections caused by *Candida albicans* and current treatment options

Candida spp. are normal colonizers of the gastrointestinal and genitourinary tracts, as well as the skin of healthy humans and, as commensals, inflict little to no damage to the host.[1,2] Normal anatomical barriers and innate defense mechanisms (*i.e.* saliva in the oral cavity, neutrophils in the circulation) are normally sufficient to help maintain the commensal status.[3,4] However, when normal microbiota balance is disturbed or immunity is impaired, *Candida* overgrowth can lead to host damage and establishment of the opportunistic infection.[1,2] Over the last few decades, dramatic improvements in a variety of medically related procedures and therapies have led to a dramatic increase in survival of critically ill patients. However, these advances have also increased the population of immunocompromised patients, patients with hematologic disorders/malignancies, surgery, transplantation, patients in ICU and those undergoing anti-cancer and cytotoxic therapy, diabetes, hemodialysis, mechanical ventilation, parenteral nutrition, aging patients and recipients of artificial joints and prosthetic devices,

among others, as well as notably HIV/AIDS patients; whom are all vulnerable to invasive fungal infections.[5,6]

Among all pathogenic species of *Candida, C. albicans* remains the most common etiological agent of candidiasis and accounts for approximately 50% of bloodstream *Candida* isolates in the United States.[6,7] As a pathogen, *C. albicans* can cause a variety of infections that range from superficial to life-threatening invasive candidiasis.[6] Oropharyngeal candidiasis remains the most common oral manifestation in HIV patients, and is also frequent in head and neck cancer patients, as well as transiently in individuals treated with antibiotics or corticosteroids.[8-10] Additionally, 3 out of 4 women are likely to experience at least one event of vulvovaginal candidiasis (VVC) over the course of their lifetime, and ∼5% of the female population suffers from recurrent infections (RVVC).[11] Invasive candidiasis affects more than 250,000 people worldwide every year and it is estimated to cause over 50,000 deaths.[12] Deep-seated infections may remain localized or lead to secondary candidemia (*i.e.*, dissemination through the bloodstream). Candidemia may occur after disruption of mucosal integrity after surgery

or by direct inoculation through medical devices such as catheters, with circulating yeasts able to colonize and infect virtually any internal organ of the host leading to invasive disease.[6,13] Importantly, candidemia now ranks as the third most common cause of health care-associated bloodstream infection (BSI) and is the leading cause of BSIs in the intensive care unit (ICU),[7] with incidence rates of 2–14 cases per 100,000 persons.[12]

Treatment of candidiasis, from superficial to invasive infections, relies on a limited drug arsenal, composed of 3 major classes of antifungal drugs: polyenes, azoles and echinocandins.[14,15] However, this arsenal is compromised by problems of selectivity, toxicity, and the development of resistance. The polyenes were the first class of antifungal drugs introduced into the clinic (in the1950s) and include amphotericin B and nystatin. These amphipathic molecules bind to the ergosterol in fungal cell membranes and form pores, leading to leakage of intracellular constituents and impaired protein traffic through the membrane.[14,16] Amphotericin B also exists as a large extramembranous aggregate that acts as a fungicidal sponge that kills yeast by simply binding and extracting ergosterol.[17,18] Despite great fungicidal activity against *Candida*, amphotericin B use is limited by its associated hepatotoxicity and nephrotoxicity,[14] especially in severely ill ICU patients and those undergoing hemodialysis, which actually corresponds to an important risk group for the development of invasive candidiasis. More recently, two lipid-based formulations of amphotericin B, lipid complex (ABLC) and liposomal amphotericin B, with reduced toxicity and improved pharmacokinetics, were introduced for clinical use; however they are significantly more expensive than conventional amphotericin B and not widely used.[19] Triazoles, including both first generation (fluconazole and itraconazole) and second generation (voriconazole and posaconazole), comprise the most commonly used class of antifungals. The introduction of triazoles in clinics during the 1980s and 1990s revolutionized medical mycology [19] and until the introduction of the echinocandins fluconazole was the drug of choice in the treatment of most *C. albicans* infections. All triazoles are inhibitors of C14α-lanosterol demethylase, a key enzyme involved in the biosynthesis of ergosterol; thus disturbing the integrity of the fungal cell membranes.[14] Because fluconazole is readily absorbed, has good biodistribution and the greatest penetration into the cerebrospinal fluid and vitreous humor, it is the treatment of choice for several invasive forms of candidiasis, including cystitis, ocular and central nervous system dissemination.[7] Unfortunately, a major problem with fluconazole is the emergence of resistance (including cross-resistance against multiple azole derivatives), mostly through the development of point mutations in the target enzyme or by overexpression of specific efflux pumps.[20] The newest class of antifungals available in the clinic is the echinocandins, released in early 2000. Echinocandins, including anidulafungin, micafungin and caspofungin, inhibit the synthesis of β-1,3 glucan, a major polysaccharide of the fungal cell wall and, therefore, is the only antifungal class to target an exclusive fungal component.[14] Echinocandins are the most selective and least toxic antifungal drugs and, following regulatory approval, their use in both prophylaxis and treatment has grown substantially. As a consequence of increased drug exposure, resistance has emerged and been linked to indiscriminate echinocandin use.[21] Acquired echinocandin resistance has now been reported in single isolates belonging to most *Candida* species, including *C. albicans*,[21] with the main mechanism of resistance identified as mutations in the *FKS1* gene encoding the target enzyme, glucan synthase.[22,23]

