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REVIEW

Combinatorial strategies for combating invasive fungal infections

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ABSTRACT

Invasive fungal infections are an important cause of human mortality and morbidity, particularly for immunocompromised populations. However, there remains a paucity of antifungal drug treatments available to combat these fungal pathogens. Further, antifungal compounds are plagued with problems such as host toxicity, fungistatic activity, and the emergence of drug resistance in pathogen populations. A promising therapeutic strategy to increase drug effectiveness and mitigate the emergence of drug resistance is through the use of combination drug therapy. In this review we describe the current arsenal of antifungals in medicine and elaborate on the benefits of combination therapy to expand our current antifungal drug repertoire. We examine those antifungal combinations that have shown potential against fungal pathogens and discuss strategies being employed to discover novel combination therapeutics, in particular combining antifungal agents with non-antifungal bioactive compounds. The findings summarized in this review highlight the promise of combinatorial strategies in combatting invasive mycoses.

Introduction

The discovery of antimicrobial drugs to treat infections caused by bacterial and fungal pathogens is a challenging endeavor. The golden era of antibiotics, from the 1940s to 1970s, witnessed the discovery of novel chemical scaffolds produced by bacteria and fungi that revolutionized modern medicine. However in the past 30 y only 2 mechanistically and structurally new classes of antibacterial antibiotics have reached the clinic: linezolid and daptomycin.[1] Similarly, the only novel antifungal drug class to reach the clinic in ∼30 y are the echinocandins.[2] This paucity of new antimicrobial drugs is further complicated given the widespread and ubiquitous evolution of resistance.[3] Surveys of environmental microorganisms reveal that collections of microbial resistance genes (termed the antibiotic resistome) are genetically diverse,[3-5] widespread across multiple environmental niches,[6] and pre-date the modern antibiotic era by millennia.[4] To support these conclusions, experience has shown time and again that the success of any new antimicrobial is ultimately undermined by the evolution of resistance that follows. Most recently the emergence and worldwide dissemination of resistance determinants NDM-1 and MCR-1 have breached the efficacy of carbapenems and colistin respectively, "last-resort" antibiotics in our arsenal to combat gram-negative bacterial pathogens.[7-9] Antimicrobial resistance is a serious threat to public health and as we enter a potential post-antibiotic age there is a dire need for novel treatment strategies to combat microbial infections.

Pathogenic fungi have emerged over the past decades as an increasing global threat to human health. Fungi are generally opportunistic in nature, preying on hosts with compromised immune systems due to infection with HIV or as a consequence of modern medical breakthroughs like chemotherapy and organ transplantation. They are the causative agents of a spectrum of diseases ranging from superficial infections affecting 1.7 billion individuals worldwide to invasive infections that kill 1.5 million humans per year.[10] Notably, epidemiological data for fungal infections are unacceptably poor due to a lack of standards for reporting fungal disease and problems with misdiagnosis. Thus, fungal pathogens are likely an even greater contributor to human morbidity and mortality than current estimates. Species of *Candida*, *Cryptococcus*, *Aspergillus*, and *Pneumocystis* are at the forefront of fungal infections, accounting for an

estimated 90% of human mortality cases.[10] Problems of antifungal drug resistance, both intrinsic and acquired, are common among all of these species across multiple antifungal drug classes. For example, the newest class of antifungal drug, the echinocandins, is ineffective against *Cryptococcus* species leaving the treatment of choice for cryptococcal meningitis reliant on medications developed in the 1950s that are plagued with problems of host toxicity.[11] Further, the increased deployment of the azole antifungals, both prophylactically and to treat active infections, has increased the prevalence of fluconazole-resistant *Candida* infections to ∼3,400 annually in the United States alone, warranting the Center for Disease Control and Prevention to rank fluconazole-resistant *Candida* as a serious threat, the same threat level as methicillin-resistant *Staphylococcus aureus* (MRSA).[12]

A promising strategy for combatting resistant microbes is to extend the lifespan and efficacy of our currently employed drugs by using combination therapy. Combining drugs has the potential to confer enhanced efficacy and specificity compared to individual drug treatments and can slow the evolution of resistance.[13,14] Further, by carefully selecting specific drug combinations, microbial drug resistance may not only be neutralized but also reversed through a process called selection inversion.[15] Combination therapy is already the treatment of choice for many infectious diseases including HIV,[16] tuberculosis[17] and malaria.[18] Consequently, the use of drug combinations to treat fungal pathogens has garnered considerable interest over the past several years. This review will describe the advantages of combination therapies and will highlight those antifungal drug combinations that show considerable promise in treating invasive mycoses.

Major classes of antifungal drugs

The therapeutic options for treating invasive fungal infections remain limited to only 3 structurally distinct classes of compounds. In contrast, there are over 2 dozen classes of antibacterials and 6 distinct classes of antiretroviral drugs available for clinical use.[19] This is in large part due to the close evolutionary relatedness between humans and fungi, limiting the number of unique fungal cellular targets that can be exploited for drug development.

The polyenes (Fig. 1) are broad-spectrum natural product antifungals originally discovered in the 1950s. For decades it was accepted that the amphipathic nature of the polyenes allowed them to directly interact with the membrane lipid ergosterol, resulting in pore formation, membrane permeabilization, cell leakage and ultimately cell death.[20] Recently, elegant structure activity studies concluded channel formation is not the primary killing mechanism and that polyenes instead form large extramembranous aggregates that extract the essential membrane-lipid ergosterol from the plasma membrane (Fig. 1).[21] Since ergosterol serves as a central molecular node for multiple essential cellular processes, the ultimate cause of death is likely multifactorial. Given the structural similarities between ergosterol and the functional mammalian analog cholesterol, polyenes cause serious problems of systemic toxicity and nephrotoxicity in human patients when used in the clinic.[20] Consequently, several lipid formulations of amphotericin B have been developed with improved safety profiles and these continue to be widely deployed to treat life-threatening disseminated and invasive mycoses.[22,23] Despite their long-term use in the clinic, resistance to the polyenes remains extremely rare.[2,24]

