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Small molecule based anti-virulence approaches against *Candida albicans* infections

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Abstract

Fungi are considered “silent killers” due to the difficulty of, and delays in diagnosis of infections and lack of effective antifungals. This challenge is compounded by the fact that being eukaryotes, fungi share several similarities with human cellular targets, creating obstacles to drug discovery. *Candida albicans*, a ubiquitous microbe in the human body is well known for its role as an opportunistic pathogen in immunosuppressed people. Significantly, *C. albicans* is resistant to all the three classes of antifungals that are currently clinically available. Over the past few years, a paradigm shift has been recommended in the management of *C. albicans* infections, wherein anti-virulence strategies are considered an alternative to discovery of new antifungals. Small molecules, with a molecular weight less than 900 Daltons, can easily permeate the cell membrane and modulate the signal transduction pathways to elicit desired virulence inhibitory actions against pathogens. This review dissects in depth, the discoveries that have been made with small molecule anti-virulence approaches to tackle *C. albicans* infections.
In recent years, tackling nosocomial and opportunistic infections has gained marked attention in the scientific community, due to the continued emergence of multi-drug resistant pathogens\textsuperscript{1,2}. Prominently, it has been estimated by the World Health Organization that approximately 10 million people will die due to antibiotic resistance by 2050\textsuperscript{3}. Hence, a synergistic approach involving several specialties, including microbiology, biotechnology, pharmacology, medicinal chemistry, bioinformatics, and materials science has been taken to discover innovative problems to this solution. While most such research focuses on bacterial infections, a kingdom that is often less addressed, and yet notorious, are fungi.

The yeast \textit{Candida albicans} exists as a commensal in the human microbiome in several anatomical sites such as the oral cavity, gastrointestinal tract, skin, and genital organs\textsuperscript{4,5}. The unique phenotypic plasticity of \textit{C. albicans} facilitates its survival in a variety of environmental conditions with changes in pH, temperature, and oxygen levels\textsuperscript{6}. Furthermore, the indiscriminate use of antibiotics results in the depletion of commensal bacteria from these niches, allowing the proliferation of yeasts. Immunosuppressed patients, including those with HIV, diabetes, those on chronic steroid therapy, cancer chemotherapy, infants in the neonatal ICU, elderly, and hospitalized patients are all at high risk of developing opportunistic fungal infections, which may range from superficial (mucocutaneous) to life-threatening invasive fungemia\textsuperscript{7}. Over 500 million people in the world suffer from candidiasis\textsuperscript{8} with an increasing mortality rate of 5 to 71\%\textsuperscript{9}.

\textit{Candida} species use several strategies for their existence in the host as a normal commensal, but when the equilibrium tilts in favor of the yeasts\textsuperscript{6}, they express several virulence factors including a morphological switch from yeast to fungi\textsuperscript{7,10}, adhesion, invasion, secretion of hydrolytic enzymes, thigmotropism, phenotypic switching, and biofilm formation\textsuperscript{11–13}. There are currently four classes of clinically used antifungal drugs for treating the \textit{C. albicans} infections such as polyenes, azoles, echinocandins and pyrimidine analogues\textsuperscript{14,15}. Polyenes bind to cell membrane ergosterol and cause cell lysis\textsuperscript{16}. Due to increased toxicity, their clinical use is limited. Azoles act by inhibiting the mechanism of ergosterol biosynthetic pathway, but increased emergence of resistance, particularly the development of point mutations in the target site of azoles or by the expression of efflux pumps in the \textit{C. albicans} hampers its use in treating fungal infections\textsuperscript{17,18}. Echinocandins, the most recent of the antifungals, acts by inhibiting 1,3-beta glucans synthase\textsuperscript{19} and help in cell wall biosynthesis inhibition, but mutation in the glucan synthase gene \textit{FKS1} has become a significant problem\textsuperscript{20}. Pyrimidine analogue, 5-flucytosine is
converted to 5-flurouracil by cytosine deaminase FCY1, which then inhibits the cellular division of C. albicans upon incorporation of the toxic fluorinated pyrimidine analogue into DNA and RNA. Emergence of resistance due to the overexpression of efflux pumps and mutation in the cytosine deaminase FCY1 gene has hindered its application\textsuperscript{21} (Figure 1).

\textit{Candida} species that are resistant to every clinical class of antifungal available today is a reality and will continue to be a threat and a major healthcare burden, unless investments are made in antifungal drug discovery\textsuperscript{21,22}. However, as stated above antifungal drug discovery is challenging since fungi share many common metabolic pathways with the host \textsuperscript{14,23}. In some pathogenic species such as \textit{Candida}, the mechanisms that drive the development of antifungal resistance are intimately linked to the same mechanisms that regulate growth and survival. Therefore, anti-virulence strategies, where the key virulence aspects of \textit{Candida} are inhibited, without affecting growth/survival is considered an agreeable alternative to discover new antimycotics\textsuperscript{24}.

\textbf{What are small molecules?}

With a molecular weight less than 900 Daltons, some molecules can easily permeate the cell membrane and modulate the signal transduction pathways. These properties make small molecules an ideal candidate for the development of drugs that control virulence in pathogenic species\textsuperscript{25}. They can potentially target virulence mechanisms of fungi, which harbors interesting therapeutic properties. Moreover, they go hand in hand with the current therapeutic course of antifungal drug development\textsuperscript{26} and can also be seen as a remedy for the issues caused by the existing antifungals.

In this review, we will review the progress made in the past decade on the discovery of small molecule inhibitors against \textit{C. albicans}. Over this decade a wide range of drug discovery approaches have been used by the researchers for the anti-virulence therapy. In particular the approaches for small molecule discovery include the i) use of existing drugs/pharmacologically active compounds (drug repurposing), ii) screening of large library, chemically diverse compounds (high throughput screening), iii) compounds derived from other organisms (e.g., plant/animal/microbe derived), iv) compounds with known targets i.e., compounds influencing a specific phenotype of candida (e.g., Secreted aspartyl protease inhibitors, quorum sensing inhibitors). The small molecules discussed here are categorized based on the approaches that are used to discover/develop them for their anti-virulence effects.
Old is gold: Repurposing drugs:

Drug discovery is a tedious and inefficient process, considering the need for identification of novel targets, safety issues, and the substantial associated costs. Therefore, drugs that have been approved for other applications in humans (and therefore have a proven safety profile) can be a quick route to the discovery of new antimicrobials. Drug repurposing has been developed as an expedited approach for developing new therapies in many areas of the clinical research. This type of the search i.e., identifying the drug for repurposing against a new disease happens using either drug based phenotypic screening approach or using the high throughput library of FDA approved drugs. Drug based phenotypic screening identifies successful molecules by serendipitous or clinical observations without knowing about the specific biological targets.

