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In pursuit of the ideal antifungal agent for *Candida* infections: high-throughput screening of small molecules

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Chemical compounds studied in this article: ETYA (PubChem CID: 1780); CGP-37157 (PubChem CID: 2688); shearinine D (PubChem CID: 16104602); shearinine E (PubChem CID: 44423349); HWY289 (PubChem CID: 487138); D75-4590 (PubChem CID: 948175); E1210 (PubChem CID: 16719049).

Teaser: High-throughput screening of small molecules, which enables rapid hit identification and increases hit rate, is particularly helpful in the pursuit of ideal antifungal for *Candida* infections.

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Candida infections have created a great burden on the public healthcare sector. The situation is worsened by recent epidemiological changes. Furthermore, the current arsenal of antifungal agents is limited and associated with undesirable drawbacks. Therefore, new antifungal agents that surpass the existing ones are urgently needed. High-throughput screening of small molecule libraries enables rapid hit identification and, possibly, increases hit rate. Moreover, the identified hits could be associated with unrecognized or multiple drug targets, which would provide novel insights into the biological processes of the pathogen. Hence, it is proposed that high-throughput screening of small molecules is particularly important in the pursuit of the ideal antifungal agents for _Candida_ infections.

Introduction

Candida, the major fungal pathogen in humans, is the fourth-most prevalent [s2]pathogen of nosocomial bloodstream infections surpassing most bacterial infections [1]. *Candida* infections are opportunistic and are, therefore, common among immunocompromised populations, such as neutropenic patients, patients under intensive care, organ-transplant recipients, patients with underlying malignancy, HIV patients and patients with uncontrolled diabetes. Invasive candidiasis is associated with significant mortality [2,3] and is, therefore, a serious threat to public health around the globe [4–6]. The recent shifting of the paradigm of *Candida* infections further complicates the situation and highlights the need for novel classes of antifungal agents, especially those with new mechanisms of action [7,8]. However, the progress of antifungal drug discovery has been lagging. The last first-in-class antifungal drug, caspofungin, was approved more than a decade ago in 2001 [9]. One of the reasons for the slow progress is the eukaryotic nature of fungi, which carry substantial similarities to human cells limiting the number of fungal-specific drug targets [10–12]. To accelerate the progress of antifungal drug discovery, the hit identification rate has to be significantly increased. High-throughput screening is a powerful tool that incorporates synthetic chemistry and combinatorial chemistry to provide rich sources of small molecules of diverse structure to increase the hit rate for discovering novel antimicrobial lead compounds.

The need for novel antifungal agents

The recent changes in the epidemiology of *Candida* infections have pressured the healthcare sector and highlighted an urgent need for novel classes of antifungal agents with different chemical structures and cellular targets [7,8]. These issues include the increased incidence of

invasive candidiasis, the shifting species distribution of *Candida* infections, the emergence of antifungal resistance and the limitation of the current arsenal of antifungal agents.

Increased incidence of invasive candidiasis

The secular trends of incidence of *Candida* infections, in particular *Candida* bloodstream infections, have been increasing over the past few decades across the globe [13–22] (Table 1). This could be attributed to the increased number of immunocompromised patients due to the growing widespread use of medical procedures, for instance the use of immunosuppressive drugs and broad-spectrum antibiotics, as well as invasive surgical procedures such as solid organ transplantation [23].

Shifting species distribution of* Candida *infections

Candida albicans, the predominant disease-causing *Candida* species, possesses various virulence factors that render it the most pathogenic of all the *Candida* species. Recently, the continued increase in invasive candidiasis caused by non-*albicans Candida* (NAC), such as *Candida dubliniensis*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis*, has been recognized [5,24–28]. This could be partly caused by the improvement in the sensitivity of species identification methods, as well as the indiscriminate use of antifungals [29]. Emergence of NAC raised a concern because they are often associated with antifungal resistance and higher mortality [30,31]. For instance, *C. glabrata* and *C. krusei* are intrinsically less susceptible to fluconazole [28], and lower susceptibility for amphotericin B and 5-fluorocytosine has also been observed in *C. krusei* [32].

