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Discovery of a Novel Dibromoquinoline Compound Exhibiting Potent Antifungal and Antivirulence Activity that Targets Metal Ion Homeostasis

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Abstract

Globally, invasive fungal infections pose a significant challenge to modern human medicine due to the limited number of antifungal drugs and the rise in resistance to current antifungal agents. The vast majority of invasive fungal infections are caused by species of *Candida*, *Cryptococcus*, and *Aspergillus*. Novel antifungal molecules consisting of unexploited chemical scaffolds with a unique mechanism are a pressing need. The present study identifies a dibromoquinoline compound (**4b**) with broad-spectrum antifungal activity that inhibits growth of pertinent species of *Candida* (chiefly *C. albicans*), *Cryptococcus*, and *Aspergillus* at a concentration as low as 0.5 µg/mL. Furthermore, **4b**, at a subinhibitory concentration, interfered with expression of two key virulence factors (hyphae and biofilm formation) involved in *C. albicans* pathogenesis. Three yeast deletion strains (*cox17*, *ssa1*, *aft2*) related to metal ion homeostasis were found to be highly-sensitive to **4b** in growth assays indicating the compound exerts its antifungal effect through a unique, previously unexploited mechanism. Supplementing the media with either copper or iron ions

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Supporting Information. Supplementary Tables 1–4 (*S. cerevisiae* heterozygous deletion strains identified as sensitive to **4b** via chemogenomic profiling, MIC results for **4b** evaluated against yeast in medium supplemented with copper or iron, aqueous solubility data for compound **4b**, and fungal strains description); Supplementary Figures 1–5 (*C. albicans* percent hyphae inhibition, *C. albicans* percent biofilm inhibition, yeast chemogenomic profiling results for *aft2* and *cox17* deletion strains and their sensitivity to 1800 other compounds, HPLC trace for compound **4b**, and dynamic light scattering analysis of **4b** in water and in DMSO).

reversed the strain sensitivity to **4b** further corroborating that the compound targets metal ion homeostasis. **4b**'s potent antifungal activity was validated *in vivo*, as the compound enhanced survival of *Caenorhabditis elegans* infected with fluconazole-resistant *C. albicans*. The present study indicates **4b** warrants further investigation as a novel antifungal agent.

Graphical Abstract

Keywords

Candida; chemogenomic profiling; antifungal; hyphae; biofilm; *C. elegans*

Pathogenic yeast (namely *Candida* and *Cryptococcus*) and molds (primarily *Aspergillus*) are responsible for an array of fungal infections that claim more than 1.5 million human lives each year ¹. The leading source of superficial (oropharyngeal mycosis and vaginal yeast infections) and invasive fungal infections worldwide are caused by *Candida*; for example, *Candida albicans* is one of the top five causative agents of bloodstream infections in hospitals ². Furthermore, a significant rise in invasive non-*albicans* *Candida* infections (caused by *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*) has been observed worldwide recently ^{3,4}. Another yeast, *Cryptococcus* (namely *C. neoformans* and *C. gatti*) infects more than one million human patients annually, resulting in over 600,000 deaths ⁵. Individuals infected with HIV are at increased risk to acquire cryptococcal infections including pneumonia and meningitis ⁶. Co-infection with HIV and cryptococcal meningitis is especially problematic in resource-limited regions such as sub-Saharan Africa, where mortality rates can exceed 40% ⁷. The third fungal pathogen noted above, *Aspergillus* (namely *A. fumigatus*), is responsible for more than 300,000 infections annually ⁸. *Aspergillus* are primarily responsible for pulmonary infections in immunocompromised patients including those receiving solid organ transplants ⁹. The challenge posed by pathogenic fungi continues to remain an ongoing global problem both in resource-limited regions (where few antifungal drugs are available for use) and developed nations where aggressive treatments for other diseases (such as cancer) may predispose susceptible patients to invasive fungal infections ¹⁰.

Invasive fungal infections are challenging to treat in part due to the few antifungal drugs available to use clinically. Three structurally-distinct antifungal drug classes are most frequently administered to treat systemic infections – azoles, polyenes, and echinocandins

¹¹. In comparison, there are twice as many antiretroviral drug classes and more than four times as many antibacterial drug classes currently used clinically. All three antifungal drug classes inhibit fungal cell membrane/wall synthesis (ergosterol synthesis or $\beta(1,3)$ -d-glucan synthesis) or directly bind to the cell membrane (polyenes bind to ergosterol), inducing pore formation ¹¹. However, many of these antifungal drugs suffer from significant drawbacks including a narrow spectrum of activity, toxicity to host tissues, and poor pharmacokinetic profiles ¹². Compounding the challenge further, fungal isolates have emerged that exhibit resistance to current antifungal drugs via a variety of mechanisms including decreased affinity for the target molecule, increased expression of drug efflux pumps (decreasing the intracellular concentration of antifungal agents), and formation of biofilms that are recalcitrant to the effect of many antifungal drugs (including those in the azole and polyene antifungal classes) ^{3, 412, 13}. This highlights the necessity for development of new antifungal therapeutics. Remarkably, in the past three decades, only one novel antifungal class has been developed ¹⁴. The difficulty in developing novel antifungal agents is due in large part to the fact that cellular features that are potential targets for antifungal therapy are also present in mammalian cells, thus giving rise to potential undesirable side effects ^{10, 14, 15}.