It has been estimated that 10-16 years are required for the development of a drug using the traditional approach while drug repurposing takes only 3-12 years, significantly reducing the time and costs. Also, since the pharmacokinetic (PK), pharmacodynamic (PD), and toxicity profile of drugs are already available, drug repurposing reduces the risk of failure of compounds in clinical development making repurposing an ideal route for bench-to-bedside translation of antifungal drugs. The schematic illustration (Figure 2) highlights the advantages of pursuing drug repurposing over traditional drug discovery methods.

Quinacrine (QNC), an anti-malarial drug was found to be effective against the growth of Saccharomyces cerevisiae and Cryptococcus neoformans. Following this, Kulkarny et al., 2014 explored the effect of QNC on C. albicans biofilms and filamentation. Interestingly, QNC inhibited C. albicans biofilms at 256 µg/ml and prevented yeast-to-hyphal transition even in echinocandin-resistant clinical isolates in neutral and alkaline pH by causing defects in endocytosis by accumulating in the vacuoles of C. albicans. However, in acidic pH, no effects were observed due to the protonation of QNC, which prevented vacuolar accumulation. QNC also disrupted the mature C. albicans biofilms by synergizing with sublethal doses of amphotericin B and caspofungin. Finasteride, a drug used for treating benign prostatic hyperplasia was found to have anti-biofilm and anti-filamentation effects against C. albicans without affecting its growth. However, finasteride is broken down to inactive metabolites prior to excretion and thus, the oral administration of finasteride for the treatment of urinary candidiasis has limited use. An alternative approach would be to deliver it as a topical formulation at the site of infection. Iron acquisition plays a major role in C. albicans pathogenesis and adaptation to the different host niches. Deferasirox, an FDA approved iron chelator reduced the invasion of C.
albicans in oral epithelial cells and significantly reduced fungal burdens in a murine model of oropharyngeal candidiasis.  

The acetylcholine receptor agonist pilocarpine hydrochloride (PHCL), an anti-glaucoma drug modulates the uncharacterized cholinergic receptor of C. albicans causing anti-biofilm and anti-filamentation effects. PHCL also reduced the pathogenesis of C. albicans in a Galleria mellonella infection model. Azoles have belonged to the antifungal armamentarium for the last four to five decades and yet only in 2019, an azole compound aripiprazole, which is used to treat schizophrenia, was identified to have an antibiofilm and the anti-filamentation effect against C. albicans. Aripiprazole significantly inhibited 90% of C. albicans biofilms at 50 µg/ml and abolished the hyphal formation at 100 µg/ml. However, in a high dose (250 µg/ml) they inhibited the growth of the C. albicans possibly by acting as an enzyme inhibitor thus inhibiting the lanosterol 14α-demethylase enzyme in the sterol biosynthetic process. As noted, aripiprazole has found to have concentration dependent effects and a similar mechanism to the other existingazole compounds, for which several clinical strains of Candida sp., were found to demonstrate resistance. 

Combinatorial therapy of FDA-approved non-antifungal drugs and antifungal drugs have also been shown to be effective against C. albicans biofilms. For instance, sub-inhibitory concentrations of the off-patent drugs drospirenone (contraceptive), perhexiline maleate (antianginal agent), and toremifene citrate (anticancer agent) were found to be “enhancers” by increasing the activity of antifungal drugs amphotericin B and caspofungin synergistically (FICI<0.5) against C. albicans biofilms both in vitro and in vivo (Caenorhabditis elegans) without affecting the growth. A group of sulfa antibacterial compounds has reversed the azole-resistant strains, likely by inhibiting the folate pathway and not by inhibiting the efflux pumps, which also mediate the development of resistance in C. albicans. Even though drug repurposing involves low risks, costs and time compared to the de novo development, one of the major limitations in developing them for anti-virulence therapy is appropriate dosing regimens. The ultimate goal is attainable only if the effective concentration of the drug for the new therapy falls in the same range as those of the original therapy. But if the efficacy of the drug is seen at an increased dosage compared to that of recommended doses specified in the original PK profile, adverse side effects or toxicities might occur. Despite this, such a compound might provide a new lead chemical scaffold for new drug development. Some of the
compounds that have been discovered using the lead optimization approach for the anti-virulence therapy against *C. albicans* have been discussed here.

**Lead optimization approach**

The emerging and the promising repurposing methods for the identification of novel anti-virulent small molecules, uses FDA approved drugs as a starting point/a scaffold for the development of the derivatives which will exhibit the drug-like properties and have known pharmacological profiles. This approach is termed as selective optimization of side activities (SOSA)\(^{36}\). Montoya MC *et al.*, used the SOSA approach for the development of the antimalarial drug, mefloquine (MEF) and its novel entities as antifungals. Sublethal concentrations of the drugs inhibited the filamentation of the *C. albicans*. MEF has a long half-life and maintains the high serum levels when administered orally making it as an ideal candidate for the treatment of disseminated candidiasis\(^{37}\). Haloperidol, an antipsychotic drug was found to have antifungal effects and has been used a scaffold/lead compound for the development of the novel compounds using the scaffold hopping approach\(^{38}\). Several benzocyclane derivatives were designed and optimised using haloperidol as a lead compound and tested for the anti-virulence properties. The lead derivative named B10 were found to inhibit the morphological transition and biofilm formation in FLC-resistant isolates\(^{39}\). Such novel chemical scaffold will require further testing of the pharmacokinetic and pharmacodynamic as well as safety profiles.