Targeting virulence represents an attractive new approach for antifungal drug development

As mentioned above, since fungi are eukaryotic, the development of antifungal agents is complicated by the limited number of selective targets that can be exploited for drug development, leading to the exceedingly short arsenal of drugs. Moreover, the antifungal pipeline is sparse and there are few new drugs in sight.[19,24] Irrespective of their targets and mechanism of action, conventional antifungal drugs act by inhibiting growth or killing the fungal cells; in either case they pose a high degree of selective pressure which is ultimately responsible for the emergence of resistance. An attractive alternative is to target virulence factors that are specific to *C. albicans*. For the purpose of this review, we define virulence as the ability of the fungus to cause active disease, and virulence factor as a *C. albicans* component or process that actively participates in causing damage to host tissues or promotes infection.[25,26] In essence, such an anti-virulence approach will "disarm" *C. albicans* from its capacity to cause infection, thereby preventing the transition to the pathogenic state or reverting it back to harmless commensal status. Of course, development of such anti-virulence approaches requires specific knowledge of *C. albicans* pathogenicity, which is complex and multifactorial in nature and, as an opportunistic pathogen, depends on a delicate balance between virulence attributes and host responses.[3] Fortunately, this has been an area of intensive research during the last few decades, leading to the identification of multiple factors and mechanisms that represent major contributors to the pathogenic potential of this fungus, as recently reviewed.[27] Thus, the time is now right to try to take advantage of all the information accumulated during these years and to apply this accrued knowledge to more translational endeavors for the development of novel antifungal therapeutics.

The concept of anti-virulence therapy emerged in the bacterial field mainly as a potential solution to overcome antibiotic drug resistance, and has gained traction in the last few years, having been fully embraced as a desirable alternative that is increasingly being explored and exploited in the development of novel classes of antibacterials.[28,29] However, the same feeling is not true in the case of antifungal drug development, which is still dominated by a somewhat unrealistic expectation that any new antifungal must have a broad spectrum of action and be able to kill or inhibit the growth of most, if not all, pathogenic fungi. In stark contrast, and by definition, anti-virulence strategies are effective against a much more limited range of microorganisms (only those that share the same virulence factor) and thereby display a narrow spectrum of action.[29]

There are many different advantages of targeting virulence as opposed to cell viability and some of these may be particularly appealing in combating fungal infections. Perhaps most importantly, in the case of antifungals, due to the paucity of selective targets in the eukaryotic fungal cell, the identification of virulence factors can substantially expand the number of potential targets that can be exploited for antifungal drug development and, in the process, lead to entirely new classes of drugs with new mechanisms of action. Moreover, because of its narrow spectrum of action, and anti-virulence approach should not alter the natural host's microbiota, and this could be of critical importance in the case of normal commensals such as *C. albicans*. Finally, stripping fungal cells of their virulence without threatening their existence should exert a much reduced selection pressure for drug resistance mutations, which has been a major problem in *C. albicans*, particularly with respect to the azole derivatives.

