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REVIEW

Antifungal adjuvants: Preserving and extending the antifungal arsenal

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ABSTRACT

As the rates of systemic fungal infections continue to rise and antifungal drug resistance becomes more prevalent, there is an urgent need for new therapeutic options. This issue is exacerbated by the limited number of systemic antifungal drug classes. However, the discovery, development, and approval of novel antifungals is an extensive process that often takes decades. For this reason, there is growing interest and research into the possibility of combining existing therapies with various adjuvants that either enhance activity or overcome existing mechanisms of resistance. Reports of antifungal adjuvants range from plant extracts to repurposed compounds, to synthetic peptides. This approach would potentially prolong the utility of currently approved antifungals and mitigate the ongoing development of resistance.

Introduction

The incidence of life-threatening invasive fungal infections has increased significantly in recent decades. This is the result of several factors including a rise in immune suppressive disorders and aggressive medical intervention that has led to a growing population of immune compromised individuals. These individuals are particularly susceptible to infection by fungal pathogens which are typically opportunistic in nature, although it is worth noting that immune competent individuals are also at risk in some cases. The prevalence of these infections is particularly concerning, especially when considering their high associated morbidity and mortality even with therapeutic intervention. Depending on the medical resources available and the site of infection, mortality rates range from 20–40% for *Candida albicans*, 20–70% for *Cryptococcus neoformans*, and 50–90% for *Aspergillus fumigatus*.[1,2]

However, despite the global health burden these infections present, there are very few treatment options available. One universal limitation confronted by those attempting to develop compounds with antifungal activity is that fungi are eukaryotes and therefore share many of the same biochemical processes as their host. This results in difficulties in identifying targets that provide sufficient fungal selectivity. The limited number of distinct targets being exploited by current antifungals is a serious concern as it increases the likelihood for the development of cross-resistance. At present, there are only 4 classes of systemic antifungals, each with their own array of limitations in activity, spectrum, and patient toxicity.

Additionally, antifungal development efforts have not kept pace with the rapid increase in incidence of these infections. The pressing need for new systemic antifungals has resulted in an increased research efforts, both in academia and industry. While this is an encouraging sign, these efforts will require time to progress from bench top to bedside. In fact, it is estimated that the average drug takes 12 y from discovery to approval, although in the field of antifungal development, 30 y may be more realistic.[3,4]

As a means of addressing the limitations of current treatment options and to help mediate the development of resistance to current and future antifungals, there is growing interest in combining antifungal therapies with compounds that either enhance the antifungal's activity directly or that alter the pathogen's susceptibility through circumventing resistance mechanisms. This approach has been used successfully in other fields, most notably in the field of oncology where the rapid development of drug resistance and the need for selectivity closely parallel the situation observed with fungal pathogens.

Limitations of antifungal therapy for serious fungal infections

There are currently only 4 classes of systemic antifungals available: polyenes, a pyrimidine analog, azoles, and

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echinocandins. Only one representative of the first 2 classes are used to treat invasive disease, amphotericin B and 5-flucytosine, respectively. While amphotericin B has been used for over 50 y and remains the gold standard for the treatment of some fungal diseases, its use is limited by lack of oral bioavailability and significant toxicity.[5] While new lipid-based formulations have been developed that improve toxicity, they are often cost-prohibitive.[5] 5-Flucytosine has potent activity and excellent fungal selectivity but is associated with significant toxicity.[6] However, its mechanism of action predisposes it to the rapid development of resistance so it is relegated to use solely in combination therapy.[7] The azoles are the most prolific and widely used class, due to their broad spectrum of activity and limited toxicity profiles.[8] However, they are fungistatic and therefore depend on the host immune response to resolve the infection. The inability of the azoles to kill the invading pathogen affords the fungus an opportunity for adaptation and development of resistance. Moreover, this antifungal class is associated with a number of drug-drug interactions.[9] The echinocandins are the newest class of antifungals and are beginning to supplant the azoles as the agents of choice for candidiasis since they are fungicidal and associated with limited drug-drug interactions, however, they are only available as an intravenously administered formulation and resistance is developing.[10] Echinocandins are also limited by a narrower spectrum of activity than the azoles and lack activity against *Cryptococcus*, a pathogen that kills nearly 650,000 people annually, and several other medically important fungi.[2]

Potential for improving effectiveness of existing antifungal agents

In contrast to the limited repertoire of antifungals, there are abundant reports of compounds, ranging from traditional herbal remedies to FDA-approved medications, that enhance antifungal activity or mitigate resistance. This primarily occurs through inhibition of fungal stress responses that are required for survival in the presence of antifungals or increasing intracellular drug concentrations by either increasing cell permeability or decreasing drug efflux.[11-13] Less frequently, combinations that inhibit or disrupt biofilm formation have also been reported. While reports exist for a wide range of fungi and a plethora of compounds, in this review, we have chosen to focus on the most prevalent human fungal pathogens and examples where the interaction has been mechanistically examined.