**High-throughput screening (HTS)**

As discussed earlier, the antifungal pipeline is sparse due to the limited number of selective drug targets that can be explored against *C. albicans* infections due to its eukaryotic nature. Thus, targeting the key virulence aspects of *C. albicans* i.e., biofilm formation and filamentation, without affecting growth/survival would be an excellent alternative option\(^{40,41}\). There are several small molecules with “drug-like properties” that have been identified through HTS to be exploited as a therapeutic option against *C. albicans* infections\(^{42}\). In HTS, large scale compound libraries can be screened rapidly in a cost-effective way, which leads to the identification of novel hit/lead compounds and desirable drug-like properties targeting the anti-virulence approach of *C. albicans* (Table 1).

*C. albicans* biofilm adhesion, development, and maturation have been illustrated in the figure 3. Once the yeast/planktonic form adhere to a substrate (adherence), they activate the multiple core regulators of the filamentation pathway and undergo morphological transition to elongated hyphal form (initiation and development). Then, these hyphal form cells elongate and proliferate...
further forming a mature biofilm (biofilm maturation) (Figure 3). The compounds that have been screened using the high-throughput approach target either one of these stages or all three stages and are discussed here.

To thwart the adhesion of *C. albicans* to abiotic surfaces such as polystyrene, over 30,000 small molecules were screened from DIVERSet library, Chembridge database. This led to the identification of antifungal compound that was christened filastatin, which showed remarkable ability to prevent or reverse the adhesion of *C. albicans* to polystyrene surfaces, silicone elastomers and human lung cells by affecting the transcriptional activation of *HWP1* (Hyphal wall protein) promoter which in turn plays a major role in initial hyphal formation. Filastatin also prevented the formation of hyphae by affecting multiple signaling pathways depending on the type of hyphae-inducing media: spider medium (cAMP/PKA pathway), serum (Ras1 pathway), and GlcNAc medium (cph1, Efg1 pathway) which has direct influence on hyphal morphology. Moreover, it reduced pathogenesis in both nematode and a mouse model of vulvo-vaginal candidiasis. As an important translational extension of their study, Fazly et al., have also shown that filastatin prevents the adhesion of *C. albicans* to several biomaterials such as dental resins, silicone elastomers and bioactive glasses. Due to the above mentioned properties, filastatin appears to be an ideal candidate for biofilm-inhibitory coatings on prosthesis and medical devices.

Filamentation is considered a high-value target, as it is a major virulence factor in *C. albicans*. The yeast-to-hyphal switch facilitates colonization, biofilm formation, invasion, and inflammation. Pierce *et al.*, screened over 20,000 small molecules from the NOVACore library (Chembridge) with “stringent drug like properties” and identified that diazaspiro-decane structural analogs (Table 1) influenced both *C. albicans* biofilm formation and filamentation, without affecting growth. Notably, it reduced virulence in mouse models of oral candidiasis and hematogenously disseminated candidiasis. They also identified that *C. albicans* reference strain (SC5314) and a clinical isolates from HIV*+* individuals could not develop resistance to the selected “lead” compound.

Similarly, Romo *et al.* screened 20,000 small molecules from the DIVERSet library, Chembridge and discovered two biaryl-amide derivatives (Table 1) that were able to inhibit *C. albicans* filamentation at a very low micromolar concentration. Notably, these compounds arrested hyphal development in most of the hyphae inducing signaling pathways including Efg1 (GlcNac), cAMP-PKA (spider), Cph2 and Tec1 (Lee’s media), pH induced (RPMI-1640), Hog1 (DTPA induced), hydroxyurea induced filamentation and also reduced virulence in mouse
models of candidiasis\textsuperscript{40,46}. Interestingly these compounds exhibited anti-biofilm efficacy against \textit{C. albicans} in the proliferation and maturation phases but not in the adhesion phase. Global transcriptomic studies on one of these lead molecules showed significant downregulation of all the core filamentation network genes\textsuperscript{47}.

Lohse \textit{et al.} screened over 30,000 small molecules from the Chembridge small molecule diversity library and found that compound CB17 ((1-\{2-(2-methylphenoxy)-3-pyridinyl\}-N-(3-pyridinylmethyl)methanamine) inhibited biofilm adhesion and disrupted the mature biofilms but didn’t inhibit the biofilm development\textsuperscript{48}. This study clearly demonstrated the need for testing the effect of the compounds on different stages of biofilm formation, avoiding the risk of overlooking compounds based on only one assessment criteria that may eventually lead to the loss of promising compounds \textsuperscript{49}.

\textbf{Compounds screened via HTS have synergistic interaction with existing antifungals}

The failure of conventional antifungals to eliminate/prevent biofilm development and virulence, the need of high dosage of these antifungals to eliminate infections, and the resulting toxicities and drug resistance, make combinatorial therapy an attractive strategy to explore. Drug combinations will rule out the “single-drug single-target” concept, which helps in reducing the required drug dosage, reducing toxicity, and potentially increases the efficacy of treatment due to multiple targets\textsuperscript{50}. For instance, the antifungal compound clotrimazole does not reduce the metabolic activity of \textit{C. albicans} biofilms, but when combined with the library of compounds (~120,000) screened from Molecular Libraries Small Molecule Repository (MLSMR), 14 compounds were found to have anti-biofilm effects without exerting cytotoxicity. But the resistance development assay and the \textit{in vivo} efficacy of the compound has not been studied. Further studies on these clotrimazole potentiators needs to be validated for development into topical application for candidiasis and vaginitis\textsuperscript{51}.

Vila \textit{et al.} screened the Pathogen Box chemical library (Medicines for Malaria Venture [MMV], Switzerland) to identify inhibitors of \textit{C. albicans} biofilm formation and identified six compounds\textsuperscript{52}. Interestingly, out of these six, three compounds were able to reduce the metabolic activity of preformed biofilms. The compound that had more potent antibiofilm activity was 2-Methyl-3-\{4-methyl-1-piperazinyl\}(2-thienyl)methyl]-1-Hindole (MMV688768) (Table \textbf{2}). Although this compound had less activity against planktonic cells, its activity against preformed biofilms was found to be profound. Hence, the authors safely concluded that this compound targets a process (or processes) that plays a preponderant role in the survival of \textit{C. albicans} cells within a
biofilm compared to their planktonic counterparts, but the targeted processes need to be explored further. Moreover, *in vitro* assays against preformed biofilms proved that the combination of this compound with fluconazole resulted in 89% reduction in cell viability, which was significantly greater than the either drug alone. Thus indicating synergy and confirming the potential for a combinatory approach using MMV688768 and fluconazole in the clinical setting\textsuperscript{52}.