Emergence of antifungal resistance

The emergence of antifungal resistance, which is one of the major reasons for antifungal treatment failure, has been regarded as a significant clinical problem [33]. The increase in the

numbers of high-risk populations has raised the frequency of prophylactic treatment. Prolonged exposure to the existing antifungals increases the selection pressure and, as a result, drug resistance has become increasingly common from originally susceptible species [34,35]. This phenomenon further restricts the available choice of treatment from the limited arsenal of antifungal agents.

Current antifungal agents and their limitations

Up until 2012, existing antifungal agents for systemic candidiasis were mainly divided into four classes: polyenes, azoles, echinocandins and pyrimidines [8] (Table 2). The classes of antifungal agents refer to their distinct mode of action. In addition to the limited number of available antifungal agents, there exist limitations for each antifungal class.

Polyenes. The first antifungal, nystatin, isolated from *Streptomyces noursei*, was discovered in 1950 through screening various cultures of actinomycetes from soil for antifungal activity [36]. However, owing to its toxicity when injected intravenously and the lack of oral bioavailability, nystatin remains a topical antifungal agent [37]. Amphotericin B was developed in 1953 from a screening of *Streptomyces* cultures for antifungal activity [38]. Amphotericin B binds directly to ergosterol, the component of the fungal plasma membrane, and thereby causes the leakage of intracellular potassium and magnesium [39]. It is by far the most potent antifungal agent for systemic candidiasis, and has been regarded as the 'gold standard' [40]. Amphotericin B has a broad spectrum and fast onset of activity [41,42]. However, it is associated with adverse nephrotoxicity that is cumulative and might not be reversible [43]. Enhanced versions of amphotericin B have been developed by lipid formulations to allow various administration routes and reduced toxicity [44,45].

Pyrimidines. Flucytosine (or 5-fluorocytosine) is a synthetic compound, which was originally synthesized in 1957 as a potential antitumor drug; however, its antifungal activity was later discovered and it was used to treat candidiasis from 1968 [46,47]. Flucytosine is converted to 5-fluorouracil in fungal cells, which then inhibits DNA and RNA synthesis. Because its target is absent in mammalian cells, flucytosine is fungal-specific and has a narrow spectrum of activity [46,48]. Flucytosine is very often used in combination with amphotericin B and azoles rather than monotherapy because of the high prevalence of intrinsic and acquired resistance observed in *Candida* spp. [49,50].

Azoles. Antifungal activity of azoles was first described in 1944 [51]; however, the first azole antifungal, clotrimazole (topical antifungal), was not developed until 1958 [52]. An azole is a fungistatic antifungal agent that depletes ergosterol in the fungal cell membrane by inhibiting the enzyme 14α-demethylase [53]. Various derivatives have been developed since to accommodate the growing burden of fungal diseases in the populace. These subclasses include imidazoles and triazoles [52,54]. Imidazoles, except ketoconazole, are ineffective systemically and are associated with toxicity. Therefore, imidazoles have been replaced by triazoles as a first-line treatment of systemic candidiasis. Second-generation triazoles, such as voriconazole, have a broader spectrum of activity than the first-generation triazoles (fluconazole and itraconazole) [55]. Despite being the first choice of therapy, popular usage and fungistatic properties of triazoles have led to a high incidence of resistance. Additionally, azoles are slower regarding onset of action than amphotericin B [56].

Echinocandins. Echinocandins are the latest class of antifungal and inhibit the synthesis of β-1,3-glucan, an important component in the fungal cell wall [57]. The first member in this class, caspofungin, was approved by the FDA in 2001, two decades after the introduction of the preceding antifungal class, the azoles, in 1981 [9]. Echinocandins were discovered from the screening of natural products produced by fungal fermentation for antifungal activity in 1974 [58]. Similar to polyenes, echinocandins are fungicidal and fast-acting. Exposure to caspofungin for just five minutes causes the uptake of propidium iodide in *Candida* [42]. This antifungal class has a narrow spectrum of activity because it is only active against *Candida* spp. and *Aspergillus* spp. [57]. Besides having a narrow spectrum, echinocandins have to be administered intravenously as a result of poor oral bioavailability [57]. However, an orally bioavailable analog (enfumafungin) is currently under development [59].