Ideally, a new antifungal agent should be broad-spectrum, be safe to host tissues, and exert its antifungal effect through a unique mechanism ¹³. To this end, we present the outcomes of a screen of an in house library of compounds for development as antifungal agents. This activity led to the discovery of a series of quinolone scaffold-based compounds that were earlier investigated by our group and found to display moderate anticancer activity (Figure 1) ¹⁶. Quinolones have been investigated primarily as potent antibacterial agents; however, several recent studies have demonstrated that quinolone compounds and natural products can possess antifungal activity ^{17–21}. In line with the aforementioned, our screening endeavor unmasked the highly potent antifungal activity residing in members of this chemotype family of compounds. The present study evaluates the antifungal effect of our disclosed dibromoquinolines. Special focus was given to the most potent compound in this study (**4b**) where its antifungal activity was examined against relevant species of *Candida*, *Cryptococcus*, and *Aspergillus*. This study also encompasses evaluation of **4b**'s toxicity against mammalian cells, evaluation of **4b**'s ability to interfere with key virulence factors expressed by *C. albicans*, investigation of the antifungal and antivirulence mechanism of **4b** using chemogenomic profiling and growth assays, and validation of **4b**'s potent antifungal activity *in vivo* in a *C. elegans* model of *C. albicans* infection.

RESULTS AND DISCUSSION

Investigation of dibromoquinoline compounds' antifungal activity against *Candida albicans*

The antifungal activity of 10 quinolone compounds was initially evaluated against *C. albicans* P60002 (Table 1). Nine compounds were found to be inactive against *C. albicans* (minimum inhibitory concentration, MIC, exceeded 64 μ g/mL). A single compound, **4b**, exhibited potent antifungal activity against *C. albicans* (MIC = 0.5 μ g/mL) and was superior to fluconazole (MIC > 64 μ g/mL). Thus, this compound was selected for further analysis. The exact molecular basis for the difference in antifungal activity observed for **4b** in relation

to the other nine compounds has not been fully elucidated. Experimental investigations into underlying causes and details thereof are currently underway.

Compound **4b** inhibits growth of both yeasts and molds

A critical limitation of many antifungals is they are unable to target multiple species of pathogenic fungi. For example, certain first- and second-generation azole antifungals (namely fluconazole) have activity against *Candida albicans* and *Cryptococcus*; however, they are generally ineffective against molds such as *Aspergillus niger* and *A. fumigatus*¹⁴. Further compounding the problem, non-*albicans* *Candida* species such as *C. glabrata* and *C. krusei* are resistant to azole antifungal drugs (chiefly to fluconazole)¹⁴. Echinocandins, on the other hand, can inhibit growth of species of *Candida* and *Aspergillus* but lack potent activity against species of *Cryptococcus*¹⁴. Thus finding antifungal compounds with broad-spectrum activity against *Candida*, *Cryptococcus*, and *Aspergillus* is needed.

As noted earlier, quinoline-based synthetic compounds and natural products have previously been found to possess antifungal activity. However, most compounds developed or isolated thus far exhibit moderate to weak antifungal activity against *C. albicans* (MIC ranges from 8 to 512 µg/mL) and are inactive against species of *Cryptococcus* and *Aspergillus*^{17–20}. Thus improving the potency of quinoline-based antifungals and expanding their spectrum of activity is a key component to their development as antifungal agents. Therefore, we moved to investigate **4b**'s antifungal activity against a wide-spectrum of *Candida*, *Cryptococcus*, and *Aspergillus* species.

Compound **4b** was first evaluated against thirteen clinical isolates of *Candida albicans* and non-*albicans* species including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (Table 2). **4b** inhibited growth of *C. albicans* at concentrations ranging from 0.5 to 1 µg/mL. The compound maintained its potent activity against strains of *C. albicans* exhibiting high-level resistance to fluconazole (MIC > 64 µg/mL), including *C. albicans* NR-29368, NR-29446, ATCC MYA-573, and ATCC 64124. Additionally, **4b** exhibited potent antifungal activity against non-*albicans* species of *Candida* including *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* (MIC ranging from 0.06 to 1 µg/mL). **4b** was superior to fluconazole against strains of *C. glabrata* and *C. parapsilosis*. Furthermore, the compound (MIC = 0.5 µg/mL) was superior to both fluconazole (MIC > 64 µg/mL) and 5-Fluorocytosine (MIC ranging from 8 to 16 µg/mL) against clinical isolates of *C. krusei*, a fungal pathogen noted for its resistance to several antifungal drugs (including to azoles and polyenes)²².

When evaluated against species of *Cryptococcus*, including *C. gattii* and *C. neoformans*, **4b** was once again superior to fluconazole (Table 2). **4b** inhibited growth of both *C. gattii* and *C. neoformans* at a concentration of 0.5 µg/mL. Fluconazole, in contrast, inhibited growth of the same strains at concentrations four-to-sixteen fold higher than the MIC for **4b**. A similar pattern was observed when **4b**'s antifungal activity was assessed against species of *Aspergillus* (Table 2). **4b** inhibited growth of *A. brasiliensis*, *A. niger*, and *A. fumigatus* at a concentration of 0.5 µg/mL. These strains exhibited high-level resistance to fluconazole (MIC > 64 µg/mL), as anticipated. Thus, **4b** appears to demonstrate highly potent and broad-spectrum activity against both pathogenic yeasts and molds.

4b is a fungistatic agent against *C. albicans*

After confirming the dibromoquinoline **4b** exhibited potent, broad-spectrum antifungal activity, it deemed of interest to investigate if **4b** simply inhibited fungal growth (is fungistatic) or killed fungi (is fungicidal). A survey of literature pertaining to quinoline or quinolone antifungal agents revealed that some compounds exhibit fungistatic activity while other compounds exhibit fungicidal activity against *C. albicans*²³. To investigate this matter further, compound **4b** and the control antifungal drug 5-Fluorocytosine were examined against *C. albicans* in a time-kill assay (Figure 2). Compound **4b** exhibited a fungistatic effect against *C. albicans*, as no reduction in fungal CFU was observed over a 24 hour timeframe. This matched the behavior observed with 5-Fluorocytosine, a known fungistatic antifungal drug, against *C. albicans*²⁴. At 10 × MIC, **4b** and 5-Fluorocytosine did not generate a three-log₁₀ reduction in fungal CFU, indicating that compound **4b** is a fungistatic agent (particularly against *C. albicans*).