*Mitochondrial and vacuole inhibitors screened via HTS*

The fungal mitochondria are an attractive target for anti-virulence therapy; however, several *in vitro* and *in vivo* studies should be conducted showing little or no harmful effects against human mitochondria. Garcia et al., screened about 678 small molecules that attenuated invasive hyphal growth in *C. albicans*, by affecting various filamentation pathways. The lead compound halogenated salicylanilide (HAS), tri-chloro-salicylanilide and its analog niclosamide, (Table 3) an FDA-approved anti-helminthic drug was found to have anti-filamentation and anti-biofilm effects. Using transcriptomic analyses, they concluded that this effect is possibly due to binding of HAS to Mge1 (a mitochondrial import complex)\textsuperscript{53}.

Screening a total of 50,240 small molecules, one study identified inhibitors of yeast-to-hyphal transition. Small molecule 21 (SM-21) the lead compound (Table 3), inhibited hyphal morphogenesis and biofilm adhesion to denture surfaces. It also showed fungal specificity, relatively low toxicity to human oral keratinocytes, reduced tongue lesions in oral and improved the survival rate in systemic candidiasis mouse models\textsuperscript{54}. Wong et al., unraveled the mechanism of action for SM-21 using *C. albicans* haploid strains, showing that the compound acts on mitochondria by inhibiting ATP synthesis machinery specifically on fungal cytochrome and ATPases which affects the ATP production and increases the reactive oxygen species (ROS) production leading to cell death. As it affects growth, *C. albicans* haploid strains developed resistance to SM-21 and the resistant strains shown increased SAP activity\textsuperscript{55}. The use of the haploid strains helps in uncovering both mechanism of action and *C. albicans*’ resistance mechanism against the small molecule, making this haploid model ideal for drug development.

On the other hand, Vacuole Disrupting chemical Agents (VDA) were identified from a chemical library, to targeting a particular role/function in *C. albicans* that is important for virulence. *C. albicans* vacuoles play a predominant role in yeast-to-hyphae transition and vacuole deficient *C. albicans* mutants are unable to tolerate stresses induced by host cells\textsuperscript{56}. Thus, *Candida* vacuoles mediate its survival within the host cells and the morphological transition which
facilitates invasion. Tournu et al., screened for VDAs from three libraries such as Prestwick Phytochemical Library, Prestwick Chemical Library, GreenPharma Natural Products Library. Significantly, a wide range of compounds such as azoles, ionophore, statin and non-steroidal anti-inflammatory drugs (NSAIDs) specifically cyclooxygenase inhibitors (Table 3) were identified to have this property of vacuole disruption. These molecules caused physiological stress to the fungal cells and inhibited their hyphal formation, one of the main virulence characteristics.

**Repurposing drugs screened via HTS**

The advantages of drug repurposing have been described in the previous sections. The bis-biguanide alexidine dihydrochloride (AXD) was found to have potent antifungal, antihyphal and antibiofilm activities against all the fungal pathogens tested including *C. albicans* reference strain SC5314 as well asazole-resistant clinical isolates. In combination with existing antifungals, AXD reduced the MIC of fluconazole and amphotericin B, clinically used first-line antifungal drugs, ironically considered expendable for biofilm treatment, thereby emphasizing their utility as an antibiofilm combination drugs. Furthermore, AXD displayed low toxicity to mammalian cells and eradicated biofilms from mice central venous catheters, highlighting its potential as a pan-antifungal drug, a drug that is effective against several fungal strains and pathogens.

Piperazine-type phenothiazine derivatives, trifluoperazine (TFP, dopamine receptor agonist) and CGS 1266B (serotonin 1β receptor agonist) have been shown to affect *C. albicans* hyphal morphogenesis and biofilm formation by affecting the proteins of the fluid-endocytosis pathway and vesicular transport (Vacuolar ATPase) without affecting cell growth. The compound TFP and CGS1266B (Table 4) particularly affected Rcy1, Vps15 proteins they are essential for membrane protein recycling by endocytosis and protein transport to vacuole respectively.

Silesset et al. screened a small molecule library consisting of 1,200 off-patent drugs of the Prestwick Chemical Library and they identified auranofin (antirheumatic), benzbrumaron (vasodilatory), pyrvinium pamoate (antiparasitic effects) drugs having novel anti-biofilm effects (Table 4). These 3 novel antibiofilm drugs inhibited and prevented the biofilms of *C. albicans*.

Similarly, Lohse et al. screened Pharmakon 1600 repurposing library found that, nisoldipine and nimodipine (a calcium channel inhibitor), paroxetine hydrochloride (serotonin uptake inhibitor), and dexlansoprazole (a proton pump inhibitor) had significantly affected biofilm adhesion, development and disrupted mature biofilms of *C. albicans* without affect the planktonic form.
Another fascinating approach in HTS is the use of animal models for screening potential antifungal drugs\textsuperscript{62}. Specifically, the similarity in the virulence factors involved in the infection of several fungal pathogens in the \textit{C. elegans} model is similar to virulence factors during mammalian infection, the use of \textit{C. elegans} for screening anti-microbial small molecules has been a widely explored approach. Although some compounds could be potentially missed due to their nematocidal activity, we cannot ignore the fact that this is an exciting approach to identify molecules that would affect, modulate or even enhance the immune responses in the host\textsuperscript{62}. Over 80,000 repurposing drugs were screened using these \textit{C. elegans} animal model for its antibacterial effects and among one of the hit compounds BAY11-7085, an anti-infective drug was further investigated for its effects against \textit{C. albicans} biofilms\textsuperscript{63}. Notable, BAY11-7085 had significant effect on all the phases of biofilms (adhesion, development, and maturation) and increased the survival rate of \textit{C. albicans} infected \textit{C. elegans} model. But the mechanism of action of the BAY11 7085 for its effect on both bacteria and the fungi needs to be elucidated further\textsuperscript{64}.

