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A complex game of hide and seek: the search for new antifungals[†]

Huy X. Ngo^a, Sylvie Garneau-Tsodikova^a, and Keith D. Green^a

Keith D. Green: keith.green@uky.edu

^aUniversity of Kentucky, Department of Pharmaceutical Sciences, 789 South Limestone Street, Lexington, KY, USA. Fax: 859-257-7585; Tel: 859-218-1686

Abstract

Fungal infections directly affect millions of people each year. In addition to the invasive fungal infections of humans, the plants and animals that comprise our primary food source are also susceptible to diseases caused by these eukaryotic microbes. The need for antifungals, not only for our medical needs, but also for use in agriculture and livestock causes a high demand for novel antimycotics. Herein, we provide an overview of the most commonly used antifungals in medicine and agriculture. We also present a summary of the recent progress (from 2010–2016) in the discovery/development of new agents against fungal strains of medical/agricultural relevance, as well as information related to their biological activity, their mode(s) of action, and their mechanism(s) of resistance.

Graphical Abstract

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Correspondence to: Keith D. Green, keith.green@uky.edu.

Introduction

Fungal infections have become a major burden for society, especially with the current rise in the number of immunocompromised patients. *Candida albicans* is the major pathogen and is responsible for 50–70% of cases of total fungal infections worldwide.¹ Among the fungal infections, invasive candidiasis is one of the most common nosocomial fungal diseases with an estimate of 250,000 cases and more than 50,000 deaths worldwide per year.^{1, 2} Additionally, *Aspergillus* strains are also the culprits of numerous disease states. For instance, approximately 2.5% of adults with asthma also suffer with allergic bronchopulmonary aspergillosis (ABPA).³ According to the Centers for Disease Control, out of these roughly 4.8 million patients with ABPA, 400,000 end up with chronic pulmonary aspergillosis (CPA).³ As recently as 2012, in the USA, a multistate outbreak of fungal meningitis and associated diseases (749 reported cases) were discovered to be due to steroid injections contaminated with fungal pathogens prepared by a single compounding pharmacy.⁴ Based on some experts' opinions, fungal diseases are expected to become even more prominent in the near future as a result of global warming and the rise of fungal strains that are capable of surviving and propagating at mammalian body temperature.⁵

The impact of fungi extends far beyond direct human infections. The main human food sources, comprised of livestock and plants, are also hosts for a variety of fungal pathogens. In fact, there are historical examples of food sources contaminated by fungi, which have presented and continue to present challenges to human civilization. A dark period in the history of the USA was the Salem witch-trials. A well-known scientific explanation for the reported afflictions was that the alleged victims unknowingly consumed mouldy bread contaminated with a fungus called *Claviceps purpurea*, more commonly known as ergot. This fungus, infecting the head of rye and other grains, produces psychotropic natural products.⁶ In the nineteenth century, fungal diseases in crops were responsible for multiple famines. For example, the Irish Potato Famine resulted from a phytopathogenic fungus, *Phytophthora infestans*, which led to innumerable deaths from starvation and triggered the emigration of an estimated half a million Irish citizens to the USA.^{7–9} Currently, the fungus *Puccinia graminis tritici* Ug99, responsible for stem or black rust disease on wheat, is threatening to wipe out the world wheat production.¹⁰ Fungal diseases also affect livestock. Mastitis in dairy cattle is an inflammation of the udder often caused by infectious microorganisms. Recently, a group from Poland identified milk samples of mastitic cows to be contaminated with *Candida parapsilosis*, a pathogen capable of infecting humans.^{11, 12}

It is therefore clear that fungal afflictions have arisen in various links of the food web throughout history (Fig. 1A). Over the ages, fungi have come out of their hiding places and humans have sought new ways to overcome these pathogens. We are currently in an era where resistance to all the currently used antifungal agents is fast emerging. Thus, there is a need for new, safe, and effective antifungals to combat¹³ these pathogens. Unlike the case of antibacterials, our repertoire of antifungals is limited, and the antifungal pharmaceutical pipeline is running dry. It is important to note that the most clinically useful antifungal classes (polyenes and azoles) were discovered at least 40 years ago and the azoles are used in livestock and plants as well. While also important, the literature on antifungal usage in livestock is limited and will not be directly addressed herein. On the contrary, since the

introduction of the most commonly used antifungals, there has been an explosion in the number of manuscripts related to antifungals since the 1960s, as exemplified by the increase from ~443 and 39 to 4934 and 1088 manuscripts in PubMed and Scifinder, respectively, from 1960 to 2015 (Fig. 1B). This outburst in interest in antimycotics inspired us to compound a comprehensive account of the current literature related to this expanding field of research.

In this review, as a mean to demonstrate how modern scientists are seeking novel antifungals from a myriad of hiding places, we summarize the latest discoveries and developments of antifungal compounds (from 2010–2016) against medical and agricultural-relevant fungal strains as well as describe their biological activity, modes of action, and mechanism(s) of resistance when available. We also provide an overview of the currently employed antifungal agents in medicine and agriculture.

Current antifungals in use

The antifungal drug pipeline has received contributions from many chemical classes: natural products, synthetic organic molecules, and inorganic compounds (Fig. 2). Collectively, synthetic organic molecules have been the predominant class in terms of applications in both humans and crops.^{14, 15} There is currently an alarmingly high number of molecules used in crops that are either structurally or pharmacologically the same as the FDA-approved drugs utilized in medicine. This observed crossover could potentially contribute to widespread fungal resistance in the future. In the following sections we discuss the antifungal compounds currently used in both humans and agriculture. The most common antifungal targets include fungal ergosterol (*e.g.*, the 14 α -demethylases), β -1,3-D-glucan synthase, *N*-myristoyltransferase, aminoacyl tRNA synthase, chitin synthase, elongation factor, and the secreted aspartic protease. Detailed information about each of these targets can be found in a recent review article,¹⁶ and therefore will not be covered in great details herein.

Antifungal agents used in medicine

The first conjugated polyketide (PK) natural product, nystatin (NYS), discovered to display antifungal activity, was isolated from *Streptomyces noursei* (named for the dairy farmer William Nourse) in 1950.¹⁷ Although this discovery was a monumental achievement in the field of medical mycology, it was apparent that, due to its toxicity, NYS would not be an ideal drug for systemic fungal infections, a complication for healthcare providers at the time. Hence, new antifungal compounds capable of treating systemic fungal diseases were necessary. However, this discovery put the spotlight on antifungal natural product isolation, specifically the polyenes. Eventually, amphotericin B (AmB), a heptaene PK, was isolated in 1953 from a soil sample from Venezuela.¹⁸ The discovery of AmB was essential considering that it was the first drug that could be used to treat deep-seated systemic fungal infections.⁷ Both NYS and AmB act on fungal cells by binding to ergosterol and forming a transmembrane channel leading to monovalent ion leakage.¹⁹ Approximately 60 years after the isolation of AmB, this polyene compound still remains a major cornerstone of several treatment guidelines for invasive fungal infections.²⁰ Despite its potent antifungal activity, AmB has severe off-target effects and is often reserved for life-threatening infections.^{21–23}

Patients treated with AmB frequently experience infusion-related adverse effects and/or dose-limiting nephrotoxicity.²⁴ The liposomal formulations of AmB have alleviated some of its toxicity concerns, but not to a satisfactory extent.²⁴ And in addition, AmB formulations are required to be administered parenterally.

In fact, it took 30 years after the isolation of AmB for the first oral antifungal agent, fluconazole (FLC), to be introduced for clinical use. FLC is part of the azole class of antifungals that inhibit 14 α -demethylase (ERG11 for *Candida spp.* or CYP51 for *Aspergillus spp.*), an enzyme essential in the biosynthesis of a major component of the fungal cell membrane, ergosterol *via* lanosterol.¹⁹ Inhibition of 14 α -demethylase causes the accumulation of toxic methylated sterols, eventually leading to fungal cell death.²⁵ Unlike AmB, FLC exhibits excellent oral bioavailability, predictable linear pharmacokinetics, broader scope of affected tissues, and fewer adverse effects.^{26, 27} As a result, FLC quickly became one of the most prescribed antifungal agents in clinics worldwide. However, some fungal strains, such as *Candida glabrata*, *Candida krusei*, *Aspergillus spp.*, *Fusarium spp.*, and the *Mucorales* were found to exhibit intrinsic resistance to FLC.¹⁴ Consequently, to expand the clinical utilities of azoles, new FLC analogues with broader spectrum of activity, such as itraconazole (ITC), voriconazole (VOR), and posaconazole (POS), were developed and introduced in 1992, 2002, and 2006, respectively (Fig. 2B).^{28, 29} ITC and POS contain modified and extended hydrophobic side chains, which allow for additional interactions with the enzyme targets, leading to an enhanced spectrum of activity.^{30, 31} These azole compounds have been essential in our fight against many types of fungal diseases. However, azoles have their own shortcomings. Despite their tremendous clinical utilities, they also inhibit the human cytochrome P450 (CYP) enzymes interfering with the metabolism of many other concomitant drugs. Indeed, to correct the problem of azole-related drug interactions, many research groups have been designing novel azole analogues that selectively target the fungal 14 α -demethylase enzyme (See the modification of current antifungals and other FDA-approved drugs section).