Compound 4b exhibits limited toxicity to mammalian cells

Toxicity is a key characteristic to examine early in drug discovery to validate that molecules with good activity against the target pathogen are not harmful to host tissues. Several currently approved antifungal drugs are challenging to use clinically because of known toxicity concerns to human tissues¹⁴. For example, a notable drawback of amphotericin B is its significant toxicity to human patients. Recently, a less toxic lipid formulation of amphotericin B was synthesized¹⁴ but its cost precludes its use in developing nations where systemic fungal infections are endemic. Thus finding antifungals that are safe to mammalian tissues are needed.

The safety profile of **4b** was evaluated against three different mammalian cell lines. The compound was found to be non-toxic to human keratinocytes (HaCaT) up to a concentration of 32 µg/mL (Figure 3a). This represents a 64-fold difference between the concentration where **4b** inhibits growth of strains of *Candida*, *Cryptococcus*, and *Aspergillus* evaluated, and the highest concentration where the compounds are safe to mammalian cells. The compound was found to be non-toxic to human colonic epithelial cells (HRT-18) up to a concentration of 64 µg/mL (Figure 3b). This represents a 128-fold difference between the concentration where **4b** inhibits growth of most fungal isolates tested and where toxicity is observed against HRT-18 cells. Compound **4b** was found to be safe to kidney epithelial cells (Vero) at the highest concentration tested, 128 µg/mL (Figure 3c). This represents a 256-fold difference between the concentration where **4b** inhibits growth of *Candida*, *Cryptococcus*, and *Aspergillus* strains we evaluated, and the highest concentration where the compound was evaluated against Vero cells. The promising safety profile of **4b** observed against mammalian cells indicates the compound warrants further investigation.

Compound 4b interferes with hyphae formation in *C. albicans*

Fungal pathogens express a host of virulence factors including adhesins, invasins, and cytolytic toxins that contribute to infection in host tissues²⁵. A key virulence factor expressed by *C. albicans* that contributes significantly to its pathogenicity is the transition of yeast cells to branched filamentous cells called hyphae or pseudohyphae. This cellular

morphogenesis plays a critical role in the invasion of host tissues, damage to the mucosal epithelia, escape from host immune cells, and dissemination into systemic circulation²⁶. Additionally, the conversion of *C. albicans* from yeast to hyphae is linked to expression of specific adhesins that permit the formation of complex microbial communities, called biofilms, which are recalcitrant to treatment with most antifungal drugs²⁷. Furthermore, though yeast cells play an important role in disseminating an infection in the host, hyphae are critical for *C. albicans* to kill host cells/tissues²⁸. Thus, inhibition of the yeast-to-hyphae transition plays a key role in negating a key virulence factor expressed by *C. albicans*.

We evaluated the ability of **4b** to interfere with hyphae formation in *C. albicans*. Fungi were grown in RPMI-1640 medium for three hours, in the presence or absence of antifungal agents, at subinhibitory and inhibitory concentrations, to examine the formation of hyphae. As presented in Figure 4, untreated cultures exhibited branched filamentous cells characteristic of hyphae. Remarkably, cells treated with $\frac{1}{2} \times \text{MIC}$ (0.25 $\mu\text{g/mL}$) of **4b** exhibited more than 80% inhibition of hyphae formation (Supplementary Figure 1). Complete inhibition of yeast-to-hyphae morphogenesis by **4b** was observed at 1 $\mu\text{g/mL}$, a concentration that was found to be non-lethal to *C. albicans*. **4b** proved superior to 5-Fluorocytosine in inhibiting the yeast-to-hyphae transition. *C. albicans* treated with $\frac{1}{2} \times \text{MIC}$ of 5-Fluorocytosine resulted in less than 50% inhibition of hyphae formation. The presence of hyphae and pseudohyphae was visible in at least 50% of cells treated with 5-Fluorocytosine even at concentrations exceeding $4 \times \text{MIC}$ (0.5 $\mu\text{g/mL}$). The inability of 5-Fluorocytosine to completely inhibit hyphae formation is in agreement with previous reports^{29, 30}. 5-Fluorocytosine interferes with RNA and DNA synthesis in fungi. Polak *et al* demonstrated that germination of yeast to hyphae can occur even in the presence of a drug that interferes with RNA synthesis²⁹. Germ-tube formation is a process that is not dependent on DNA synthesis but is mainly dependent on the availability of carbohydrates to the fungal cell^{29, 30}. Thus, a potential advantage of **4b** over antifungal drugs such as 5-Fluorocytosine is the compound's ability to interfere with hyphae formation, a critical virulence factor important for *C. albicans* pathogenesis.

The quinoline derivative **4b** exhibits potent antibiofilm activity against *C. albicans* biofilm

The successful inhibition of *C. albicans* hyphae formation by **4b** led us to evaluate its ability to also interfere with biofilm formation and disrupt pre-formed mature *C. albicans* biofilm. Biofilms are complex structures composed of microbes, polysaccharides, and extracellular DNA that can attach to the surface of medical devices (such as catheters, shunts, dentures, and prosthetic devices) and lead to recurring infections in afflicted patients¹². *C. albicans* is the most frequently isolated pathogen from fungal biofilms³¹. Of note, *C. albicans* biofilms are highly resistant to treatment with azole antifungals, including newer drugs such as voriconazole and posaconazole; remarkably, cells residing in biofilms are nearly 1000-fold more resistant than planktonic cells to the effect of fluconazole³². The presence of polysaccharides such as β -1,3-glucan and extracellular DNA within the biofilm matrix have been proven to contribute to the poor antifungal activity of certain drugs (such as fluconazole) against *C. albicans* biofilms^{33, 34}. Thus, antifungal agents possessing antibiofilm activity against mature biofilms are needed.

