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Synergistic combinations of antifungals and anti-virulence agents to fight against *Candida albicans*

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Candida albicans, one of the pathogenic *Candida* species, causes high mortality rate in immunocompromised and high-risk surgical patients. In the last decade, only one new class of antifungal drug echinocandin was applied. The increased therapy failures, such as the one caused by multi-drug resistance, demand innovative strategies for new effective antifungal drugs. Synergistic combinations of antifungals and anti-virulence agents highlight the pragmatic strategy to reduce the development of drug resistant and potentially repurpose known antifungals, which bypass the costly and time-consuming pipeline of new drug development. Anti-virulence and synergistic combination provide new options for antifungal drug discovery by counteracting the difficulty or failure of traditional therapy for fungal infections.

Introduction

Candida albicans, one of the leading opportunistic fungal pathogens, caused high mortality rate especially in immunocompromised and high-risk surgical patients.[1,2] *C. albicans* locate in the oral cavity, digestive tract and genital region as the commensal flora in more than half of the healthy population. When given pathogenic opportunity, *C. albicans* is responsible for more than 50% of human candidiasis, including 2 major types of infections, superficial infections (nonlethal), such as oral or vaginal candidiasis; and systemic infections (~40% mortality).[3,4] Systemic infections caused by *C. albicans* have become a serious public health threaten in immunocompromised patients, organ transplantations, non-trauma emergency surgery, massive chemotherapy and implantable medical devices during the past several decades.[3-6] The development of antifungal drug discovery is relative slower than antibacterial antibiotics, and antifungal drug resistance reduce the efficacy of known antifungals.[7] The lack of new antifungal drugs and the limited therapeutic options call for new strategies to find novel antifungal candidates.

Synergistic drug combination has been proved to be a valid and pragmatic strategy to seek drugs with novel mode of actions. It can potentially reduce the dose of single drug usage with increased drug-efficacy, and subsequently lower the drug toxicity. The practice of targeting 2 or more drug targets simultaneously is consistent with the philosophy that a disease is a systematic and complicated outcome caused by multi-effects. Furthermore, the development of drug resistance can be slowed down by the multi-target strategy. There are 3 different phases for synergistic antifungal drug combinations, *in vitro* testing, *in vivo* animal model validations, and the clinical trials. Using two or more antifungal drugs to control severe invasive fungal infections has been adopted in clinic for a long time. The first application of synergistic therapy forinvasive candidiasis is flucytosine and amphotericin B. The flucytosine monotherapy usually caused drug resistance and unexpected side effects, while amphotericin B compromised these problems.[8,9] This combination was recommended by Infectious Diseases Society of America (IDSA) guidelines for the treatment of candidiasis among patients in selected situations, including those with serious and deep-seated candidal infections involving the central neuron system (CNS) infections, endovascular infections and serious intra-abdominal candidiasis.[10] There are also some cases which are well and widely used in clinic (**Table 1**).

An alternative approach of antifungals is to target virulence factors which opens a new pipeline for antifungal drug discovery.[11] It extends the range of potential drug targets from "essential processes (growth)" to "virulence processes (pathogenesis)". The strategy of targeting pathogen specific virulence can also help to preserve the host normal commensal microbiome.[12] In addition, the effective synergistic combinations between anti-virulence agents and low dose of toxic antifungal drugs provide more potent controls against fungal infections, which are more efficacious but less toxic than the use of single drugs. In this review, we summarized the mode of actions of antifungals and fungal drug resistance,

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Table 1. *Selected synergistic combinations against *C. albicans in vitro* and *in vivo*.