An alternative strategy that has been used to overcome the problem of selectivity towards the fungal enzymes, is the development of another class of compounds, the allylamines, which inhibit a different target in the ergosterol biosynthetic pathway. The first FDA-approved allylamines included terbinafine (TER) and naftifine (NAF), which were put on the market in 1996 and 1988, respectively (Fig. 2).³² These compounds inhibit squalene epoxidase, the enzyme responsible for generating a sterol upstream of ergosterol.³³ TER has been shown to accumulate more in the skin and nail beds relative to blood plasma, possibly due to its lipophilicity.³⁴ Thus, TER and the other allylamines are highly effective against dermatophytes and employed for the treatment of onychomycosis and cutaneous fungal infections.³⁵

Despite the improvements brought by the allylamines, in order to further differentiate between mammalian and fungal cells, their distinct cell walls have been targeted by the echinocandins. This latest milestone in medical mycology provided us with caspofungin (CFG), micafungin (MFG), and anidulafungin (AFG), which were introduced in 2001, 2005, and 2006, respectively.^{36, 37} More specifically, these compounds target glucan synthesis, which is the most notable aberration between fungal and mammalian cells, by inhibiting the

β -1,3-D-glucan synthase. The polymer β -glucan is an essential component of many fungal cell walls.³⁸ As a result of this unique mechanism of action, the echinocandins addressed an important scientific challenge in medical mycology, which is selective toxicity against the eukaryotic fungal cell.³⁹ Indeed, when comparing the rate of treatment discontinuation due to adverse effects, the aforementioned echinocandins were found to be more tolerable than AmB in all formulations, ITC, and VOR.⁴⁰ The echinocandins are fungicidal against most *Candida spp.* and fungistatic against *Aspergillus spp.* Unfortunately, they are generally not active against Zygomycetes, *Cryptococcus neoformans*, or *Fusarium spp.*³⁶ Recent work by Nett and Andes provides a full account of the antifungals currently approved and used in the medical field, their spectrum of activity, resistance, pharmacology, clinical indications of use, toxicities, and any drug-drug interactions.⁴¹ Although good antifungal agents exist, based on the number of affected individuals and the number of deaths from candidiasis, aspergillosis, and cryptococcal meningitis, among other diseases (Fig. 3), it is clear that new and improved antifungals are urgently needed.

Antifungal agents used in agriculture

The need for agricultural antifungals became prevalent over 170 years ago. Between 1840 and 1845, the Irish potato famine was at its peak, and eventually led to approximately one million deaths.⁸ Around that time, experimental combinations of calcium-containing inorganic materials (lime), brine, sulfur dust, and copper sulfate were widely used for such applications. Copper sulfate was actually found to be toxic to certain crops. It was believed that lime could reduce the toxicity of copper sulfate. In 1885, the botanist Pierre-Marie-Alexis Millardet of the University of Bordeaux discovered that the mixture of lime and copper sulfate, the “Bordeaux mixture” was potent in combating downy mildew.⁸ The Bordeaux mixture and a lime-sulfur mixture were the first plant fungicides with broad applications.

Due to the popularity of the Bordeaux mixture, the early twentieth century began the era of do-it-yourself and proprietary mixtures.⁴² These mixtures were mostly modifications of the Bordeaux mixture, which typically contained some combinations of copper sulfate, copper carbonate, copper ammonia, lime, and water.^{8, 42} It slowly became apparent that the efficacies of these inorganic mixtures were insufficient. To achieve the desired therapeutic effect, toxic concentrations and frequent re-applications were required.¹⁵ Thus, there was an obvious need for more efficacious agricultural fungicides. From a restaurant in New York City, plant pathologist James G. Horsfall and his friend Donald F. Murphy rationalized that sulfur was a fungicide, therefore, organic sulfur compounds would also possess fungicidal activity.⁴³ Murphy convinced his company to send 100 chemical samples including multiple organo-sulfur compounds to Horsfall for screening. Eventually, the dithiocarbamate compound ethylenedithiocarbamate (also termed nabam) was identified.⁴³ These dithiocarbamate compounds attack fungal cells at multiple sites and their mechanism(s) of action are still ambiguous. This first series of dithiocarbamates was the tipping point leading to the switch from inorganic to organic fungicides for crops.

Currently, the fungicide market is dominated by many organic compounds, such as triazoles and strobilurins (Fig. 4).¹⁵ Since first launched in 1973, the triazoles, along with other

demethylase inhibitors (DMIs), make up the largest class of fungicides.¹⁵ These compounds are similar to the azoles used in medicine (e.g., FLC, ITC, POS, and VOR), which act *via* inhibition of the 14 α -demethylase in ergosterol biosynthesis. The agricultural triazole fungicide class includes: tebuconazole (TBC), epoxiconazole (EPC), propiconazole (PCZ), etc. This class is highly popular because of its broad spectrum of activity and low rate of resistance, thus far. As the triazole fungicides and azole antifungal medicines share many similarities in terms of their chemical structures and have the same pharmacological target, this could be a concern for possible widespread fungal resistance.

The currently used semisynthetic strobilurins, which were introduced to the market in 1996, represent the second largest chemical class of plant fungicides. Their core structures are derived from a group of natural products called β -methoxyacrylic acid from wood-rotting fungi such as *Oudemansiella mucida* or *Strobilurus tenacellus*.⁴⁴ They display broad-spectrum activity and are highly efficacious.¹⁵ The fungicidal activity of strobilurin is mediated by their ability to reversibly inhibit mitochondrial respiration by specifically targeting the Q_o site of cytochrome b.⁴⁵ Some popular strobilurins currently used are: azoxystrobin (AXN, Fig. 2B), dimoxystrobin, and fluoxastrobin. Strobilurins are interesting due to their unique mechanism of action and chemical structures that are distinct from those of currently employed medicinal antifungal compounds, which is the ultimate challenge for the discovery and development of crop fungicides.

Morpholines, while not a large part of consumer fungicides, provided a novel target of action. Flumorph (IUPAC = 4-[3-(3,4-dimethoxyphenyl)-3-(4-fluorophenyl)-1-oxo-2-proprenyl] morpholine) was developed in 2000. This compound is excellent at controlling *P. infestans*, *Phytophthora capsici*, *Pseudoperonospora cubensis*, and *Plasmopara viticola* infections of various plant species. More recently, Si and co-workers determined that flumorph is able to penetrate the cells and disrupt the organization of F-actin, which leads to alterations in cell-wall deposition.⁴⁶

Development of resistance to fungicides not only triggers global public health issues, but also threatens the safety of food products.⁴⁷ Treatment with echinocandins, normally reserved for human, can sometimes stimulate chitin synthesis, which in turn causes the fungi to be more resistant to antifungal treatment.⁴⁸ As a result, this pan-usage of echinocandins could increase the rate of resistance development.

Antifungal resistance

Much like their bacterial counterparts,^{49, 50} the number of fungi resistant to the current antifungals and fungicides is rapidly increasing. Below, we briefly summarize the main mechanisms of resistance for human pathogens and some phytopathogens. Although phytopathogens are abundant, the literature examining their mechanisms of resistance to fungicides is sparse, at best. For full coverage of the resistance mechanisms found in human fungal pathogens, please refer to the recent review by Sanglard on resistant fungal pathogens.⁵¹

There are two types of resistance found in fungi: (i) acquired resistance through exposure to antifungal agents and (ii) intrinsic resistance, a genetic predisposition that precludes the

activity of a particular antimycotic agent. The former has been reported for nearly every major fungal pathogen.⁵² Molecularly, resistance occurs in one of three ways:⁵¹ (i) decrease in effective drug concentration, (ii) drug target alterations, and (iii) metabolic bypasses. A decrease in intracellular concentration can be achieved in several ways including active efflux, target overexpression, and compartmentalization through the formation of biofilms.⁵¹ One recent case reported in phytopathogens is the development of resistance in *P. capsici* due to a mutation in the *CesA3* gene, involved in cellulose synthesis.⁵³ Drug targets are altered primarily through mutation of the given enzyme (*e.g.*, 14 α -lanosterol demethylase and β -1,3-glucan synthase) so that the antifungal agent no longer efficiently prevents the enzyme from performing its dedicated task.⁵¹ Metabolic bypasses can include alternative metabolic or shunt pathways that convert the toxic metabolites, created by antifungal treatment, into a secondary metabolic or excretion route.⁵⁴ In the following sections, we discuss the new antifungal compounds that either overcome these methods of resistance, or avoid the currently targeted metabolic pathways.