As noted earlier, the expression of hyphae by *C. albicans* contributes to this pathogen's ability to form biofilms. Given **4b** demonstrated the ability to interfere with hyphae formation in *C. albicans*, we postulated that this compound would also be capable of inhibiting biofilm formation. *C. albicans* was incubated with **4b**, at subinhibitory concentrations, and the biofilm mass was stained with crystal violet. As expected, we found **4b** notably decreased *C. albicans* biofilm formation by more than 70% at $\frac{1}{8} \times$, $\frac{1}{4} \times$, and $\frac{1}{2} \times$ MIC (Supplementary Figure 2), the same concentrations where significant hyphae inhibition was noted.

In addition to interfering with biofilm formation, we investigated **4b**'s effect on mature biofilms. Utilizing the XTT assay, we evaluated the effect of increasing concentrations of **4b** and amphotericin B (potent antibiofilm agent) on the metabolic activity of *C. albicans* present in mature biofilm. Both agents exhibited a concentration-dependent reduction of *C. albicans* metabolic activity, as presented in Figure 5. **4b**, at 4 $\mu\text{g/mL}$, reduced metabolic activity of *C. albicans* present within the biofilm by more than 50%. This concentration was equal to the compound's MIC against planktonic cells at this inoculum size, confirming the potent antibiofilm effect of **4b**. Amphotericin B, at 1 $\mu\text{g/mL}$, reduced cellular metabolic activity in excess of 60%. This is in agreement with a previous report³⁵. Thus, in addition to **4b**'s ability to interfere with the yeast-to-hyphae transition, the compound also exhibits the ability to inhibit biofilm formation and to prevent additional *C. albicans* biofilm formation in the setting of an intact biofilm.

Compound 4b interferes with metal ion homeostasis in fungi

The potent antifungal and antivirulence activity of **4b** led us to next investigate the compound's potential mechanism of action. Chemogenomic profiling was employed to investigate the antifungal mechanism of action of **4b**. Although chemogenomic profiling has not been used for *Candida* or developed for *Cryptococcus*, the method is well-established as a viable tool for target identification of small molecules in the yeast *Saccharomyces cerevisiae*³⁶. The method relies on the principle that the targeted activity of a small molecule is reduced in the corresponding heterozygous deletion strain. Hence, the targeted activity and corresponding deletion strains have increased growth sensitivity compared to a genome-wide set of deletion strains. We have previously applied chemogenomic profiling to identify biological pathways targeted by an FDA-approved drug, auranofin, and a molecule in clinical trials, ebselen, which were found to possess antifungal activity^{37, 38}. Utilizing this approach, 34 heterozygous diploid deletion strains were sensitive to **4b** (Supplementary Table 1). Several genes encoded proteins involved in maintaining metal ion homeostasis and metal trafficking, protein synthesis, and proteins involved in key regulatory pathways (such as the MAP kinase pathway).

Interestingly, four genes identified as potential targets of **4b** – *sbe22*, *fkf1*, *rim101*, and *ecm33* are involved in *S. cerevisiae* bud growth and construction/maintenance of the yeast cell wall. Expression of *ECM33* and *RIM101* genes play a critical role in the expression of key virulence factors in yeast. Notably, Ecm33 and Rim101 are important for *C. albicans* to undergo the yeast-to-hyphae transition in *in vitro* studies^{39, 40}. Furthermore, inhibition of *ECM33* has a negative impact on *C. albicans* biofilms. Decreased expression of *ECM33*

correlated with a notable decrease in *Candida* biofilm formation on the surface of medical catheters in previous studies⁴⁰. Thus, the interference of yeast-to-hyphae transition and biofilm disruption exhibited by **4b** may be attributed to its interference with Ecm33 and Rim101 function.

The 34 mutant strains identified as potential targets of **4b** were subjected to growth curve experiments in order to pinpoint strains that were highly-susceptible to the effect of **4b** and thus the main target(s) for the compound. The growth of the wild-type (BY4743) *S. cerevisiae* strain relative to the heterozygous deletion mutant strain, in the presence of a subinhibitory concentration of **4b** (7.5 µg/mL; 13.8 µM), was determined to identify the most sensitive strains. A shift in the time required to reach the mid-logarithmic stage of growth (OD₆₀₀ ~ 0.30) between the wild-type and mutant strains was categorized as significant. From this analysis, 31 of the 34 mutant strains exhibited a nearly identical growth curve pattern to the wild-type strain, as depicted by the growth curves for the *isu1* and *yjr039w* strains in Figure 6a. Although these strains were identified in the chemogenomic screen, they are either not sensitive to **4b** or maybe sensitive under different conditions and higher compound concentrations.