Targeting filamentation for antifungal drug development in *C. albicans*

C. albicans filamentation and its role in virulence

C. albicans is polymorphic and capable of undergoing reversible morphological transitions between single-cell ellipsoid budding yeast and different filamentous forms, including pseudohyphae (chains of elongated cells with constrictions at the septa) and true hyphae (which posses parallel walls and grow by continuous apical extension and septation).[30-32] These morphogenetic conversions greatly contribute to disease, and although both yeast and filaments are normally observed during infection, they play distinct functions with yeast cells participating in early phases of the infectious process (*i.e.* adhesion and dissemination) and filamentous forms responsible for invasion and damage to tissues and organs, leading to pathology and potentially

death.[33-35] Thus, because of its predominant role during the disease process, of all putative *C. albicans* virulence factors, without any question filamentation is the one that has received the most attention and has been the focus of a majority of the research conducted on this fungus. Early experiments revealed that genetically defined mutant strains locked in the yeast morphology, and therefore unable to filament, were avirulent in a murine model of *C. albicans* invasive infection.[36] Subsequent studies using regulatable strains in which morphogenetic conversions could be controlled both *in vitro* and *in vivo* provided compelling evidence for the role of filamentation in the progression to active infection,[35,37] as well as genetic validation that filamentation could be targeted in the development of new antifungal agents.[38] Furthermore, although filamentation is coordinately regulated with other virulence factors [39-41], the observation that virulence of the Δ*hgc1* mutant (see below) is heavily attenuated in the murine model of invasive candidiasis provides further credence to the notion that filamentation *per se* constitutes a major virulence factor.[42] Although a systematic screen of a collection of *C. albicans* deletion strains pointed to a lack of correlation between filamentation and virulence,[43] we note that the authors looked only at infectivity (not active disease), and we and others have shown that yeast cells can extravasate and infect target organs without necessarily lead to mortality.[13,35] Figure 1 shows extensive filamentation of *C. albicans* cells associated with characteristic invasive kidney lesions in infected mice with hematogenously disseminated candidiasis.

Control of *C. albicans filamentation by multiple signaling pathways*

Filamentation can be triggered by a multitude of stimuli that act through multiple complex signal transduction pathways, and readers are referred to excellent, detailed
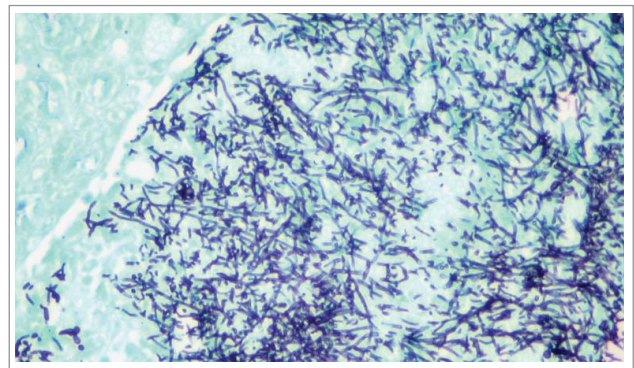
Figure 1. *C. albicans* filamentation in kidney tissues of infected mice. Fungal elements are visualized in kidney tissues from mice with invasive candidiasis by using the Grocott-Gomori methenamine-silver (GMS) stain.