New antifungal leads

Drug discovery and development is a strenuous and costly process. Despite the increased investment in pharmaceutical research and development (R&D), the overall productivity has been depressing and attrition rates continue to skyrocket due to physicochemical, efficacy and, safety problems.^{55–57} An analysis of a large database containing 28,000 R&D projects from companies based in the USA, Europe, and Japan revealed that R&D projects for systemic anti-infective drugs had a success rate of 3.92%, which unfortunately was part of the bottom five in terms of the rate of success among the various therapeutic categories.⁵⁷ For the pharmaceutical industry, anti-infective discovery is simply not as attractive in terms of economic compensation as the discovery of drugs for chronic diseases. As a result, the antifungal discovery pipeline has been sparse.⁵⁸ For instance, the gold-standard therapy for the deadly cryptococcal meningitis, as of 2016, is still comprised up of two of the oldest antifungals, AmB and flucytosine (5-FC), which were both approved back in 1950's.⁵⁹ In order to stimulate the antifungal drug discovery, diverse methods are currently being used and a plethora of ideas have been explored as to where to seek out new antifungals from numerous hiding places. However varied these ideas and methods may be, they can be sorted into two main groups with several subsets: (i) modification, combination, and repurposing of current antifungals or other FDA-approved drugs and (ii) seeking out new sources of antifungal agents.

Modification, combination, and repurposing of current antifungals and other FDA-approved drugs

There are 3 major ways by which scientists are trying to circumvent antifungal resistance: (i) modifying the currently used antifungals to overcome the mutations or modifications that rend them inert, (ii) combining the antifungals with other compounds, from various origins, to lower the effective concentrations needed, and (iii) using other FDA-approved compounds, with antifungal properties, to treat fungal infections.

i. Modification of current antifungals and other FDA-approved drugs—Since the approval of FLC in 1990, the azoles have been a staple of many antifungal therapies. However, azole-related drug interactions continue to cause problems for patients and healthcare professionals. Efforts have been allocated to generate new FLC analogues that solely target the fungal CYP enzymes. A major challenge in azole's design is that human CYP enzymes are homologous to the fungal 14 α -demethylases. Most notably, they both share a conserved heme-iron motif. A group from Viamet Pharmaceuticals recently rationalized that the main problem of azoles leading to azole-related drug interactions is mostly due to the non-specificity of the metal-binding heterocycles (triazoles and imidazoles).⁶⁰ Hence, they screened different nitrogen-containing heterocycles and identified the tetrazole moiety to display less affinity for human CYP enzymes. These tetrazole analogues were generated and shown *in vitro* to display much greater selectivity for the fungal CYP enzyme, 14 α -demethylase.⁶¹ Two of these promising tetrazole analogues, VT-1161 (**1a**, Fig. 5) and VT-1129 (**1b**, Fig. 5) are currently undergoing clinical trials. Both of these compounds have demonstrated potent and broad-spectrum antifungal activity in various animal models.^{62–64} Besides the tetrazole analogues, some groups are still working on optimizing the FLC scaffold. Emami and co-workers recently reported a series of 18 triazole alcohols by replacing the 1,2,4-triazol-1-yl group of FLC with 4-amino-5-aryl-3-mercapto-1,2,4-triazole.⁶⁵ Some of the analogues from this library showed promise by demonstrating ng/mL activity against five pathogenic fungal strains (*C. albicans*, *C. glabrata*, *C. neoformans*, and *A. fumigatus*).

Besides inhibition of ergosterol biosynthesis, an alternative and innovative strategy for the development of antifungals is to physically disrupt the fungal cell membrane. Over the past five years, the strategy of adding alkyl chains to hydrophilic molecules has been highly efficient in generating a variety of amphiphilic compounds capable of disrupting fungal cell membranes. In general, the more lipophilic compounds with alkyl-chain attachments have been shown to be more active against fungi than their unmodified counterparts. For example, the aminoglycosides neamine (**2a**, Fig. 5),⁶⁶ tobramycin (**2b**, Fig. 5),^{67, 68} and kanamycin A (**2c**, Fig. 5) and B (**2d**, Fig. 5),^{69, 70} have been alkylated and found to display antifungal activity, while the parent drugs do not. Additionally, the strategy of generating membrane-disrupting amphiphilic molecules displays one major advantage over the traditional method of modifying existing azoles: there is less likelihood of resistance for these compounds. Indeed, since these amphiphilic molecules insert themselves through the cell membrane, they are likely unaffected by efflux pumps. These compounds have been discussed in detail in some excellent recent reviews,^{71, 72} and are, therefore, not extensively covered herein.

Another antifungal molecule that also targets the fungal cell membrane is AmB. AmB is potent, but its clinical efficacy is greatly hindered by its adverse effects. Thus, AmB would be an ideal molecule for further optimization for improved tolerability. While chemically modifying AmB could be a valid strategy, it has not been widely investigated due to the synthetic complexity of the core scaffold of this molecule.^{73, 74} Only two reports on the semisynthesis of AmB or NYS derivatives, where different sugar side-chains have been appended to AmB itself, are found in the literature. These compounds did not display improved antifungal activity and only modest improvements in toxicity. Historically,

formulations of AmB, such as the lipid complex, have been clinically successful and displayed improved efficacy and safety over the non-formulated AmB deoxycholate.⁷⁵ Additionally, silver nanoparticles are more exotic vehicles that also have been explored as a novel formulation of AmB.⁷⁶ Alone, colloidal silver has been used as an antibiotic for some time,⁷⁷ and, recently has been shown to enhance the antimicrobial activity of other antibiotics.⁷⁸ However, due to the silver's irreversible side effects, it is often only used topically.⁷⁹ In 2015, Gruszecki and co-workers combined silver nanoparticles with AmB in an effort to generate not only a better antifungal, but also one with better selectivity. When tested against three fungal strains, *C. albicans*, *Aspergillus niger*, and *Fusarium culmorum*, the silver nanoparticle-AmB combination was found to inhibit the growth of the fungi better than the combination of the two components alone.⁷⁶ Analogously to the development of the AmB-lipid complexes and AmB liposome, a recent publication by Nokhodchi and co-workers detailed the development of two solid lipid nanoparticle (SLN)-FLC delivery systems.⁸⁰ A SLN is a biodegradable lipid-based drug delivery system that is solid at room and body temperatures.⁸¹ The work explored FLC-derivatized nanoparticles to treat both susceptible and resistant *Candida spp.*, and found that the new drug delivery system improved the MIC values for both types of *Candida spp.*

In addition to being popular drug delivery vehicles, inorganic metals were also recently proposed by Ebenso and co-workers in a creative strategy for repurposing the plant fungicide dithiocarbamate (**3**, Fig. 5). Metals, complexed with *N*-methyl-*N*-phenyl dithiocarbamate have been tested as novel antifungal agents, analogous to the zinc pyrazinone complex used to combat pathogenic bacteria. The dithiocarbamate ligand was synthetically complexed with several transition metals (*e.g.*, Co^{II}, Mn^{II}, Ni^{II}, Cu^{II}, and Na, a non-transition metal as a control) and the antifungal activity of the complexes compared to that of FLC.⁸² The cobalt complex showed comparable activity to that of the known azole against *C. albicans* and two *Aspergillus spp.*

ii. Combination of current antifungals and other FDA-approved drugs—In order to avoid the pitfalls of (i) going through the lengthy and costly processes of clinical studies and FDA approval, and (ii) the potential of the derivatives of FDA-approved drugs to rapidly experience resistance, one could argue that the development of an efficacious combination therapy using the existing antifungal drug repertoire would be a more efficient and economical strategy. Currently, there are two ways in which combination therapy is being used for antifungal treatments: the combination of two drugs (i) with divergent targets, either within or outside the same metabolic pathway, and (ii) displaying synergy and reduced toxicity.

Specifically, recent work by Garneau-Tsodikova and coworkers investigated combination therapy with a slight twist. In this study, various azoles (FLC, VOR, etc.) were combined with a modified FDA-approved aminoglycoside, tobramycin.⁶⁷ The combination of the azole with the amphiphilic aminoglycoside resulted in a lower MIC value for both compounds. This also allowed azole-resistant *Candida spp.* to be effectively killed using azoles in the presence of the modified aminoglycoside. This is an example of the first type of combination therapy. The azole targets the ergosterol biosynthesis, while the modified tobramycin targets the cell membrane. Likewise, a derivative of geldanamycin, 17-AAG (**4**,

Fig. 6C), originally designed as an anticancer agent, has recently been shown to improve the activity of FLC in many biological models.⁸³ The work suggested that inhibition of the fungal heat shock proteins, the mammalian target of 17-AAG, improved the efficacy of FLC. Additionally, the antidepressant, sertraline (**5**, Fig. 6C), was found to work in combination with FLC, appearing to target DNA translation in fungi.⁸⁴ This combination worked particularly well in a study of cryptococcosis in mice.