Data from	Wild type or resistance#	Synergistic combination	Reference
In vitro Animal	Wild type and Resistance	Beauvericin + Clotrimazole, or Fluconazole, or Itraconazole, or Ketoconazole, or Miconazole	Unpublished
In vitro	Wild type and Resistance	Rapamycin + peptides	Unpublished
In vitro	Wild type and Resistance	Ketoconazole + Avilamycin	Unpublished
In vitro	Wild type and Resistance	Ketoconazole + Spinosad	Unpublished
In vitro	Wild type and Resistance	Nitroimidazoles + Amphotericin B	83
In vitro	Wild type and Resistance	Histatin 5 and its analogs Amphotericin B	84
In vitro	Wild type and Resistance	Tetrandrine + Ketoconazole	85
In vitro	Wild type and Resistance	Fluconazole + Thymol, or Carvacrol	86
In vitro	Wild type and Resistance	Geldanamycin + Fluconazole	87
In vitro	Wild type and Resistance	Pure polyphenol curcumin I + Fluconazole, or Miconazole, or Ketoconazole, or Itraconazole, or Voriconazole, or Nystatin or Amphotericin B	88
In vitro	Wild type and Resistance	Berberine + Fluconazole	89-91
In vitro	Wild type and Resistance	Fluconazole + alverine citrate, or Caspofungin, Latrunculin-A, or Wortmannin, or Fenpropimorph	92
In vitro	Wild type and Resistance	Tunicamycin + FK506, or Cyclosporine-A	92
In vitro	Wild type and Resistance	Wortmannin + Tunicamycin, or FK506	92
In vitro	Wild type and Resistance	Posaconazole + Caspofungin, or FK506	93
In vitro	Wild type and resistance	Amphotericin B + Terbinafine	94
In vitro	Wild type and Resistance	Flucytosine + Econazole, or Miconazole	95
In vitro	Wild type and Resistance	Fluvastatin + Fluconazole, or Itraconazole	96
In vitro	Wild type and Resistance	FK506 + Fluconazole, or Voriconazole, or Itraconazole	97
In vitro	Wild type and Resistance	Retigeric acid B + Fluconazole, or Ketoconazole, or Itraconazole	98
In vitro	Wild type and Resistance	Lactoferrin + Fluconazole, or Itraconazole	99
In vitro	Wild type and Resistance	Farnesol + Fluconazole, or Ketoconazole, or Miconazole, or Amphotericin B	100
In vitro	Wild type and Resistance	Amiodarone + Fluconazole, or Voriconazole, or Itraconazole	101
In vitro	Resistance	Eugenol + Amphotericin B, or Fluconazole	102
In vitro	Resistance	Honokiol + Fluconazole	103
In vitro	Resistance	Glabridin + Fluconazole	104
In vitro	Resistance	Tioconazole + Butylated hydroxyanisole	105
In vitro	Resistance	Baicalein + Amphotericin B	106
In vitro	Resistance	Curcumin+ R6G, or Ketoconazole, or Itraconazole, or Miconazole	107
In vitro	Resistance	Anidulafungin + Posaconazole, or Amphotericin B	108
In vitro	Resistance	Caspofungin + Posaconazole, or Micafungin	108
In vitro	Resistance	Minocycline + Fluconazole	109
In vitro	Resistance	Baicalein + Fluconazole	110
In vitro	Resistance	Ofloxacin + Fluconazole	111
In vitro	Biofilm and Planktonic cells	Fluconazole + FK506, or Cyclosporine A	112
In vitro	Biofilm and Planktonic cells	Berberine + Miconazole	113
In vitro	Biofilm and Planktonic cells	Aspirin + Amphotericin B	114
In vitro	Biofilm	Tyrocidines + Amphotericin B, or Caspofungin	115
In vitro	Biofilm	Amphotericin B + Drospirenone, or Perhexiline, or Toremifene	116
In vitro	Biofilm	Caspofungin + Drospirenone, or Perhexiline, or Toremifene	116
In vitro	Biofilm	Amphotericin B + N-acetylcysteine, or EDTA, or Ethanol, or Talactoferrin	117
In vitro	Biofilm	Fluconazole + N-acetylcysteine, or EDTA, or Ethanol, or Talactoferrin	117
In vitro	Biofilm	Terpenes + Fluconazole	118
In vitro	Biofilm	Doxycycline + Fluconazole	119
In vitro	Biofilm	Silver nanoparticles + nystatin, or chlorhexidine digluconate	120
In vitro	Biofilm	Doxycycline + Fluconazole	121
In vitro	Biofilm	Shearinines D (3) and E (4) + Amphotericin B	122
In vitro	Biofilm	Verapamil + Fluconazole, or Tunicamycin	123
In vitro	Biofilm	Cyclosporine A + Fluconazole, or Voriconazole, or Amphotericin B, or Caspofungin	124
In vitro	Biofilm	Amphotericin B + Rifampicin, or Clarithromycin	125
Animal	Wild type and resistance	Posaconazole + Caspofungin, or FK506	126
Animal	Resistance	Amphotericin B + Caspofungin	127
Animal	Resistance	Tetrandrine + Ketoconazole	128
Animal	Wild type	Berberine + Amphotericin B	129
Animal	Wild type	Cilofungin + Amphotericin B	130
Animal	Wild type	Antimicrobial peptides + Caspofungin	131
Animal	Wild type	Nikkomycin Z + R 3783	132
Animal	Wild type	Amphotericin B + Ketoconazole, or 5-Fluorocytosine	133
Animal	Wild type	5-Fluorocytosine + Fluconazole, or Itraconazole	133