and in depth reviews on this topic.[44,45] Signals able to stimulate filamentation include neutral pH, 5% CO_2, body temperature (37°C), serum, nutrient availability as well as embedded and microaerophilic conditions. Certain human hormones such as estrogen and progesterone are also known to stimulate filamentation and biofilm formation leading to vulvovaginal candidiasis (VVC).[46] The ability of C. albicans to respond to a multitude of signals allows its adaptation to the environmental conditions presented by the host. The filamentation process in C. albicans is tightly regulated, while simultaneously being highly dynamic, occurring under a wide range of environmental conditions.[2,45,47] The intricate regulation of hyphal initiation and extension is further complicated by the convergence of multiple signaling pathways on the same or different transcription factors, as well as several transcription factors converging on similar targets. Filamentation is subject to both negative and positive regulation. Negative regulation is carried out by Tup1 acting in combination with Nrg1 or Rfg1, or by Rbf1 alone.[45] Indeed, modulation of NRG1 using a tetracycline regulatable promoter has revealed that C. albicans cells overexpressing NRG1 grow exclusively as yeast under any hypha-enducing condition.[35] Furthermore, deletion strains of C. albicans lacking TUP1, NRG1 or both are locked into a filamentous form.[48,49] Positive regulators of filamentation include Efg1, Cph1, Tec1, Czf1, Hgc1, Ume6, Brg1, and Rim101.[45,50-53] These positive regulators are able to respond to signals transmitted though different filamentation pathways or are able to further relay the signal from multiple pathways. Efg1 is responsible for inducing filamentation in response to pathways stimulated by N-Acetyl Glucosamine (GlcNac), pH (by interacting with Rim101), CO_2 (through the activation of the cAMP cascade stimulated by Cyr1),[54] as well as serum and appears to be the principal filamentation regulator under most environmental conditions.[45,48] Hyphal induction through Cph1 requires a mitogen-activated protein kinase (MAPK) pathway, which can be activated by low nitrogen levels. Moreover, both the cAMP and MAPK cascades are stimulated by Ras1, which further displays the complexity of the signaling circuitry.[45] Czf1 stimulates filamentation via a signaling pathway involving Dck1 and is triggered by embedded and hypoxic conditions.[55] Hgc1 is known to form a complex with Cdc28 and together they are able to induce hyphal growth by phosphorylating Efg1, leading to the repression of the cell separation machinery [56] and it has been proposed that Ume6 drives filamentation through this Hgc1 pathway.[50] The GATA-family transcription factor Brg1 has also been shown to play a crucial role in filamentation.[51] An overexpression strain of BRG1 was shown to filament under yeast growth conditions (YPD at 30°C) with a concomitant upregulation of hypha-specific genes such as ALS3, HWP1, and ECE1.[51]

Stimulation of the signaling transduction pathways described above leads to the induction of hypha-specific genes. Among these, ALS3, ECE1, and HWP1 which have recently been identified as part of a core filamentation response (CFR) network composed of 8 genes.[57] This network also includes HGT2, IHD1, RBT1, DCK1, and the open reading frame orf19.2457, whose function is unknown. These CFR members were only upregulated in the early and late filamentation responses and not during the initial stage of germ tube formation.[57] Using different media conditions, individual filamentation pathways can be stimulated and studied. Media such as Lee's is able to induce filamentation through the Cph2 and Tec1 pathway, Spider medium, which is carbon limiting, will induce filamentation through the cAMP pathway, while GlcNAc mediium is able to induce filamentation through Efg1.[44]

Filamentation modulates host immune responses during C. albicans infection

Cells of the immune system as well as epithelial and endothelial cells sense and respond to C. albicans yeast and filamentous cells in different ways, ultimately evoking distinct immune responses that can either help in the resolution of infection or contribute to pathology.[3] Perhaps the best example of this is how mucosal epithelial cells are capable of discriminating between yeast cells and hyphae, with the former stimulating protective responses and the latter resulting in an overly exacerbated inflammatory response, which contributes to the pathology of both vaginal and oral candidiasis.[58,59] Therefore, one additional advantage of an anti-virulence treatment that acts by inhibiting C. albicans filamentation would be the ensuing modulation of host immune responses in a manner that favors the host in fighting the infection. As such, under certain circumstances an anti-virulence strategy can be somewhat comparable to the use of live attenuated vaccines to induce protective immune responses.

The search for small molecule compounds with inhibitory activity against C. albicans filamentation

Although historically numerous molecules have been reported to exert an inhibitory effect on the C. albicans yeast to hypha transition, very few of these compounds were tested for their effects on virulence in vivo,[60] and there were also some potential concerns about their overall toxicity. Thus, to date it has been difficult to translate these basic science discoveries into new therapies to combat C. albicans infections. In the last few years several groups have implemented more targeted efforts

using high content phenotypic screens for the identification of small molecule inhibitors of *C. albicans* filamentation.