While it is difficult to predict or even find combinations of two compounds that will prove to be synergistic, this method is a promising area of research. As we will describe in the alternative sources for novel antifungal agents section, natural products are a good source of bioactive molecules. In a recent report 5-hydroxy-7,4'-dimethoxyflavone (**6**, Fig. 6C) was isolated from *Combretum zeyheri* (bushveld tree) and found to have antimycotic activity on its own.⁸⁵ However, this flavone displayed better activity and synergy when combined with the currently FDA-approved miconazole (MCZ). When used with ciprofloxacin, there was also a greater accumulation of the fluoroquinolone in the pathogens, indicating that the compound increased the permeability of the fungi.

The second type of combination therapy involves one compound sensitizing the pathogen to a currently used antifungal agent. Monk and co-workers discovered a peptide sensitizer from a library of D-octapeptides, with no antifungal activity of their own, that accumulated at the cell surface and avoided the cellular detoxification processes.⁸⁶ The peptide allowed for FLC to affect fungi that were previously resistant to the drug. A group working out of California has also found that a simple benzaldehyde, 2-hydroxy-4-methoxybenzaldehyde (**7**, Fig. 6C), was able to sensitize filamentous fungi to other simple phenols (*e.g.*, carvacrol and thymol).⁴⁷ The study found that using a lower concentration of the two phenolic compounds combined, resulted in lower MIC values than the compounds used individually in *Aspergillus spp.*, *Penicillium spp.*, and *Saccharomyces spp.*

iii. Repurposing of drugs approved by FDA for other indications—Novel drugs are always valued, especially when dosage is simple and off-target effects are minimal. Nonetheless, with the progression of newer and cleaner drugs through clinical trials and into therapeutic use, many drugs have become outdated and are no longer actively prescribed. These compounds can sometimes have functions for ailments other than the original indication(s).¹³ Likewise, drugs actively prescribed could also have targets outside of their current scope of use. Re-tasking or drug repurposing is an attractive avenue, since most, if not all, of the side effects of a particular drug are known and much of the clinical testing is complete, allowing for a less expensive investment and a quicker turn around time from bench to bedside.

In line with the drug repurposing idea, a library of 1,581 containing FDA-approved drugs and drugs approved abroad were tested for antifungal activity against *C. albicans* in serum.⁸⁷ It is important to note that FLC is only moderately active against *C. albicans* when grown in serum. From the library of approved drugs, 15 were active: six were known antifungals, five were antimicrobials and antiseptics, and four were other multifunctional drugs. Of the compounds identified, only octodrine (**8**, Fig. 7) was better at inhibiting the growth of yeast on serum over yeast extract-peptone-dextrose broth.⁸⁷ A second library of known drugs, in

high-throughput screen (HTS) format, was used to look for activity against *C. albicans* biofilms and found that two compounds, not previously known to have antifungal activity, were excellent at reducing the pre-formed biofilms of *C. albicans*.⁸⁸ Auranofin (**9**, Fig. 7), a gold containing anti-inflammatory used for rheumatoid arthritis, and pyrvinium pamoate (**10**, Fig. 7), an antiparasitic, were found to both prevent and decrease the biofilm formation of the *C. albicans* tested.⁸⁸

While HTS of drug libraries is one way of finding drugs worth repurposing, another strategy is to use known antibiotics and tailor them to specific microbes (*e.g.*, aminoglycoside antibiotics being altered to work on fungi instead of bacteria as outlined in modification of current antifungals and other FDA-approved drugs section). While β -lactams are traditionally used for bacterial infections, a novel β -lactam attached to aromatic rings (**11**, Fig. 7) has been found to inhibit the 14 α -demethylase and cAMP pathway in *C. albicans*.⁸⁹ The compound proved to have MIC values in the mid to high $\mu\text{g/mL}$ range. Additionally, the triphenylethylene tamoxifen (**12**, Fig. 7), a known selective estrogen receptor modulator used in the treatment of breast cancer, has been highlighted as a novel antifungal treatment specific to *C. neoformans*.⁹⁰ Tamoxifen is also known to be a kinase inhibitor and to interfere with the calcium homeostasis and by proxy calcineurin, which is required for *Cryptococcus spp.* pathogenesis at normal body temperatures and above.

From these examples, it is clear that modifying and/or repurposing current treatments represent valid ways of seeking new antifungal agents. However, one must not forget that many more scaffolds offered by Nature remain to be investigated.

Alternative sources for novel antifungal agents

Tempering the use of currently available antifungal agents could help alleviate some of the problems (*e.g.*, resistance) resulting from their heavy usage. However, considering the limited repertoire of antifungals, relying exclusively on this strategy would be foolhardy, and novel sources of antimycotics should be sought out. Nature has provided a virtual cornucopia of molecules to discover, emulate, or decorate. The search of novel antifungals is a ubiquitous challenge that scientists are attempting to hurdle. Common compounds that are being examined are (i) natural products, (ii) extracts and essential oils of medicinal plants, (iii) symbiotic organisms known as endophytes, and (iv) synthetic organic molecules. In this section, we present the discoveries that scientists have made from examining these areas for novel antifungals.

i. Natural products—A detailed analysis of all FDA-approved drugs from 1981 to 2010 revealed that 34% of the compounds were either natural products or inspired by natural products.⁹¹ From the anticancer taxol to the antibiotics streptomycin and azithromycin, Nature has provided complex and biologically active compounds to treat diseases that have plagued humanity. It is therefore clear why these molecules have been highly sought after, replicated, and used in medicine. Specifically, natural products have contributed greatly to medical mycology. Besides the polyenes, the echinocandins represent another drug class with great clinical utilities, being highly valued because they lack the drug interactions and adverse effects observed with the azoles and the polyenes.⁶³ Using the semisynthesis

approach, the novel echinocandin ASP9726 (**13a**, Fig. 8) was generated from the natural product FR901379 and found to inhibit the hyphal growth of *Aspergillus spp.*^{92, 93} ASP9726 was further evaluated in guinea pigs and shown to be well tolerated and efficacious against invasive pulmonary aspergillosis.⁹⁴ Another novel echinocandin, currently being evaluated in clinical trials, is bialfungin (also known as CD101 IV or SP3025), which was developed by Cidara Therapeutics.⁶³ Bialfungin (**13b**, Fig. 8) is unique compared to other echinocandins due to its long-lasting effects. The additions of ASP9726 and bialfungin to the echinocandin class have been exciting and intriguing for clinicians and patients as the echinocandins are known to be highly efficacious against invasive candidiasis and aspergillosis. However, one disadvantage of this class of compounds still remains unaddressed. The entire class of glucan synthase inhibitors is made up of echinocandins, which are cyclic peptide drugs and only available in parenteral formulation. An orally active glucan synthase inhibitor would be highly valuable clinically as a step-down therapy. Additionally, orally active drugs are often associated with improved safety and patient compliance, and are more economical. Scynexis, Inc. recently developed SCY-078 (formerly known as MK-3118) (**14a**, Fig. 8), a semisynthetic analogue of enfumafungin (**14b**, Fig. 8).⁹⁴ Enfumafungin is a hemiacetal triterpene glycoside isolated from *Hormonema sp.*⁹⁵ Unlike the glucan synthase inhibitors currently used in the clinic, SCY-078 was found to be orally available and displayed *in vivo* efficacy in a murine invasive candidiasis model. Interestingly, SCY-078 retained *in vitro* antifungal activity against clinical isolates that are resistant to current echinocandins.⁹⁶ Thus, the clinical benefits of SCY-078 can potentially extend beyond its oral bioavailability.

Bacteria and fungi have been a common source of natural products, mainly due to the general ease and simplicity of growth and maintenance. Similarly to the echinocandins, surfactins represent another class of cyclic peptides. Surfactin compounds, isolated from *Bacillus subtilis*, have found many uses in medicine. Recently Zhao and co-workers have used them to inhibit the growth of a the plant pathogen *Fusarium moniliforme* which affects maize, tomatoes, peanuts, bananas, peppers, cotton, and wheat, to name a few.⁹⁷ These researchers engineered a new surfactin by recombining the genes responsible for making the compound in the native bacteria. While the surfactins, under normal circumstances, do not inhibit the growth of filamentous fungi, a simple deletion of D-leucine at one of two positions was able to provide activity against *F. moniliforme* and disrupt the hyphae of the fungi. Also working with *B. subtilis*, Ghribi and coworkers isolated and characterized a cyclic lipopeptide that showed excellent activity against *Rhizoctonia bataticola* and *Rhizoctonia solani*, omnipresent soil-born phytopathogens.⁹⁸ The lipopeptide was shown to weaken sclerotial integrity, which led to hyphal fragmentation and granulation, and eventually shrivelling and cell death at a concentration of 0.04 mg/mL. *Paenibacillus elgii*, a closely related genus to *Bacillus*, is known to produce a variety of active antifungal compounds that fall into many classifications. Most recently, a group from South Korea isolated methyl-2,3-dihydroxybenzoate (**15**, Fig. 8) from *P. elgii* HOA73 and this benzoate was shown to completely inhibit the growth of *Botrytis cinerea* and *R. solani* and slow the growth of *P. capsici* and *Fusarium oxysporum* f.sp *lycopersici*.⁹⁹ In a recent report, a new polypeptide of undetermined origin was obtained from the fermentation of an unidentified fungal strain. This novel compound was tested against several strains of *Candida spp.*,

Aspergillus spp., and *Trichophyton spp.* and displayed activity against all three genera.¹⁰⁰ Macrocyclic trichothecenes (**16**, Fig. 8) are a type of polyketides that have been isolated from both fungi and plants. Their bioactivity has been documented since the mid 1940s and have been extensively explored.¹⁰¹ More recently, this family of compounds has displayed activity against the three of the major human pathogens, *C. albicans*, *A. niger*, and *T. rubrum*, with distinct differences in their activity with each fungus.