(*continued on next page*)

Table 1. *Selected synergistic combinations against *C. albicans in vitro* and *in vivo*. *(Continued)*

Data from	Wild type or resistance#	Synergistic combination	Reference
Animal	Biofilm	Fluconazole + FK506, or Cyclosporine A	112
Clinic trials	Clinical	Amphotericin B + 5-Fluorocytosine	134-137
Clinic trials	Clinical	5-Fluorocytosine + Azoles	138
Clinic trials	Clinical	Amphotericin B + Azoles	139
Clinic trials	Clinical	Terbinafine + Itraconazole or Fluconazole	140
Clinic trials	Clinical	Mycograb + lipid-associated amphotericin B	141

*Because strains, culture conditions, susceptibility testing methods and models used to define synergy in these studies were different, results from different literatures may be contrary to each other, only synergy reports were selected in this table.
#Strains were labeled as wild type if they were not claimed as resistance explicitly in the reference. For clinical trials, the infection sources are not distinguished, all labeled as clinical.

highlighted the synergistic screening for new antifungal agents, we also introduced the recent progress on anti-virulence factor research in antifungal drug discovery and prospected the strategy to fight against *C. albicans* with synergistic combinations of antifungals and anti-virulence agents.

Mode of Actions of Antifungals and Drug Resistance

Currently, the clinical anti-candidiasis therapeutic drugs are limited to few classes including polyenes, azoles, allylamines and echinocandins. These antifungal drugs usually target essential processes of *C. albicans* which cause the evolution of drug resistance rapidly such as azoles and echinicandins, while the resistance to amphotericin B (a polyene antifungal) is rare. The antifungal drug targets can be confined to the following distinct pathways (**Fig. 1**): **a) ergosterol and ergosterol biosynthesis**. Ergosterol is a key component in fungal cell membrane and similar to human cholesterol. It plays an important role in fungal cell growth. Polyene drugs, such as amphotericin B, can bind to ergosterol and lethally cause leak of cell components by forming channels on the fungal cell membranes.[13,14] Azoles are another class of antifungals targeting ergosterol biosynthesis. Fluconazole, for instance, functions through targeting lanosterol 14α-demethylase, which is a core enzyme encoded by *ERG11* in ergosterol biosynthesis.[15,16] **b)** ***β*(1 3)-D-glucan synthesis**. Fungal cell wall containing mannan, chitin, and α- and β-glucansis another attractive drug target because there is no counterpart in mammalian cells. Echinocandins can lead to cell death by inhibiting β(1 3)-D-glucan synthesis and consequently disrupting the fungal cell wall integrity.[17] **c) Nucleic acids synthesis**. Biosynthesis of macromolecules, such as DNA and RNA, are also adopted as antifungal targets. The clinical used antifungal drug, 5-fluorocytosin (5-FC), a fluorinated pyrimidine analog, can be transported into cells and finally converted into 5-fluorodeoxyuridine monophosphate (5-FdUMP) or 5-fluorouracil triphosphate (5-FUTP) to inhibit RNA or DNA synthesis.[18] Besides the drug targets list above commonly used in clinic, there are also some other targets identified for antifungal drug discovery. **d) Protein synthesis**. A potential candidate for new fungicidal development named sordarin is proved that it can inhibit the elongation process of protein synthesis in yeasts by stabilizing the ribosome/EF2 complex but do not affect the protein synthesis machinery in mammalian cells.[19] **e) Mitosis**. The antifungal drug, griseofulvin, used both in animals and humans to treat fungal infections of the skin (commonly known as ringworm) and nails, was reported it can bind to tubulin, interfering with microtubule function, thus inhibiting the fungal cell mitosis.[20] **f) Mitochondria**. The antifungal candidate arylamidine was demonstrated that it can selectively accumulated in *C. albicans* via transporter-mediated systemsand disrupted yeast mitochondrial function.[21,22] Although current antifungals functioning depend on above discussed pathways, more are imperative to be discovered in the future as the pace of antifungal drug resistance continues to increase.

