

Chemical tools to probe antifungal targets

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Abstract

Chemical probes are selective, reversible small molecules that enable temporal control of protein function in cells and are central to target identification, pathway interrogation, and chemical validation in drug discovery. Compared with genetic approaches, they offer tunable target engagement, rapid perturbation, and direct assessment of druggability, thereby bridging basic biology and therapeutic development. Despite their widespread use in human biology, chemical probes that interrogate molecular machineries in pathogenic fungi remain scarce. This limitation is particularly significant given the increasing incidence of life-threatening fungal infections in immunocompromised patients and the urgent need for new antifungal targets and therapeutic strategies. In this review, we summarize 17 small-molecule chemical tools that probe 13 antifungal targets across *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and the model yeast *Saccharomyces cerevisiae*. These compounds represent a foundational chemical toolkit for fungal research and provide a framework for developing next-generation chemical probes or antifungal agents in future studies.

Introduction

Infectious diseases are threats to human health, ranking among the top four leading causes of global death from 1990 to 2021 ¹. Most life-threatening infections are caused by pathogenic bacteria and viruses ^{2,3}. Fungi, on the contrary, usually lead to mild superficial (e.g., skin) infections ⁴. Although severe fungal infections are rare in healthy individuals, immunocompromised patients exhibit increased susceptibility to systemic fungal infections ^{5–7}, and the number of immunocompromised individuals rose during the 20th century. Since early 1980s, the incidence of AIDS (acquired immune deficiency syndrome) resulted in the rising number of people with weakened immunity ^{8,9}. The increasing use of special medical treatments (e.g., chemotherapy) also increases the number of immunocompromised patients vulnerable to fungi ^{10,11}. These facts indicate the need for antifungal treatments for immunocompromised people. Moreover, climate change in recent decades makes some fungal species adapted to higher temperature, enabling these fungi to grow in human bodies where temperature is higher than that of the atmosphere ^{2,12,13}. Currently, the annual death toll of fungal infections is estimated as 3.8 million, with the mortality rate of 58.5% ¹⁴, but only four categories (flucytosine, azoles, polyenes, echinocandins) of antifungal drugs are used in clinical treatments ¹⁵. The limited classes of antifungal drugs pose a medical challenge for treating fungal infections, and the expansion of the antifungal pipelines is urgently needed.

Over the past decade, substantial efforts have been devoted to expanding the antifungal drug pipeline ^{15–17}. One major strategy has been target-based drug discovery, which seeks to identify and exploit new, druggable molecular targets to enable the development of novel antifungal agents ^{18,19}. This approach is founded on the premise that therapeutic efficacy can be achieved by modulating specific molecular machineries using small-molecule tools ²⁰. In principle, any biological macromolecule, including proteins or RNA, that mediates a pharmacological effect may serve as a potential target ²⁰. Structural information for such targets further accelerates medicinal chemistry optimization and rational drug design ^{18,21}. Despite these advantages, target-based discovery remains costly and high-risk, with expenditures reaching millions of euros before clinical testing and escalating to hundreds of millions of dollars during clinical phases I–III ²². Given these substantial investments, early assessment of target suitability is critical to minimize late-stage failures. Target validation has therefore emerged as a central step in modern drug discovery.

Target validation typically involves both genetic and chemical approaches ²³. Genetic validation tests whether perturbation of a candidate target—such as gene deletion or knockdown—produces the desired pharmacological phenotype ²³. Positive results support subsequent chemical validation, which demonstrates that small-molecule tools can engage the target *in vivo* and phenocopy the effects observed genetically ^{23,24}. From a chemical biology perspective, these small-molecule tools are termed **chemical probes**—compounds designed to selectively interact with defined targets

and modulate target-associated phenotypes ^{25–28}. Chemical probes may function as inhibitors or as targeted degraders, such as proteolysis-targeting chimeras (PROTACs) ²⁹. Importantly, chemical probes differ conceptually from therapeutic drugs: probes are optimized for mechanistic specificity and target interrogation, whereas drugs are optimized primarily for clinical efficacy and safety ²⁸. Together, robust genetic and chemical validation establishes confidence in target tractability and provides a strong foundation for subsequent target-based antifungal drug development.

Over the past fifteen years, our laboratory has conducted extensive target-validation studies in pathogenic fungi ^{30–36} and consistently observed that many genetically validated targets fail to advance to chemical validation due to the lack of suitable chemical probes. This bottleneck reflects a broader imbalance in the chemical biology field. Although chemical probes have been actively developed since the early 2000s ^{37–39}, the vast majority of efforts have focused on human proteins, exemplified by large-scale initiatives such as Target 2035 ^{40,41}. In contrast, probes directed against antifungal targets remain largely absent. At the time of writing, no compounds are explicitly annotated as antifungal chemical probes in major community resources, including the Chemical Probes Portal (<https://www.chemicalprobes.org/>) and the Structural Genomics Consortium (<https://www.thesgc.org/chemical-probes>) databases. Thus, canonical repositories currently provide little direct support for chemical validation in fungal systems. While several reviews have summarized the discovery of antifungal compounds ^{15,42}, few have evaluated whether these molecules meet the standards of high-quality chemical probes, such as demonstrated target engagement, selectivity, and mechanistic clarity. Consequently, potentially valuable tools for fungal biology may be overlooked. Beyond their role in target validation, the availability of chemical probes also shapes research priorities: investigators tend to focus on targets for which tractable chemical tools already exist ^{43,44}. This trend, together with historically limited funding for fungal research ⁴⁵, further constrains progress in antifungal target discovery. Developing and recognizing robust chemical probes for fungal targets could therefore both enable rigorous validation and stimulate broader scientific and financial investment in the field.