Besides bacteria and fungi, another option for finding natural products with antifungal activity is to examine the secondary metabolites that plants produce naturally. For example, potatoes produce glycoalkaloids and phenolic acids that display antifungal activity, particularly when in combination with each other. In 2015, a study was published showing that α -chaconine (**17**, Fig. 8) combined with caffeic acid (**18**, Fig. 8) had better antifungal activity than α -chaconine alone, and was active against fungal strains resistant to α -chaconine, including *Pyrenophora teres* f. *teres*, *Fusarium graminearum*, *A. niger*, *Penicillium roqueforti*, and *Mucor plumbeus*.¹⁰² β -Caryophyllene (**19**, Fig. 8), was isolated from the essential oil of *Aquilaria crassna*, a tree once used in the perfume industry. The extracts were narrowed down and fractionated until a singular compound with antimycotic properties was left.¹⁰³ The assays indicated that the β -caryophyllene was as efficient at killing the fungi (*A. niger*, *Penicillium citrinum*, *Rhizopus oryzae*, and *Trichoderma reesei*) as kanamycin, and this property was attributed to its strong antioxidant activity. Isoliquiritin (**20**, Fig. 8), isolated from the liquorice plant, has many pharmacological uses. Hu and coworkers showed that this compound does indeed inhibit the mycelial growth of *Peronophythora litchii*, *P. capsici*, *Sclerotinia sclerotiorum*, and *Cladosporium herbarum*, and has a toxic effect on the viability of the pathogens' cells as well.¹⁰⁴ Two natural products were recently isolated from leaves of the Mayan medicinal plant *Cestrum schlechtendahlia* (pepper tree or pepper plant).¹⁰⁵ The isolated saponins (**21**, Fig. 8) were shown to be active against the human pathogens *C. albicans* and *C. neoformans*, as well as *Saccharomyces cerevisiae*. Of importance was the ability of the saponin to effectively kill the *C. albicans* D10 strain, which is known to be resistant to the commercial antifungals AmB and ketoconazole (KTC). In addition, the compound was also active against the grain phytopathogen *F. graminearum*. Geraniol (**22**, Fig. 8) is a natural terpenoid found in many plant extracts. The antifungal activity of this small molecule has been noted many times. Earlier this year, work by Singh and co-workers demonstrated that geraniol was linked with the signalling pathway of calcineurin, which leads to disruption of the cell wall.¹⁰⁶ There are also indications of activity in mitochondrial function, iron homeostasis and genotoxicity in *C. albicans*. An oxidized geraniol molecule with promising antifungal activity is geranate. Recently, Mitragotri and coworkers reported a eutectic mixture made up of choline (**23**, Fig. 8) and geranate (**24**, Fig. 8) called CAGE.¹⁰⁷ CAGE exhibited broad-spectrum antimicrobial activity against a number of multidrug-resistant fungi while displaying minimal toxicity against human keratinocytes and mice. Another terpene, cremenolide (**25**, Fig. 8) has recently been isolated from *Trichoderma cremeum*, a very prominent genus of soil fungi that are well known for their generation of bioactive secondary metabolites.¹⁰⁸ This compound was shown to inhibit the radial mycelial growth of *F. oxysporum*, *B. cinerea*, and *R. solani*, while promoting the growth of seedlings.

Phenolic acids and other aromatic compounds are found throughout natural products. Many of these display antifungal activity. However, due to a recent review article published,¹⁰⁹ we will only focus on the ones that have been reported in the last year. Cinnamaldehyde (**26**, Fig. 8), a natural aromatic compound, has recently found uses as an antimicrobial. It was shown that this compound has dose-responsive activity against *Aspergillus spp.* by lowering the levels of lipid peroxidase and reduced glutathione, forcing the cells to be in a state of oxidative stress. This is also thought to change the cellular structure, particularly in the hyphae, resulting in cell death.¹¹⁰

ii. Extracts and essential oils—While efforts to identify and synthesize novel antifungals represent an important area, an alternative way to finding new treatments is to examine homeopathic remedies that have been used for centuries. The use of homeopathic and/or herbal medicine has been controversial among the scientific community despite the discovery of an antiparasitic in *Artemisia annua* (sweet wormwood) that won its discoverer the 2015 Nobel Prize in Medicine.¹¹¹ Recently, many scientists have examined essential oils and extracts for bioactive compounds, including antifungals. Since commercial antifungal agents are either not available or too expensive for certain global populations, an alternative for these individuals is to turn to professionally prescribed holistic or homeopathic remedies. Herein, we will discuss promising plant extracts and essential oils.

Several studies have examined the extracts of various plants ranging across several botanic families and from disparate locations across the globe. Many of the plants in Africa are found to be rich in biologically active compounds. One study tested the extracts of multiple parts of plants found in the pharmacopoeia of the Côte D'Ivoire.¹¹² While no individual plants were identified or compounds characterized, *C. albicans spp.* were found to be sensitive to all the extracts tested. Methanolic extracts from *Cleistopholis patens* ("salt-and-oil" tree), exhibited significant activity against *C. albicans*, *A. fumigatus*, and *C. neoformans*.¹¹³ The alkaloid 3-methoxysampangine was found to be responsible for the antifungal activity. The sampangine scaffold is an azaoxaporphine alkaloid, which was first discovered from the stem bark of *Cananga odorata* (cananga tree).^{114, 115} Sheng and co-workers utilized scaffold hopping and structural simplification strategies to generate analogues of sampangine with improved antifungal activity as well as drug-like properties.^{116, 117} The aqueous, methanolic, and acetone extracts of three plants (*Combretum cafferum* (bushwillow tree), *Salix capensis* Thunb (cape silver willow), and *Schotia latifolia* Jacq. (a legume)) used for the treatment of livestock disease in South Africa were examined for antimycotic activity.¹¹⁸ From these extracts, the methanolic and acetone fractions had the best fungicidal activity, while the aqueous extract was more fungistatic when tested against *Alternaria alternaria*, *A. niger*, *Mucor hiemalis*, *Penicillium chrysogenum*, and *Schizophyllum commune*. The *Artemisia herbaalba* (desert wormwood) essential oil has also been tested for antifungal activity.¹¹⁹ The best antifungal activity was observed with the dermatophyte fungi *T. rubrum* and *Epidermophyton floccosum*. The extracts also displayed minimal effects on mammalian cells.

The methanolic extracts of *Calibrachoa x* hybrid (nightshades), originating in Europe, have been tested for antimicrobial activity.¹²⁰ The extracts showed excellent inhibition of the

growth of several strains of fungi, including *Aspergillus spp.*, *Penicillium spp.* and *Candida spp.*

From the Americas, *Flourensia cernua* (American tarwort) extracts have been examined for various toxicities and were found to have antifungal activity.¹²¹ The hexanes extract was consistently more active against *Candida spp.* than the essential oil. The main components of the extract were α -pinene, 3- γ -carene, and limonene. A Brazilian group took the idea of extracts further and examined the resinous mixture from various parts of plants (propolis), which is processed by bees to construct their hives.¹²² The researchers collected several types of propolis from around the country and found that one particular type of propolis had excellent fungistatic properties against several *Candida spp.* with comparable MIC values to the known antifungal NYS.