Interestingly, three heterozygous diploid strains (*cox17* , *ssa1* , *aft2*) did exhibit a notable shift in the growth curve compared to the wild-type strain, when exposed to **4b** (Figure 6b). Cox17 and Aft2 play key roles in maintaining metal ion (namely copper and iron) homeostasis in yeast and protecting cells from the effects of oxidative stress. Ssa1 is a Hsp70 chaperone that has been implicated responding to varied types of stress including sensing of redox stress²¹. A search of a yeast chemogenomic database⁴¹ with yeast heterozygous sensitivity data for 1800 compounds indicated that both *cox17* and *aft2* heterozygous deletion strains are highly sensitive to deferasirox (Supplementary Figure 3). Deferasirox is a FDA approved drug with metal ion chelating properties that is used to treat iron overload⁴². Deferasirox has tridentate central 1,2,4-triazole rings that chelate iron, whereas **4b** also has a similar 1,2,4-triazole ring which may explain the ability of **4b** to affect deletion strains with disrupted metal ion homeostasis. In addition, it is striking that **4b** has an 8-hydroxyquinoline moiety, which also is reported to have metal ion chelating ability^{10, 12}. This intriguing link between **4b**, established metal chelating compounds and the *cox17* and *aft2* heterozygous deletion strain sensitivity warrant further investigation to determine if the antifungal effect is mediated by a metal ion chelation mechanism.

In order to further corroborate that **4b** exerts its antifungal effect by targeting copper and ferrous ion homeostasis, the MIC of **4b** was evaluated in the presence of increasing concentrations of copper (CuSO₄) or iron (FeSO₄). A noticeable increase in the MIC was observed for **4b** with increasing concentrations of CuSO₄ or FeSO₄ (Supplementary Table 2). In the presence of 50 µM CuSO₄, the MIC of **4b** against both *S. cerevisiae* and *C. albicans* increased significantly to 128 µg/mL. The compound was found to be inactive (MIC > 128 µg/mL) against both yeast strains when exposed to media supplemented with 100 µM CuSO₄. A similar result was observed for **4b** in the presence of increasing concentrations of FeSO₄. In the presence of 100 µM FeSO₄, the MIC of **4b** against both *S. cerevisiae* and *C. albicans* increased to 128 µg/mL. The compound was found to be inactive (MIC > 128 µg/mL) against *S. cerevisiae* in the presence of 500 µM FeSO₄ and **4b** was

inactive against *C. albicans* in the presence of 1 mM of FeSO₄. This approach suggests that **4b** exerts its antifungal effect in yeast primarily by targeting metal ion homeostasis.

Compound **4b** enhances survival of *C. elegans* infected with *C. albicans*

Many promising compounds exhibiting potent *in vitro* activity often fail when evaluated *in vivo* in animal models of infection. Given **4b**'s promising antifungal activity *in vitro*, combined with its potent antivirulence activity against *C. albicans* hyphae and biofilm formation, it was critical to evaluate whether the compound's activity could be retained *in vivo*. *C. elegans* is an excellent animal model for early-stage evaluation of promising anti-infective and antivirulence agents prior to evaluating agents in mammalian models such as rodents^{38, 43–46}. A challenge of translating antifungal compounds to clinical applications are issues pertaining to toxicity and rapid metabolism. *C. elegans* expresses cytochrome P450 enzymes that are homologous to mammalian enzymes, thus providing early insight into potential issues with rapid metabolism of novel compounds⁴⁷.

To evaluate the *in vivo* antifungal efficacy of **4b**, *C. elegans* were infected with a highly-virulent, fluconazole-resistant strain of *C. albicans*. After a short infection period (90 minutes), worms were washed and subsequently treated with **4b** (8 × MIC), the fungistatic agent 5-Fluorocytosine (8 × MIC), fluconazole, or left untreated. Worms were checked daily for viability and the number of live worms was recorded. As presented in Figure 7, more than 40% of worms in the untreated group died after one day and less than 5% were alive after the third day. All worms in the untreated group were dead by the fourth day post-infection. A similar result was observed for worms treated with fluconazole. Nearly 30% of worms died one day after treatment, and more than 80% of worms had died by the second day. All worms receiving fluconazole treatment were dead by the third day post-infection. Infected worms treated with either 5-Fluorocytosine or **4b** exhibited prolonged survival. All worms treated with either 5-Fluorocytosine or **4b** remained alive one day after infection. Remarkably, more than 90% of worms treated with **4b** continued to remain alive three days after infection. Nearly 80% of worms receiving **4b** treatment were alive four days after infection when the experiment was concluded. The results further validate the potent antifungal activity of **4b** *in vivo* against *C. albicans* and opens the door for future evaluation of **4b** in a rodent model of fungal infection.

In conclusion, the present study characterizes a novel dibromoquinoline compound (**4b**) that exhibits broad-spectrum antifungal activity against pathogenic yeast (*Candida* and *Cryptococcus*) and molds (*Aspergillus*) (MIC of 0.5 µg/mL). The compound possesses a promising safety profile as it is non-toxic to mammalian cells (keratinocytes) up to a concentration of 32 µg/mL. In addition to its potent antifungal activity, **4b** is capable of interfering with the yeast-to-hyphae transition in *C. albicans* and exhibits potent antibiofilm activity against mature *C. albicans* biofilm. Deeper investigation of **4b**'s antifungal mechanism via chemogenomic profiling revealed the compound interferes with genes that regulate metal ion homeostasis and bud (and hyphae) growth/cell wall maintenance. Furthermore, **4b**'s potent antifungal activity is validated *in vivo* as the compound enhanced survival of *C. elegans* infected with a highly-virulent strain of *C. albicans*. The present study provides evidence that **4b** represents a promising quinoline scaffold-based new lead with

potent broad-spectrum antifungal activity that warrants further evaluation for development as an antifungal and antivirulence agent.

METHODS

Synthesis of compounds

The dibromoquinolone compounds were prepared using previously reported procedures¹⁶. The complete chemical characterization and purity of the tested compounds has been previously reported¹⁶. HPLC data of **4b** is provided in Supplementary Figure 4. All compounds were dissolved in dimethyl sulfoxide (DMSO) to achieve stock 10 mg/mL solutions. Dynamic light scattering data for **4b** in water and DMSO is provided in Supplementary Figure 5.