Along with the antibiotics development, drug resistance evolved successively. Several drug resistant mechanisms were found in *C. albicans* (**Fig. 1**). **a) Target overexpression**. Antifungal drug resistant strains can over produce drug targets to blunt the efficacy of antifungals. **b) Targets alteration**. Under the selective pressure of antifungals, target mutated cells with decreased the binding affinity of antifungals survive and develop as resistant strains. **c) Drug sequestration**. Fungal pathogens are capable of separating antifungal drugs from their targets, either by keeping drugs out of cells, such as forming biofilms, or by hijacking the invaded drugs into sub-cell structures.[23-25] **d) Enhanced drug efflux**. There are 2 types of drug efflux pumps identified in *C. albicans, Candida* Drug-Resistance (CDR) pump and Major Facilitator Superfamily (MFS) efflux pump. Both have been implicated in antifungal drug resistance among the putative transporter genes identified in the *C. albicans* genome.[26,27] **e) Blocking of antifungal drug entry**. Pathogens can set up barriers to reduce or stop the antifungal drug entry to decrease the intracellular drug concentrations. Apart from the mechanisms discussed above, clinical isolates also developed other mechanisms to overcome or bypass the action of antifungals such as chromosome aneuploidy (e.g. increase the copy of target genes on some chromosome) and phenotype transition (**Fig. 1**).[28,29] Moreover, clinical isolates usually occupy more than one drug resistant mechanisms resulting in multi-drug resistance, which cause therapeutic failure of current antifungal drugs.[30]