It is important to apply adequate screening methods to small-molecule compound libraries because appropriate selection procedures are the key to successful screening. The HTS procedure is more advantageous for screening the molecules that are already available in the market which are known to have pharmacological effect and desirable drug like properties. Additionally, the main effect of these small molecules on human cells are already known and described in the database of the manufacture. Therefore, it may be easier to translate these compounds to the clinic as they are expected to have fewer side effects. Even though several groups have identified anti-virulence compounds which affect multiple targets/pathways through high-throughput screening in the past decade, none of them have yet been translated to clinical application. The major factors for this failure are poorly validated drug targets which causes undesirable interactions with the host system, not considering physiological condition in preliminary screening, a limited follow up, lack of using appropriate animal models, and unpredictable toxicities. Once the hits/leads are identified by primary screening, chemical synthesis approaches can be used to develop several analogues which may have superior effects than the original scaffold.
Plant – derived small molecules

Plant-derived bioactive natural products are known to be source of several medicinal products and many molecules have been applied in therapeutic applications\textsuperscript{65,66}. They are considered as a “gold mine” in drug development, especially antibiotics owing to their minimal toxic effects and increased efficacy\textsuperscript{67}. Many researchers have explored the anti-candida activity of plant-derived bioactive molecules which have also been reviewed elsewhere\textsuperscript{65,67,68}. One of the major advantages of plant derived bioactive molecules is that they have enormous scaffold diversity and structural complexity. This natural product reservoir is enriched with unexplored bioactive molecules which gives the researchers the broader area of chemical scaffolds compared with the small molecule libraries. Initially, the bioactive molecules are screened by performing biological screening for the “crude extracts” to identify the bioactive “lead” extract, which then fractionated using several bioactivity guided fractionation/mass-spectrometry techniques to isolate the bioactive molecules. The bioactive molecules, based on their structure are classified further into terpenoids, alkaloids and flavonoids (Table 5, Figure 4). These specifically exert anti-virulence effects by interacting with the specific downstream regulatory pathways of candida that control hyphal morphogenesis, biofilm development and maturation and are discussed here in this section.

\textbf{a) Terpenoids}

Hinokitol (\(\beta\)-thujaplicin) isolated from \textit{Chamacyparis taiwanesis} has been reported to possess a wide range of antifungal properties. It has been shown to inhibit biofilm formation of both fluconazole-susceptible and resistant strains of \textit{C. albicans}, by down-regulation of adhesive genes (\textit{HWP1, ALS3}) and transcriptional regulators of cAMP pathway (\textit{CYR1, RAS1})\textsuperscript{69}. Other terpenoids such as thymol isolated from \textit{Thymus vulgaris}, carvacrol isolated \textit{Origanum vulgare} and eugenol isolated from \textit{Eugenia caryophyllata} which were found to be similar at a structural level due to the presence of functionalized groups such as hydroxyl, alkyl and methyl groups were found to have lower activities when compared with hinokitol. But, all these terpenoids, were considered to have similar mechanism of action for affecting the biofilm formation that is by disrupting the regulation of cAMP-PKA pathway\textsuperscript{69}.

Nepodin, a derivative of naphthalene isolated from roots of \textit{Rumex crispus} displays structural similarity to the above-mentioned compounds. Nepodin was found to inhibit the biofilms of FLU-resistant \textit{C. albicans} in polystyrene, and silicone catheter substrate. It also affected the hyphal morphogenesis, mainly due to the 5-fold downregulation of genes such as \textit{ECE1, HWP1, and...}
UME6 determined by RNA-seq. But surprisingly apart from having structural similarity to the terpenoids the mechanism was found to be different, i.e., none of the downstream regulators of c-AMP PKA pathway was affected which authors concluded as the complex mechanistic process of nepodin70.

b) Alkaloids

An alkaloid, tetrandrine, inhibits C. albicans biofilm formation and morphogenesis without affecting fungal growth. In vivo, it prolonged the survival of C. albicans infected Caenorhabditis elegans and normal hyphal growth was restored with the addition of cAMP. This indicates that tetrandrine inhibits hyphal morphogenesis through the Ras1p-cAMP-PKA pathway71. Lycorine hydrochloride, an alkaloid isolated from the herb Lycoris radiata has proven anti-cancer properties. Notably, it also demonstrates anti-biofilm, anti-filamentation, and anti-adhesive properties against C. albicans interestingly by inhibiting the hyphal formation in varying media conditions such as spider medium, GlcNAC medium, and in RPMI 164072. Piperine, , was shown to inhibit C. albicans biofilm formation, hyphal morphogenesis and disturbs mature biofilms with no effect on C. albicans growth and metabolism. It was also found to inhibit the in vivo C. albicans colonization and prolonged the survival rate of C. albicans-infected Caenorhabditis elegans by affecting the various biofilm associated genes such as HWP1 and ALS3, drug resistance related genes such CDR2 and CDR4. Furthermore, it showed no toxicity to human buccal epithelial cells and diminished potential for drug resistance development. With all these desirable properties, piperine can be considered a distinct anti-virulence candidate for management of candida infections73.

c) Flavonoids

Quercetin, a flavonoid has been shown to inhibit biofilm development and filamentation of C. albicans, in addition to increasing its susceptibility to FLC in FLC resistant clinical strains isolated from Vulvovaginal Candidiasis patients. This synergism enhanced the property of both drugs even in resistant strains and in murine models by preventing hyphae formation and reduced fungal burdens in the vagina74. In another study, it was shown that Quercetin acts as a sensitizer by increasing the susceptibility of FLC-to-FLC resistant isolates by increasing the production of farnesol. Farnesol, a quorum sensing molecule at high concentrations inhibited hyphal development and biofilm density of C. albicans. Quercetin-mediated suppression of biofilm formation is reversed in the farnesol defective ΔCzF1 mutant strain of C. albicans, indicating that Quercetin acts by increasing the production of farnesol thus suppressing the
various virulence factors\textsuperscript{75}. The effects of the farnesol on modulating the \textit{C. albicans} virulence is discussed in later sections.