The azoles are synthetic compounds first introduced as antifungals in the 1980s. They function by disrupting ergosterol biosynthesis through inhibition of the cytochrome P-450-dependent enzyme lanosterol 14-α-demethylase (Fig 1).[2] Subsequent ergosterol depletion and accumulation of toxic ergosterol precursors result in induction of severe membrane stress on the cell, thus inhibiting growth.[2] Azoles are chemically classified as either imidazoles if they possess 2 nitrogen atoms in the azole ring, or triazoles if they have three. Imidazoles are generally employed to treat superficial infections and only the triazoles fluconazole, itraconazole, voriconazole, and posaconazole are approved as drugs for systemic infections due to their favorable pharmacokinetic and safety profiles.[20] Given their low toxicity, they are widely used in the clinic as an initial therapy for most fungal infections, and as a prophylactic treatment for high-risk patients.[22,25,26] Unfortunately, given their widespread use, resistance to the azoles is widespread particularly in *Candida* species.[12]

The echinocandins are the newest class of antifungal to reach the clinic, first entering the market in 2001. These molecules are large semi-synthetic lipopeptides that inhibit the cell wall enzyme complex β-1,3-D-glucan synthase, disrupting cell wall integrity and resulting in fungal cell death (Fig. 1).[27] Currently, there are 3 echinocandin drugs available: caspofungin, anidulafungin, and micafungin. Although echincoandins are well tolerated with little to no side effects, they are poorly absorbed when administered orally and completely ineffective against *Cryptococcus* or *Fusarium* species.[27] Echinocandin resistance has been reported in both laboratory and clinical settings, and the incidence of echinocandin resistant infections continues to rise.[28,29]

Finally, although the rapid emergence of resistance to the fluorinated pyrimidine flucytosine precludes its use as a single agent, it is often combined with amphotericin B. This antifungal drug combination is the standard treatment for infections caused by *C.*
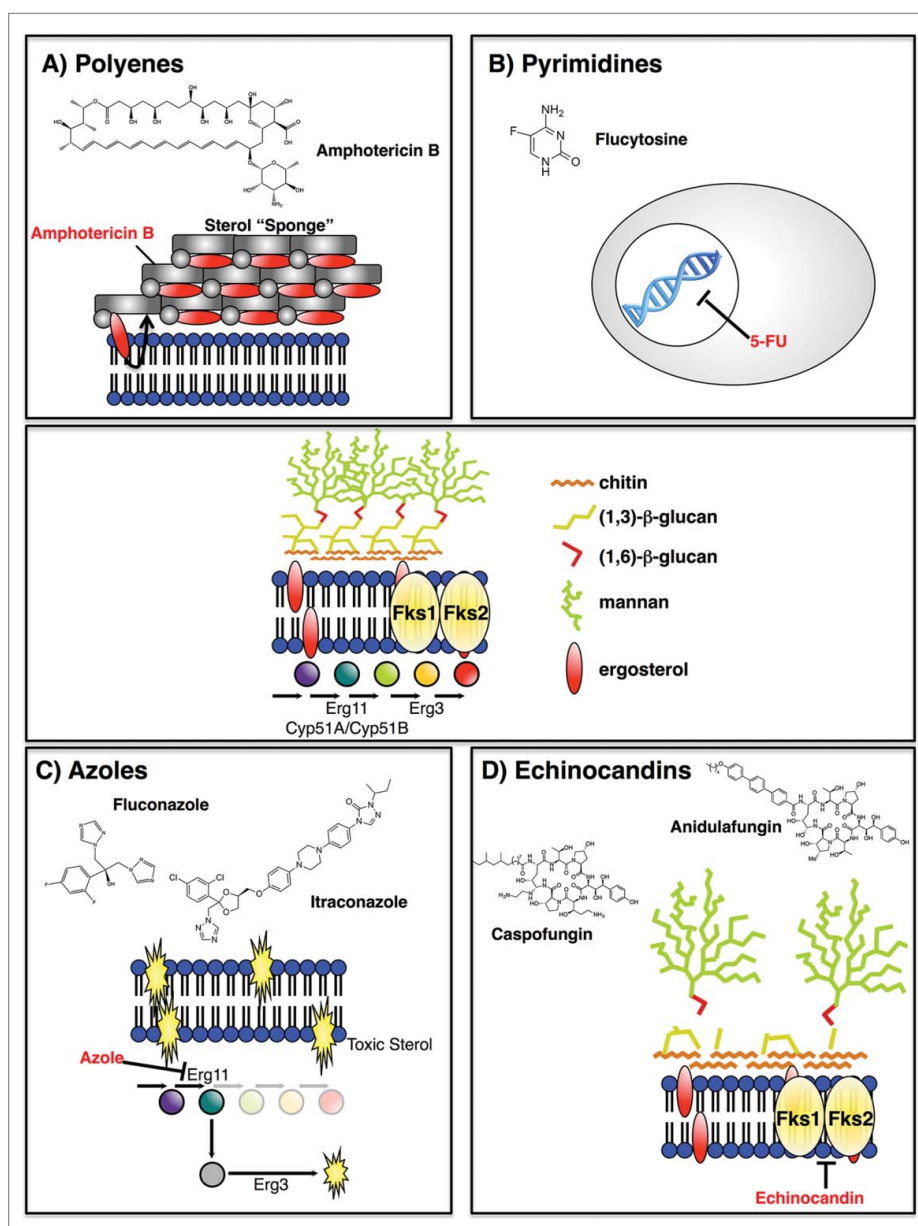
Figure 1. Structures and mechanisms of action of clinically relevant antifungal drugs. Fungal cells consist of a phospholipid membrane bilayer containing the sterol ergosterol. Ergosterol is synthesized within the cell in the endoplasmic reticulum. Fungi also possess a complex cell wall consisting of (1,3)-β-D-glucans covalently linked to (1,6)-β-D-glucans as well as chitin, mannans, and cell wall proteins (center panel). (A) Polyenes such as amphotericin B primarily exist in the form of large extramembranous aggregates that extract ergosterol from lipid bilayers. Structure of amphotericin B is shown. (B) Pyrimidines such as flucytosine becomes rapidly deaminated in the cytosol to generate 5-fluorouracil (5-FU) by fungal specific cytosine deaminases. 5-FU acts as a potent antimetabolite that causes RNA miscoding and inhibits DNA synthesis. Structure of flucytosine is shown. (C) The azoles function by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by *ERG11* (*C. albicans* and *C. neoformans*) or *cyp51A* and *cyp51B* (*A. fumigatus*), causing a block in the production of ergosterol and the accumulation of a toxic sterol (yellow sunburst) produced by Erg3. This toxic sterol exerts a severe membrane stress on the cell. Structures of the clinically used azoles fluconazole and itraconazole are shown as examples. (D) The echinocandins act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase (encoded by *FKS1* in *C. albicans*, *C. neoformans*, and *A. fumigatus* and by *FKS1* and *FKS2* in *C. glabrata* and *S. cerevisiae*) and thereby cause a loss of cell wall integrity and severe cell wall stress. Structures of the clinically used echinocandins caspofungin and anidulafungin are shown as examples.