The ideal antifungal [s3]agent for *Candida* infections

Deducing from the pros and cons of the existing antifungals discussed above, it is tempting to speculate the properties of an 'ideal' antifungal agent for *Candida* infections. Although such antifungal agents might be unattainable in reality, it could serve as a guideline for designing newer drug discovery and development programs to seek optimal antifungals. A promising lead compound should display properties close to the ideal antifungal agent in the preclinical development stage, and this would also be crucial in increasing the success rate in clinical trials. The ideal antifungal agent, besides potent antifungal activity, should possess several properties as outlined below [60,61] (Table 3). An ideal antifungal agent for *Candida* infections should have a broad spectrum of activity (within the *Candida* genus), no resistance, an ideal pharmacokinetic and pharmacodynamic profile and no toxicity or side effects.

Broad spectrum of activity within the* Candida *genus

Broad-spectrum antifungal agents, which are effective against a wide range of species in the fungus kingdom, are traditionally preferred, because this allows empirical therapy and the start of treatment before the species identification of the responsible pathogen [62]. Recently, it has been suggested that molecules with narrow-spectrum antifungal activity could be of significant value and should not be neglected [63,64]. Narrow-spectrum antifungals that are effective against certain fungal genera would enable the rational use of antifungals and, therefore, would slow the emergence of resistance strains. Various sensitive diagnostic methods are now available that allow rapid species identification from blood cultures to couple with the use of narrow-spectrum antifungals [65,66]. Therefore, an ideal antifungal agent for *Candida* infections should possess a broad spectrum of activity within the *Candida* genus.

Absence of resistance

The ideal antifungal agent should not associate with intrinsic or acquired resistance. Intrinsic resistance can be easily avoided by screening the lead compounds with all the *Candida* species but acquired resistance is an inevitable outcome of evolution; however, its rate of development is regulated by several artificial factors and can be slowed down. First, because fungistatic antifungal agents cannot completely eradicate the pathogens, survivors are selected for after exposure and resistance then arises. Complete elimination of the pathogens is clearly a safer option; therefore a fungicidal drug is preferred. Furthermore, drugs with multiple targets were associated with higher toxicity and were not preferred in the past; however, drugs that strongly inhibit a single step in a pathway are likely to promote selective pressure and resistance development [67]. Therefore, to minimize the emergence of acquired drug resistance, the ideal antifungal agent should be fungicidal and possess multiple cellular drug targets.

Ideal pharmacokinetics profile

The four determinants of pharmacokinetics are absorption, distribution, metabolism and excretion (ADME) [68]. The ideal antifungal agent should have adequate bioavailability via intravenous and oral routes of administration to ensure flexibility. Amphotericin B and caspofungin are administered intravenously only, as a result of poor oral bioavailability [57]. According to Lipinski's Rule of Five, it is estimated that membrane permeability and oral bioavailability can be achieved by molecules with not more than five hydrogen-bond donors and ten hydrogen-bond acceptors, Log*P* <5 (a quantitation of the compound's lipophilicity) and molecular weight <500 Da [69,70]. Synthetic small molecule compounds have an advantage over natural products here, because the molecular properties of natural products cannot be controlled and the Rule of Five is often violated.

The ideal antifungal for systemic candidiasis should be well distributed to various organs and exhibit a long plasma half-life to maximize and prolong its effect. Invasive candidiasis is often seen deep in the organs of the patients; therefore good tissue penetration of the drugs is necessary. For example, flucytosine diffuses well into the tissue [46].

The metabolites of the ideal antifungal should not be toxic. Therefore, the pathway of metabolism has to be investigated. Imidazoles are known for severe drug–drug interactions by inhibiting cytochrome P450 enzymes [71]. The cytochrome P450 superfamily is essential for catalyzing drug metabolism, and the inhibition of such enzymes will result in accumulation of the drugs, leading to toxicity [72].

Antifungal drug discovery

Drug discovery is a lengthy process and it is estimated to take 14 years for the launching of a new systemic antibiotic onto the market after its initial discovery from high-throughput screening [73]. Preclinical drug discovery and development is the most challenging part that requires an extended period of time. Despite much effort invested in drug discovery and development, only 20% of all projects proceed to clinical trial, and just 10% of these successfully pass through the latter hurdle [74]. These failures are mainly biological (poorly validated hits) and/or chemical (undesired chemical properties that lead to toxicity) [74].