Catechins extracted from \textit{Camella sinensis} (tea leaves) inhibited hyphal formation by reducing mRNA expression levels of hyphae specific genes such as \textit{HWP1}, \textit{ALS3}, \textit{CPH1}, \textit{SAP} 4-6 but interestingly \textit{RAS1} was not affected, but disturbed the downstream effectors of \textit{RAS1}, mainly Cek1 phosphorylation in the MAPK pathway and cAMP synthesis in CAMP-PKA pathway\textsuperscript{76}. Morin, a compound found in various medicinal plants showed significant inhibition of \textit{C. albicans} virulence factors \textsuperscript{77}. It inhibited biofilm formation (up to 90%), yeast to hypha transition, phospholipase, protease, and exopolysaccharides production with no fatal effect on fungal cells. \textit{In vivo} efficacy was also assessed using \textit{C. albicans} infected zebrafish animal model where morin increased the survival rate with a significant reduction in fungal load compared with untreated animals\textsuperscript{77}. Likewise, \textit{Boesenbergia rotunda} extracts pinostrobin and pinocembrin inhibited \textit{C. albicans} biofilm formation without altering the growth of planktonic cells. All stages of biofilm formation including adhesion, biofilm development and maturation were inhibited in a concentration-dependent manner with decreased \textit{ALS3} mRNA level\textsuperscript{78}.

d) \textit{Anthraquinone and other plant derived products}

Purpurin, an anthraquinone isolated from \textit{Rubia tinctorum} L., affects the \textit{C. albicans} biofilm development and filamentation. Purpurin significantly reduced the biofilm formation, but it was less effective in eradicating mature biofilms, due to the inherent diffusion limitation imposed by the biofilm architecture\textsuperscript{79}. The genes \textit{ALS3}, \textit{HWP1} that are essential for adhesion of \textit{C. albicans} to the substrate, are also downregulated, indicating their effects on biofilm formation.

Similarly, other plant derived compounds such as essential oils, \textit{α}-longipinene and \textit{linalool}\textsuperscript{80}, 6-gingerol and 6-shogaol\textsuperscript{81} and cedar leaf essential oil, camphor, and fenchone derivatives\textsuperscript{82} were assessed for their anti-virulent potential. They were found to be effective in inhibiting \textit{C. albicans} hyphal and biofilm formation without affecting the growth of planktonic cells. They also reduced \textit{in vivo} \textit{C. albicans} virulence in \textit{Caenorhabditis elegans}.

4-hydroxycordoin, an isopentyloxychalcone also demonstrated antibiofilm properties by inhibiting \textit{C. albicans} formation (by 85%) and yeast to hypha transition without affecting \textit{C. albicans} growth\textsuperscript{83}. Furthermore, plant derived oleic acid poses anti-virulence effects by inhibiting filamentous growth, biofilm formation, secreted aspartyl proteinases (SAPs) and lipase production of \textit{Candida species}. It also reduced the ergosterol content without affecting
the viability and the growth of fungal cells. Gene expression and proteomic analysis of oleic acid treated cells suggest that oleic acid treatment may result in oxidative stress and affects glucose metabolism, lipase production, iron homeostasis and ergosterol and amino acids biosynthesis. Other compounds such as trans-cinnamaldehyde and coumarin also have been reported to have antibiofilm effects against *C. albicans*.

**e) Polyphenols**

Curcumin, a polyphenol isolated from the rhizome of *Curcuma longa* is one of the well-studied compounds for its anti-adhesive, anti-biofilm, anti-filamentation, and anti-candidal effects against *C. albicans*. The anti-candida effects were observed at higher concentrations, possibly by perturbing the cell wall integrity affecting the calcineurin and MAPK pathways. Curcumin prevented the adhesion and biofilm formation of *C. albicans* to Poly-methyl methacrylate (PMMA), mostly used in denture materials, by affecting the major adhesins ALS1 and ALS3. Concentrations around 100 µg/mL and 200 µg/mL are needed to inhibit the growth and biofilms of *C. albicans* respectively. Despite the fact that curcumin demonstrates antifungal and antibiofilm properties through several mechanisms, their hydrophobicity precludes optimal bioavailability, poor solubility and potential reduced efficacy *in vivo*. Hence to overcome these difficulties our group recently explored the curcumin-sophorolipid nanocomplex which improved curcumin bioavailability, stability, and solubility. It also retained biofilm inhibitory effects and anti-filamentation property but at very low concentration of 9.37 µg/mL. Considering that these compounds hold the “generally regarded as safe (GRAS)” category status by FDA, urgent studies are needed to develop innovative delivery methods so that these molecules can be rapidly translated to clinical applications.

Apart from the plant derived compounds, fungal metabolites, and by-products such as mycotoxins are produced by some fungi and moulds to maintain equilibrium in the dynamic ecosystem and considered as harmful or toxic to other microbes, these microbe-derived small molecules were also found to have anti-virulence properties against *C. albicans*. Although natural products have several advantages, the unmodified form may possess passable efficacy or poor ADME (absorption, distribution, metabolism, excretion) properties. It is often required to synthetically modify and optimize to achieve full efficacy. Also, translating the natural products for the clinical application requires enough of the analogue. Fortunately, there are many recent advances, in total chemical synthesis, Biosynthetic engineering (Exploiting the producing organism at their biosynthetic pathway) etc., which ultimately leads to the development of analogues with superior pharmacological activity.
Insights into anti-virulence approaches

Virulence is the ability of a pathogen to cause disease. Virulence determinants are defined as fungal factors such as morphological transition, phenotypic switching, biofilm formation, secretion of hydrolytic enzymes, adhesin expression, and invasion of the host cell surface, all of which can actively cause damage to host tissues. Anti-virulence therapies are tailored at disarming the pathogen by stripping of its virulence potential. For instance, impeding the adherence of microbial cells will hamper their biofilm formation and force them to survive in the planktonic state which will eventually make them vulnerable for clearance by the host immune system and/or antimicrobial treatment. By selectively targeting the virulence mechanisms instead of employing fungicidal or fungistatic approaches, will create a weaker selective pressure for the development of antimycotic resistance.

The development of anti-virulence approaches need intensive research on the mechanisms of virulence and identification of virulence factors, which can reveal several potential targets that may contribute to the developments of newer classes of antifungals. Moreover, this non-lethal alternative is most suitable to the commensal C. albicans thus preserving the host microbiome. While the approach has a certain advantage, there are potential challenges in its clinical translation. One key challenge to this approach is that the timing of drug administration must coincide with the time at which the targeted virulence factor actively promotes disease. This poses a challenge in the clinical setting for the rapid and accurate diagnosis prior the use of such antifungals. Research in this area is still nascent and it remains unknown if fungi will evolve strategies to resist the anti-virulence strategies.