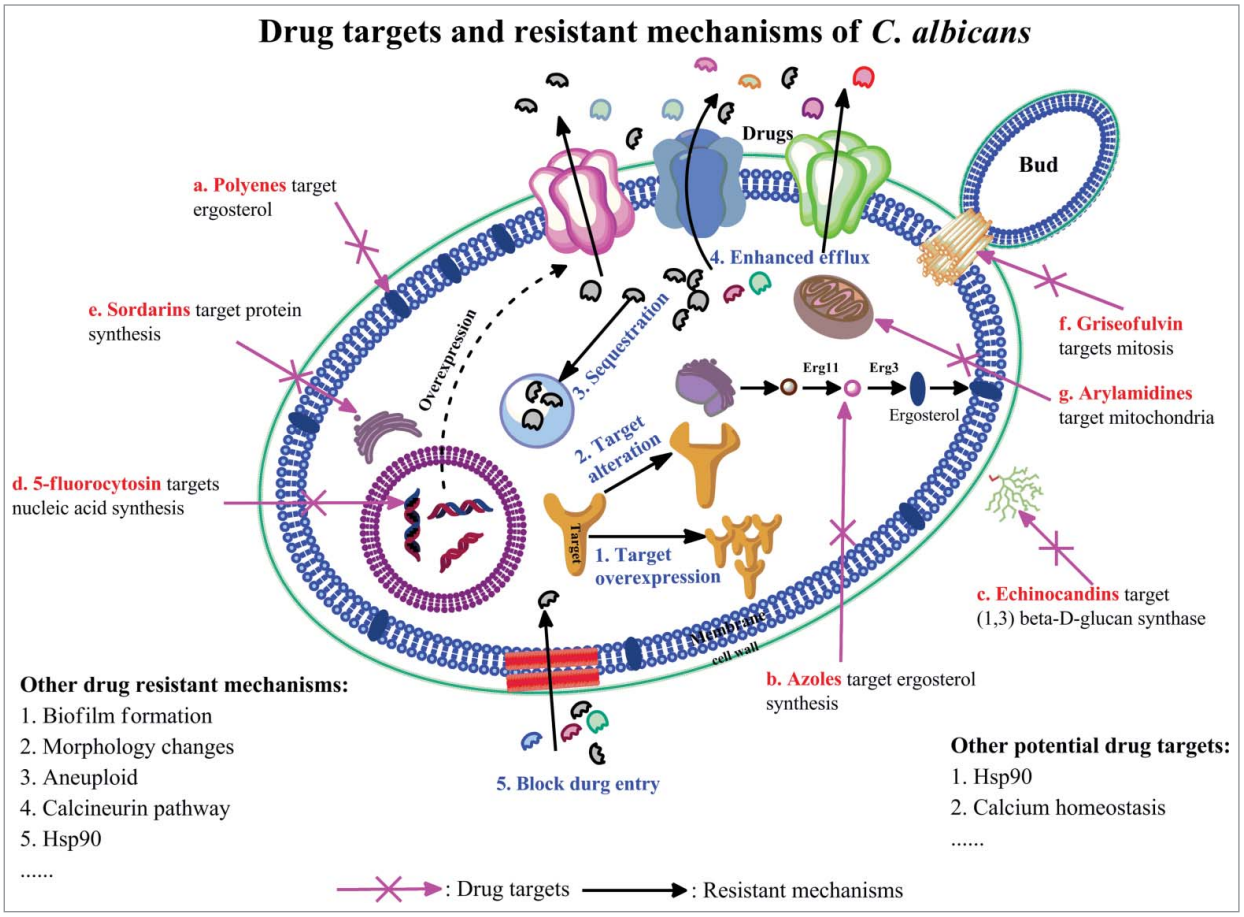

Figure 1. Antifungal drug targets and related drug resistant mechanisms of *C. albicans* Antifungal drugs and their targets: (**A**) polyenes, target is ergosterol; (**B**) azoles, target is ergosterol biosynthesis; (**C**) echinocandins, target is β(1 3)-D-glucan synthesis; (**D**) fluoropyrimidine, target is nucleic acids synthesis; (**E**) sordarins, target is protein synthesis; (**F**) griseofulvin, target is mitosis; (**G**) and arylamidines, target is mitochondria. The general drug resistant mechanisms in *C. albicans* include: **1**. target overexpression; **2**. targets alteration; **3**. drug sequestration; **4**. enhanced drug efflux; and **5**. blocking of antifungal drug entry.

Seeking Synergistic Combinations to Fight Against *C. albicans*

The challenge of decreased public health and lack of new drugs urgently call for new pharmaceutical strategies, one of which is drug combination research. The generally accepted criterion to determine whether a combination is synergistic or not is to calculate the FICI (Fractional Inhibitory Concentration Index) value by the formula: FICI = ($MIC_{drug\ A\ in\ combination}$/$MIC_{drug\ A\ alone}$) + ($MIC_{drug\ B\ in\ combination}$/$MIC_{drug\ B\ alone}$) in which A and B mean 2 drugs used in the combination. When FICI > 4, the combination is antagonism, while FICI < 0.5 means synergy, and the combination effect is additive when FICI between 0.5 and 4.[31]

In order to increase the hit rates of synergistic combinations, sample the unexploited expanse of bioactive chemical space, repurpose the old drugs which are even out of market, and accelerate the antifungal drug development pipeline, we establish a high throughput synergistic screen (HTSS) platform to discovery new antifungal drugs with novel mode of actions.[32-34] For the first time, we construct a database named Antifungal Synergistic Drug Combination Database (ASDCD) to assemble published synergistic antifungal combinations.[35] Here we highlight some synergistic combinations against *C. albicans* in *in vitro* studies, *in vivo* animal models, and clinical trials (**Table 1**). In accordance with their mode of actions, these synergistic combinations can be categorized into several groups, including 1) antifungal drugs and drug efflux pump inhibitors, 2) antifungal drugs and drug resistant efflux pump reversers which can increase the entrance and accumulation of antifungal drugs, and 3) antifungal drugs and cell wall or cell membrane disrupting agents which enhance antifungal drugs penetrating the cell barriers.

For example, beauvericin, identified from our biodiversity and taxonomy guided marine microbial natural product library, synergized with several azole drugs such as ketoconazole, miconazole against *C. albicans* including drug resistant isolates by inhibiting the ABC transporters (Tong et al., unpublished).[33] Beauvericin was also demonstrated for taking part in the inhibition of FK506 and cyclosporin in calcineurin pathway which is essential for the virulence of *C. albicans* (Tong et al., unpublished). Berberine, another natural product from our library, was confirmed that it can reverse the efflux

function of *MDR1*, a major facilitator of *C. albicans*, in fluconazole resistant isolates and further sensitize *C. albicans* to azole drugs (Sun et al., unpublished). The combinations of azoles with beauvericin or berberine, were confirmed for their effect of killing of *C. albicans* in both *in vitro* and systematic infectious mouse model *in vivo* studies (Tong and Sun et al., unpublished).[33]

Most of these studies were carried out *in vitro*, without considering the physicochemical and biopharmaceutical properties of the drug combinations as well as the physiological environment *in vivo*. Thus, more pre-clinical performances need to be evaluated for clinical usage. However, these synergistic combinations set up the example for synergistic combinations to fight against *C. albicans*.