neoformans, C. gattii, and other less common fungal species.[10,11] Upon entry into the cytosol, flucytosine (also referred to as 5-fluorocytosine) becomes rapidly deaminated to generate 5-fluorouracil (5-FU) by fungal-specific cytosine deaminases.[30] 5-FU acts as a potent antimetabolite that inhibits DNA synthesis and causes RNA miscoding, which ultimately impairs protein synthesis (Fig. 1).

Drug combinations to expand the therapeutic repertoire

Advantages of drug combinations

Combining antifungal drugs for treatment of invasive fungal infections has numerous advantages over monotherapy. In particular, drug combinations can delay or even prevent the development of resistance by rapidly reducing the pathogen population. Furthermore, it generally requires several mutations to accumulate to confer resistance to 2 drugs rather than one. Another combination strategy is the administration of an antibiotic in combination with an inhibitor of resistance enzymes. β-lactam antibiotics are routinely combined with inhibitors of serine β-lactamases, for example.[31] Various studies have highlighted the potential of reversal of antibiotic resistance through combination treatment.[15] Suppressive drug combinations have been shown to invert selective pressure to disfavor drug resistant mutants.[32] The type of drug interactions can change in response to mutations[33] and drug combinations can become synergistic in resistant mutants.[34] Selection inversion can also occur when resistance to one drug also confers sensitivity to the second drug, a phenomenon called collateral sensitivity.[35] Most of these studies were done in vitro and need to be validated in vivo. Further, synergistic drug combinations allow for lower doses of each drug and shorter treatment duration reducing host-toxicity.[13,14] Finally, compound combinations might improve fungicidal efficacy through synergy and result in greater therapeutic effect and broader activity than can be achieved with either drug alone.[36]

The concept of synthetic lethality and its application to drug combinations

An additional advantage of combination therapy is that is has the potential to unveil a plethora of additional antifungal targets given that eukaryotic genomes are proven to be highly interconnected and functionally redundant. This is especially true for combinations of antifungal drugs with non-antifungal bioactive compounds. The complexity of cellular networks and the multifactorial nature of many diseases suggest that multi-component therapies might be more effective than single agents. Biological systems contain many features such as crosstalk, feedback and feed-forward loops which systems biology is only beginning to unravel. The functional redundancy and extensive buffering in biological networks is evident from the fact that only ∼1,000 of the ∼6,000 genes in budding yeast are essential.[37] Systematic screens have explored this robustness by mapping genetic interactions in the model organism *Saccharomyces cerevisiae*.[38] A genetic interaction between 2 genes is observed when a phenotype caused by a mutation in one gene is exacerbated by a mutation in another gene such that the combined effect exceeds the sum of the individual effects.[39] Synthetic lethality represents the extreme case of a negative genetic interactions.[1,40] Genome-wide analyses uncovered that most single deletion strains are sensitive to additional perturbations, such as a second genetic perturbation[38] or environmental and chemical stresses.[41] The mapping of over 200,000 genetic interactions suggests that effective antifungal therapies may require the inhibition of multiple cellular targets simultaneously.[42-46]

For example, in *S. cerevisiae* the echinocandin target *FKS1* and its paralog *FKS2*, encode the biosynthetic enzyme for (1,3)-β-D-glucan synthesis. Genetic interaction networks have highlighted that *FKS1* is synthetic lethal with *CHS3*, a chitin synthase required for the synthesis of chitin,[38] and pharmacological inhibition of chitin synthases with nikkomycin strongly potentiates caspofungin against numerous fungal pathogens.[47,48] Further, *FKS1* is synthetic lethal with *CNB1*, the regulatory subunit of calcineurin and calcineurin inhibitors exert potent synergy with echinocandins against diverse fungi.[49] While these examples highlight the power of genome-wide genetic interaction maps at predicting small molecule interactions, targeting 2 genes that genetically interact does not always result in successful pharmacological synergy.[50,51] However, computational approaches have been developed that predict small molecule synergies based on gene expression data[52] and chemical-genetic interactions[51] that can increasingly be deployed to guide combination drug development.