Enhancing activity

One approach to improving antifungal therapy is by enhancing the activity of existing antifungals. The most prevalent example of this is through converting a fungistatic monotherapy into a fungicidal combination. This may aid in resolving infections rapidly and limit the development of drug resistance.[14] In some cases, these combinations also improve antifungal activity against resistant strains and isolates, without directly impacting the mechanism(s) of resistance.

Iron homeostasis inhibitors

Iron is essential for a variety of biological processes including oxygen transport, electron transfer, and DNA synthesis and is required for all heme containing proteins. However, excess free iron can be toxic so the concentration and availability of iron is tightly regulated by the host, such that iron is often growth limiting for pathogens during infection.[15] Host mechanisms of iron sequestration result in free iron concentrations of approximately 10^{-18} M in serum.[16] Since iron acquisition is crucial for survival, microbes have developed an array of mechanisms to meet this need, including within the host. While these mechanisms are sufficient to support pathogen growth, iron is often still a limiting nutrient and the addition of small molecule iron chelators can disturb the balance of iron acquisition and utilization.

Several structurally-distinct iron chelators have been shown to have antifungal activity including doxycycline and other tetracycline antibiotics, deferasirox, and lactoferrin.[17-19] However, while standalone activity generally occurs at concentrations above those therapeutically achievable, doxycycline has been shown to enhance the activity of various azoles at much lower concentrations.[17] Azoles inhibit lanosterol demethylase, a key component of the ergosterol biosynthesis pathway. When confronted with this challenge, fungi upregulate several of the ergosterol biosynthesis genes to compensate for the inhibition of lanosterol demethylase.[20] Many of these enzymes require heme cofactors to function and their upregulation increases the cell's demand for iron. Thus constricting the availability of iron inhibits an azole stress response resulting in improved antifungal activity. Additionally, iron chelation has been shown to improve outcome in combination with amphotericin B in a murine model of aspergillosis indicating that the need for iron to tolerate drug stress is not an azole specific phenomenon.[18]

Calcium homeostasis inhibitors

Calcium acts as a second messenger and its release can trigger a range of biological responses in eukaryotes, depending on concentration and location. Calcium signaling is central to multiple processes including cell

division, polarized growth, and stress responses in fungi, including those that occur in response to drug exposure.[21] As such intracellular calcium levels are very tightly controlled and adjusted through the expression and activity of a network of transmembrane pumps at both the plasma membrane and the vacuole, the primary site of intracellular calcium storage.[22] Disruption of the delicate balance between calcium import and storage can result in impaired signaling and an inability to respond appropriately to antifungals.[21]

Depletion of calcium levels, either by chelating extracellular calcium with ethylene diamine tetra-acetic acid (EDTA) or inhibition of calcium importers with benidipine and nifedipine, have been reported to enhance azole activity against *C. albicans*.[23,24] Alternatively, treatment of various fungi with low doses of amiodarone, an antiarrhythmic, results in a rapid increase in the cytosolic calcium concentration through inducing calcium influx and the release of internal calcium stores.[25] While amiodarone alone has modest antifungal activity, its use has been shown to reduce the MIC of fluconazole even against highly resistant strains of *C. albicans* in vitro.

Calcineurin/calmodulin inhibitors

The calmodulin/calcineurin signaling pathway is highly conserved in eukaryotes. In response to stress, calcium is released and binds to calmodulin, which then binds to and activates the phosphatase, calcineurin. Calcineurin in turn dephosphorylates the transcription factor Crz1, which then translocates from the cytoplasm to the nucleus and transcribes a variety of stress related genes. While the components of this pathway are highly conserved, there has been significant rewiring such that the signals this cascade responds to and the processes it is required for vary among fungi. In *Candida albicans* calcineurin is required for growth in serum, for *Cryptococcus neoformans* this pathway is essential for growth at elevated temperatures, and in *Aspergillus fumigatus* it is essential for conidia formation. However in all 3 pathogens mentioned above, calcineurin signaling plays an essential role in antifungal tolerance. Thus, it is not surprising that inhibition of the pathway by genetic or chemical manipulation result in decreased virulence and hyper-susceptibility to various antifungals in *Candida, Cryptococcus*, and *Aspergillus*.[26]

Calcineurin inhibitors

Cyclosporin A and FK506 (tacrolimus) are both immunosuppressives that bind to cyclophilin A and FKBP12, respectively, and form an inhibitory complex with calcineurin.[27,28] These drugs are frequently used in organ transplant recipients and there is some clinical evidence that patients receiving calcineurin inhibitor based immunosuppressive therapy develop fewer invasive fungal infections than those on other regimens.[26]