Anti-Virulence Factors to Discover New Antifungal Drugs

Virulence factors are attributes of pathogens and generally considered not essential for pathogen survival *in vitro* but functioning and causing damage to host during infections.[36,37] *C. albicans* express several virulence factors that contribute to its pathogenicity. These factors include environmental adaptation factors, adhesins, morphogenesis, secreted enzymes, phenotype switching, and biofilms.[38-40] Virulence factors has also been considered as potent antifungal targets.[41-43] The fundamental behind targeting virulence is instead of killing, hindering pathogens to cause any harm to the host. Several advantages can be expected from the drug discovery strategy by targeting virulence factors: a) it extends the range of potential drug targets from 'essential processes' to 'virulence processes' and enlarges the number of potential drug targets; b) it reduces direct selection on fungal cells which ultimately fosters resistance; c) the strategy of targeting pathogen-specific virulence preserves the host microbiome which is important for normal commensals, whereas broad-spectrum antifungals can cause host microbiota unbalance, such as in gut and mouth.[12]

Among the virulence factors identified, secreted hydrolytic enzymes, filamentation and the ability to form biofilms are recognized as the main virulence factors contributing to the pathogenesis of candidiasis. Many studies have demonstrated the potency of anti-virulence agents in the antifungal trials with these virulence factors.

Anti-Secreted Hydrolytic Enzymes

One class of the candidates for anti-virulence drugs is protease inhibitors. In the treatment of HIV infections, Hoegl et al. found that the potent HIV protease inhibitors showed a favorable influence on the frequency of mucosal candidiasis in HIV infected patients.[44] Further study revealed that this phenomenon were not completely due to partial or total reconstitution of the immune status as originally presumed, but rather due to a direct inhibitory activity of these compounds against secretary aspartic proteases, Saps, a major virulence factor contributed to invasiveness from *C. albicans*.[45] The HIV proteinase inhibitors, such as saquinavir and indinavir, which already showed potency in the cure of candidiasis, can be carefully selected and considered as potential candidates for anticandidal virulence agents.[46,47] Phospholipases are another major *C. albicans* secreted enzymes contributed to invasiveness during infections. Ganendren et al. reported that phospholipases substrates analogs such as alexidine dihydrochloride and 1,12 bis-(tributylphosphonium)-dodecane dibromide had a relatively broad antifungal activity against *C. albicans, Cryptococcus neoformans, Aspergillus flavus in vitro*.[48] These phospholipid inhibitors could be attractive molecules for further development of anticandidal agents.

Anti-Morphogenesis

C. albicans is a polymorphic fungus and is able to transform its morphologies between yeast and filamentous forms. Filamentation not only represents a virulence trait itself, but it is also coordinately regulated with other virulence factors associated with cellular morphology.[36,49] The evidence of filamentation in *C. albicans* virulence was derived from the gene disrupted strains locked in yeast morphology.[50] By using tet-NRG1 strain with a tetracycline-regulatable promoter system (morphology is controlled by the presence or absence of doxycycline), Saville et al. demonstrated that the filamentation of *C. albicans* was associated with virulence and mortality.[51] Moreover, the authors provided a proof of concept that inhibition of filamentation represents an attractive target for the development of new antifungal drugs. An increasing number of small molecules has been reported that are able to modulate morphogenetic conversions and inhibit filamentation. These are mainly regulators of the yeast-to-hyphae transition for *C. albicans* such as phenazines and homoserine lactones from *Pseudomonas aeruginosa*, mutanobactins from *Streptococcus mutans* and capric acid secreted by *Saccharomyces boulardii*, farnesol and other autoregulatory alcohols that act as quorum sensing molecules produced by *C. albicans* itself, retigeric acid, and bisbibenzyls.[52-58]