In this review, we reassess reported antifungal compounds through the lens of chemical probe criteria and identify 17 molecules that represent promising starting points for probe development. These compounds provide new opportunities for chemical **target** validation and offer a foundation for accelerating the discovery of next-generation antifungal therapeutics. **To avoid ambiguity, here we emphasize that this review is not intended to summarize advances in antifungal drug discovery, but rather to evaluate antifungal agents from a chemical biology perspective. Importantly, chemical probes are not necessarily drugs or lead compounds ²⁸. Instead, they are tools designed to interrogate biological function. Accordingly, the central question addressed here is whether the observed antifungal activity arises from specific probe-target interactions (on-target effects)**

supported by a defined mechanism of action, rather than from general antifungal properties such as minimum inhibitory concentration (MIC) values or spectrum of activity.

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Literature survey of chemical tools probing antifungal targets

To date, no research articles have explicitly characterized compounds as chemical probes for antifungal targets. To address this gap, we conducted a systematic literature survey to identify small molecules that functionally probe antifungal targets. Given the enormous diversity of fungi — estimated at 2.2–3.8 million species ⁴⁶ — it is neither practical nor necessary to survey the entire kingdom. Instead, we focused on the three highest-priority human fungal pathogens defined by the World Health Organization in 2022 ⁴⁷— *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans*. We additionally included the model yeast *Saccharomyces cerevisiae*, whose conserved molecular machineries provide valuable mechanistic insights transferable to pathogenic fungi ⁴⁸. Because the goal of this review is to support target validation, we restricted our analysis to targets that are genetically validated and essential for fungal growth (Fig. 1). Although chemical probes can encompass multiple modalities, including PROTAC degraders ²⁹ and nanobodies ^{49,50}, we focused specifically on small-molecule inhibitors, which currently represent the most established and accessible class of probes in fungal research. Consistent with the defining feature of chemical probes—well-characterized mechanisms of action ²⁸ — we selected inhibitors with experimentally determined protein-ligand complex structures, data of antifungal activity, and biochemical evidence of target engagement.

Accordingly, we examined protein–ligand complex structures in the Protein Data Bank and compiled ligands with reported inhibitory activities ($IC_{50}/K_i/K_d$) for further evaluation. Compounds lacking evidence of direct target engagement or antifungal activity were excluded. To emphasize emerging opportunities rather than established therapeutics, we also excluded well-characterized targets such as ergosterol and β -glucan biosynthesis, as well as clinically approved antifungal drugs (e.g., polyenes, azoles, and echinocandins) ¹⁵, even though some of these agents could technically satisfy chemical probe criteria. Applying these primary filtering criteria (Fig. 1)—including genetic validation of the target, the availability of small-molecule inhibitors, resolved protein-inhibitor complex structures, and demonstrated target engagement—we identified 17 candidate inhibitors from the literature.

Assessment of potential chemical probes

After identifying 17 candidate inhibitors from the literature, we evaluated whether they meet the standards of high-quality chemical probes. The chemical biology community has established general criteria for probe quality ^{26,28} — such as high potency, selectivity, target engagement, and favorable physicochemical properties — exemplified by the Chemical Probe Portal (<https://www.chemicalprobes.org/>). However, these standards were developed primarily for human targets, and equivalent guidelines for fungal systems are lacking. We therefore adapted these principles to antifungal research, taking into account challenges specific to fungi, particularly the

conservation^{51,52} between fungal and human proteins that raises the risk of host toxicity¹⁵, as well as the cellular permeability barrier caused by the fungal cell wall^{53,54}. Our assessment focused on four key aspects (Tables 1, 2): potency, mechanistic validation, selectivity, and compound quality. First, with respect to potency, we required inhibitors to exhibit nanomolar biochemical activity ($IC_{50}/K_i/K_d < 100$ nM), ideally supported by resolved target–ligand complex structures. At the cellular level, compounds were expected to inhibit fungal growth with direct evidence of target engagement, thereby linking biochemical activity to phenotypic outcomes. However, given that the fungal cell wall imposes a significant barrier to compound permeability^{53,54}, the relationship between biochemical potency and antifungal efficacy is often uncoupled. Therefore, we did not impose strict thresholds on antifungal activity (MIC/MEC), recognizing that even high-quality chemical probes may display modest apparent activity in whole-cell assays. To strengthen mechanistic validation and ensure on-target effects, we prioritized cases where orthogonal inhibitors (structurally distinct compounds targeting the same protein) or inactive analogues (structurally related but inactive controls) were available⁵⁵. For selectivity, compounds were required to demonstrate at least 30-fold specificity within the fungal proteome and over human orthologues, minimizing the risk of off-target effects. Where available, evidence of *in vivo* efficacy in animal models was considered supportive but not mandatory. Moreover, compounds were expected to exhibit ≥ 30 -fold selectivity within the fungal proteome and over human orthologues, and, where possible, demonstrate efficacy in animal models. Finally, regarding compound quality, we excluded pan-assay interference compounds (PAINS)⁵⁶ and favored molecules with acceptable physicochemical properties, such as $cLogP < 5$ ⁵⁷, to ensure suitability as chemical probes.

Chemical tools that probe enzymes of *C. neoformans*

C. neoformans is a major opportunistic fungal pathogen responsible for approximately 181,100 deaths annually⁵⁸ and ranks among the top three pathogens on the WHO priority list⁴⁷. A defining virulence feature of this organism is its polysaccharide capsule surrounding the cell wall, which is essential for pathogenesis^{59,60}. From the literature, we identified four inhibitors (1, 2, 3 and 4 in Table 1) with evidence of target engagement that may serve as chemical probes for *C. neoformans* enzymes.