Mechanisms of drug interactions

Various efforts have been made to classify mechanisms of drug interactions.[53-55] However, many drug combinations have multiple mechanisms and do not fall cleanly into a single category. The combination of 2 drugs might be synergistic because compound one is effective against a disease on its own and the second drug increases its effective concentration at the target site (Fig. 2).[54] In this case the second drug does not have a direct effect of its own. It could affect pharmacokinetics of the first drug by increasing the rate or extent of absorption or the distribution. It could also target the first drug to the intended site of action or slow down metabolism or elimination of the first drug. This type of interaction has also been described as the bioavailability model.[50] Targeting of the fungal cell membrane by azoles and amphotericin B may explain synergism of these drugs with flucytosine.[56,57] Alternatively, 2 drugs that target different stages of the same biological pathway or even the same protein (i.e., the same disease component) can also
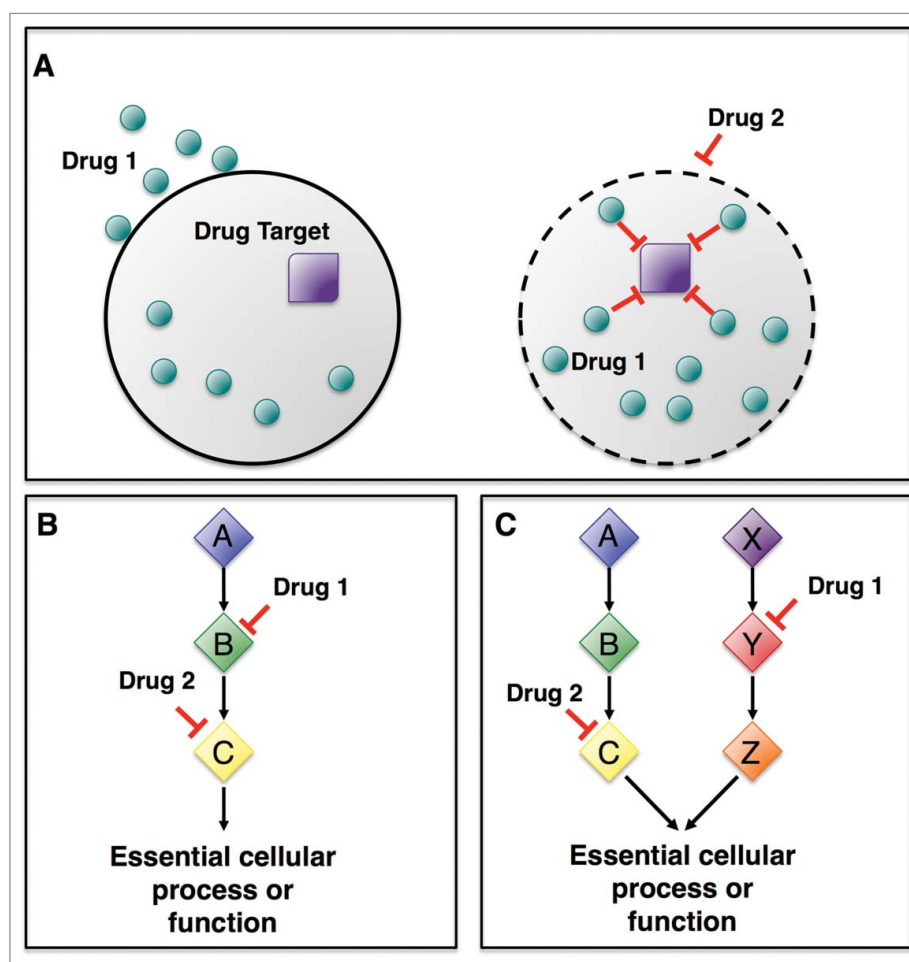
Figure 2. Mechanisms of drug potentiation. (A) Drug potentiation through the bioavailability model suggests 2 drugs will be synergistic if one drug's action (Drug 2) helps another drug's availability (Drug 1) in the target cell. This could be achieved by increasing a drugs entry into the cell, by decreasing a drug's degradation or by affecting the pharmacokinetics of a drug within a host. (B) Alternatively, 2 drugs that target different stages of the same biological pathway can enhance pharmaceutical efficacy when combined, often in an additive manner. These combinations allow greater effects and/or reduced toxicities due to lower single doses. (C) A final model for drug synergy, the parallel pathway inhibition model, suggests 2 drugs will be synergistic if they target 2 proteins on parallel pathways that converge on an essential process.

increase pharmaceutical efficacy when combined, generally in an additive manner (Fig. 2). These combinations allow greater effects or reduced toxicities due to lower single doses. Both, terbinafine and azoles, target fungal ergosterol biosynthesis and impair the function of the cell membrane and when combined can improve efficacy.[58,59] Finally, combination treatment with drugs that target separate cellular pathways converging on the same essential biological function can result in synergistic drug interactions, analogous to synthetic lethal interactions between 2 genes as mentioned above (Fig. 2). The drug interaction network of 21 antibiotics revealed synergistic interactions between specific classes of antibiotics that target different cellular functions.[60] Combinations of echinocandins with azoles and polyenes are synergistic and target the fungal cell wall and membrane simultaneously.[61,62]

Concepts and terminology for combination therapy

Various models and concepts have been developed to study and assess antimicrobial compound combinations *in vitro* and *in vivo*.[53,63] These tools have been reviewed extensively in the context of the debate about the definition of synergy.[63] In this section we will discuss the 2 main mathematical models that underlie most approaches developed to evaluate the effect of compound combinations. Both approaches consist of a conceptual model used to predict the expected result for a combination and the phrases used to categorize the results.