Traditionally, antimicrobial drug discovery is based on phenotype-based screening assays of natural products, the so-called ‘classical pharmacology’ or ‘forward pharmacology’ [75,76]. It refers to the screening of small molecules that could result in a certain phenotype, for example inhibition of growth or cell death. This approach is, however, considered to be relatively irrational.

During the genomic era, with the advances of recombinant technology and synthetic organic chemistry, the target-based approach (also called reverse pharmacology or rational drug design) has become the dominant paradigm [64,77]. The term target-based approach refers to the rational and careful design of a predefined drug target (i.e. a specific gene or protein of interest). It is then followed by either *de novo* synthesis of chemical compounds or the screening of a library of natural products, as well as synthetic small molecules that effectively interact with the predefined drug target. Interestingly, it has been shown that the classical phenotype-based approach still produces more first-in-class small molecule drugs than this seemingly more rational target-based approach [78]. The two main reasons for this could be: (i) the inadequate understanding of the mechanisms of disease, which might lead to the poor validation of the predefined drug target that the target-based approach relies heavily on [77]; (ii) other possible binding sites might be ignored by the *de novo* synthesis of lead compounds, which would lead to undesirable toxicity that is only discovered in later stages of drug development [79]. Moreover, the target-based approach cannot accommodate complex molecular pathways that involve several genes or proteins. The limitations of the target-based approach have led to a search for alternative and optimal strategies in the post-genomic era.

High-throughput screening of small molecules with antifungal activity

Phenotype-based and target-based drug discovery approaches involve the screening of small molecule libraries. It could be hypothesized that, given a large enough number of small molecules in the library for screening, more hits can be identified. Coupled with modern high-throughput screening technology, rapid hit identification is enabled and the hit rate is likely to increase significantly. Subsequently, with a careful hit-to-lead process and lead optimization, eventually the ideal antifungal agent will be discovered. In other words, the

focus of antifungal drug discovery should be placed on the quantity and quality of the small molecules from chemical libraries and the translation of hit-to-lead and lead-to-drug [80].

High-throughput screening of small molecules is advantageous to antifungal drug discovery. (i) The utilization of small molecules can bridge drug discovery with chemical biology to study biological processes of the pathogens. (ii) Synthetic organic chemistry and combinatorial chemistry have allowed the rapid and cost-effective generation of a large amount of compounds with diverse structures [76,81]. (iii) Most importantly, high-throughput phenotype-based screening of small molecules with antifungal activity could allow the identification of hits that target multiple proteins [82].

Quantity and quality of small molecules

Small molecules can be defined very differently according to application. In antimicrobial drug discovery, small molecules are defined as non-peptide organic compounds that are synthetic or obtained from natural product extracts and are of low molecular weight (~200–500 Da) according to Lipinski's rule [69], thus binding to biopolymers such as proteins and nucleic acids and altering their normal functions [82–85]. Small molecules are also used in these disciplines to probe biological pathways and gain new insights into unclear mechanisms [85,86]. Academic investigators have an advantage over the developers from the pharmaceutical companies by acquiring and coupling the first-hand knowledge from basic research with drug development [80,87]. The data of small molecules obtained from probing assays, including chemical structure and predicted solubility, are now stored in open databases, such as ChemBank, PubChem (currently contains information of over 700 000 compounds) [88,89] and ChemDB (contains over 4 million small molecules) [90].

Theoretically, there is an estimation of up to 10^{60} different chemical structures in the chemical space [91,92]. Although not all of these theoretical structures can be successfully

synthesized, it is suggested that an ideal small molecule library should contain all the small molecules that can be synthesized, fulfill the Rule of Five and interact with biopolymers [80].

There are two main sources of small molecules: commercial libraries and in-house libraries. There are many commercially and publicly available small molecule libraries that are made up of many thousands of existing drugs, extracts of natural products and bioactive compounds of known or unknown action. These libraries provide a convenient source of ready-to-use small molecules. To fit different screening assays, in-house small molecule libraries can be generated, and this should be done objectively in a diversity-oriented or target-oriented manner [93]. A diversity-oriented small molecule library comprises compounds that are diverse in structure and, thus, increases the probability that a lead compound could be discovered. A target-oriented small molecule library contains analogs that are built around a specific structure, called the scaffold, to optimize the binding to the target [94]. The quality of small molecule libraries, which governs the successful rate of hit identification, is achieved by the purity or lead-like characteristics of the small molecules [95].