Targeting other virulence mechanisms:
Proteases
C. albicans possesses numerous virulence factors that enable the fungus to colonize, invade the host, evade the host immune response and cause disease. One of the main virulence attributes produced by C. albicans, is the secreted proteases enzymes, specifically secreted aspartyl proteinases (SAPs), phospholipases (PLs) and lipases (LIPs). These are three families of hydrolytic enzymes that are linked to virulence in C. albicans. SAPs (SAP 1-10) are the best characterized hydrolytic enzymes and of these, SAPs 1-8 are secreted extracellularly into the media while SAP9 and SAP10 remain bound to the fungal cell surface. The PL family has four classes (A, B, C and D). Only PL members of class B have been detected extracellularly...
and contribute to virulence, with PLB1 constituting the major component of secreted PLBs. LIP1-10 are lipases, which is are believed to facilitate penetration into host tissues in addition to nutrient acquisition for fungal growth. It has been shown that deletion mutants of SAP1, SAP2, and SAP3 showed significantly attenuated virulence compared to the wild type strains. Therefore, using therapeutics that target the secreted hydrolytic enzymes would be of a great value in targeting C. albicans virulence and pathogenicity.

One notable application of this is the introduction of aspartyl protease inhibitor-type HIV (HIV-PI) as a component of highly active retroviral therapy (HAART) in HIV+ patients. This category of immunocompromised patients is highly predisposed to oropharyngeal candidiasis. Significantly, the addition of the protease inhibitor resulted in a remarkable decline in opportunistic infections, disease progression and HIV associated mortality. The intended action of the HIV-PIs is to inhibit the viral proteases essential to produce the mature infectious form of the virus. Although, the associated reduction in candidiasis was mainly attributed to the improved host immunological status, the direct antifungal effect of HIV-PIs against C. albicans SAPs was also demonstrated in vitro. This was further supported by the fact that C. albicans SAPs and HIV aspartic protease belong to the same family. In fact, HIV-PIs have been shown to have a protective effect against Candida colonization in HIV infected patients.

By contrast, one recent study showed that HIV-PIs containing antiretroviral therapy was significantly associated with oropharyngeal Candida colonization in HIV patients. This difference could be attributed to the fact that different types of HIV-PIs have variable effect on Candida SAPs. Despite the reported antifungal properties of HIV-PIs, their use as therapeutics targeting non-HIV candidiasis may not be feasible owing to the serious side effects associated with these drugs including toxicity, accumulation of intracellular free cholesterol, lipid and insulin resistance. Using the SPECS 3D database, a total of 28,700 small molecules were screened by various virtual screening methods and 35 compounds were found to interact with the SAP2 protease. From these small molecules, using a combination of in vitro screening and Structure Activity Relationship (SAR), several structural analogues were synthesized. Compound 23h showed better activity and is inactive against human proteases. Although this compound, when used alone, resulted in the survival of only 10-20% fluconazole-resistant Candida infected mice, it resulted in 50% survival rate when combined with fluconazole. This demonstrates for the first time that the combination therapy of SAP2 inhibitors and fluconazole is an effective strategy to combat the drug resistance.
molecules in the family of protease inhibitors have been shown to inhibit biofilm formation and disturb mature biofilms alone and also in combination with subinhibitory concentration of traditional antifungals\textsuperscript{119}. Furthermore, it has been shown that not all Candida SAPs have the same sensitivity to protease inhibitors. Aoki and colleagues\textsuperscript{120} identified SAP7 as being insensitive to Pepstatin A, a classical protease inhibitor. As a result, developing a potent protease inhibitor capable of inhibiting all Candida SAPs would be highly advantageous.

**Quorum sensing**

Quorum sensing (QS), a signaling mechanism used by C. albicans to communicate amongst themselves, and is directed by soluble molecules that are secreted into the environment in a density-dependent manner\textsuperscript{121}. These molecules in the extracellular environment positively and negatively influences both biofilm development and morphogenesis \textsuperscript{122–124}. For instance, Farnesol (3,7,11-trimethyl-2,6,10-dodecatriene-1-ol) the first QS molecule that has been studied extensively in Candida was known to block the transition of yeast to fungi at higher cell densities thereby inhibiting hyphae formation\textsuperscript{121,125,126}. Farnesol inhibits the hyphal transition by targeting the RAS1 pathway, specifically affecting Cyr1 and cAMP signalling\textsuperscript{127,128}. Some antifungal molecules stimulate farnesol production through affecting the ergosterol biosynthesis in fungi. For instance, naturally derived products such as trans-cinnamaldehyde, and a phenolic compound bisbibenzyl was known to increase farnesol production by inducing the expression of encoding phosphatase DPP3, which plays an essential role in farnesol synthesis by conversion of farnesol pyrophosphate to farnesol\textsuperscript{85,129}. Stimulation of farnesol production by these small molecules then affected both biofilms and hyphal morphogenesis of C. albicans.

Apart from small molecules that induce the production of farnesol, a recent study demonstrated the use of the medium chain fatty acid named nonanoic acid, which mimics the farnesol and has anti-virulence properties\textsuperscript{130}. However, farnesol may also induce the biofilm-related infections by promoting the formation of the yeast cells within the mature biofilms, that are then easily dispersed. These dispersed cells have been shown to promote the propagation of C. albicans cells into the bloodstream\textsuperscript{121,131}.

The other quorum sensing molecule that is produced from C. albicans is Tyrosol (2-[4-hydroxyphenyl] ethanol) a tyrosine derivative, that is known to regulate both biofilm formation and hyphal development\textsuperscript{132}, conversely to the farnesol. Both quorum sensing molecules controls their expression in a density-dependent manner, i.e., at low cell densities, tyrosol is expressed and at high cell densities, farnesol is produced (Figure 6). Thus, the compounds modulating,
tyrosol production may help in eliminating the infection at low cell density state itself. Along with these two major QSM the other molecules that have been identified in Candida spp. and are not extensively studied are tryptophol and phenylethyl alcohol which were also found to inhibit cell growth and germ tube formation similar to that of farnesol. All these studies clearly demonstrate that QSM either have a positive or negative regulation on the morphogenesis of C. albicans.