Disruption of calcineurin function has minimal effect on *Candida albicans* growth under normal laboratory conditions. However, these strains are sensitive to growth under a variety of stress conditions, including growth in serum, and attenuated in virulence.[29] As such, inhibiting calcineurin has significant *in vivo* potential. Additionally, when combined with azoles there is a potent synergy and a fungicidal effect.[30] This enhanced activity has been observed with several azoles and against other *Candida* species that are intrinsically more resistant to the azoles, *C. glabrata* and *C. krusei*.[31]

In *Cryptococcus*, it has been shown that calcineurin signaling is required for growth at elevated temperatures.[32] It is therefore not surprising that calcineurin inhibitors have anticryptococcal activity when tested at 37°C, but not at 24°C.[33] While FK506 is active alone, it also enhances the activity of caspofungin and the fluconazole and result in fungicidal combinations which is a particularly important outcome for the treatment of cryptococcosis.[34,35] In the case of fluconazole, this enhanced activity is not due to calcineurin inhibition but its ability to inhibit multidrug efflux pumps which would result in higher intracellular fluconazole concentrations. FK506 has also been shown to enhance the activity of caspofungin against *Cryptococcus*, in a fully calcineurin dependent manner, although not to a clinically relevant extent since there is only a modest increase in susceptibility and *Cryptococcus* is inherently resistant to echinocandins.

Loss of calcineurin activity in *Aspergillus* results in defects in hyphal extension and invasive growth. *In vivo* this defect manifests as reduced host tissue damage and significantly reduced mortality.[36] Alone, calcineurin inhibitors have minimal in vitro activity against *Aspergillus* species. However, in combination with either azoles or echinocandins there was enhanced, fungicidal activity.[37,38]

Finally, several groups have independently examined clinical isolates of *C. albicans, C. neoformans*, and *A. fumigatus*, from patients receiving long-term calcineurin-inhibitor based immune suppression.[39-41] No differences in susceptibility to various calcineurin inhibitors were detected, indicating that resistance is not being selected for in this patient population. Despite these positive interactions in vitro, the potent immune suppressive effects of cyclosporine A and FK506 limit their potential for use as adjuvants with antifungal therapy.[26] For this reason, non-immunosuppressive analogs of both compounds have been developed and investigated.[33] These fungal specific analogs retain their synergistic activity in vitro, however there is little in the literature to indicate

that they are efficacious *in vivo* and it appears that they are no longer being pursued.

Calmodulin inhibitors

More recently, it has been shown that inhibiting the calcineurin pathway further upstream also results in enhanced azole activity both in *Candida* and *Cryptococcus*.[42] Structurally diverse calmodulin inhibitors have been shown to enhance fluconazole activity, and some combinations resulted in fungicidal activity as demonstrated by time-kill assays.[43] Some of the most promising of these are the triphenylethylene estrogen receptor antagonists, tamoxifen and toremifene. Tamoxifen and toremifene both have antifungal activity on their own at clinically relevant concentrations, but when combined with fluconazole at subinhibitory concentrations produce a fungicidal effect. The combination of subinhibitory concentrations of fluconazole and tamoxifen has been shown to reduce brain fungal burden in a murine model of cryptococcosis.[44]

Hsp90 inhibitors

Heat-shock protein 90 is a molecular chaperone and regulator of a variety of stress responses, including calcineurin signaling, in many fungi. It is also required for several morphogenic changes associated with host environmental adaptation.[11] Recently, it has been shown that depletion of Hsp90 interferes with the development of resistance to azoles and echinocandins.[45,46] As such, inhibition of Hsp90 by the natural product geldanamycin and synthetic analogs, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) and 17-(allylamino)-17- demethoxygeldanamycin (17-AAG) which are being developed as anti-tumor agents, has gained favor as a potential therapeutic strategy.

The addition of any of the above named Hsp90 inhibitors to the growth media reduced the concentration of fluconazole required to inhibit *C. albicans* growth, even though they have no detectable growth inhibitory activity alone. The combination of geldanamycin with fluconazole has also been shown to be fungicidal against *C. albicans* within 24hrs by time-kill analysis. In a *Galleria mellonella* larval model of systemic candidiasis, the combination of either 17-AAG or 17-DMAG with fluconazole resulted in complete rescue and survival of the *G. mellonella*. Additionally, there are reports of Hsp90 inhibitors preventing *C. albicans* biofilm formation, although there are currently no studies to the best of our knowledge that explore the impact of Hsp90 inhibitors on established biofilms. In *Aspergillus*, inhibition of Hsp90 function enhances the activity of both caspofungin and voriconazole in vitro. However, in a similarly designed *G. mellonella* experiment, the combination of geldanamycin and caspofungin prolonged larvae survival but had minimal impact on total mortality.[47]

Selective serotonin reuptake inhibitors

Sertraline is a commonly prescribed selective serotonin reuptake inhibitor (SSRI), a class of compounds used to treat a variety of psychological disorders including depression. Its antifungal activity was first described in 2001 when 3 women receiving sertraline therapy to treat premenstrual dysmorphic disorder experienced remission of their recurrent vulvovaginal candidiasis.[48] In vitro testing confirmed that sertraline exhibited fungicidal activity against several *Candida* strains. It was later confirmed that sertraline, as well as several other SSRIs, have activity against *Aspergillus* as well.[49] Unfortunately, the MICs against these organisms were relatively high compared to established serum levels and interest in translating sertraline to antifungal use stalled.