Plants also produce specific compounds that give them their identity. In particular, many aromatic plants have an individual oil, or mixture of oils that is responsible for the characteristic smell that is known as that plant's essential oil. These oils also contain biologically active molecules and have been mined for novel drugs and scaffolds. These compounds have also been tested as antifungals. Essential oils from *Cymbopogon citratus* (lemongrass) and *Ocimum basilicum* (basil) were tested for antifungal activity and showed the ability to reduce the mycelial growth of the *Phytophthora spp.* tested.¹²³ Likewise, the essential oil of *Zataria multiflora*, a thyme-like plant, was tested against strains of *Candida spp.* isolated from the oral cavity and compared to the activity of sodium hypochlorite (NaOCl).¹²⁴ The study concluded that the essential oil was found to be an acceptable antifungal considering the hazardous nature of NaOCl. The essential oils of four *Myrtus communis* (myrtle plants) species have been examined for their antimycotic activity against a host of phytopathogenic fungi.¹²⁵ The oils containing α -pinene, limonene, 1,8-cineole, linalool, myrtenyl acetate, and linalyl acetate, showed a broad range of activity against the 20 fungi tested (0–100% growth inhibition). The essential oil of the fruit of *Coriandrum sativum* (cilantro/coriander) has been examined for antifungal activity.¹²⁶ The main constituents of the oil were linalool, geraniol, and neryl acetate. Several *Candida spp.* and *Microsporum canis* were tested against the essential oil. While the compounds were effective at killing or halting the growth of the fungi, they displayed MIC values much larger than those for known antimycotics. However, as this is a mixture of volatile compounds, testing of a single compound may provide better data. The authors were particularly interested in the treatment of animals, as many of the fungi found on animals are resistant to azoles.¹²⁷ Garlic oil was employed to subdue the growth of *Candida spp.* and avoid the issue of biofilm formation,¹²⁸ which can drastically change the efficacy of drugs, especially those formed by *C. albicans*. The oil was found to severely damage some of the organelles, mainly the vacuoles, mitochondria, and the storage granules.

Just as with the FDA-approved compounds, researchers have investigated the potential to repurpose holistic treatments. A series of plants from sub-Saharan Africa, known to have antiparasitic properties, were recently tested for their antifungal properties.¹²⁹ Leaf extracts from 13 plant species were tested against the three most prevalent fungal pathogens *C. albicans*, *C. neoformans*, and *A. fumigatus*. Several extracts showed good antifungal activity

after both 12 h and 24 h of growth. Extracts were particularly active against *A. fumigatus* and *C. neoformans*.

A particularly rich and under-cultivated source of secondary metabolites, dung-inhabiting fungi, has been recently reviewed.¹³⁰ Many species of fungi have been identified from solid excrement and analyzed for secondary metabolites with antifungal properties. Compounds isolated ranged in class including benzoquinones, polyketides, sesquiterpenes, depsipeptides, and terpenoids.

iii. Isolation of compounds from endophytes—Perhaps an overlooked area for antifungal compounds is the symbiotic organisms that exist in Nature. Many plants and other organisms live in a balanced equilibrium with one or more fungal or bacterial species that benefit both organisms. In particular, many species of plants contain endophytes that help them grow, prevent infections, and, in return, the plants give them a home. Endophytic fungi are microorganisms that reside either within the tissue or on the surface of plants without harming the development or health of the host. These organisms are known to produce some rare and novel secondary metabolites with notable pharmacological properties.¹³¹ It is likely that these fungi produce secondary metabolites that protect their hosts from other forms of colonization.

Dendrobium officinale (orchid) is a known plant in the Chinese pharmacopoeia. Recently Han and co-workers examined this for endophytic fungi and isolated five compounds from one fungal strain, DO14, belonging to the genus *Pestalotiopsis*.¹³² These compounds showed various antifungal activities against the four major pathogenic genera (*Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., and *Trichophyton* spp.), while being significantly less toxic against mammalian cells. Another endophytic fungus that produced antifungal compounds was isolated from the *Eleusine coracana* (finger millet) plant.¹³³ Three different fungi resembling the genera *Aspergillus* spp., *Penicillium* spp., and *Phoma* spp. were isolated from *E. coracana* and were found to produce compounds active against the phytopathogen *Fusarium* spp. by disrupting the hyphae of these filamentous fungi. The endophyte *Trichoderma kongiopsis*, isolated from the medicinal plant *Panax notoginseng*, was found to produce koniginins, which showed both antifungal and herbicidal activity.¹³⁴ A more extensive examination of the koniginins showed that while koningiopisin B, koningiopisin C, and koningiopisin H all had antibiotic activity on their own, they displayed stronger activity in their mixed form (koningiopisin D-H).¹³⁵ The endophyte, *Guignardia* sp., from the flowering plant *Euphorbia sieboldiana*, has been examined for its antifungal meroterpenes and dioxolanone derivatives.¹³⁶ Out of 17 compounds, four were found to inhibit fungal growth, including the known compound guignardone B. These compounds were also tested in combination with FLC. When in combination, compounds that showed no antifungal activity against *Candida* spp. were active, while the MIC value of FLC decreased. The compounds were also tested for disruption of the *Candida* spp. biofilm and showed excellent disruption when used in combination with FLC. An asexual endophyte, *Epichloë* sp., has attracted much attention over the past 30 years for its known benefits in forage grasses, rye, and fescue.¹³⁷ In particular the *Epichloë* sp. XH03 isolated from *Festuca sinensis*, a cool-season grass important to cattle, showed broad inhibition of the

phytopathogenic species *Alternaria alternata*, *Bipolaris sorokiniana*, and *Cochliobolus lunata*, in both saprotrophic and biotrophic growth.¹³⁸

Plants can also form endophytic relationships with bacteria. Recently, endophytic strains of *Streptomyces* from the transitional medicinal plant *Arnica montana* were isolated and analyzed for biological activity.¹³⁹ In total, five strains of *Streptomyces* were found, and extracts from each strain were analyzed. Cycloheximide, a known antifungal,¹⁴⁰ was isolated from two of the strains. These isolates proved to be active when tested for antifungal activity with *C. parapsilosis* and *Fusarium verticillioides*.

In addition to plants, other life forms have symbiotic relationships with microbes. Recently the *Mytothecium* sp. Z16 fungi isolated from *Argyrosomus argentatus*, a species of fish, was shown to produce compounds that have antifungal properties,¹⁴¹ one novel and five known compounds. The best compounds isolated from the fungus were roridin A and verrucarins A with activity similar to that of KTC against the pathogenic fungi *A. niger*, *T. rubrum*, and *C. albicans*. The medicinal cactus, *Opuntia humifusa* was the object of a recent phylogenetic analysis of its endophytic fungi.¹⁴² 17 distinct species were discovered from a menagerie of 108 isolates. The extracts of six of these isolated fungi showed varied antifungal activity. NMR analysis led to the isolation of 5-methylmellein, which was tested against seven phytopathogens and showed growth inhibition for several strains including *Peltula obscurans* and *Plasmopara viticola*.

iv. Novel synthetic scaffolds—While Nature provides an excellent cornucopia of molecules that can be used in an almost infinite pattern, sometimes these compounds are not effectively, efficiently, or easily obtained. For these instances, organic synthesis provides a means to produce large quantities of compounds to test or even use for treatment. Unmistakably, the azoles and allylamines are some excellent organic small-molecule drugs. Additionally, as discussed previously, in terms of crops, the majority of the plant fungicides (triazoles, morpholines, benzimidazoles, and thiocarbamates among others) currently used are synthetic organic small-molecules. While lacking the chemical complexities of natural products for potent and specific target binding, small molecules possess unique advantages in that they are easy to synthesize, modify, scale up, and more likely to possess drug-like characteristics and predictable pharmacokinetics. In this section, we are highlighting some promising novel synthetic antifungals that are currently being developed and categorize them by their respective molecular targets. These promising compounds not only give us hope in the fight against pathogenic fungi, but also demonstrate the creativity and brilliance of the medicinal chemists and mycologists of our generation.

Since the discovery of penicillin by Sir Alexander Fleming, the bacterial cell wall has been one of the primary targets for many FDA-approved and experimental antibacterials. In comparison, targeting the fungal cell wall remains fairly unexplored. In fact, the echinocandins, sometimes known as the “penicillin of antifungal agents”, were not introduced to the market until 2001 when CFG, the first fungal cell wall inhibitor, was approved by the FDA.³⁶ As exemplified by the safety and efficacy of the echinocandins, the fungal cell wall is a highly valuable target due to the lack of homologous structure in mammalian cells. Despite their clinical success, one drawback of the echinocandins is that

CFG, AFG, and MFG are only available as intravenous drugs.¹⁴³ This clinical need paved the way for the discovery of a class of orally active small-molecule glucan synthase inhibitor called the piperazinyl-pyridazinone (**27**, Fig. 9), which was reported by Merck in 2011.¹⁴⁴ These compounds were initially identified in a high-throughput screen (HTS) of the Schering-Plough compound collection and optimized by SAR studies.^{145, 146} The lead compounds were found to display oral efficacy in a murine model of *C. glabrata* infection. Furthermore, at least one piperazinyl-pyridazinone analogue displayed good MIC values (4 µg/mL) against *C. neoformans*, which is an improvement over the current echinocandins in terms of antifungal spectrum.¹⁴⁴ Another glucan synthase inhibitor is an oxathiolone-fused chalcone called AMG-148 (**28**, Fig. 9).¹⁴⁷ AMG-148 exhibited potent MIC values (1–4 µg/mL) against clinical *C. albicans* strains. Many small-molecule cell wall modifiers have been identified and could potentially supplement the current class of echinocandins as a step-down therapy in the treatment of invasive candidiasis. Unlike the echinocandins and other natural-product glucan synthase inhibitors, these small molecules are structurally simpler and thus, could potentially become more cost-effective therapeutic options for patients.