Inhibitors 1 and 2 targeting sterylglucosidase 1 (Sgl1). Sgl1 hydrolyzes ergosterol 3 β -D-glucoside, a sterylglucoside that accumulates under stress⁶¹, and is essential for fungal growth⁶² while lacking a human orthologue, making it an attractive and potentially selective antifungal target. Inhibitor 1 was discovered through high-throughput screening of ~50,000 compounds⁶² and blocks the Sgl1 active site with micromolar potency ($IC_{50} \approx 1$ μ M)⁶². Treatment of 1 phenocopies *sgl1* deletion⁶², leading to intracellular accumulation of the substrate and growth inhibition⁶².

Importantly, the compound shows *in vitro* antifungal activity and protects against brain cryptococcal infection in a mouse model, supporting *in vivo* target engagement ⁶². A structurally distinct molecule (inhibitor **2**) was reported as an orthogonal inhibitor, strengthening on-target confidence, although no inactive control has been described ⁶².

Inhibitor 3 targeting acetyl-CoA synthetase (Acs). Acs generates acetyl-CoA ⁶³ and is required for *C. neoformans* growth when acetate is the sole carbon source ⁶⁴. Inhibitor **3**, identified from a screen of ~55,000 compounds ⁶³, inhibits Acs activity (IC_{50} = 8.6 μ M) and suppresses fungal growth under acetate-dependent conditions (MIC = 2 μ g/mL) ⁶³, consistent with the genetic essentiality of Acs ⁶⁴. Moreover, because Acs is synthetically lethal with ATP-citrate lyase, the compound phenocopies this synthetic lethality ^{63,65}, providing indirect evidence of target engagement. However, probe quality remains limited by modest selectivity over the human orthologue ($\geq$ 2-fold only reported) and the absence of inactive or orthogonal control compounds.

Inhibitor 4 targeting the calcineurin signalling pathway. Protein phosphorylation modulates multiple biological events ⁶⁶, and its reversal by protein phosphatases is equally critical for controlling fungal physiology ⁶⁷. Calcineurin is a Ca^{2+} /calmodulin-dependent phosphatase that is critical for the virulence ⁶⁸ and sexual cycle ⁶⁹ of *C. neoformans*. FK506 (inhibitor **4** in Table 1), a natural product secreted by *Streptomyces tsukubaensis* ^{70–72}, binds to the FK506 binding protein (FKBP12) ^{73,74}, and the resulting FKBP12-FK506 complex binds to calcineurin ⁷⁴, disrupting the downstream signalling pathway ⁷⁵. FK506 inhibits the growth of *C. neoformans* (MIC = 12.5 μ g/mL), and this inhibitory effect is attenuated upon deletion of either calcineurin or FKBP12 ⁷⁶, providing strong genetic evidence for on-target activity mediated through the FKBP12-calcineurin axis. Although FK506 itself shows limited antifungal efficacy in murine models ^{74,77}, the non-immunosuppressive analog APX879 improves survival in a mouse model of cryptococcosis ⁷⁴, supporting the translational potential of targeting calcineurin *in vivo*.

Collectively, these compounds provide valuable chemical tools for interrogating the function of Sgl1, Acs and calcineurin in *C. neoformans*.

Chemical tools that probe enzymes of *C. albicans*

C. albicans is a major opportunistic pathogen capable of switching between yeast and hyphal morphologies, with the hyphal form driving tissue invasion and virulence ⁷⁸. It is responsible for approximately 200,000 deaths annually ⁷⁹. From the literature, we identified three inhibitors (**5**, **6** and **7** in Table 1) with evidence of target engagement that interrogate distinct cellular processes, including protein synthesis, chromatin regulation, and signal transduction (Table 1).

Inhibitor 5 targeting glutamyl-tRNA synthetase (Gln4). Aminoacyl-tRNA synthetases are essential for protein biosynthesis and represent attractive antifungal targets ⁸⁰. Genetic repression of Gln4 improves survival in mouse candidiasis models ⁸¹, supporting its essentiality. Inhibitor **5**

was identified by phenotypic screening from 9600 compounds ^{81,82} and acts as an allosteric inhibitor of Gln4 with nanomolar potency (IC_{50} = 106 nM). It suppresses *C. albicans* growth (MIC = 0.7 µg/mL) ⁸¹, and its activity correlates with Gln4 expression levels ⁸². The binding pocket of inhibitor **5** is gated by a loop (G493 to M496) ⁸². Other fungi (e.g., *A. fumigatus* and *S. cerevisiae*), in which Gln4 orthologues carry a point mutation (M496L) in this gate-keeping loop, are not sensitive to inhibitor **5** ⁸². These results provide strong evidence of on-target action. The compound also reduces virulence in *C. elegans* and mouse models ⁸². However, inactive controls or orthogonal probes have not been reported.

Inhibitor 6 targeting BET bromodomain protein (BD1). Eukaryotic DNA wraps histones and forms nucleosomes, which make up of chromatin ⁸³. Acetylation of histone loosens chromatin, modulating the expression of certain genes ^{84,85}. One family of proteins that carry out this modulation is bromodomain containing proteins, readers of acetyl groups on histones ^{86,87}. In *C. albicans*, the bromo- and extra-terminal domain (BET) protein contains two bromodomains, BD1 and BD2. ⁸⁸. Although deletion of either domain alone is tolerated, simultaneous loss of both is lethal, indicating that bromodomain function is essential for fungal viability ⁸⁸. A BD1-selective inhibitor (Table 1, inhibitor **6**, IC_{50} 1.7 µM) was identified from a ~80,000-compound library by high-throughput screening ⁸⁸. Structural studies showed that the compound occupies the acetyl-lysine binding pocket and competes with the native substrate ⁸⁸. Cellular target engagement is supported by genetics: strains lacking or inactivating BD2 are hypersensitive to the inhibitor, whereas restoration of BD2 rescues growth, indicating that growth inhibition results from BD1 blockade ⁸⁸. A derivative of inhibitor **6** displays reduced toxicity toward mammalian cells (HeLa and IMR90) ⁸⁸. However, inactive controls or orthogonal inhibitors have not been reported.