Anti-Adhesion

Biofilms are structural microbial communities attached to a surface or encased in a matrix of material. They provide the potential to initiate or prolong infections by providing a safe environment for cells for local tissue invading, new infection sites seeding and drug resisting.[7] *C. albicans* is one of the biofilm forming species and most clinical manifestations of candidiasis are linked to biofilm formation.[59,60] The exopolymeric anti-adhesion strategy can be either targeting biofilm matrix or cell dispersion. Martins et al. demonstrated that addition of DNase improves the anti-biofilm activity of some antifungal drugs as extracellular DNA is a component of the *C. albicans* biofilm matrix.[61] Another possible strategy is to target dispersion, as cells dispersed from the biofilms are responsible for dissemination, extravasation and establishment of deep-seated candidiasis.[62]

Molecules targeting the specific genes or proteins in the regulatory of yeast cell dispersions could be consider as a candidate for anti-biofilm agent. It is notable that, because of the intimate link between filamentation and biofilms, drugs that modulate *C. albicans* morphogenesis also could potentially inhibit the development biofilms. This assumption could be also applicable to other virulence factors as the pathogenicity of *C. albicans* is multifactorial and delicate to the host and environment conditions.

Combinations of Anti-Virulence Agents With Antifungals

Though promising, the approach of targeting virulence is still at the start of the antifungal drug development pipeline. One of the drawbacks of the anti-virulence agent application is that most of the genes encoding the major virulence factors are non-essential and *C. albicans* express different virulence factors during the pathogenic process. A pragmatic strategy is to identify anti-virulence agents in conjunction with antifungal therapy, which not only increases the clearance of fungal pathogens, but also reduces the pathogenicity and decreases the toxicity of antifungal drugs by lowering their dosages. Here we highlight some important applications of anti-virulence agents combined with antifungals in *in vitro* studies.

Synergistic Activities of Inhibitors from Calcineurin Pathway

Calcineurin is proved essential for virulence of *C. albicans*.[63] Mutations from catalytic subunit Cmp1 and regulatory subunit Cnb1 cause the hypersensitivity to environmental stresses and are avirulent in mouse model of disseminated candidiasis.[64,65] The deletion of genes from the calcineurin pathway resulted in loss of tolerance to several antifungal agents.[64,66] CyclosporineA (CsA) and FK506, 2 immunosuppressive drugs which can inhibit calcineurin signaling by binding to the cyclophilin and FKBP12 respectively, have been proved for their repurposing usage in antifungal treatment by synergizing with various antifungals which mainly are azoles.[67] These synergistic effects possibly result from the cell membrane damage and accumulation of toxic sterols when *C. albicans* is treated by azoles, while calcineurin pathway is essential for the response to these stresses.[68] This proved concept opens the chapter to combine inhibitors from these 2 pathways: calcineurin signaling pathway and egosterol biosynthesis pathway. For example, radicicol and geldenamycin show potent synergistic antifungal activities with azoles by inhibiting the functions of Hsp90, a molecular chaperone to calcineurin.[69] The inhibitors of *PKC1* (regulates cell wall integrity) render *C. albicans* hypersensitive to azoles and echinocandins by regulating the PKV signaling cascade involved with calcineurin and Hsp90.[70] Another small heat shock protein Hsp21, similar to Hsp90, which plays important roles in environmental adaption and virulence potentiates antifungal drug tolerance in *C. albicans*, while null mutants become sensitive to antifungal drugs including terbinafine, clotrimazole, bifonazole, nocadazole and caspofungin.[71,72] These results indicated the synergistic potential between antifungal drugs and Hsp21 inhibitors.

Anti-Biofilm Agents Sensitize *C. albicans* to Antifungals

Biofilms formed by *C. albicans* are resistant to most of the commonly used antifungal drugs.[73] Susceptibility studies have revealed that biofilms formed by *C. albicans* can be resistant to various antifungal drugs even thousand times than the planktonic cells.[74,75] Currently, there is not a signal antifungal antibiotic was found effective against biofilm related *C. albicans* infections at low concentrations. Meanwhile, higher concentrations of the antifungal drugs are not advisable because of side effects due to toxicity. Thus, combination of drugs with different mode of actions inhibiting multiple cellular targets would be a wise strategy against biofilms. In 2002, for the first time, Khun et al. demonstrated the unique combination activity of lipid formulations of amphotericin B and echinocandins against *Candida* biofilms.[76] More efforts have been taken in this prospective area since then and a summary of these works can be found in the recent review article by Bink et al.[77]