Hit-to-lead and lead optimization

Hits are defined as the positive output of a screen having sufficient potency and are of known structure. Some of the hits identified from the primary screening can be false positives. Therefore, a secondary screen (hit validation) is required for validation to ensure that the resulting phenotype was caused by the responsible small molecule.

Leads are defined as the molecules, on top of the potency, with sufficient potential (e.g. pharmacokinetics, specificity, patentability) to be developed as drugs [96]. The lead compounds should be tested in an effective way to eliminate the ones that are unlikely to be

developed early in the development process. The lead compounds can be optimized by chemical modifications to increase potency and to ensure *in vivo* efficacy [80].

Although the discovery of hits is accelerated by high-throughput screening, there seems to be a big gap in the bridging of hits to lead compounds. About 60% of the small molecule drug discovery projects have failed as a result of poor lead optimization (because the lead does not possess drug-like properties) [74]. In addition, there seems to be a lack of medicinal chemists to translate the promising hits into leads [97]. Therefore, it has been suggested that the future of drug discovery should be focused on assessing the 'therapeutic utilities' of the leads [98].

Animal models

Animal models have contributed significantly to the study of the pathogenicity of *Candida* and new therapies [99,100]. *In vivo* models for systemic and oral candidiasis are well established [101,102]. Infection is established in systemic candidiasis murine models by injecting the fungal cells intravenously, and the mice eventually die of progressive sepsis [103]. Yeast cells are mostly retrieved from the kidneys [104]. Although neutropenia is often induced in the murine model to mimic the immune status of the high-risk immunocompromised population, systemic candidiasis can also be induced in non-neutropenic mice.

A vast number of new drug candidates fail in the *in vivo* studies owing to undesirable toxicity and/or poor pharmacokinetics. Therefore, the *in vivo* efficacy of a lead compound should be tested thoroughly in the early stages of antifungal drug development. It is important to note that *Candida* is not a constituent of the normal microbiota of mice [99]. Also, there exist variations between the immune systems of mice and the human host [105]. Therefore, the outcome of a drug could differ in mice and in humans. This difference can be best demonstrated by triazole, which is metabolized faster in mice than in humans by cytochrome

P450, thereby increasing the *in vivo* efficacy of azole in mice [99]. Importantly, *in vitro* minimum inhibitory concentration does not correlate with the outcome of the therapy. A detailed pharmacodynamics–pharmacokinetics profile is therefore needed for downstream clinical trials.

Potential new drug targets

One of the reasons for the slow progress in this field is the eukaryotic nature of fungi. This is a major hurdle in development of antifungals because they carry substantial similarities to human cells. Therefore, finding a suitable fungal-specific drug target is not a trivial task.

Cell wall

The cell wall has remained an attractive area of study as an antifungal drug target, because it comprises a number of key molecules that are not present in humans. The cell wall is an essential *Candida* component and is the first contact site of the pathogen with the host [106]. The *C. albicans* cell wall is rich in carbohydrate, and mainly comprises glucose (glucans), *N*-acetyl-D-glucosamine (chitin) and mannose (mannan) [107]. Beta-glucan is the most abundant constituent, followed by mannan and chitin [107]. The inner layer is enriched with chitin and the outer layer enriched with mannoprotein [108]. Two classes of antifungal agents, echinocandins and nikkomycin Z[s4], target the fungal cell wall. Echinocandins target the β-1,3-glucan biosynthesis and nikkomycin Z targets chitin synthesis [109].