The first model was defined by Siegfried Walter Loewe and is based on the assumption of additive interactions.[64] Loewe assumed that an agent cannot interact with itself, and consequently, Loewe additivity is observed when an

agent is combined with itself and is the term used for indifferent combinations (Fig. 3B). In contrast, Chester Ittner Bliss assumed that the relative effect of a drug at a specific concentration is independent of the presence of another drug and Bliss independence is used to describe indifferent combinations (Fig. 3C).[65] For both, the additive and the multiplicative model, if the effect of a combination is better than expected the combination is said to be synergistic, and results worse than expected indicate antagonistic combinations. Many sophisticated approaches have been developed to deal with the complex nature of compound interactions that are based on the additive or multiplicative models just described.[63]

Quantification of compound interactions in the laboratory is traditionally done by calculation of fractional inhibitory concentration (FIC) index (FICI) based on data obtained with the checkerboard method, a simple 2-dimensional array of serial dilutions of 2 test compounds (Fig. 3A).[66] Other methods to assess compound interactions include E-test, time-kill and disk diffusion assays.[67,68] Due to its simplicity, the checkerboard method is the most commonly used approach to assess compound interactions *in vitro*. Checkerboard results are usually interpreted by constructing an isobologram based on dose pairs that give a specified effect (Fig. 3B). The axes of this graph are the doses of the 2 drugs, and
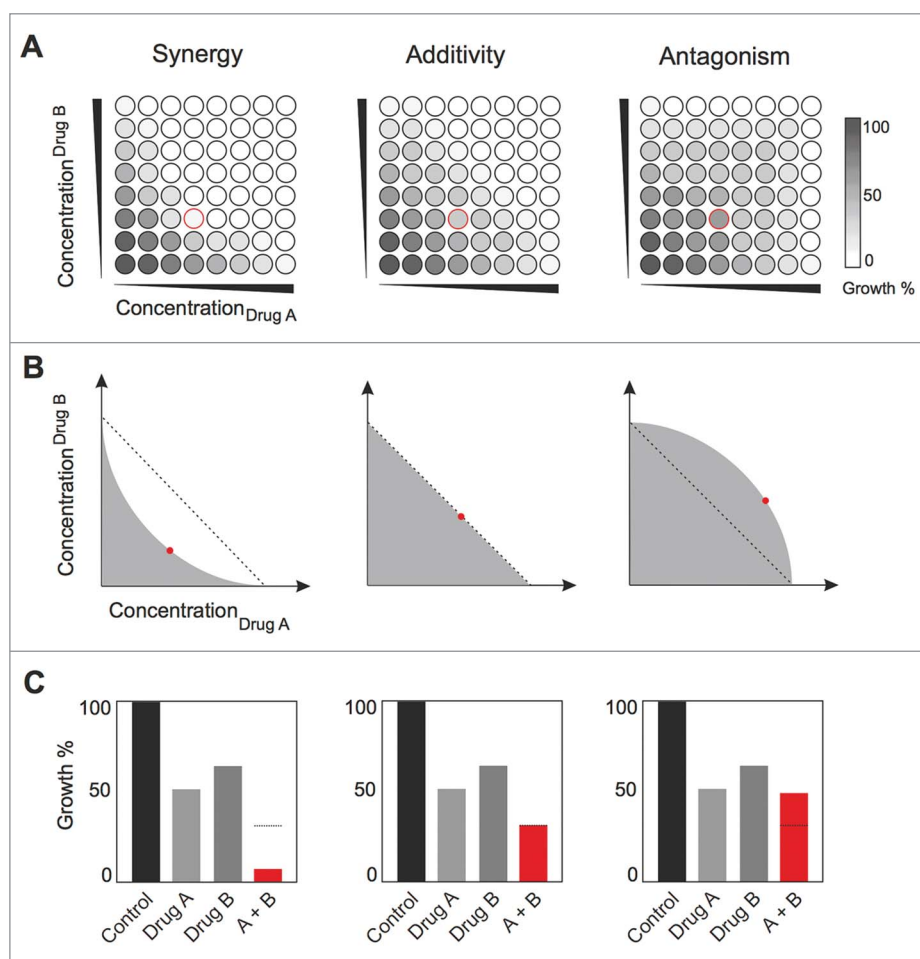

Figure 3. Loewe additivity and Bliss independence to define drug interactions. (A) Checkerboards that are representative of synergistic, indifferent and antagonistic drug interactions. Concentrations of drugs A and B increase along the x and y-axis, respectively. (B) Representative isoboles for synergistic, additive and antagonistic drug interactions based on the Loewe model. The x and y-axis represent the concentration of drugs A and B necessary to achieve a defined growth inhibition level *X*. The dashed line indicates additivity and is constructed by connecting the IC_X values for drugs A and B. Concentration pairs for drugs A and B used in combination to achieve the same growth inhibition *X* are added to this plot (red dots). The position of these points below, on or above the line result in concave, linear or convex isoboles and indicate synergistic, indifferent or antagonistic drug interactions, respectively. (C) Examples of drug interactions that are synergistic, indifferent and antagonistic according to the Bliss independence model. Growth in the presence of drugs A and B (red bars) is compared to the expected growth in the presence of the drug combination (E_{combo}) based on treatment with drugs A and B alone: $E_{combo} = G_{Drug\ A} {}^* G_{Drug\ B}$ (indicated by dashed line). If the observed growth is less than expected, a drug combination is classified as synergistic. Conversely, more growth than expected indicates an antagonistic drug interaction.

the axial intercepts represent the dose of each agent that when used alone cause growth inhibition. If the selected effect is half the maximum effect (E_{max}), then the intercepts are the ED_{50} values. A linear isobole represents the expected concentration pairs when the 2 compounds do not interact ('indifference'). Deviation below the linear isobole indicates synergism and deviation in the other direction is indicative of antagonism (Fig. 3B). The deviation from indifference can be quantified by calculating FICI values. The FICI is defined as the sum of the FICs of each individual drug tested:

$$FICI = FIC_{Drug\ A} + FIC_{Drug\ B}$$

$$= \frac{MIC_{Drug\ A\ in\ combination}}{MIC_{Drug\ A\ alone}} + \frac{MIC_{Drug\ B\ in\ combination}}{MIC_{Drug\ B\ alone}}$$

In terms of the Loewe additivity model, we see indifference when FICI $= 1$, which is the same as to say that combining equal amounts of 2 indifferent compound yields the same effect as doubling the amount of a single compound. In terms of the isobole, this represents a point on the linear isobole.