Inhibitor 7 targeting yeast casein kinase II (YCK2). Kinases participate in signal transduction in cells via protein phosphorylation ⁶⁷. Deletion of yeast casein kinase II (YCK2), a serine/threonine kinase, leads to impaired cell wall and reduced virulence in *C. albicans* ⁸⁹. Phenotypic screening of a kinase inhibitor library (736 compounds) resulted in GW461484A (Table 1, inhibitor **7**), a YCK2 inhibitor with the IC_{50} value of 110 nM ^{90,91}. Overexpression of YCK2 confers resistance to inhibitor **7**, and repression of YCK2 increases the inhibitor **7** sensitivity ⁹⁰. These results indicate that inhibitor **7** inhibits YCK2 in *C. albicans* cells. Two derivatives reduce the fungal burden in a mouse candidiasis model ⁹¹. Although orthogonal probes are unavailable, structure–activity relationship studies provided inactive analogues as controls ⁹¹. Collectively, these compounds offer initial chemical tools to interrogate essential pathways in *C. albicans*, with inhibitor **5** currently showing the strongest combination of potency, genetic support, and *in vivo* efficacy.

Chemical tools that probe enzymes of *A. fumigatus*

A. fumigatus is the only filamentous pathogen on the WHO fungal priority list ⁴⁷ and is a leading cause of invasive aspergillosis. From the literature, we identified two inhibitors with evidence of target engagement that probe essential signalling and lipid-modification pathways in this organism.

Inhibitor 4 also targets calcineurin phosphatase in *A. fumigatus*. Similar to *C. neoformans*, calcineurin is also critical for the growth and virulence of *A. fumigatus* ⁹². FK506 (inhibitor 4 in Table 1) inhibits calcineurin with submicromolar affinity ($K_d = 356$ nM) and suppresses *A. fumigatus* growth at low concentrations (MEC = 0.016 μ g/mL) ^{74,93}. Target engagement is supported genetically, as a point mutation (F88H) within the binding pocket reduces drug sensitivity ⁷⁴. Although no orthogonal inhibitors have been described, structure–activity relationship studies yielded an analogue (JH-FK-05) with lower affinity to the target molecule ($K_d = 6.73$ μ M), and this compound also shows reduced antifungal activity (MEC = 2 μ g/mL), serving as an inactive control of FK506 ⁹³.

Inhibitor 8 targeting N-myristoyltransferase (NMT). NMT catalyzes N-terminal myristoylation ⁹⁴ and is essential for growth and cell wall integrity in *A. fumigatus* ³². Zelenistat, a clinical-stage anticancer compound ⁹⁵, inhibits NMT by occupying the active site ³². Repression of NMT sensitizes cells to the compound, supporting on-target activity ³². However, inactive controls and orthogonal probes have not yet been reported. Given the extensive medicinal chemistry developed for NMT inhibitors in *Trypanosoma brucei* ^{32,96}, these scaffolds may provide a useful starting point for future optimization of fungal-selective probes.

Collectively, calcineurin and NMT inhibitors provide initial chemical tools to interrogate essential signaling and lipid-modification pathways in *A. fumigatus*, although further development of selective and orthogonal probes is needed.

Chemical tools that probe enzymes in *S. cerevisiae*

Although non-pathogenic, *S. cerevisiae* is the best-characterized fungal model system and shares highly conserved molecular machineries with pathogenic fungi ⁹⁷. Consequently, chemical tools validated in *S. cerevisiae* often provide transferable starting points for antifungal target discovery ⁴⁸, in agreement with the fact that pharmaceutical industry (e.g., Novartis Pharma AG) uses *S. cerevisiae* as a platform for the early-stage drug discovery ⁹⁸. From the literature, we identified nine targets and nine corresponding inhibitors (9–17 in Table 2) spanning lipid metabolism, membrane trafficking, protein folding, and protein synthesis.

Inhibitors targeting lipid metabolism and membrane biogenesis. Aureobasidin A (Table 2, inhibitor 9), a cyclic peptide isolated from *Aureobasidium pullulans* ^{99,100}, exhibits broad-spectrum antifungal activity against *A. fumigatus*, *C. albicans*, and *C. neoformans* ¹⁰¹. Its target is inositol phosphorylceramide (IPC) synthase, a key enzyme in sphingolipid biosynthesis and absent in humans ^{102,89}. Genetic evidence supports target engagement: the F158Y mutation ¹⁰³ or

overexpression of IPC synthase confers resistance ^{102,103}, and loss of the enzyme is lethal in *S. cerevisiae* ^{102,104}. Aureobasidin A directly inhibits IPC synthase activity (IC_{50} = 32 nM) ¹⁰⁵. The inhibitory mechanism was clarified by a co-crystal structure, which showed disruption of substrate recognition ¹⁰⁵. Together, these data establish aureobasidin A as a well-defined chemical probe of IPC synthase, with demonstrated efficacy in a mouse candidiasis model ¹⁰¹. Although no inactive controls or orthogonal inhibitors have been reported, structure–activity relationship studies provide a basis for derivative design.

Glycosylphosphatidylinositol acyltransferase (GWT1), required for GPI-anchor biosynthesis ¹⁰⁶, represents another validated target ¹⁰⁷. BIQ, an antifungal compound against *S. cerevisiae*, was identified by phenotypic screening ¹⁰⁸. The scaffold of BIQ was optimised into manogepix (Table 2, inhibitor **10**), a derivative that inhibits (IC_{50} 300 nM) the *S. cerevisiae* GWT1 by blocking the active site ^{109–111}. Mutation of a residue adjacent to the binding pocket (V168) reduces antifungal activity ^{111,112}, confirming on-target effects. Moreover, manogepix (E1210) reduces fungal infections in several murine models ¹¹³. Currently, no inactive controls and orthogonal inhibitors of manogepix were reported, but the structure–activity relationship studies of manogepix facilitates the exploration of inactive control compounds ¹¹⁴.