Turbidimetric solubility analysis for compound **4b**

The aqueous solubility of **4b** was evaluated using a standard turbidimetric solubility assay in phosphate-buffered saline (PBS), using a previously described method⁴¹. Briefly, serial dilutions of **4b** and control drugs (tamoxifen and verapamil) were prepared in dimethyl sulfoxide. These solutions were diluted 100-fold into PBS in a 96-well plate to attain final dilutions equivalent to 50–400 μ M. All drug concentrations were prepared using triplicate samples and were allowed to stand for two hours before measuring the absorbance of each well at 540 nm via a spectrophotometer. Blank readings were determined by measuring the absorbance of the drug-free solvent system. The solubility limit was reported as the highest experimental concentration with no evidence of turbidity. An absorbance value greater than “mean + 3 $\times$ standard deviation of the blank” was considered as indicative of turbidity influenced by precipitate formation upon PBS dilution. Using the criteria outlined by Guha *et al.*⁴², test agents were characterized as exhibiting low aqueous solubility (< 10 μ g/mL), medium solubility (10 μ g/mL – 60 μ g/mL), or high solubility (>60 μ g/mL). The solubility result for **4b** is presented in Supplementary Table 3.

Fungal strains and reagents used in this study

Candida, *Cryptococcus*, and *Aspergillus* isolates were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) and BEI Resources (Manassas, VA, USA). Strain information is presented in Supplementary Table 4. HaCaT cells were purchased from AddexBio (San Diego, CA). Fluconazole and 5-Fluorocytosine were purchased commercially and dissolved in DMSO to prepare respective stock solutions (10 mg/mL). Yeast extract peptone dextrose (YPD), RPMI-1640 for MIC determination, 3-(N-morpholino)propanesulfonic acid (MOPS), phosphate-buffered saline (PBS), DMEM and MEM for cell culture assays, fetal bovine serum (FBS), fetal horse serum, and 96-well plates were all purchased from commercial vendors.

Determination of minimum inhibitory concentration (MIC)

The broth microdilution assay was used to determine the MIC of compounds and control antifungal drugs (fluconazole and 5-Fluorocytosine) in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute for yeasts (M27-A3)⁴⁸ and molds (M38-A2)⁴⁹. Plates containing fungal isolates with **4b**, fluconazole, or 5-

Fluorocytosine were incubated at 37 °C for at least 24 hours for *Candida* spp. and *Aspergillus* spp. *Cryptococcus* spp. were incubated for 72 hours before the MIC was determined by visual inspection.

Time-kill assay against *C. albicans*

An overnight bacterial suspension of *C. albicans* P60002 cells was diluted to 4.2×10^4 colony-forming units per milliliter (CFU/mL). The suspension was exposed to either compound **4b** or 5-Fluorocytosine at concentrations equal to either $5 \times \text{MIC}$ (in triplicate) or $10 \times \text{MIC}$ (in triplicate) in RPMI-1640 medium supplemented with MOPS. A sample (100 μL) was obtained 0, 2, 4, 6, 8, 12, and 24 hours after incubation with test agents at 37 °C and subsequently serially diluted in PBS. An aliquot of each dilution was transferred to YPD agar plates and incubated at 37 °C for at least 20 hours. The number of CFU was subsequently enumerated.

Evaluation of compound **4b**'s toxicity against mammalian cells

Compound **4b** was examined (from 16 to 128 $\mu\text{g/mL}$) against human keratinocyte (HaCaT), human colonic epithelial cells (HRT-18), and monkey kidney epithelial cells (Vero) to determine the potential toxic effect to mammalian cells *in vitro*, as described elsewhere⁵⁰. HaCaT cells were cultured in DMEM supplemented with 10% FBS at 37 °C with CO₂ (5%). HRT-18 cells were cultured in RPMI-1640 medium supplemented with 10% fetal horse serum at 37 °C with CO₂ (5%). Vero cells were cultured in MEM supplemented with 10% FBS, 1 mM sodium pyruvate, and penicillin-streptomycin at 37 °C with CO₂ (5%). Control cells received DMSO alone at a concentration equal to that in drug-treated samples. The cells were incubated with test agent (in triplicate) in a 96-well plate at 37 °C with CO₂ (5%) for two hours. The assay reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for four hours. Absorbance readings (at OD₄₉₀) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the viability of DMSO-treated control cells (average of triplicate wells $\pm$ standard deviation). The toxicity data was analyzed via a two-way ANOVA, with post hoc Sidak's multiple comparisons test ($P < 0.05$), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Examination of *C. albicans* yeast-to-hyphae inhibition

C. albicans P60002 ($\sim 6.4 \times 10^5$ CFU/mL) was suspended in RPMI-1640 medium supplemented with MOPS in order to induce germ-tube formation, as described elsewhere⁵¹. Compound **4b** and 5-Fluorocytosine, at subinhibitory concentrations (ranging from 0.13 to 1 $\mu\text{g/mL}$) were added to a 96-well plate containing the fungal suspension and incubated at 35 °C for three hours. The morphology of the cells was examined using a Nikon TiS Wide-Field microscope (40 $\times$ objective). Images were captured using the NIS-Elements Microscope Imaging Software (Nikon Metrology Inc.). Cells were classified either as budded/non-filamentous or filamentous cells (that included both true hyphae and pseudohyphae)⁵¹. Cells from at least two different fields of view were counted to determine

the number of yeast and filamentous cells in order to calculate the percent inhibition of hyphae formation. Data were analyzed via a two-way ANOVA with post-hoc Dunnet's test for multiple comparisons utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Biofilm investigation

An overnight suspension of *C. albicans* P60002 was centrifuged ($4000 \times g$ for five minutes), washed with PBS, and adjusted to a starting inoculum of $\sim 5.0 \times 10^5$ CFU/mL in RPMI-1640 supplemented with MOPS.