In antifungal combination studies, it is interesting that the results of combination against planktonic cells are not always match with that of the biofilms but the utilization of anti-biofilm agents usually sensitize *C. albicans* to antifungals. For example, combination of fluconazole and amphotericin B has synergistic effects on planktonic growth of *C. albicans* but does not alter the activity of amphotericin B against biofilms.[78] While in another research, Mohd et al. found that the phytocompound eugenol, a potential anti-biofilm agent against pre-formed biofilms and the formation of biofilms alone, exhibited a synergistic interaction with fluconazole against biofilms.[79] The SMIC (sessile MICs) of fluconazole could be reduced down to 32-fold. The tested compounds were added before the biofilm formed and inhibit its development which would be of interest for combating recalcitrant infections involving *Candida* biofilms. The results showed a varying level of attenuation of biofilm formation by planktonic *Candida* cells in the presence of anti-biofilm compounds and drugs in a dose-dependent manner. The authors suggested that the anti-biofilm agent, eugenol, target cell membranes in both planktonic (higher sterol content) and sessile (lower sterol content) cells of *C. albicans*, and that their mode of action remains unaffected by the phenotypic variation in the ergosterol content exhibited by planktonic and sessile cells.[79] Other candidates for combination therapies such as chloroquine and cyclosporine also showed potency in the sensitization of *C. albicans* to conventional antifungal drugs.[80,81] It is notable that most of the reports dealing with studies of *C. albicans* biofilm susceptibility for antifungal combination treatment were *in vitro*. Studies *in vivo* and clinical are need to perform imperatively for real applicable combination treatments of candidiasis.

Host Model in Synergistic Antifungal Drug Discovery

Despite the increasing knowledge of *C. albicans* virulence and the discovering of synergistic combinations, it has been not possible to harness all the information for the development of new drugs and therapies for candidiasis. Moreover, the great majority of these combination studies were done *in vitro*, lacking of evidence for the effects *in vivo* and clinical, which limited the eventual development of anti-candidiasis drugs with some general concerns about potency and potential toxicity. Thus, host models are necessary to be introduced for comprehensively studying and understanding virulence factors and their interactions with antivirulence agents. In antifungal drug discovery, besides screening antifungal compounds *in vitro*, an alternative approach is to screen molecules based on host-pathogen interactions *in vivo*. Breger et al. developed a high-throughput *in vivo* assay with *Caenorhabditis elegans* for antifungal screening.[82] In a screen of 1266 compounds with known pharmaceutical activities, 15 were identified that prolonged survival of nematodes infected with *C. albicans* and inhibited filamentation and biofilm formation of the fungus. Considering *C.elegans* is invertebrate organism with only innate immunity, other vertebrate animals should be employed as host model in synergistic antifungal study in the future. The utilization of such host model in anti-infection drug discovery can help to identify not only antifungal or anti-virulence agents but also host immunomodulatory active compounds which could also expand the library of synergistic combinations.

Conclusion

The antifungal drug discovery pipeline has declined substantially over the past decades. The concept of synergistic therapy breaks the historical paradigm, "one-drug-one-target dogma", by targeting different targets and pathways in the disease network. And the synergistic combination of anti-virulence agents and antifungal drugs was proved to be a promising way to combat *C. albicans*, especially the drug-resistant strains, by targeting both pathogenic process and the cell growth. However, the discovery of such synergistic combinations based on experimental methods by testing a large number of combinations, which is a formidable challenge in terms of costs and time-consuming. Therefore, discovery of synergistic drug combinations based upon known combinations and advancements of fungal pathogen genomics with computational prediction science prospects a new direction in antifungal drug discovery and therapy.

To date, rare synergistic combinations have been proved for the efficacy and safety based on animal models and clinical trials. More pre-clinical evaluation and investigations need to be carried out in the future and the mode of actions of these synergistic combinations should be deciphered. Though there is a long road from *in vitro* assay to clinical usage, we believe that an approach abiding by the integral concept of incorporating disease process and synergistic bioprospecting strategy gives a promising prospect for the fight against fungal pathogens, even the multi-drug resistant ones.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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