The lipid transfer protein Sec14p, which regulates phosphatidylinositol and phosphatidylcholine trafficking ^{98,115}, is also essential for fungal growth ^{116,117}. A phenotypic screening identified compound **1** showing antifungal activity against *S. cerevisiae*, and deletion of Sec14p results in hypersensitivity to compound **1** ⁹⁸. Optimization of this compound led to inhibitor **11** that binds to the lipid-binding pocket of Sec14p with IC_{50} of 6.7 μ M ⁹⁸. Mutants in the inhibitor binding pocket reduce the antifungal activity of inhibitor **11** ⁹⁸. These results show that the target of inhibitor **11** is Sec14p, and inhibitor **11** preferentially inhibits the growth of *S. cerevisiae* over human cells ⁹⁸. The inactive control of inhibitor **11** was identified in the structure–activity relationship investigation ⁹⁸, and a structurally distinct compound (Table 2, inhibitor **12**) also inhibits Sec14p with evidence of target engagement, being the orthogonal inhibitor of **11** ^{118–120}.

Inhibitors targeting protein folding. Heat shock protein 90 (Hsp90) is an essential molecular chaperone that facilitates protein folding and cellular proteostasis ¹²¹. Loss of Hsp90 function is lethal in *S. cerevisiae* ¹²², highlighting its critical role in viability. Two natural products, geldanamycin (Table 2, inhibitor **14**) and radicicol (inhibitor **13**), directly bind Hsp90 and inhibit substrate processing by occupying the ADP-binding pocket ^{123,124}. Both compounds suppress *S. cerevisiae* growth, and mutations that disrupt their binding pocket reduce antifungal activity, confirming Hsp90 as the cellular target ^{125,126}. Radicicol additionally potentiates fluconazole efficacy in a *C. elegans* model of cryptococcal infection ¹²⁷. Geldanamycin and radicicol function as orthogonal inhibitors, and structure–activity relationship studies of both scaffolds provide opportunities to develop inactive controls ^{128–131}.

Inhibitors targeting protein synthesis. Ribosome-mediated protein synthesis is essential for all organisms ¹³², and disruption of translation is lethal to fungi. Early studies in *S. cerevisiae* showed that loss of ribosomal integrity compromises cell viability ¹³³. Eukaryotic ribosomes harbour three tRNA-binding sites (A, P and E). Cycloheximide (Table 2, inhibitor **16**), an antibiotic produced by *Streptomyces griseus* ^{134,135}, blocks mRNA translocation by occupying the ribosomal E site ^{136,137}. Consistently, cycloheximide suppresses *S. cerevisiae* growth, and mutation of its binding pocket (P56Q) reduces antifungal activity ¹³⁸, confirming the ribosome as its cellular target. Anisomycin ¹³⁹ (Table 2, inhibitor **17**) serves as an orthogonal inhibitor of cycloheximide ^{140–142}. Structure–activity relationship studies of these two inhibitors (**16** and **17**) provide avenues to explore inactive controls ^{143,144}. Moreover, elongation of protein biosynthesis is assisted by elongation factor 2 (eEF2) (Fig. 2), which is indispensable for the viability of *S. cerevisiae* ¹⁴⁵. The natural product sordarin (inhibitor **15**) disrupts eEF2 function by preventing its association with the ribosome ^{146,147}. Mutations in the sordarin binding pocket diminish antifungal activity ^{147,148}, validating eEF2 as the target. Moreover, sordarin derivatives reduce *C. albicans* and *A. fumigatus* infections in mouse models ¹⁴⁹, and structure–activity relationship analyses further support the development of inactive controls ¹⁵⁰.

As discussed above, our primary purpose in reviewing *S. cerevisiae* is to inform target selection for pathogenic fungi. Accordingly, we summarized compounds **9–17** that modulate essential *S. cerevisiae* proteins (Table 2) as potential starting points for antifungal probe development. Several of these targets are already validated in pathogens. Hsp90 is now widely recognized as an antifungal target, and recent efforts have improved the selectivity of fungal Hsp90 inhibitors over the human homolog ¹⁵¹. Ribosomes, which are indispensable for protein synthesis, likewise represent attractive antifungal targets. Notably, cycloheximide binds directly to the 80S ribosome of *C. albicans* and has been used as a chemical tool to interrogate ribosomal function ¹⁵². The cycloheximide-binding pocket contains residues absent in human ribosomes (Fig. S5) ^{152,153}, suggesting that pathogen selectivity may be achievable through structure-guided design. For the remaining targets lacking protein–inhibitor complex structures in pathogenic fungi (*A. fumigatus*, *C. albicans*, and *C. neoformans*), we constructed homology models of their orthologues. Comparison of inhibitor-binding pockets between *S. cerevisiae* enzymes and the corresponding pathogen models revealed high structural conservation. For example, GWT1 orthologues, in *A. fumigatus*, *C. albicans* and *C. neoformans*, display nearly identical folds, with fewer than three non-conserved residues in the manogepix-binding site (Fig. S1A), consistent with the observed antifungal activity of manogepix against these pathogens ¹⁵⁴. Similar conservation was observed for IPC synthase (Fig. S2), Sec14p (Fig. S3), and eEF2 (Fig. S4). Together, these analyses indicate that inhibitors originally developed against *S. cerevisiae* enzymes can serve as transferable chemical starting points for targeting conserved orthologues in pathogenic fungi.

Inhibitors that partially meet the primary filtering criteria

Apart from the 17 compounds discussed above, we also identified inhibitors that partially meet our primary filtering criteria (Fig. 1) but are still promising candidates of chemical probes.