When reproducible variations from an FICI of 1 are observed, the inherent inaccuracy of MIC methodologies and the question of biological relevance must be considered. Thus, conservative interpretation of results has been proposed by restricting interpretations of "synergy" to FICI ≤ 0.5 and "antagonism" to FICI > 4 while $0.5 <$ FICI ≤ 4 indicates "indifference."[69] These interpretative categories are based on the assumption that checkerboard assays are carried out with 2-fold dilutions of compounds and that the range of experimental error for MIC determination is within one dilution step. For a result to be synergistic and achieve a FICI ≤ 0.5, the MIC of both drugs has to decrease at least 4-fold. Importantly, a FICI of 0.5001 does not indicate synergy, meaning that if the MIC of one drug only decreases 2-fold, even if the FIC of the other drug decreases significantly, the interaction is reported as indifferent since the FIC of the first drug will be 0.5. Conversely, antagonism is observed when the MIC of at least one drug increases 4-fold. Since the condition for antagonism is FICI > 4, the situation where one drug dramatically increases the MIC of the other drug is included in the definition. As mentioned above, it is hard to assess drug interactions based on a single number (like the FICI) because interactions between compounds are not usually smooth and linear.

Combination therapy with antifungal drugs

The efficacy of various antifungal drug combinations against different fungal pathogens has been assessed

in vitro, in vivo, and in clinical settings.[53,70-72] An advantage of in vitro studies is the ease of the methodology and analysis, making it feasible to test a variety of drug combinations against multiple fungal pathogens. However, checkerboard assays only assess the susceptibility of the fungus itself to the drug combination precluding host factors and pharmacokinetic parameters from being taken into consideration.[73] Factors that contribute to clinical efficacy of a drug combination include fungal virulence and resistance, the host immune condition and their interaction with the therapeutic agents. In addition, in vitro results for activity of specific drug combinations against specific fungi often differ between laboratories.[53] The checkerboard dilution method is most commonly used to assess drug interactions in vitro.[69] However, this method has demonstrated poor inter-laboratory reproducibility due to the fact that it is not standardized. Animal models of invasive fungal infections allow for pharmacokinetics of the administered drugs, tissue burden, and rate of clearance can be assessed. However, animal models generally do not account for a suppressed immune system and pharmacokinetics in animals differ from this in humans.[73] Despite their limitations, in vitro and in vivo studies are important for framing new hypotheses. Carefully designed clinical trials constitute the ultimate reference for treatment guidelines. The downsides of clinical data include the heterogeneous nature of subjects, the variety of fungal pathogen isolates, as well as comorbidity and dose verification across different centers. Since clinical data are the most informative with respect to treating systemic fungal infections, the following sections will focus on clinical studies that assess antifungal-antifungal drug combinations.

Cryptococcus

Current recommendations for the treatment of cryptococcal meningitis consists of 3 phases: an initial induction therapy for 2 weeks, followed by consolidation therapy for 8 weeks, and subsequent maintenance therapy for 6–12 months or until restoration of host immunity.[74] Treatment options for cryptococcosis are limited to amphotericin B, flucytosine, and fluconazole, alone or in combination. The induction therapy is an efficient fungicidal regimen, typically consisting of amphotericin B with flucytosine, and consolidation and maintenance therapies are usually monotherapies of fluconazole.[74] The other triazoles show activity against cryptococcal isolates in vitro,[75] but robust studies with these agents are not available yet. The combination of amphotericin B and flucytosine is the 'gold standard' for treatment of cryptococcal meningitis in the Infectious Disease Society of America and the World Health Organization treatment guidelines.[74,76] Several

clinical studies have demonstrated superior efficacy of this combination using different mycological endpoints and observed faster cerebral spinal fluid sterilization, fewer relapses and lower mortality.[11,77-81] Patients receiving the amphotericin B and flucytosine combination need to be closely monitored for renal dysfunction, however studies on cryptococcal meningitis had previously shown that this combination reduces toxicity of the polyene component because clinicians can give lower amphotericin B doses for a shorter time.[77,82] Further, there is evidence that lower doses of flucytosine are just as fungicidal.[83] Flucytosine availability is limited in settings where disease burdens and mortality are highest and the inferior combination of amphotericin B and fluconazole is often an alternative in settings with limited resources.[23,75]

Higher order combinations of antifungal agents have been tested against *Cryptococcus neoformans*. The combined effect of amphotericin B, flucytosine and fluconazole has been assessed in a mouse model of cryptococcal meningitis and in clinical settings,[81,84,85] however, the triple combination did not outperform amphotericin B and flucytosine.

Candida

The echinocandins demonstrate fungicidal activity against most *Candida* species and are therefore generally recommended as primary therapy.[25,86] Treatment with fluconazole is suggested as an alternative initial therapy. Combination treatment is not recommended for invasive candidiasis (IC) in the latest guideline published by the Infectious Diseases Society of America, since combinations of antimycotic agents are generally not more effective against *Candida* species compared to single agents.[25] The largest clinical trial involved 219 non-neutropenic patients with mostly *C. albicans* infections and found similar clinical outcomes for fluconazole alone compared with fluconazole and amphotericin B treatment.[87] There is limited evidence of effective combinations against IC. For example, amphotericin B in combination with flucytosine has been shown to be more effective than fluconazole at treating peritonitis.[88] Further, the combination of amphotericin B and flucytosine has also been shown to be more effective than amphotericin B alone for treatment of *Candida* meningitis, an uncommon manifestation of systemic candida infections.[89]