Moving on from the fungal cell wall to the cell membrane, the ergosterol biosynthesis is one of the most valuable fungal targets. Moving on from the fungal cell wall to the cell membrane, the ergosterol biosynthesis is one of the most valuable fungal targets due to its role in the cell membrane.^{148, 149, 150} Nitrogen-containing heterocycles are traditionally good sources of small-molecule inhibitors of the ergosterol pathway. Thus, numerous ergosterol biosynthesis inhibitors contain imidazoles, triazoles, and morpholine functional groups. In 2010, Ando and co-workers identified another nitrogen-containing heterocycle, the aminopiperidine (**29**, Fig. 9) as a potential ergosterol-inhibiting pharmacophore.¹⁵¹ Two aminopiperidines were found to be 10-fold more potent than FLC. Biochemical and genetic analyses indicated that these compounds inhibit the enzyme Erg24p of the ergosterol biosynthetic pathway. Interestingly, the authors suspected that the compounds could act as a dual inhibitor by also inhibiting Erg2p also involved in ergosterol biosynthesis. Another novel 14α-demethylase inhibitor is compound SYP-Z048 (**30**, Fig. 9), which contains a combination of pyridine and isoxazoline moieties.¹⁵² This compound was designed for use as an agricultural fungicide. After extensive testing against several phytopathogenic fungi, SYP-Z048 was shown to be a broad-range antifungal agent and to work as both a protectant and curative. Besides utilizing a nitrogen heterocycle as the pharmacophore, an alternative approach is to design inhibitors that mimic the substrate or intermediate of the enzymes of the ergosterol biosynthetic pathway. In 2011, Murgich and coworkers developed steroid analogues that inhibited enzymes in this pathway.¹⁵³ They reported four sterol hydrazine analogues (**31**, Fig. 9) capable of inhibiting the 24-sterol methyltransferase enzyme with potent fungicidal activity against *Paracoccidioides brasiliensis*.¹⁵³ The approach of targeting non-CYP enzymes in the ergosterol biosynthetic pathway is exciting because it is a novel strategy to generate compounds that can mimic the clinical utilities of the azoles without theazole-related drug-drug interaction drawbacks.

Besides targeting ergosterol biosynthesis, the fungal cell membrane can also be targeted by using molecules that can physically disrupt it. Amphiphilic aminoglycosides with long alkyl

side-chains have been a topic of many research investigations and were found to selectively disrupt fungal cell membranes.^{66–70} Analogously, similar amphiphilic compounds derived from trehalose (**32**, Fig. 9) were synthesized by Fridman and co-workers and were found to be non-specific membrane permeabilizers, which work on both bacteria and fungi.¹⁵⁴ Notably the compounds show consistent activity against most of the *Candida spp.* tested, showing activity against azole-sensitive and azole-resistant species. Similarly to the amphiphilic trehalose and aminoglycoside derivatives, other groups have also investigated coupling linear alkyl chains to other small-molecule heterocyclic scaffolds. These compounds coupled with linear alkyl chains may also act on fungal cells' membrane. The imidazolium salts (**33**, Fig. 9) have been studied for various applications. In 2015, Fuentesfria and co-workers explored their use as antifungals.¹⁵⁵ Of particular interest were the dermatophytes. The 11 imidazolium salts tested inhibited the dermatophyte fungi (*Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*), with the most effective compounds having either a decyl or palmitoyl side chain. The study also varied the counter ion and found the chloride and mesyl salts to have the best activity, especially when combined with the palmitoyl side chain. In addition, these compounds displayed no major toxic effects. Later, the same group examined the effect of the imidazolium salts on the phytopathogen *F. graminearum*.¹⁵⁶ They observed that the antifungal activity was mostly modulated by that the counter ion. Another molecule containing long linear alkyl chains is phosphatidylethanolamine. Phosphatidylethanolamine-*N*-amino acid derivatives (**34**, Fig. 9) have recently been investigated as antimicrobials.¹⁵⁷ These compounds were tested against *S. cerevisiae*, *C. albicans*, and *A. niger* and were found to be most active against the latter two species, with the phenylalanine derivative being the most active. Esterquats are quaternary amine salts that are capped with either one or two alkylated esters (**35**, Fig. 9). A series of these compounds was synthesized and tested for antimicrobial activity by Penumarthi and co-workers.¹⁵⁸ They found that six compounds showed anticandidal activity. Additionally, a series of novel macrocyclic amidinoureas (**36**, Fig. 9), resembling the common macrolide antibiotics, was synthesized and tested for antimycotic activity.¹⁵⁹ These compounds also contain the long linear alkyl moieties. They were tested against 116 clinical isolates consisting of seven *Candida spp.* Two compounds were shown to have excellent activity, being more active than FLC and active against FLC-resistant strains. The compounds showed activity similar to that of AmB. Different size cycles were made and showed species selectivity, with a 14-membered ring having better activity against *C. albicans*, *Candida guilliermondii*, and *C. parapsilosis*, while the larger macrocycle (15-membered) was more active against *Candida tropicalis* and *Candida kefyr*.

As an alternative to the whole-cell screening approach, some researchers prefer the target-based strategy. In this strategy, an essential enzyme for cell survival would be selected, and then the researchers would seek out a specific inhibitor for that enzyme. Many agricultural antifungals (e.g., carboxin and boscalid) target the ubiquinone-binding site of succinate dehydrogenase (SDH).^{160–162} This enzyme also provides an excellent target in plant-based fungal infections, as SDH is an essential enzyme of the tricarboxylic acid (TCA) cycle. Hence, inhibition of SDH would be lethal to the cell. Krawczyk and co-workers have synthesized 2H-1,4-benzoaxazin-3(4H)-one derivatives (**37**, Fig. 9) as potential inhibitors of SDH.¹⁶³ This scaffold was selected based on its structural similarity to the commercially

available fungicides. Two synthesized compounds showed excellent activity against seven strains, based on growth of the phytopathogens' hyphae. Additionally, another SDH inhibitor developed by a Japanese company, 2-(3,5-dimethyl-1*H*-pyrazol-1-yl)-5-methylphenol (ME1111, **38**, Fig. 9), was found to display antifungal activity against *Trichophyton spp.*, a causative organism of onychomycosis,¹⁶⁴ with MIC values in the mid to high ng/mL range.¹⁶⁵

Aside from these compounds with well-validated mechanisms of action and molecular targets, there have also been other molecules found to display promising antifungal activity, but their mode(s) of action remain an enigma. One such compound is T-2307 (**39**, Fig. 9), an arylamidine discovered by Toyama Chemical Co., Ltd.^{63, 166} T-2307 is highly fascinating due to its ability to accumulate precipitously faster in *C. albicans* cells over mammalian cells, which eventually leads to collapse of the mitochondrial membrane potential.^{167, 168} The efficacy of T-2307 has been validated in a murine model of fungal infection.¹⁶⁶ Deepak and co-workers synthesized 2-azetidinonyl-5-(2-benzoylphenoxy) methyl-1,2,4-oxadiazoles (**40**, Fig. 9) to combat the fungal infection of seeds, and found two promising compounds, which were recommended for use in finger millet.¹⁶⁹ A series of metal-complexed (*E*)-*N*-(4-(2-hydroxybenzylideneamine) phenylsulfonyl) acetamide Schiff bases (**41**, Fig. 9) was synthesized and tested against *Aspergillus spp.* and *Candida spp.*¹¹⁹ The metals used included Ag^I, Cd^{II}, Ce^{III}, Co^{II}, Cr^{III}, Fe^{III}, Ni^{II}, and Pb^{II}. The MIC values for the complexes were slightly better than those for AmB. However, the complexes did not seem to be more active than the ligand alone.