A decade later, sertraline was rediscovered as a potentiator of the antifungal effect of fluconazole against several yeast species, including *Cryptococcus*.[50] In humans, sertraline inhibits the 5-hydroxytryptamine transporter and in tumor cells has been shown to inhibit translation by disrupting signaling through the mTOR pathway. However, there are no clear fungal orthologs for these targets so genome wide screens were conducted to identify the mechanism(s) by which sertraline exerts its antifungal effect. The cumulative results indicate that sertraline has a variety of effects on fungal cells including altering membrane organization, vesicle transport, and inhibiting protein translation.[51,52] Follow-up studies confirmed that it possesses potent anti-cryptococcal activity alone at concentrations that are similar to the levels that can be achieved in the cerebrospinal fluid and the brain in humans, an extremely relevant niche given the neurotropism of *Cryptococcus*. This activity, as well as the additive effect in combination with fluconazole, has been demonstrated in a murine model of cryptococcosis.[52] Efforts to progress sertraline as an adjunctive therapy for cryptococcosis are on-going and thus far have resulted in a dose finding clinical study.[53]

Statins

Statins are a class of drugs that are commonly prescribed for the treatment of high cholesterol. It has however been observed that they have a variety of other properties including antioxidant, anti-inflammatory, and antimicrobial activity. These compounds have been shown to inhibit the activity of fungal HMG-CoA reductase.[54] HMG-CoA reductase functions in the isoprenoid

pathway which is upstream of the ergosterol biosynthesis pathway. Alone, the concentrations of statins required to inhibit fungal growth are not therapeutically achievable but have been shown to reduce MICs when used in combination with various azoles.

Although there are some discrepancies as to the underlying mechanism(s) of the antifungal effect of statins, it is likely a multifactorial effect. Inhibition of the isoprenoid pathway reduces the availability of the substrates required for sterol synthesis which is then further inhibited by the azoles. It has also been shown that treatment of *C. albicans* with lovastatin results in downregulation of multiple genes whose products are required for sterol biosysnthesis.[55] Finally, there is some evidence to suggest that treatment with statins alters membrane fluidity and may therefore increase cell permeability resulting in higher intracellular drug accumulation.[54]

The interaction between a panel of commercially available statins and several azoles against 2 *Candida* and *Aspergillus* species has been explored. Although the exact nature of the interaction depended on the specific statin/azole/strain combination, antagonism was never observed and the majority of the interactions resulted in growth inhibition at reduced azole concentrations.[56] Notably, this enhancement of azole activity occurred at statin concentrations that are achievable in human serum.

Nonsteriodal anti-inflammatory drugs

Prostaglandins are small lipid molecules that are produced and secreted by a variety of organisms, including humans and some pathogenic fungi, that serve as signaling molecules. In humans, prostaglandins are produced by the activity of 2 cyclooxygenase isozymes, COX-1 and COX-2, which are inhibited by a variety of anti-inflammatory drugs including many over the counter pain relievers. *C. albicans* and *C. neoformans* both produce and secrete prostaglandins and although their function is not fully understood, they may regulate gene expression and serve a role in host immune modulation. Furthermore, addition of prostaglandins to *C. albicans* cultures has been shown to induce filamentation. Treatment with various COX inhibitors decreases the ability of both *C. albicans* and *C. neoformans* to produce and secrete prostaglandins.[57] Additionally, they reduce fungal viability, although it is not clear if the decrease in viability is due to the loss of prostaglandins or an additional role of cyclooxygenases in fungal metabolism. While their standalone antifungal activity occurs at concentrations much higher than serum concentrations of people on anti-inflammatory regimens, several of these compounds have been shown to be synergistic with fluconazole against *C. albicans*.[58] High-dose ibuprofen causes direct damage to the cytoplasmic membrane which likely underlies its ability to synergize with fluconazole.[59]

Plant extracts and essential oils

There are extensive reports of the antifungal activity of various plant extracts, particularly from aromatic herbs. The majority of this work has been conducted using crude extracts and the active component or components are not clearly identified or well characterized. However, in some cases, purified constituents responsible for activity have been identified and studied.