Targeting virulence factors

Although none of the current antifungal drugs target virulence factors of the yeasts, targeting virulence factors as therapeutic options has been proposed as a new paradigm for antifungal drug discovery [11,110,111]. This is a compelling paradigm because virulence-inhibiting small molecules would exert much lower selective pressure and, hence, lead to less

resistance, as compared with growth inhibitory small molecules [11,110]. Moreover, virulence factors are usually pathogen-specific and, therefore, these small molecules would be narrow spectrum with only a few side effects [11,110,112]. Yet, there are some disadvantages that render this paradigm arguably controversial. For example, nonfilamentous *C. albicans* mutants were shown to be avirulent in a mouse infection model [113]; therefore, it is hypothesized that disabling hyphal formation, one of the major virulence factors of *C. albicans*, would result in attenuation of virulence in *C. albicans*. As a result, small molecules that inhibit hyphal formation would be tremendously narrow spectrum and would only be effective for two *Candida* species that are able to form true hyphae: *C. albicans* and *C. dubliniensis* [114]. Although *C. albicans* is the predominant species of *Candida* infections, as discussed above the incidence of infections caused by non-*albicans* species is on the rise. Different virulence factors can be required by *Candida* depending on the type and site of infections [114,115]. For example, the deletion of the gene *CHK1* results in impaired hyphal formation and attenuated virulence in a systemic candidiasis mouse model [116]; but, discouragingly, it does not affect the virulence in a vaginal candidiasis (mucosal candidiasis) rat model [117]. Therefore, it is suggested that some virulence-inhibiting small molecules could possibly be limited to certain species and type of or site of infection.

By contrast, one of the most important questions in this context is whether virulence-inhibiting small molecule inhibitors will be effective in immunocompromised patients [110], who constitute the major group of the population prone to *Candida* infections. *In vivo* studies are awaited to examine the consequences of the presence of avirulent pathogens in the host. Therefore, virulence-inhibiting drugs might only be able to act as a prophylactic or an adjuvant therapy [118], or as a pretreatment for medical devices that are at high risk of *Candida* colonization.

Novel small molecule antifungals

A literature search was performed to look for novel small molecule antifungals (excluding new derivatives of existing antifungal agents). A handful of small molecule antifungals were identified and their discovery approach, antifungal activities, spectrum of activity, mechanism of action and their latest development are reviewed and classified below. However, these small molecules were evaluated mainly for their *in vitro* inhibition of growth, virulence factors, such as yeast-to-hypha transition, or biofilm formation. *In vivo* evaluation of these molecules is, at best, scant.

Virulence processes

Yeast-to-hypha transition- and biofilm-inhibiting small molecules. Toenjes *et al.* have studied a ChemBridge™ small molecule library that contained 72 molecules using a microplate-based morphological screening assay [119]. These researchers noted that seven of the small molecules (five noncytotoxic small molecule inhibitors and two structural derivatives of unknown nature) inhibited yeast-to-hypha transition without affecting normal budded growth, possibly by affecting the expression of hyphal-specific gene *HWP1* [119].

In another study, a microplate-based morphological screening assay was performed to find fungicidal small molecules from the BIOMOL Institute of Chemistry and Cell Biology (ICCB) Known Bioactives collection with 480 small molecules with known cellular targets and processes [120]. The screening revealed 53 molecules that were cytotoxic to *C. albicans*, and 16 that were yeast-to-hypha inhibitors.

By investigating the efficacies of 21 of the 23 yeast-to-hypha small molecules from the above studies (excluding the two structural derivatives), it was suggested that they either act on the Efg1[s5] or Cph1 pathways [121] that govern the filamentation of *C. albicans* [122].

Biofilm inhibition of the 23 yeast-to-hypha-inhibiting small molecules from the two studies above (including the structural derivatives) was examined and three (ETYA, CGP-37157, buhytrinA) were found to be biofilm inhibitors [123]. ETYA, an inhibitor of eicosanoid synthesis, was the most potent out of the three, followed by CGP-37157, which affects calcium homeostasis, and buhytrinA, a novel molecule with no known target.

Nineteen biofilm-inhibiting small molecules. More recently, 19 *C. albicans* biofilm inhibitors (effective alone or in synergy with clotrimazole) were discovered by screening 120 000 molecules from the NIH Molecular Libraries Small Molecule Repository [124]. The underlying mechanism of action of these small molecules is not known.

Shearinines D and E: biofilm inhibitors. Isolated from *Penicillium* spp., shearinines D and E suppress *C. albicans* biofilm formation by blocking yeast-to-hypha transition. These molecules also enhance the antibiofilm activity of amphotericin B [125].