Another important total synthetic scaffold with antifungal activity that has been recently described was the *N*-substituted-1,3-thiazolidin-4-one.^{170–172} A large library of these *N*-substituted-1,3-thiazolidin-4-one analogues was generated and evaluated *via* the broth microdilution method against 22 clinical *Candida* spp. Compared to the currently used topical and systemic antifungal drugs, some of the best *N*-substituted-1,3-thiazolidin-4-one analogues displayed similar or better MIC values. These compounds also displayed toxicity against the mammalian Hep2 cell line at concentrations much higher than the antifungal or therapeutic concentrations. Interestingly, these compounds possessed strong selectivity for fungal cells, as none of the analogues were antibacterial.^{170, 172} Furthermore, some of the analogues (**42**, Fig. 9) with potent antifungal activity were used as “Training-Set” molecules for ligand-based pharmacophore virtual screening, which led to the identification of some coumarin hit compounds with antifungal activity.¹⁷¹

High-throughput screening

As it is difficult to create new organic scaffolds from scratch, HTS has gained popularity in the past ~15 years to identify leads for further chemical modifications, and therefore a discussion on the discovery of antifungals by HTS is warranted. Although sparse in the recent literature, we were able to identify a few innovations in HTS technology within the context of antifungal drug discovery. Recently, a library of 1,280 compounds was tested against *C. albicans*, from which only five were confirmed hits.¹⁷³ When testing different cell loads for the resistance of these compounds, only one, sanguinarine chloride was found to be active at both high and low cellular concentrations. Sanguinarine chloride showed activity

against adhesion, and was still active, albeit to a lower extent on the biofilm stage of growth. Based on previous reports, it is thought that sanguinarine chloride suppresses $\text{NH-}\kappa\text{B}$ and modulates several protein kinases.

Roemer and co-workers recently published a report in which they screened a library of natural products to identify antifungals with potentially novel cellular targets.¹⁷⁴ From these efforts, several compounds were identified, including yefafungin and aspirochlorine, both targeting protein synthesis, 12-deoxy-hamigerone interfering with microtubule assembly, campafungin inhibiting hyphal growth, dretamycin disrupting iron homeostasis, and fellutamide C and D inhibiting the proteasome.¹⁷⁴ Recently, by screening 361,675 molecules, Krysan and co-workers identified the benzothiourea scaffold, which inhibited the cell wall integrity pathway.¹⁷⁵

Given the usefulness of HTS in drug discovery and its recent success in identifying novel antifungals, it is surprising that it has not been exploited more broadly. Thus, it is pretty much a wide-open area of research for scientists interested in antifungal discovery.

Novel antifungal targets

In addition to finding new antifungal drugs for the currently known targets, another strategy to overcome resistance consists of identifying new molecular targets for inhibition. One such target, which was discovered ~10 years ago, is Upc2, a member of the fungus-conserved $\text{Zn}_2\text{-Cys}_6$ binuclear cluster transcription factors.¹⁷⁶ This protein has homologues in many pathogenic fungi and it induces the overexpression of ergosterol biosynthetic genes in response to azole treatment. After developing a genetic and cellular-based assay, the authors identified several activators and several inhibitors of Upc2. However, the compounds did not kill the fungi, but did inhibit their growth.¹⁷⁷

The conventional strategies of targeting the fungal cell wall or the biosynthesis of ergosterol have been historically effective by either killing or inhibiting the growth of the pathogenic fungi. However, these approaches tend to produce strong selective evolutionary stress, allowing the microbes to evolve into drug-resistant strains.^{178, 179} Along with the increased understanding of fungal pathogenesis, a new strategy of targeting fungal virulence factors was proposed.^{178, 180} The glycosylphosphatidylinositol (GPI)-anchored proteins are virulence factors facilitating adhesion of fungal cells to hosts' mucosal and epithelial surfaces.^{181, 182} The adhesion of *Candida* cells to host has been determined to be a key process during pathogenesis.¹⁸³ E1210 (**43**, Fig. 10) was reported as an orally active isoxazole-based small-molecule compound, which inhibited the enzyme GWT1, a key inositol acyltransferase involved in GPI biosynthesis.^{184, 185} E1210 displayed potent activity against many pathogenic *Candida spp.*, *Aspergillus spp.*,¹⁸⁶ *Fusarium spp.*,¹⁸⁷ and *Scedosporium spp.*^{63, 188} Despite its broad spectrum of activity, E1210 was actually not as potent against *C. krusei* and members of *Mucorales in vitro*.^{63, 187, 188} Lindquist and co-workers identified gepinacin as another small-molecule inhibitor of GPI acyltransferase with low toxicity to mammalian cells.¹⁸⁹ Gepinacin (**44**, Fig. 10) displayed potent activity against many *Candida spp.*, but was inactive against *C. krusei* and *A. fumigatus*.¹⁹⁰ Other GWT1 enzyme inhibitors identified were the picolinamide analogue G884 (**45**, Fig. 10) and the benzoate analogue G365 (**46**, Fig. 10).¹⁹⁰ However, both G884 and G365 were not active

against *C. krusei* and *C. glabrata*. G884 was also inactive against *A. fumigatus*.¹⁹⁰ Another enzyme in the GPI biosynthesis that has been studied as a potential drug target is Mcd4, an ethanolamine phosphotransferase responsible for transferring phosphoethanolamine to the first mannose molecule during GPI biosynthesis.^{191, 192} The terpenoid lactone compounds M743 (**47a**, Fig. 10) and M720 (**47b**, Fig. 10) were reported to inhibit Mcd4 while displaying potent antifungal activity against *Candida spp.* and *A. fumigatus*, which were comparable to those of CFG and AmB.¹⁹⁰ Overall, molecules that can inhibit GPI biosynthesis have great potential as novel drug classes that may act as either as monotherapy or may be used in conjunction with another fungicidal agent.

Other methods

While the search for novel antifungals is an ongoing process, an alternative to finding small molecules for the treatment of crops to prevent blight outbreaks is to grow the plants in the presence of another organism, which will result in commensalism. A recent study has examined the use of *Chromobacterium* sp. C61, a chitinase-excreting bacteria under different field conditions to prevent the blight of peppers caused by *Phytophthora*.¹⁹³ This species of bacteria produces a chitinase, which prevents the growth of several types of phytopathogens. Further investigation of the bacteria showed that the production and excretion of the chitinase was dependent on the amount of bacteria present, *i.e.*, a quorum sensing response.

There has also been extensive studies on soil *Actinobacteria*, which showed that several *Streptomyces spp.* are able to control the populations of phytopathogens and other toxin-producing fungi that are detrimental to both plants and humans.¹⁹⁴ Admittedly, the idea of adding bacteria to food crops is not the most appealing thought; this investigation does however provide evidence for the need for new methods to treat fungal outbreaks.

Perspective and conclusions

Based on the information we have presented here, there is one concerning aspect of note, the same class of compounds being used on multiple links of the food web (azoles or other DMIs) is distressing and a possible avenue for fungi to become resistant to many antimycotics. The search for novel antifungals has bridged many scientific disciplines from organic synthesis to systems biology and many novel antifungal candidates have been discovered, along with new formulations and combinations of known antimycotics. While development of new compounds to fill the antifungal pipeline is trickling, it is greatly encouraging to see that that researchers are seeking out antifungal compounds from hiding places throughout the world. The field of antifungal discovery is experiencing a renaissance. It is therefore a great era for young and experienced scientists alike to carve their niche in this field of research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A. Schematic showing that fungi can infect not only humans, but affect humans at multiple points of the food web. Lines indicate connections through food sources. Examples of pathogenic fungi are written adjacent to each point or connection. **B.** Line graph showing the increase in the publication of antifungal papers.

A. Timeline showing the introduction to market (year of introduction into parentheses) of the antifungals for human use (top) and for use on crops (bottom) discussed in this review. AmB = amphotericin B, 5-FC = 5-fluorocytosine, NYS = nystatin, MCZ = miconazole, NAF = naftifine, FLC = fluconazole, KTC = ketoconazole, ITC = itraconazole, TER = terbinafine, CFG = caspofungin, VOR = voriconazole, MFG = micafungin, POS = posconazole, AFG = anidulafungin, CBM = carbendazim, PCZ = propiconazole, TBC = tebuconazole, FPN = fenpropidin, DTM = dimethomorph, AXN = azoxystrobin, KS-M = kresoxim-methyl, ECZ

= epoxiconazole, FLU = flumorph. **B.** Structures of antifungals currently used in the clinic and as fungicides.

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Fig. 3. Bar graphical representation of the number of individuals living with and deaths caused by fungal infections. Data for this graph were obtained from the Global Action Fund for Fungal Infections website (www.gaffi.org).

Fig. 4.
Pie chart showing the percentage market shares of agricultural fungicides in 2005.

Fig. 5.
Structures of compounds **1–3** from the modified FDA-approved drugs section.

Fig. 6. Cartoon depiction of the two different types of drug combination **A**. Two compounds inhibiting two separate targets (purple and green ovals), and **B**. One compound sensitizing the fungi to the second, shown is drug Y causing the fungi to be sensitive to drug X. **C**. Structures of compounds **4–7** used in combination with FDA-approved antifungals.

Fig. 7. Structures of the FDA-approved and like compounds **8–12** discussed in this section that were found to display antifungal activities.

Fig. 8. Structures of compounds **14–26**, some of the natural products and natural product mixtures that were observed to have antifungal properties.

Fig. 9. Structures of compounds **27–41**, novel synthetic compounds displaying antifungal activity.

Fig. 10.
Structures of compounds **42–46b** that inhibit new targets in antifungal metabolism.