Aspergillus

Voriconazole is currently the recommended first line therapy for invasive aspergillosis (IA),[90] improving clinical outcomes for patients with transplantation and leukemia (response rate 53%) while reducing severe side effects from amphotericin B.[91] Isavuconazole, a newer triazole, has been approved for treatment of IA and is better tolerable than voriconazole.[92] Echinocandins have been approved for salvage therapy, but given their fungistatic activity against *Aspergillus*, efficacy is only 33% in severely immunocompromised patients.[93] The search for effective therapeutics is therefore ongoing. A systematic review of 7 observational studies and one randomized controlled trial (RCT) up until 2011 included data for 1071 patients and found evidence for combination antifungal therapy for primary IA to be conflicting and stresses the need for well-designed RCTs.[94] Retrospective reporting is susceptible to biases because outcomes in patients with different underlying diseases are compared, combination treatments are used in cases of severe disease, and selective reporting of positive outcomes.[73] The weak support for combination treatment is most likely due to the variety of antifungal combinations tested, the nature of IA investigated (6 studies looked at primary IA and 2 at salvage therapy for IA) and the heterogeneity of patient populations, treatment regimens and endpoints. Meta-analysis of 16 studies (including 1833 patients) comparing combinations of echinocandin with triazoles or amphotericin B against non-echinocandin monotherapy revealed that combination treatment improves clinical outcomes in salvage settings.[95] Due to the lack of definite proof that combination therapy is beneficial,[96,97] initial combination therapy is not routinely recommended for IA, but is considered in high-risk patients.[90]

Combinations of antifungal and non-antifungal drugs

Given the complex genetic landscape that exists in eukaryotic genomes coupled with the therapeutic superiority of the amphotericin B with flucytosine drug combination highlighted above, there is broad interest to identify other molecules that enhance the efficacy of our current antifungals. While none are yet in clinical use, a subset of the most promising drug combinations that show efficacy against pathogenic fungi are elaborated on below.

HSP90 inhibitors

One of the best-characterized examples of compounds capable of potentiating our current antifungals includes inhibitors of the essential molecular chaperone Hsp90. In pathogenic fungi, targeting Hsp90 function as an antifungal strategy holds considerable promise given that it governs crucial cellular responses to drug-induced stress.[98-100] In *S. cerevisiae, C. albicans, C. neoformans* and *C. gattii* pharmacological inhibition of Hsp90 impairs the evolution of azole resistance and potentiates

azole activity *in vitro*.[101-103] Moreover, in *C. albicans, C. glabrata* and *A. fumigatus*, Hsp90 enables basal tolerance and resistance to the echinocandins.[104-107] In *Caenorhabditis elegans* and murine models of fungal pathogenesis, combination therapy with Hsp90 inhibitors with caspofungin or fluconazole improves survival upon infection with *A. fumigatus* or *C. albicans* and *C. neoformans*, respectively.[103,108] Furthermore, in mouse models of *C. albicans* disseminated disease, genetic compromise of fungal *HSP90* expression reduces virulence[109] and enhances the therapeutic efficacy of fluconazole and caspofungin.[104,108] Similarly, genetic compromise of *A. fumigatus* Hsp90 abrogates virulence in a murine model of invasive aspergillosis.[107] Finally, in an *in vivo* rat catheter model of *C. albicans* biofilm infection, clinically relevant Hsp90 inhibitors such as 17-AAG transform fluconazole from completely ineffective to highly efficacious without showing any signs of host toxicity.[110] Thus, efforts to create fungal specific Hsp90 inhibitors holds considerable promise as a combination agent with multiple antifungals for the treatment of diverse fungal pathogens.

Calcineurin inhibitors

Hsp90 is one of the most highly connected hubs in cellular networks, interacting with an estimated 10% of the yeast proteome.[111,112] Chemical genomic studies in *C. albicans* identified a multitude of Hsp90 genetic interactors important for cellular responses to azoles and echinocandins,[113] suggesting that Hsp90 may have pleiotropic effects on circuitry governing drug resistance. One well-characterized Hsp90 client protein important for governing responses to antifungal-induced stress is the protein phosphatase calcineurin.[104] In pathogenic fungi, calcineurin regulates a myriad of physiological processes including cell cycle progression, cation homeostasis, morphogenesis, virulence, and antifungal drug responses.[49,114] Pharmacological inhibition of calcineurin in *C. neoformans* abrogates growth at 37°C,[115] potentiates the activity of both azoles and echinocandins in *C. albicans*,[101,102,104,116,117] and renders the fungistatic azoles fungicidal against multiple *Candida* species.[36] Further, calcineurin inhibitors cyclosporine A and FK506 act synergistically with azoles against *C. albicans* biofilms both *in vitro* and *in vivo*, implicating calcineurin as a key modulator of azole resistance during biofilm growth.[118] In *A. fumigatus*, calcineurin inhibitors enhance echinocandin activity, and transform the fungistatic activity of caspofungin to fungicidal.[119,120] Calcineurin inhibitors also show potent activity against azole- and echinocandin-resistant strains of *A. fumigatus*.[121] Recently, it was shown that calcineurin orchestrates dimorphic transitions, antifungal drug responses, and virulence of the fungal pathogen *Mucor circinelloides*.[122,123] The challenge that remains for antifungal drug discovery is to develop fungal specific calcineurin inhibitors or to identify fungal-specific calcineurin effectors that can be targeted pharmacologically.

Lysine deacetylases and lysine acetyltransferases

Additional cellular targets that have garnered considerable attention as an antifungal combination target are lysine deacetylases (KDACs) and lysine acetyltransferases (KATs). These enzymes catalyze the removal or addition of acetyl groups from lysine residues present on histones and other cellular proteins. The broad-spectrum KDAC inhibitor trichostatin A (TSA) potentiates azole activity in *C. albicans*.[124,125] Further, a Hos2 KDAC inhibitor, MGCD290 (Mirati Therapeutics, San Diego, CA, USA), displays synergistic activity with azoles and echinocandins against diverse *C. albicans* drug-resistant clinical isolates *in vitro*.[126,127] Additional *in vivo* studies coupled with promising preliminary clinical trials have supported the use of MGCD290 in combination with fluconazole to treat *C. albicans* infections.[128] KATs also modulate azole resistance. Genetic impairment of *C. albicans ADA2*, which encodes a component of the Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex, confers hypersensitivity to fluconazole due to impaired upregulation of efflux pumps Cdr1 and Mdr1.[129] Further, deletion of the *C. albicans* KAT gene *RTT109* confers hypersusceptibility to macrophages, altered metabolic gene expression, and induction of a weaker inflammatory response, culminating in attenuated virulence in a murine infection model.[130,131] Deletion of *RTT109* also confers increased sensitivity to echinocandins, and other genotoxic stresses such as hydroxyurea and methyl methane sulfonate.[130] Thus, these KATs represent an excellent pharmacological target for future drug development.