Screening for compounds that could inhibit the *C. albicans* yeast to hyphae transition in response to carbon limitation in Spider media, Toenjes *et al.* identified 5 novel small molecules and 16 molecules with known mammalian targets.[61,62] These known compounds were inhibitors of protein kinases, protein phosphatases, Ras signaling pathways, G protein-coupled receptors, calcium homeostasis, nitric oxide and guanylate cyclase signaling, and apoptosis in mammalian cells. Interestingly several of these molecules were also capable of inhibiting filamentation in other *Candida* species and *Aspergillus fumigatus*, suggesting a somewhat broad spectrum of action. Since the original screen used the carbon-limiting hyphal-inducing signal of Spider media, it was implied that the identified compounds inhibited *C. albicans* filamentation by blocking the Efg1 pathway, although subsequent evaluation of their activity in different hyphal-inducing media indicated that some of the inhibitors may act through multiple signaling pathways.[63] The group also performed chemical epistasis analyses to provide further insights into mechanism of action and potential molecular targets.[63] However, none of these inhibitors were tested in infection models.

A high throughput phenotypic screening of 30,000 small molecules from a commercial library (DIVERset, Chembridge) in search for compounds that prevent adhesion of *C. albicans* to polystyrene surfaces identified several bioactive molecules.[64] Besides attachment to plastic, several compounds also inhibited adhesion to epithelial cells, *C. albicans* morphogenesis and biofilm formation. The main leading compound was termed "filastatin" (see Fig. 2 for structure) based on its strong and long-lasting inhibition of filamentation.[64] Filastatin blocks the transcriptional induction of the hyphal-specific *HWP1* promoter and subsequent experiments indicated that filastatin acts downstream of multiple (but not all) signaling pathways; in particular filastatin blocks hyphal formation induced by serum, Spider media, and GlcNac, but not by the genotoxic stress agent hydroxyurea. Of note, filastatin exhibited antifungal activity in a nematode model of *C. albicans* infection and also in an *ex vivo* mouse model of vulvovaginal candidiasis.[64]

A cell-based phenotypic screen was performed using 3 different chemical libraries from the National Cancer Institute's Open Chemical Repository collection (Natural set, Structural Diversity set, and Challenge set), which identified several compounds with inhibitory activity against *C. albicans* filamentation.[65] To screen for filamentation the authors took advantage of the tight control of morphogenetic conversions in the genetically
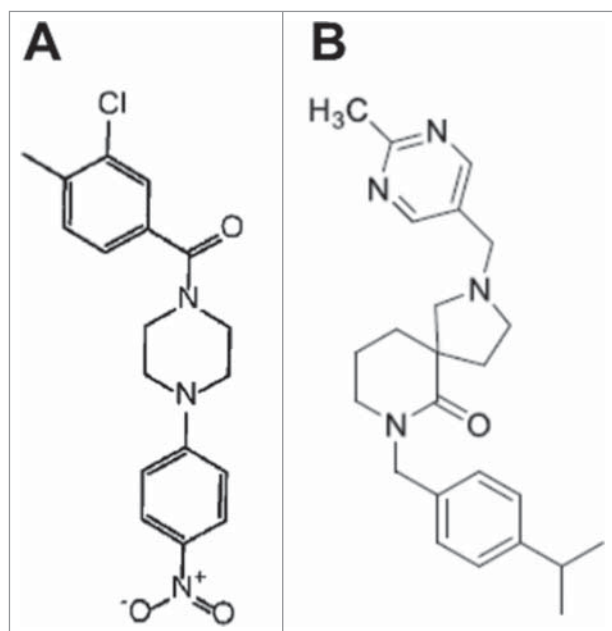
Figure 2. Chemical structures of filastatin (A) and compound 61894700 (B).

engineered *C. albicans* *tet-NRG1* strain,[35] in order to develop an easy, inexpensive and robust screen for inhibitors of filamentation using a 96 well microtiter plate-based assay. Of the 2,293 compounds screened, there were a total of 17 confirmed hits identified as inhibitors of filamentation in *C. albicans*. The antibiotic activity of several of these compounds had already been previously described, but the screen also identified a few novel compounds. Nevertheless, most of the compounds identified as hits were cytotoxic, which severely limits their potential as candidates for the development of new antifungal drugs. Because of this reason, their *in vivo* efficacy was not further examined.