Covalent inhibitors targeting fungal enzymes. Inhibitors mentioned above are non-covalent inhibitors. In recent years, covalent inhibitors attract rising interests in probing specific targets¹⁵⁵. One example is the identification of cysteine modifiers of the Prp8 intein¹⁵⁶, a self-splicing protein embedded within the essential spliceosome factor Prp8 in *C. neoformans*^{157,158}. Inteins are absent in humans, making this a highly selective antifungal target^{159,160}. Covalent inhibitor 6G-318S disrupts Prp8 intein splicing (IC_{50} = 5.8 μ M) through modification of the active-site cysteine¹⁵⁶. This covalent inhibitor inhibits the *C. neoformans* growth (MIC = 0.6 μ g/mL), with the accumulation of unspliced Prp8 precursor¹⁵⁶. Overexpression of wild-type Prp8 intein confers resistance to 6G-318S¹⁵⁶. These data reveal that 6G-318S is on target. Although no structural data show the intein-inhibitor mode of recognition, these covalent inhibitors reveal a novel avenue to probe Prp8 in *C. neoformans*. Another case of covalent inhibitor is the identification of isothiazolones targeting phosphoglucomutase, an enzyme that catalyzes the reversible conversion of glucose-6-phosphate and glucose-1-phosphate, which is further converted to UDP-GlcNAc, precursor of the cell wall chitin³⁴. Genetic research shows that phosphoglucomutase is essential for the viability of *A. fumigatus*³⁴, and the enzyme activity of phosphoglucomutase is inhibited by covalent modification of a surface cysteine (C353), using isothiazolone-based covalent inhibitors³⁴. This cysteine (C353) is not conserved in the human phosphoglucomutase, and isothiazolones indeed exhibit selectivity to the *A. fumigatus* phosphoglucomutase over the human orthologue³⁴, suggesting an avenue to develop inhibitors with selectivity. Due to the broad thiol reactivity of isothiazolone¹⁶¹, these covalent inhibitors are not used as chemical probes even though they inhibit the growth of *A. fumigatus*³⁴. The future research can focus on the identification of alternative thiol reactive covalent inhibitors targeting C353 of phosphoglucomutase, from which chemical probes are likely to be achieved.

Inhibitors identified by virtual screening. Computational tools facilitate the identification of inhibitors¹⁶². An example is the identification of dihydroorotate dehydrogenase (DHODH), a rate-limiting enzyme in the fungal pyrimidine biosynthesis pathway, essential for RNA/DNA synthesis and cell wall formation^{163,164}. With low sequence homology (~30%) to the human orthologue, it represents a highly selective antifungal target¹⁶⁴. Olorofim, a pyrrole-based compound developed by F2G Ltd., was identified through antifungal drug discovery efforts and specifically inhibits *Aspergillus fumigatus* DHODH with potent nanomolar activity (IC_{50} = 44 nM)^{165,166}. It shows no detectable inhibition of human DHODH, supporting selectivity. Olorofim has demonstrated efficacy against azole-resistant *Aspergillus* species and is currently in Phase III clinical trials^{165,167}.

Recently, virtual screening against a homology model of AfDHODH identified two novel scaffold inhibitors, CHEMBL1509241 and ZINC67300323, with micromolar IC_{50} values (16.9 μ M and 57.3 μ M, respectively), providing new chemical starting points for further optimization¹⁶⁸. No orthogonal inhibitors or inactive controls are described for these novel scaffolds, but the clinical validation of olorofim establishes DHODH as a *bona fide* antifungal target. Although no experimentally determined structures demonstrating the binding mode of these inhibitors, these compounds reveal avenues to target DHODH.

Natural product-based inhibitors. Natural products are one of the sources of antifungal agents¹⁶⁹. One example is a series of natural products that inhibit family 18 chitinases, enzymes that remodel chitin in the fungal cell wall¹⁷⁰, a well-characterized antifungal target¹⁷¹. Argifin and argadin are natural product cyclopentapeptides isolated from *Gliocladium* and *Clonostachys* spp., respectively^{172,173}. X-ray crystallography reveals that both inhibitors mimic the chitin substrate^{174,175}. Argifin inhibits *Aspergillus fumigatus* chitinase B1 (AfChiB1) with an IC_{50} of 27 nM, while argadin shows a K_i of 20 nM against *Serratia marcescens* chitinase B¹⁷⁶. Both compounds exhibit reduced activity against human chitotriosidase (e.g., argifin IC_{50} = 4.5 μ M), indicating potential selectivity for fungal enzymes¹⁷⁵. However, their large molecular weight (>600 Da) and peptidic backbone limit drug-likeness and stability¹⁷⁷. To overcome this limitation, a caffeine derivative (bisdionin C) was designed as a submicromolar inhibitor against AfChiB1^{178,179}. Probably due to the redundancy of chitinases^{180,181}, these natural products do not inhibit the growth of *A. fumigatus* and therefore do not meet the requirement of chemical probes, but they still provide starting points to target the chitin remodeling in the fungal cell wall.

Inhibitors targeting ribozymes. Ribozymes are RNAs with enzymatic activity¹⁸². Recent studies show that group II introns are self-splicing ribozymes found in fungi but absent in mammals, making them a selective antifungal target¹⁸³. A small molecule inhibitor (compound 1) was identified by high-throughput screening¹⁸³. The optimization of compound 1 resulted in intronistat A with antifungal activity against *S. cerevisiae* and *Candida parapsilosis* (MIC = 2 - 4 μ g/mL)¹⁸³. Target engagement is demonstrated by the resistance of the intronless strain and accumulation of precursor via the inhibitor treatment¹⁸³. Although no experimentally determined structure showing the mode of actions of intronistat A, this compound demonstrates that ribozymes can also be explored as antifungal targets.