**Future perspectives and conclusion**

Invasive and mucosal candidiasis affects approximately 500 million people worldwide with the increasing mortality rate of 30-50% even when treated. The challenge posed for the development of novel anti-fungals is mainly due to the eukaryotic nature of fungi, limiting drug targets. Sparse availability of antifungals, toxicity, and emergence of resistance causes extreme difficulties in treating these infections, clinically. Anti-virulence approaches provide a hope that they may reduce/eliminate the selective pressures that has been imposed on the pathogens which led to the failure of the conventional antifungals. Antifungals usually kill all the commensal and beneficial fungi causing dysbiosis, a pernicious side effect of the antifungals which can be bypassed using anti-virulence strategies. Finding specific targets for the fungal pathogens has always been difficult, but an increased understanding of pathogenesis and host-microbe interaction and the completion of the C. albicans genome project, has uncovered several targets for the development of anti-virulence drugs.

One of the major questions to address here, is that even though several novel antifungal molecules targeting anti-virulence approaches has been discovered in the last decade, as discussed in this review in detail, why have none of the molecules reached the market? The main answer for this question apart from time and budget constraints is that failure of these molecules in preclinical/clinical trials. Thus, in the preliminary screening using the phenotypic assays, incorporating more reliable physiological settings is essential. This will hopefully reduce the likelihood of overlooking compounds. Of course, as anti-virulence therapy is yet to be explored in the clinical setting, there is still no understanding of whether all this investment and time will lead to anti-virulence therapies.

As there is a pressing need for the novel antifungal compounds, and to achieve rapid translation into the clinical setting,"repurposed drugs" are an excellent candidate as their pharmacological properties are established reducing time and cost. Also use of artificial intelligence/machine learning approaches to discover anti-virulence drugs from the pool of
compounds/libraries available may reduce the time and lead to the discovery of novel chemical scaffolds. By integrating the efforts of biologists, clinicians, pharma companies, and funding agencies, it is possible the development of novel antifungals can be achieved affordably and lead to a reduction in the healthcare burden.

References:


Legend to Figures and tables

Fig 1: Resistance mechanisms of *C. albicans* to conventional antifungals. Azoles, echinocandins, and polyenes act on the cell wall components of *C. albicans* causing cell lysis. (Left) Azoles act by inhibiting the enzyme 14α – lanosterol demethylase is essential for the synthesis of ergosterol causing loss of cell integrity. However, the development of efflux pumps prevents the intake of the drug, mutation in the gene sequence of the target enzyme, and overexpression of target reduces the efficacy of the azoles towards candida. (Center) Echinocandin inhibits 1, 3 - beta glucans synthase (FKS1, FKS2) but mutation in these genes leads to resistance development. (Right) Polyenes acts on ergosterol causing membrane leakage. Mutation in ERG3 affects its susceptibility towards Candida infections. Flucytosine affects the growth and development by converting to 5- Fluro UMP (down), this toxic pyrimidine gets incorporated into DNA and RNA. Resistance to this occurs by the increased expression of efflux pump and mutation to the genes involved in the conversion of flucytosine to 5-fluro UMP.

Fig 2: Comparison of the traditional antifungal drug discovery process versus drug repurposing. The traditional way of drug discovery begins with a) identification of the effective molecular targets and chemical synthesis of the compound, which involves optimizing the stability, yield, and solubility; b) preclinical studies and animal studies with 50:50% success: failure rates c) phase 1-4 clinical trials with just approximately 10% success rate. These methods clearly lack efficiency as they are exorbitantly expensive, time-consuming, and have an unpredictable success rate. However, drug repurposing holds the advantage due to its proven lack of or reduced toxicity and pre-existing clinical trial data which will lead to the rapid translation to the clinic when compared to the traditional approach. Anti-malarial, anti-cancer, anti-glaucoma, and anti-bacterial drugs have been discovered for their ability to act as an anti-fungal by themselves or in synergy with existing traditional antifungals.

Fig 3: Multiple targets/phases of drug development identified via HTS method. Planktonic cells of *C. albicans* adhere to a substrate to initiate biofilm development and progresses to form mature biofilms. HTS may be used to discover drugs that affect various phases of biofilm development by targeting single and multiple signaling pathways.

Fig 4: Target dependent action of plant-derived natural bioactive molecules: Compounds are characterized based on their class of bioactive compounds and listed. The advantages (pros) of plants derived bioactive molecules include “GRAS” category recognized by FDA, ease of consumption and less toxicity. Whereas the disadvantages (cons) in developing the plants’ natural products was purification, bioavailability, and solubility issues due to increased hydrophobicity.

Fig 5: Proteases in Candida and protease inhibitors. (Top) cell surface associated Secreted aspartyl proteases SAP1-3 mediate the epithelial cell adhesion. Fungal invades the epithelial cells by endocytosis and active penetration, where SAPs play a role in mediating active penetration. SAPs digests the molecules and helps in nutrient acquisition via the transporter ZRT1. SAP4-6 plays an essential role in evading the host immune responses by cleavage of complementary proteins. (Below) The developed protease inhibitors, acts by reducing the adhesion, invasion and inducing the oxidative stress.

Fig 6: Quorum sensing mechanism and inhibitors of *C. albicans*. The QS molecules produced in the high cell densities such as farnesol, phenylethyl alcohol, and tryptophol, inhibits the biofilm formation and virulence. Farnesol inhibits the *CYR1* production in the *RAS1* pathway.
thus inhibited the filamentous growth. Conversely tyrosol, which is expressed in low cell densities induces the morphogenesis for which mechanism is not fully understood.

Table 1: High throughput screening: Table represents the library and molecule screened via the High-through put screening. The lead compound name with their chemical structure and mechanism of action is reported.

Table 2: Details of compounds screened via HTS have synergistic interactions with existing antifungals. The lead compound name with their chemical structure and mechanism of action is reported.

Table 3: Details of mitochondrial and vacuole inhibitors screened via HTS. The lead compound name with their chemical structure and mechanism of action is reported.

Table 4: Details of repurposing drugs screened via HTS. The lead compound name with their chemical structure and mechanism of action is reported.

Table 5: Plant and microbe derived drugs. The compound name, source, structure with their inhibitory concentration range and mechanism of action is reported in the table.