Thymol, a major component of extracts from thyme, has been shown to negatively affect membrane integrity and reduce ergosterol content.[60] Additionally, thymol is synergistic with amphotericin B, fluconazole, and itraconazole against *C. albicans*, and synergistic, neutral, and additive, respectively, against *C. neoformans*.[61] It has been shown to be additive in combination with amphotericin B, fluconazole and ketoconazole against *A. fumigatus*.[62] The ability of thymol to undermine membrane integrity and diminish ergosterol content is likely at the root of its ability to sensitize these fungi to cell membrane perturbing antifungals. Carvacrol, also found in thyme extracts, shares this mechanism of chemosensitization.[63] Eugenol and methyleugenol, which are present in high levels in the extracts of basil and clove, have also been reported to disrupt ergosterol biosynthesis and synergize with fluconazole against both fluconazole-susceptible and resistant isolates of *C. albicans*.[64] In addition to their membrane active mechanisms, thymol and eugenol have been shown to inhibit the plasma membrane H^+ATPase resulting in cellular acidification.[65]

Several other phenolic compounds found in plant extracts, including berberine, curcumin, and dihydrobenzaldehydes have been reported to enhance fluconazole activity against *C. albicans* and *C. neoformans* through inhibition of the oxidative stress response pathway.[61,66,67] Interesting, cinnamaldehyde, which is structurally similar, acts through a distinct mechanism. In vitro assays have demonstrated that it inhibits β-(1,3)-glucan synthase and chitin synthase from *Saccharomyces cerevisiae*.[68] The combination of cell wall and membrane disruption results in improved activity of fluconazole against *C. albicans* and *C. neoformans*.[61] Allicin, a component of garlic extract, synergizes with amphotericin B and fluconazole against *C. albicans*.[69,70] The primary mechanism appears to be disruption of vacuolar function, but only when ergosterol is present in the membrane. The ability to enhance azole activity has been confirmed in a murine model of systemic

candidiasis.[70] Epigallocatechin-O-gallate, found in black tea, is a dihydrofolate reductase inhibitor which in turn inhibits ergosterol biosynthesis. In combination with amphotericin B it enhanced antifungal activity and has been shown to improve the outcome in a murine model of systemic candidiasis.[71]

Overcoming resistance

Another, complimentary approach to improving antifungal treatment is overcoming existing resistance mechanisms. This approach has been successfully used in the treatment of bacterial infections, where many therapies include inhibitors of detoxifying enzymes, and in oncology, where efflux pump inhibitors are routinely used to increase the intracellular concentration of the active agent. The most commonly encountered mechanisms of antifungal resistance are alterations in drug target sequence, increased expression of the drug target, and increased drug efflux.

Histone deacetylase inhibitors

Gene expression is a highly dynamic process with several layers of regulation including alterations in histone acetylation. In *C. albicans*, treatment with fluconazole leads to the upregulation of *ERG11* which encodes sterol demethylase, the target of the azoles, and several efflux pumps.[20,72, 73] This ability to alter gene expression in response to drug treatment increases MIC and is thought to contribute to the phenomenon of trailing growth, which is continued growth at drug concentrations well above the MIC.[74] The ability of *Candida* to adapt to growth at very high azole concentrations is a therapeutic challenge. To address this issue, the use of histone deacetylase inhibitors in combination with azoles has been explored. Alone, several HDAC inhibitors, including trichostatin A, apicidin, and sodium butyrate, had no or minimal effect on *C. albicans* growth in rich media at 35°C. Additionally, while the inclusion of a trichostatin did not reduce the fluconazole MIC at 24 hours, it had a profound impact on the extent of trailing growth. This reduction in trailing growth was also observed with itraconazole and 2 closely related *Candida* species, *C. tropicalis* and *C. parapsilosis*, although it was not observed with the more distant relatives, *C. glabrata* and *C. krusei*. Finally, it has been demonstrated that the addition of HDAC inhibitors prevents the azole induced upregulation of several ergosterol biosynthesis genes and efflux pumps.[75]

Efflux pump inhibitors

Upregulation of efflux pumps is an extremely common mechanism of antifungal drug resistance. This results in increased antifungal efflux and reduces the intracellular concentration of the active agents. As such, efflux pump inhibitors are an attractive strategy for overcoming resistance. ATP-binding cassette (ABC) efflux pumps are the predominate type that contribute to drug resistance, although major facilitator superfamily (MFS) efflux pumps also have a role. In *Candida albicans* the ABC transporters that are most commonly overexpressed in drug resistant isolates are Cdr1 and Cdr2 while overexpression of the MFS transporter Mdr1 appear to be less prevalent.[76] These pumps have varying substrate specificities and limited structural similarities, so it is not surprising that inhibitors are rarely active against both classes.[77]

ABC transporter inhibitors

As mentioned above, it has been shown that FK506 has some ABC efflux inhibitory activity, as does a structurally unrelated propafenone, GP382. To demonstrate this activity, *C. albicans* Cdr1 and Cdr2 were heterologously expressed in a *Saccharomyces cerevisiae* efflux null mutant and shown to be functional. It was then shown that the addition of FK506 could reverse ketoconazole resistance in this strain. Finally, a rhodamine efflux assay was used to confirm that treatment with either FK506 or GP382 resulted in decreased efflux of the rhodamine dye, Rh-6G, which is a known substrate of several ABC transporters.[78]