Examination of 4b's ability to inhibit *C. albicans* biofilm formation

An aliquot (150 μ L) of the fungal inoculum was transferred to each well of a 96-well tissue-culture treated plate. Compound **4b** or amphotericin B (six wells for each treatment regimen) were added and serially diluted to create a concentration gradient. The plate was incubated for 24 hours at 37 °C to permit biofilm formation. The inoculum was subsequently removed and biofilms washed once with PBS (to remove planktonic fungi). Biofilm mass was quantified (OD₅₉₅) using the crystal violet reporter assay described elsewhere ⁵².

Evaluation of 4b's activity against mature *C. albicans* biofilm

An aliquot (150 μ L) of the fungal suspension was transferred to each well of a 96-well tissue-culture treated plate and incubated for 24 hours at 37 °C to form mature biofilm. The inoculum was subsequently removed and biofilms washed once with PBS (to remove planktonic fungi). Test agents were added (in RPMI-1640 medium supplemented with MOPS), serially diluted (from 128 μ g/mL to 1 μ g/mL), and incubated with biofilm for 24 hours at 37 °C. Biofilm viability was evaluated utilizing the XTT reduction assay ⁵³.

Chemogenomic profiling of 4b against *S. cerevisiae*

Saccharomyces cerevisiae sensitivity to **4b** was initially determined against the wild-type BY4743 diploid strain. BY4743 was grown in YPD with DMSO (1%) or **4b** in concentrations ranging from 10 to 100 μ M. **4b** (55 μ M; 30 μ g/mL) delayed growth of BY4743 by 30% compared to the no drug control half-maximal optical density (OD). Chemogenomic haploinsufficiency profiling using the complete genome-wide heterozygous deletion strain pool (Thermo Fisher Scientific, Waltham, MA) was performed as previously described ³⁸. A frozen aliquot (200 μ L) of the pool was thawed and inoculated into 2 mL of YPD and grown for nine hours until OD₆₀₀ of 4.0. The culture was diluted to an OD₆₀₀ of 0.13 and either DMSO (1%) or **4b** (35 μ g/mL) was added (three replicates each, 1 mL) and grown for seven hours. The cultures were diluted to an OD₆₀₀ of 0.13 in 1 mL YPD with DMSO or **4b** (64.3 μ M; 35 μ g/mL) and grown for eight hours. Cultures were harvested and genomic DNA extracted using the YeaStar Genomic DNA kit (Zymo Research, Irvine, CA) and the unique synthetic barcodes in the genomic DNA were amplified by PCR. Additional genomic DNA samples from other cultures treated with unrelated compounds were processed at the same time and all the PCR products were normalized and pooled for Illumina HiSeq 2500 sequencing. The reads for each experiment were identified using a 5 base multiplex tag and subsequent unique barcodes in each experimental sample was cross-

referenced to a database of recharacterized barcode sequences⁵⁴. The resulting strain counts were analyzed with edgeR⁵⁵ using previously described parameters³⁸.

Saccharomyces deletion strain haploinsufficiency validation

Strains were grown and maintained on media according to standard practices⁵⁶. An overnight suspension of *S. cerevisiae* strains were diluted ($OD_{600} \sim 0.05$) and grown in the presence of **4b** (7.5 $\mu\text{g/mL}$). Growth for the wild-type (*S. cerevisiae* BY4743) and mutant strains was monitored using a spectrophotometer (OD_{600}) at indicated time points for 20 hours, as described elsewhere³⁸.

Evaluation of antifungal activity of 4b in presence of copper or iron

The MIC of compound **4b** was determined against both *S. cerevisiae* BY4743 and *C. albicans* P60002 using the broth microdilution method, as described above. To evaluate the impact of copper and iron on diminishing the antifungal effect of **4b**, media containing *S. cerevisiae* or *C. albicans* was supplemented with increasing concentrations of either copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) or ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (ranging from 50 μM to 1 mM). Microtiter plates, containing yeast in supplemented media, were incubated with **4b** for 24 hours at 35 °C before determining the MIC via visual inspection.

Evaluation of 4b in C. elegans infected with C. albicans

L4-stage *C. elegans* AU37 (sek-1; glp-4) strain (glp-4(bn2) worms were utilized to assess the efficacy of **4b** to enhance survival of *C. albicans*-infected worms *in vivo*, as described elsewhere³⁸, with the following modifications. Fluconazole-resistant *C. albicans* NR-29437 was grown to log-phase ($\sim 1 \times 10^7$ CFU/mL) in YPD broth. Worms (23–36) were added to the broth and infected with *C. albicans* for 90 minutes at 25 °C. Next, worms were harvested by centrifugation and washed with sterile PBS. The worms were subsequently treated with **4b** ($8 \times \text{MIC}$), 5-Fluorocytosine ($8 \times \text{MIC}$), or fluconazole (5 $\mu\text{g/mL}$). One group of worms was left untreated (negative control). Worms were inspected daily for viability and the number of live worms was recorded. The data are presented as percent survival of infected *C. elegans* utilizing a Kaplan-Meier survival curve generated using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical structures for dibromoquinoline compounds presented in this study.

Figure 2. Time-kill analysis of compound **4b** and 5-Fluorocytosine (both at 5 × and 10 × MIC) against *C. albicans* P60002 over a 24 hour incubation period at 37 °C. DMSO served as a negative control. Error bars represent standard deviation values.