Most recently, Pierce *et al.* performed a large-scale, whole-cell assay screen of 20,000 small molecules from the research-intensive and medicinally relevant NOVACore™ chemical library (Chembridge) to identify compounds that inhibit *C. albicans* biofilm formation.[66] The screen identified a novel hit series of diazaspiro-decane structural analogs, which was largely represented among the bioactive compounds and which is predicted to possess very favorable "drug-like" physical chemical properties. Not surprisingly, since filamentation plays a pivotal role in biofilm formation in this pathogenic fungus, an in depth characterization of the leading compound from this series (compound 61894700, Fig. 2) indicated that it also displays a potent inhibitory activity of *C. albicans* filamentation, and this activity was observed at relatively low concentrations at which the compound did not inhibit overall growth under planktonic conditions.[66] Therefore, in contrast to conventional

antifungals that target cell viability, this leading candidate seems to represent a true anti-virulence compound. Of note, serial passage experiments *in vitro* indicated that repeated exposure to the lead compound did not lead to the development of resistance, thereby confirming that this anti-virulence strategy is highly unlikely to foster the emergence of resistance.[66] The anti-filamentation activity was retained in the presence of serum, which is essential from a drug development point of view. Also, the toxicity values of this leading compound determined in a standard assay using human hepatocytes were considerably higher than the effective concentrations against filamentation, presumably indicating a good safety profile. Thus, based on its promising *in vitro* characteristics a series of experiments were performed to assess its activity *in vivo*. Results of these experiments indicated that the leading compound exhibits activity in clinically-relevant murine models of both invasive and oral candidiasis and, consistent with its effect on fungal morphology and its anti-virulence mode of action, the efficacy of the treatment was associated with inhibition of filamentation *in vivo* as assessed by histological observations.[66] In the invasive model, characteristic filamentous lesions were predominant in kidneys (the main target organ) from untreated mice, whereas isolated or small groups of mostly yeast cells were observed in kidney sections retrieved from mice treated with the leading compound. Similarly in the oral model, the tongues from untreated mice demonstrated a widespread biofilm and extensive lesions with numerous hyphae penetrating and causing damage to the epithelium, as opposed to superficially located scattered yeast cells in tongues from mice treated with this compound.[66] Importantly, the model of oral candidiasis uses immunosuppressed mice;[67,68] thereby these results would suggest that an anti-virulence strategy could potentially be used to treat certain forms of candidiasis, even in immune deficient patients.

Conclusions and outlook

Infections caused by *C. albicans* continue to represent a major challenge to an expanding population of at-risk patients. These infections carry unacceptably high morbidity and mortality rates, which clearly points to major limitations in our current antifungal armamentarium. However, the development of novel antifungal drugs is difficult due to the scarcity of specific fungal targets. The increased knowledge on *C. albicans* pathogenesis acquired through years of arduous work by multiple groups of investigators, particularly in the last 10–15 y after the completion of the initial *C. albicans* genome project, provides for an unprecedented opportunity and framework for the discovery and development of novel

antifungal drugs. Thus, two main questions remain. First, will we be able to harness this knowledge and apply these insights from *C. albicans* biology and pathogenicity in order to develop a new approach for the treatment of these infections? And second, when will this investment pay off for antifungal drug discovery?

These anti-virulence therapeutics will shift the advantage to the host by effectively devoiding this fungus of its pathogenic potential, and they will be less likely to foster the emergence of resistance, thereby expanding the number of potential targets and constituting a new paradigm for the development of drugs with novel modes of action. Another of the major advantages of an anti-virulence strategy is the maintenance of the microbiome and the normal yeast ecology, as suppression of *C. albicans* populations due to antifungal treatment contributes to the changing epidemiology of candidiasis, with the substitution of *C. albicans* by species that are intrinsically less susceptible to conventional antifungals (*i.e., C. krusei, C. glabrata*). Thus, even in the absence of direct "activity" against non-*albicans* species, it is entirely possible that the preservation of a commensal state with *C. albicans* while under treatment with an anti-filamentation compound will suppress proliferation of these other *Candida* species. An important caveat is that the successful implementation of anti-virulence approaches will also require the rapid and accurate diagnosis of *C. albicans* infections, which also remains a major challenge. Taken together, as the best studied virulence factor in *C. albicans*, filamentation represents an attractive target, already validated at the genetic level, for the development of anti-virulence approaches against candidiasis. This accumulated information, together with the implementation of high throughput/high content screenings in search for specific inhibitors of *C. albicans* filamentation, stand poised to deliver much needed novel compounds to the antifungal development pipeline.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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