Other targets

Other pharmacological inhibitors that target a variety of cellular processes have been reported to synergize with current antifungals. Many of these inhibitors are identified in the Antifungal Synergistic Drug Combination Database (ASDCD), a curated collection of 210 antifungal synergistic drug combinations involving 105 drugs from the literature (last updated 2013).[132] Such compounds include, but are not limited to, pharmacological inhibitors of ADP-ribosylation factors,[133] protein kinase C [134], fungal sphingolipids,[135] and protein translation[136] in order to potentiate fluconazole. Further, tamoxifen and other triphenylethylene-based

estrogen receptor antagonists have antifungal activity against *C. neoformans*,[137,138] and potentiate the activity of azoles, polyenes, and echinocandins in diverse fungal species.[139] Finally, the antidepressant sertraline combined with fluconazole provides improved activity relative to either drug alone in both an invertebrate model of cryptococcosis[138] and in a mouse model of disseminated cryptococcosis,[136] likely due to its membrane perturbing effects in fungi.[138]

High-throughput screens

In addition to the several promising examples highlighted above, scientists in both academia and industry have leveraged the power of high-throughput screening methodologies in order to uncover novel antifungal combinations. Compounds that enhance the activity of known antimicrobials in *S. cerevisiae* have been identified both by focused small molecule library screens and by computational methods.[140-142,51] Further, several high-throughput screening endeavors have searched for molecules that potentiate the azoles and overcome azole resistance in fungal pathogens themselves.[138,139,143,144] Such compounds that increase azole efficacy are often found to target membrane function or sphingolipid biosynthesis.[138] One such example is the antimycobacterium compound clofazimine, which induces a cell membrane stress in fungi and enhances the efficacy of fluconazole in an *in vivo Galleria mellonella* model of fungal pathogenesis.[139] Further, screens performed as part of the NIH-Molecular Libraries and Probes Screening network identified a class of indole derivatives that restore fluconazole susceptibility to resistant *C. albicans* isolates.[143] Finally, high-throughput screens using microbial extracts identified the natural product cyclic hexadepsipeptide beauvericin as a potentiator of ketoconazole against *C. parapsilosis*.[145]

In addition to employing high-throughput screens to look for agents that enhance the activity of the azoles, studies have identified molecules that potentiate the other antifungal drug classes. Recently, we screened sublethal concentrations of 6 known antifungals in combination with ~3,600 bioactive compounds against diverse fungal species to uncover synergistic drug combinations.[139] This dataset, termed the Antifungal Combination Matrix (ACM), identified ~1550 chemical combinations that abrogate fungal growth often in an antifungal- and species-specific manner.[139] The number of compounds that potentiated amphotericin B and caspofungin far exceeded the number of compounds that potentiated the azoles. Another study has employed high-throughput screens to assay >300,000 molcules in search for chemical entities that specifically perturb cell wall integrity, the mode-of-action of the echinocandins.[146] Three benzothioureas were discovered that exhibited anticryptococcal activity and acted in an additive manner with caspofungin.[146] This was profound given that the echinocandins do not show activity against *Cryptococcus* species. The molecular mechanism by which these benzothioureas elicited activity was through the inhibition of the cell wall integrity MAP kinase cascade.[146]

Targeting resistance determinants

Small molecules that target resistance determinants are often effective pairings with currently available antimicrobials to combat drug resistant isolates. Examples of such drug therapies include drug combinations of β-lactam drugs with inhibitors of β-lactamase resistance enzymes.[31] A recent study with *C. glabrata* identified a small molecule (iKIX1) that inhibits azole efflux pump expression by disrupting the interaction between the Mediator complex and the transcriptional activator Pdr1.[147] Follow-up *in vivo* studies demonstrated that iKIX1 improved the therapeutic efficacy of fluconazole against fluconazole-resistant *C. glabrata* in both *G. mellonella* and mouse models of infection.[147] It is difficult to predict which, if any, of these antifungal combinations will represent a breakthrough in the way we treat fungal infections, but it is promising to see the diverse molecular targets that are being identified.

Conclusions

Antifungal combination therapy is well established for the treatment of cryptococcosis and it is recommended for some hard to treat invasive *Candida* and *Aspergillus* infections. New classes of antifungal agents will improve the chances that combination therapies are more effective than currently available monotherapies. There are several antifungal drugs in the pipeline that target the biosynthesis of chitin, GPI anchor and heme.[148] This comes at a time where legislation including the US Food and Drug Administration's (FDA) Generating Antibiotics Now (GAIN) and the Orphan Drug Acts has been passed by government agencies to push forward the development of antifungal agents that offer increased market exclusivity and several fungal pathogens qualify for both schemes. Many studies have targeted the fungal stress response in combination with antifungal agents and the results are promising. High-throughput screens for antifungal synergistic combinations are revealing further biological pathways and chemical scaffolds that can be targeted to yield effective antifungal drug combinations. The biggest challenge for targeting these pathways

is the identification of fungal-specific compounds or effectors. In addition to new drugs, better diagnostic tools need to be developed. This will improve therapeutic outcomes because of prompt diagnosis and early treatment.[149,150] It will also allow for development of narrow-spectrum antifungals with improved antifungal activity.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

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