Small molecules that target cell wall and/or cell membrane

HWY-289. HWY-289 was discovered from the screening of 515 synthetic or semi-synthetic protoberberine derivatives and displayed the most potent anti-*Candida* activity with no toxicity in rats [126]. Protoberberine is an extract from a Korean medicinal plant that was shown to inhibit the growth of *C. albicans* by inhibiting ergosterol and chitin synthesis [127]. Multiple molecular targets of HWY-289 were reported, which included sterol 24-methyl transferase, acyl CoA, [s6]sterol acyltransferase and chitin synthase isozymes [128]. This extract also inhibited yeast-to-hypha transition by disrupting the prohyphal RAS [s7]signaling [129].

Pyridobenzimidazole derivatives. D75-4590 was discovered from a high-throughput cell-based screening of a chemical library of synthetic compounds that target β-1,6-glucan

synthesis [130]. However, owing to its unsatisfactory physiochemical profile, D75-4590 lacks significant efficacy in animal models [130]. Derivatives of D75-4590 (D11-2040 and D21-6076) are inhibitors of β-1,6-glucan synthesis with *in vivo* efficacy [131]. D21-6076 also inhibits hyphal elongation and adherence to mammalian cells [131]. D11-2040 inhibits vegetative growth and hyphal development as a single drug or in synergy with caspofungin or fluconazole [132].

Tamoxifen and structural analogs. A library with 4505 compounds of known biological activity and natural products was screened in a microplate-based setting for cell lysis inducers identifying tamoxifen and its structural analog. These agents disrupt the cell wall integrity possibly by interfering with calmodulin [133,134].

E1210. A small molecule, BIQ, was discovered from a high-throughput cell-based screening of a chemical library for cell wall glycosylphosphatidylinositol (GPI) biosynthesis inhibitors that target the GWT1[s8] protein [135]. Following pharmacological optimization of the hit, E1210 was discovered as the potential drug candidate [136]. E1210 is a fungistatic small molecule with potent activity against most *Candida* species except *C. krusei* [136]. The low cytotoxicity of E1210 against a primary human kidney cell line was comparable to that of fluconazole (IC_{50} >32 μg/ml) [136]. Through inhibition of GPI biosynthesis, E1210 also inhibits germ-tube formation, adherence and biofilm formation of *Candida* [137]. *In vivo*, E1210 is effective in mouse models of oropharyngeal and disseminated candidiasis via oral administration [138].

SCH A, SCH B, SCH C and SCH D. A high-throughput screening was performed to look for antifungal small molecules. The mechanistic studies revealed that the primary hits were β-1,3-D-glucan inhibitors [139]. In addition, the identified molecules were also effective in treating disseminated *C. glabrata* infection in a mouse model [139].

Aminopiperidine derivatives. Aminopiperidine derivatives, namely compound 1a and 1b[s9], have been shown to inhibit C-14 reduction in ergosterol synthesis [140,141]. These compounds are fungistatic *in vitro* and extend the survival of a murine model with disseminated *C. albicans* infection.

Novel mechanism of action

UK-118005. Two synthetic small molecules, UK-118005 and its structural analog ML-60218, are broad-spectrum antifungal compounds that inhibit RNA polymerase (Pol) III in yeasts and human cells [142].

ECC145 and ECC188. ECC145 and ECC188 were identified from a high-throughput screening of a synthetic small molecule library (ChemBridge™) by fitness test on *OLE1*-deleted *C. albicans*. The gene *OLE1* encodes the fungal fatty acid Δ-9 desaturase. ECC145 and ECC188 inhibit the unsaturated fatty acid synthesis and hyphal development [143]. *OLE1* is a potential antifungal target because it is essential for virulence.

MGCD290. MGCD290, currently in Phase II clinical trials, is a novel oral small molecule that inhibits the Hos2 fungal histone deacetylase (HDAC). MGCD290 enhances the activity and broadens the antifungal spectrum of azoles. When administrated to the systemic candidiasis mouse model together with fluconazole the survival of the mice increased [144,145].

Zinc homeostasis modulators. In this target-based drug discovery, a small molecule library was screened in a *Saccharomyces cerevisiae* model for fungal zinc homeostasis modulators [146]. The library contained 2000 small molecules that had been preselected to cover a wide range of biological processes and structures. The 80 small molecules that were found to affect

the zinc homeostasis in the *S. cerevisiae* screening model were further tested on *C. albicans*, and three were shown to be strong zinc homeostasis modulators.