In an effort to identify novel efflux pump inhibitors one group conducted a screen of natural products from fermentation extracts that potentiated the activity of fluconazole in strains of *C. albicans* and *C. glabrata* that overexpressed Cdr1. Several extracts were identified as enhancing fluconazole activity against the Cdr1-overexpressing strains. Active components of these extracts were isolated and several, including enniatins, beauvericins, and milbemycins, were confirmed as ABC efflux pump inhibitors in a whole cell efflux assay. Of these compounds, it was decided that the milbemycins warranted further exploration as they are also being explored as insecticides and antiparasitics. Nearly two dozen milbemycins were isolated and tested for efflux inhibition against a panel of *Candida* strains and isolates. The addition of milbemycin α_9 significantly decreased both the MIC_{50} and MIC_{90} of posaconazole against all *Candida* species tested. This broad spectrum of activity is extremely promising since the infecting species is not always known at the onset of treatment for candidiasis.[79]

Multiple large scale screens of D-octopeptide combinatorial libraries have identified ABC-efflux pump inhibitors of varying specificity. The original screen was conducted using a strain of *Saccharomyces cerevisiae* that over-expressed Pdr5p in the presence of a subinhibitory concentration of fluconazole. In this manner, KN20 was
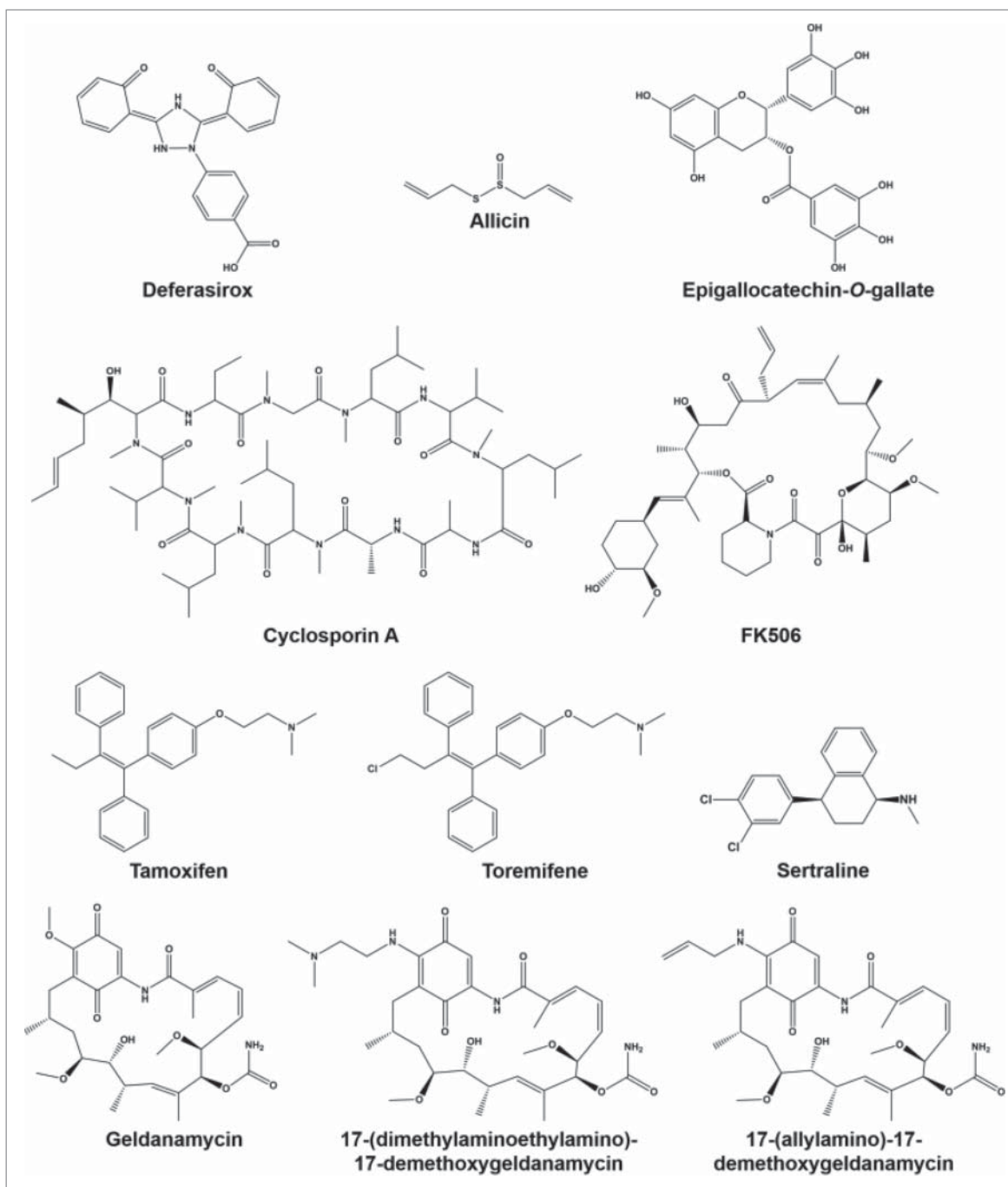
Figure 1. Compounds that have been tested *in vivo* in combination with existing antifungals and enhanced efficacy.