Concluding remarks

Pathogenic fungi pose a persistent and growing threat to immunocompromised patients, a challenge compounded by the limited number of available antifungal drug classes¹⁵. Target-based drug discovery offers a strategy to expand the antifungal pipeline; however, progress is constrained by insufficient chemical validation of proposed targets, largely due to the lack of high-quality chemical probes. Without both genetic and chemical validation, advancing drug discovery campaigns remains difficult, particularly because fungal pathogens share highly conserved molecular machinery with humans, complicating the identification of selectively toxic agents¹⁸⁴. In contrast to human biology—where chemical probes are well established—systematic efforts to develop antifungal chemical probes remain scarce. Consequently, we evaluated **seventeen** antifungal compounds with demonstrated target engagement across four fungal species (*A. fumigatus*, *C. albicans*, *C. neoformans*, and *S. cerevisiae*).

Most compounds inhibit their targets with submicromolar to nanomolar potency (>100 nM $IC_{50}/K_i/K_d$) and suppress fungal **growth with target engagement**, supporting a mechanistic link between target inhibition and antifungal activity. In addition, proteome-wide selectivity has not been established, and potential off-target effects remain uncharacterized. Consequently, these molecules do not yet meet the stringent criteria of chemical probes but instead represent valuable starting scaffolds for further optimization, **and the evidence of target engagement still contribute to the chemical validation, justifying follow-up drug discovery campaigns against these targets**. For instance, the *S. cerevisiae* Sec14p is inhibited by compounds **11** and **12** with data of target engagement (Fig. 2; Table 2). In recently years, a Sec14p-targeted compound, turbinmicin, has been demonstrated as a novel antifungal scaffold, with *in vivo* efficacy in mouse models of *A. fumigatus* and *Candida auris*¹⁸⁵. Although turbinmicin is still in the preclinical stage¹⁸⁵, this case retrospectively suggests that chemical probes enhance the chance of success in target-based antifungal drug discovery.

Among those **seventeen** evaluated antifungal compounds, most of them are identified from conventional high-throughput screening (eight compounds) and natural products (seven compounds) (Table 1 & 2). The future research can expand methods used to identify chemical probes. Because enzyme–inhibitor complex structures are available for all compounds discussed, structure-guided approaches are likely to accelerate the development of improved probes^{186,187}. Fragment-based ligand discovery, scaffold hopping, and **artificial intelligence**-assisted design offer practical routes to enhance affinity, selectivity, and physicochemical properties¹⁸⁸. **Under our stringent primary filtering criteria, only inhibitor 4 (FK506) can be applied to multiple fungi, *C. neoformans* and *A. fumigatus***. Future research can explore probe spectrums of these **seventeen** compounds.

Another key challenge of chemical probe is cellular permeability. Although most targets are intracellular, direct evidence for cell-wall penetration is lacking. While the fungal cell wall (≈ 5.8 nm porosity) can accommodate relatively large particles (e.g., amphotericin B liposomes)¹⁸⁹, the structural features that enable small-molecule uptake remain unclear. Nonetheless, observed target engagement implies that these scaffolds can access intracellular targets, providing useful starting points to investigate relationships between chemical structure and fungal cell-wall permeability.

Taken together, we identified seventeen antifungal inhibitors with validated target engagement. Although they do not yet fulfil the strict standards of chemical probes, they establish a foundation for the rational development of high-quality antifungal probes and should help accelerate target validation and antifungal drug discovery.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

KY and WF. conceptualization; KY and WF. investigation; KY and WF. formal analysis; KY and LD. writing – original draft; KY, LD and WF. writing – review and editing; WF. project administration and funding acquisition.

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Figures

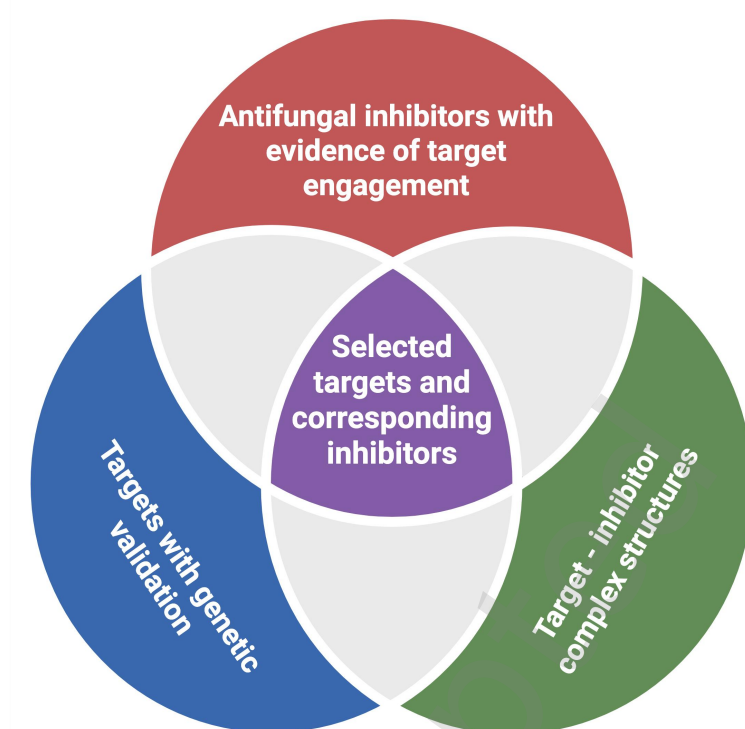


Figure 1 Selection of targets and corresponding inhibitors from literature. Light blue ellipse represents targets with evidence of genetic validation. Light green ellipse indicates targets with reported inhibitors, and corresponding target-inhibitor complex structures are reported. Light orange represents inhibitors that repress the fungal growth by inhibiting certain targets, i.e., target engagement.