Unknown mechanism

A novel antifungal, SM21, was identified by high-throughput phenotype-based screening of a library of 50 240 small molecules for growth inhibitors and hyphal formation inhibitors of *C. albicans* by our group [147]. Subsequent assays revealed that SM21 is fungicidal against a wide range of *Candida* species. *In vivo*, SM21 is able to treat systemic and oral candidiasis in murine models. The exact mechanism of action of SM21 is still to be elucidated.

Concluding remarks

The serious threat that *Candida* infections are posing to public health urgently requires rapid and effective screening programs for antifungal drug discovery. The drawbacks of the current limited arsenal of antifungal agents have prompted the search for the ideal antifungal agent targeting *Candida* infections. High-throughput screening technology, coupled with synthetic chemistry and combinatorial chemistry, has been shown to be a promising approach in providing rich sources of small molecules of diverse structures and accelerating the hit discovery rate and significantly benefitting downstream drug development.

Conflicts of interest

The authors declare no conflict of interest related to this manuscript.

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1 **Table 1. The change in incidence of *Candida* bloodstream infections in various parts of**

2 **the world from 1980 to 2009**

Area	Publication year	Study period	Type of population	Trend or change in % incidence	Refs
Spain	2012	2000–2009	General hospital	Increased	[13]
Taiwan	2011	2000–2008	Tertiary medical center	Increased	[14]
Hong Kong	2009	1998–2006	Intensive care unit	+66%	[15]
Denmark	2008	2004–2006	Multiple general hospitals covering about 65% of Danish population	+17%	[16]
United States	2008	2000–2005	General populations	+52%	[17]
Switzerland	2003	1991–2000	General hospitals covering about 80% of Swiss population	Unchanged	[18]
Canada	2002	1992–1996	Three general hospitals	+155%	[19]
Iceland	2002	1980–1999	General populations	+250%	[20]
Norway	1998	1991–1996	General hospitals	Unchanged	[21]
Taiwan	1997	1981–1993	Single general hospital	Increased	[22]

3

4

Table 3. Properties of an ideal antifungal agent for *Candida* infections

Ideal antifungal agent
Potent antifungal property
Broad spectrum of activity within the *Candida* genus
Ease of administration
No association with intrinsic or acquired resistance
Fungicidal
Nontoxic
No side effects
No drug–drug interactions

1 **Table 2. Timeline of discovery of new and current major antifungals**

Antifungal	Year of FDA approval	Molecular weight (g/mol)	Fungicidal and/or fungistatic	Route of administration	Advantage(s)	Disadvantage(s)
Polyene						
Nystatin	1954	926	Fungicidal and fungistatic	Oral and topical	Broad spectrum	No intravenous injectable formulation owing to toxicity profile for mucocutaneous fungal infections only
Amphotericin B	1958	924	Fungicidal	Intravenous only	High potency, rapid onset of action, broad spectrum	Nephrotoxicity
Amphotericin B lipid complex	1995	924			Less toxic than conventional amphotericin B	Cost
Amphotericin B cholesteryl sulfate	1996	924				
Liposomal amphotericin B	1997	924				
Pyrimidine						
Flucytosine	1972	129	Fungistatic	Oral and intravenous	Minor side effects, good tissue distribution	Narrow spectrum
Imidazole						
Ketoconazole	1981	531	Fungistatic	Oral and intravenous	N/A[s1]	Toxicity
Triazole						
Fluconazole	1990	306	Fungistatic	Oral and intravenous	Minimal toxicity	High rates of resistance, narrow spectrum of activity
Itraconazole	1992	705			Extended spectrum more than first generation	Low bioavailability
Voriconazole	2002	349				Class-related side effects
Posaconazole	2006	700		Oral only		Cost
Echinocandin						
Caspofungin	2001	1213	Fungicidal	Intravenous only	Low toxicity	Narrow spectrum: fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp.
Micafungin	2005	1292				
Anidulafungin	2006	1140				
Enfumafungin	N/A[s2]	708		Oral only		