identified. This peptide derivative inhibits efflux by Pdr1p, Cdr1p, Cdr2p, and Mdr1p. Not surprisingly, addition of KN20 sensitized a collection of *Candida* species, including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* to fluconazole.[80] It is worth noting, however, that it had minimal effect on fluconazole's activity against *C. neoformans*. It is not clear if this is due to an inability to inhibit the efflux pumps or if it is unable to penetrate the polysaccharide capsule that surrounds *C. neoformans*. More recently, a highly selective D-octopeptide inhibitor of *C.*

albicans Cdr1p, RC21, was identified by a similar screening strategy. However, this strategy employed several counter screens to eliminate hits with promiscuous activity. Addition of RC21 did not reduce fluconazole resistance in strains overexpressing a variety of other ABC efflux pumps, Mdr1p, or Erg11p but did sensitize several azole-resistant isolates of *C. albicans*.[81]

MFS transport inhibitors
While searching specifically for inhibitors of ABC transporters, another compound, cytochalasin H, was

identified which was isolated from hit extracts and shown to have modest inhibitory activity against Mdr1, a member of the major facilitator superfamily.[79]

Recently, a screen for compounds that potentiate the activity of fluconazole against an Mdr1 overexpressing strain was conducted using a synthetic small molecule library. Two, structurally similar compounds, MCC1189 and MCC1375, were identified as having minimal antifungal activity alone but enhanced fluconazole activity. Efflux assays confirmed inhibition of Mdr1-mediated efflux but not Cdr-mediated efflux. Both compounds were shown to be synergistic with fluconazole against *C. albicans* and the combination of fluconazole and MCC1189 resulted in fungicidal activity.[82]

General inhibitors

While many screens for efflux pump inhibitors are based on sensitizing cells to the presence of a subinhibitory concentration of an antifungal, usually fluconazole, one group took an alternative approach. In a repurposing screen, cells were loaded with one of 2 fluorescent efflux pump substrates and incubated in the presence of compounds in the Prestwick library. Actively effluxing cells decreased in fluorescence while those with inhibited efflux retained the fluorescent substrate and high fluorescence. This approach identified clorgyline, a monoamine oxidase A inhibitor, as a multi-efflux class inhibitor. This compound inhibited both ABC and MFS efflux pumps. Furthermore, addition of clorgyline sensitized azole resistant isolates both *C. albicans* and *C. glabrata* to fluconazole.[83]

Both thymol and carvacrol, which are discussed above, have also been shown to reduce drug efflux through disruption of the proton gradient used to drive MFS pumps.[84] This is why the degree of sensitization is greater in fluconazole resistant isolates. Baicalein, a component of many Chinese herbs, has alsobeen shown to reduce efflux in *C. albicans* and improve fluconazole activity, especially in highly resistant strains.[85]

Biofilm active combinations

As mentioned above, *Candida* biofilms are a significant therapeutic challenge. These multicellular communities form on both inert and biological surfaces and include cells in a variety of morphological and metabolic states surrounded by a dense extracellular matrix. Biofilms are inherently resistant to antifungal treatment due to both reduced drug penetration through the matrix and the presence of cells that are metabolically quiescent and not actively dividing. In addition to being difficult to treat, biofilms can act as a reservoir of fungal cells that can break off and seed infections throughout the body making their eradication crucial for the successful treatment of systemic candidiasis. To acheive this, compounds that inhibit biofilm formation, as well as those that are active against mature biofilms, are needed.

Doxycycline

As discussed above, the addition of various tetracyclines to fluconazole treatment against planktonic cells enhances antifungal activity. It has also been shown that doxycycline is synergistic with fluconazole against early stage biofilm (4–12 hours). However, mature biofilms exhibited resistance to this combination.[86] While it is tempting to assume that this enhanced activity is due to iron chelation, as is the case for planktonic cultures, that has not yet been confirmed to our knowledge.