Figure 3.

Toxicity analysis of compound **4b** against **a**) Human keratinocytes (HaCaT), **b**) Human colonic epithelial cells (HRT-18), and **c**) Monkey kidney epithelial cells (Vero). Data represent percent viable cells after exposure to **4b** (tested in triplicate) at 16, 32, 64, and 128 µg/mL using the MTS assay. Dimethyl sulfoxide (DMSO) was used as a negative control. Error bars represent standard deviation values. An asterisk (*) denotes statistical difference between **4b** and DMSO evaluated using a two-way ANOVA, with post hoc Sidak's multiple comparisons test ($P < 0.05$).

Figure 4. Effect of **4b** on inhibition of *C. albicans* P60002 hyphae formation. *C. albicans* ($\sim 6.4 \times 10^5$ CFU/mL) in RPMI-1640 medium supplemented with MOPS was exposed to **4b** (0.13 µg/mL up to 1 µg/mL), 5-Fluorocytosine (5-FC, 0.13 µg/mL up to 1 µg/mL), or left untreated for three hours at 35 °C to induce yeast-to-hyphae transition. Morphological changes were observed via a Nikon TiS inverted microscope (40× objective lens).

Figure 5. Antibiofilm activity of **4b** against *C. albicans* P60002 biofilm evaluated with the XTT assay. Mature biofilms were treated with either **4b** or amphotericin B (both in triplicate) at the concentrations presented, over a 24-hour period. The percent metabolic activity for each treatment was calculated relative to untreated wells. Error bars represent standard deviation values. Asterisk denotes statistical difference between **4b** and amphotericin B relative to the negative control (untreated wells) evaluated using a two-way ANOVA, with post hoc Dunnet's multiple comparisons test ($P < 0.05$).

Figure 6. Growth curves of *S. cerevisiae* wild type (BY4743) and heterozygous diploid deletion strains (*cox17* , *ssa1* , *aft2* , *isu1* , and *yjr039w*) in the presence of **4b** (7.5 μ g/mL) in YPD broth over a 20-hour incubation period at 37 °C. Growth curves were evaluated for differences in time to reach mid-logarithmic growth (OD₆₀₀ ~ 0.30) for the wild-type strain relative to the mutant strain. **a)** 31 of 34 mutant strains, including *isu1* and *yjr039w* , exhibited no difference in their growth pattern relative to the wild-type strain at this low concentration. **b)** In contrast, *cox17* , *ssa1* , and *aft2* mutants exhibited a notable shift in their growth curve pattern relative to the wild-type strain.

Figure 7. Examination of effectiveness of **4b**, 5-Fluorocytosine, and fluconazole to enhance survival of *C. elegans* infected with the highly-virulent, fluconazole-resistant *Candida albicans* NR-29437 strain. Adult (L4-stage) worms were infected with *C. albicans* for 90 minutes at 35 °C. Worms were washed and subsequently treated with **4b** (8 × MIC), 5-Fluorocytosine (8 × MIC), fluconazole, or left untreated. Survival of worms was monitored daily and recorded. Data are presented as a Kaplan-Meier survival curve.

Table 1

The minimum inhibitory concentration of dibromoquinoline compounds and control antifungals drugs (fluconazole and 5-Fluorocytosine) evaluated versus *C. albicans* P60002.

Compound/Drug Name	Minimum Inhibitory Concentration (µg/mL)
1a	>64
1b	>64
1c	>64
2a	>64
2b	>64
2c	>64
3a	>64
3b	>64
4a	>64
4b	0.50
Fluconazole	>64
5-fluorocytosine	0.13

Table 2

The minimum inhibitory concentration (MIC in µg/mL) of 4b, fluconazole, and 5-Fluorocytosine evaluated versus species of *Candida*, *Cryptococcus*, and *Aspergillus*.

Strain Name	Compound/Drug Name		
	4b	Fluconazole	5-fluorocytosine
<i>Candida albicans</i> NR-29351	0.50	0.50	N.D. ^a
<i>Candida albicans</i> NR-29365	0.50	0.50	N.D.
<i>Candida albicans</i> NR-29368	1	>64	0.13
<i>Candida albicans</i> NR-29446	0.50	>64	0.50
<i>Candida albicans</i> ATCC MYA-573	0.50	>64	0.25
<i>Candida albicans</i> ATCC 64124	0.50	>64	0.50
<i>Candida krusei</i> ATCC 14243	1	64	16
<i>Candida krusei</i> ATCC 34135	1	64	8
<i>Candida parapsilosis</i> ATCC 22019	0.25	1	N.D.
<i>Candida glabrata</i> ATCC MYA-2950	0.50	32	N.D.
<i>Candida glabrata</i> ATCC 66032	0.06	16	N.D.
<i>Candida tropicalis</i> ATCC 1369	0.50	1	N.D.
<i>Candida tropicalis</i> ATCC 13803	0.50	0.50	N.D.
<i>Cryptococcus gattii</i> NR-43208	0.50	2	N.D.
<i>Cryptococcus gattii</i> NR-43209	0.50	8	N.D.
<i>Cryptococcus neoformans</i> NR-41292	0.50	8	N.D.
<i>Aspergillus brasiliensis</i> ATCC 16404	0.50	>64	N.D.
<i>Aspergillus niger</i> ATCC 6275	0.50	>64	N.D.
<i>Aspergillus niger</i> ATCC 16888	0.50	>64	N.D.
<i>Aspergillus fumigatus</i> NR-35302	0.50	>64	N.D.
<i>Aspergillus fumigatus</i> NR-35301	0.50	>64	N.D.

^a N.D. = Not determined