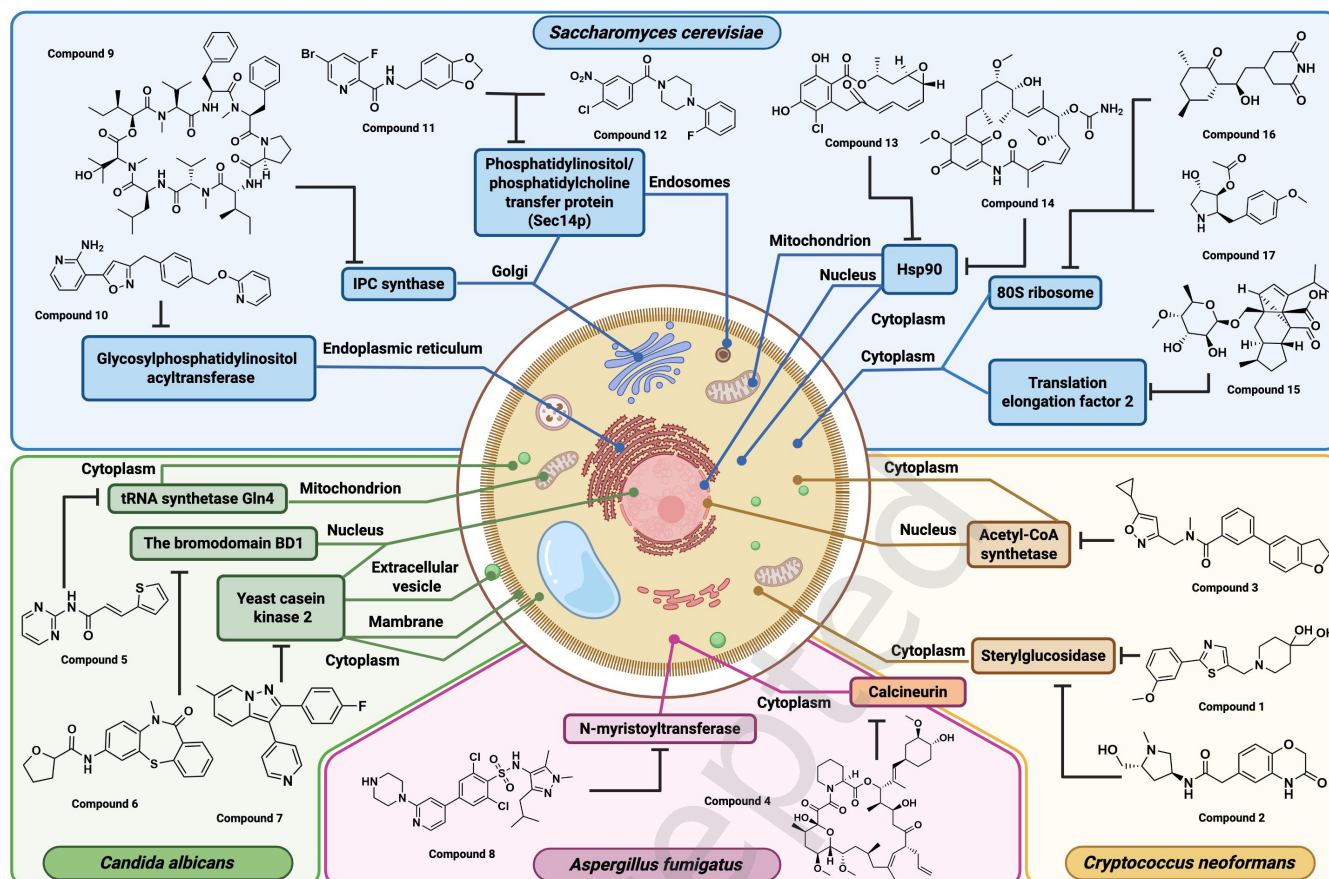


Figure 2 Compounds that target molecular machinery in fungal cells. Each colour represents a fungus. Rectangular frames indicate targets. The image was drawn in BioRender.

Table 1 Compounds that inhibit enzymes of pathogenic fungi. References of this table are in Table S2. Pan-assay interference compounds and cLogP are calculated by DataWarrior.

	<i>C. neoformans</i>				<i>C. albicans</i>			<i>A. fumigatus</i>	
Inhibitor ID	1	2	3	4	5	6	7	4	8
Genetic validation of targets									
IC50/Ki/Kd < 100 nM									
Target-inhibitor complex structure									
> 30-fold selectivity over other proteins in the proteome									
> 30-fold selectivity over the human orthologue									
With antifungal activity									
Evidence of target engagement									
Inactive control									
Orthogonal inhibitors									
cLogP < 5									
Not pan-assay interference compounds									
Antifungal efficacy in animal models									
Method of identification	HTS	HTS	HTS	NP	HTS	HTS	HTS	NP	CRP

	Yes	HTS	High-throughput screening
	No	NP	Natural product
	no information	CRP	Compound repurposing

Table 2 Compounds that inhibit enzymes of *S. cerevisiae*. References of this table are in Table S2. **Pan-assay interference compounds and cLogP are calculated by DataWarrior.**

	<i>S. cerevisiae</i>								
Inhibitor ID	9	10	11	12	13	14	15	16	17
Genetic validation of targets									
IC50/Ki/Kd < 100 nM									
Target-inhibitor complex structure									
> 30-fold selectivity over other proteins in the proteome									
> 30-fold selectivity over the human orthologue									
With antifungal activity									
Evidence of target engagement									
Inactive control									
Orthogonal inhibitors									
cLogP < 5									
Not pan-assay interference compounds									
Antifungal efficacy in animal models									
Method of identification	NP	HTS & CO	HTS & CO	CS	NP	NP	NP	NP	NP

	Yes	HTS	High-throughput screening
	No	NP	Natural product
	no information	CO	Chemical optimization
		CS	Compound selection