Calcineurin inhibitors

In addition to resulting in fungicidal activity against planktonic cells, the combination of either FK506 or cyclosporine A and fluconazole has dramatic activity against mature *C. albicans* biofilms. Interestingly, while calcineurin function was not required for the formation of apparently normal biofilms, mutants in this pathway demonstrated increased fluconazole susceptibility. In vitro biofilms treated with the drug combination not only had reduced metabolic activity measured by XTT assay compared to either drug alone, but they were also substantially thinner after 24 hours of treatment. These finding where then confirmed in an *in vivo* rat catheter model. Finally, the ability of FK506 to enhance fluconazole activity against *C. albicans* biofilms was confirmed to be calcineurin dependent.[87]

Nonsteriodal anti-inflammatory drugs

A variety of nonsteroidal anti-inflammatory drugs have been shown to inhibit *C. albicans* biofilm formation and some even have activity against mature biofilms.[88] This is likely due to their ability to interfere with prostaglandin production and decrease *C. albicans* filamentation which has a detrimental impact on adherence and the development and maintenance of biofilms. However, this occurs at relatively high concentrations, limiting their use as single agent therapies. However, it has since been shown that the addition of aspirin to amphotericin B has enhances its activity against *C. albicans* and *C. parapsilosis* biofilms.[89]

Plant extracts

The ability of several plant-derived compounds to inhibit biofilm formation and improve the activity of antifungals against mature biofilms has been investigated. One group investigated the activity of cinnamaldyhyde, citral, geraniol, and eugenol. While each compound has some

antifungal activity, their inclusion in the growth media at sub-growth inhibitory concentrations reduced biofilm formation alone, with eugenol and cinnamaldehyde showing the greatest activity. Importantly, eugenol and cinnamaldehyde were also synergistic with fluconazole against mature biofilms. Scanning electron microscopy of biofilms treated with eugenol or cinnamaldehyde showed decreased secreted polysaccharide matrix and rough, shriveled cell membranes.[90] This loss of biofilm structure and organization results in a loss of fluconazole resistance.

Repurposed compounds

Recently, a screen of the Pharmakon 1600 library was conducted to identify compounds that enhanced the activity of amphotericin B against *C. albicans* biofilms. Test compounds were included at the initiation of biofilm formation and amphotericin B was added 24 hours later. To differentiate between compounds that prevented or inhibited biofilm alone and those that enhanced amphotericin B activity, biofilms treated with the test compounds were subjected to concentration gradient of amphotericin B. Three compounds were found to enhance amphotericin B activity including drospirenone, perhexiline, and toremifine. These compounds were then shown to enhance the antifungal activity of amphotericin B and caspofungin against both *C. albicans* and *C. glabrata* biofilms. It is worth noting that perhexiline and toremifene have antifungal activity linked to the inhibition of calmodulin function, however this has mechanism has not been investigated in relation to their ability to enhance activity of known antifungals against biofilms.[91]

Conclusions

When examining the literature for this review it was surprising how many reports there were, but also how diffuse this body of work appears. The terminology used varies widely between investigators and includes enhancers, chemosensitizers, potentiators, and synergizers with often overlapping definitions.[92,93] The methodologies and strains used also vary significantly which has resulted in conflicting reports with specific combinations being reported as synergistic, additive, neutral, and antagonistic. It is our belief that as focus shifts toward identifying these combinations and the subfield of antifungal adjuvant research becomes more cohesive, standard methodologies will be established and issues with ambiguous terminology will be resolved. In many cases, a compound's ability to improve the activity of established antifungals seems to be investigated almost as an afterthought and is rarely mechanistically characterized and examined in *in vivo* studies. This is understandable and by no means meant to be disparaging to these investigators, but instead is intended to highlight the broad base of knowledge in this area with extensive possibilities for further research and development.

As screening technologies become more advanced there is growing interest in a more systematic approach to identifying multi-component therapies and these approaches may yield novel compounds that lack inherent antifungal activity.[50,51, 94] For example, this approach is at the root of the discovery of many of the compounds discussed in the overcoming resistance section of this review and is how the majority of efflux pump inhibitors where identified as well as some of the compounds in the anti-biofilm section. Another exciting strategy is the idea of targeting virulence attributes in combination with existing antifungal therapy for improving efficacy. Work toward exploiting this approach in both *C. albicans* and *C. neoformans* has been reviewed recently.[95,96]

While it is generally believed that only fungal specific structures and processes, such as the cell wall and ergosterol biosynthesis, are acceptable antifungal targets, it is worth noting the vast majority of targets exploited above have human homologs. Specificity is a concept that is rarely black and white, and it is important to realize that specificity can be built in parts.

It is encouraging to see that several groups appear to be actively pursuing this approach for improving antifungal activity and are conducting in depth mechanistic characterization efforts and exploring the effects *in vivo*. It is also promising that individuals outside of academia are beginning to take notice and embrace the idea of antifungal adjuvants as a viable path forward in the treatment of invasive fungal infections. It has now been 14 y since the newest class of systemic antifungals received FDA approval.[3] Even with state-of-the-art therapies, mortality rates for invasive fungal infections often exceed 50%. It is crucial that we not only embrace the idea of antifungal adjuvants, but push forward to move this approach from conceptually interesting to clinically applicable.

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