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New Application of Neomycin B–Bisbenzimidazole Hybrids as Antifungal Agents

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Abstract

Alkylated aminoglycosides and bisbenzimidazoles have previously been shown to individually display antifungal activity. Herein, we explore for the first time the antifungal activity (in liquid cultures and in biofilms) of ten alkylated aminoglycosides covalently linked to either mono- or bisbenzimidazoles. We also investigate their toxicity against mammalian cells, their hemolytic activity, and their potential mechanism(s) of action (inhibition of fungal ergosterol biosynthetic pathway and/or reactive oxygen species (ROS) production). Overall, many of our hybrids exhibited broad-spectrum antifungal activity. We also found them to be less cytotoxic to mammalian cells and less hemolytic than the FDA-approved antifungal agents amphotericin B and voriconazole, respectively. Finally, we show with our best derivative (**8**) that the mechanism of action of our compounds is not the inhibition of ergosterol biosynthesis, but that it involves ROS production in yeast cells.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00254. EC₅₀ values (±SDEV) for compounds **8–10**, Table S1; selectivity index (SI) and Log P values for compounds **8–10**, Table S2; % hemolysis caused by compounds **8–10** and VOR against mouse erythrocytes with error bars (±SDEV), Table S3; HC₅₀ values (±SDEV) for compounds **8–10**, Table S4; 96-well plates showing the antibiofilm activity of compounds **8–10** and VOR against strains **B** and **C** (from which the data in Table 5 come from), Figure S1; HPLC traces confirming the purity of compounds **1–6**, Figures S2–S7 (PDF)

Author Contributions

S.G.-T. and D.P.A. designed the chemical syntheses. N.T.C., N.R., and A.S. synthesized NEO–bisbenzimidazole derivatives used in this study. S.K.S. performed all of the biochemical and biological experiments. S.K.S., N.T.C., and S.G.-T. designed the overall study. N.T.C., S.K.S., and S.G.-T. analyzed data, wrote the manuscript, and prepared all figures. All authors reviewed the final manuscript.

The authors declare no competing financial interest.

Keywords

benzimidazoles; ergosterol; cytotoxicity; biofilm; hemolysis; time-kill; reactive oxygen species (ROS)

Invasive fungal infections have become a serious problem in public health due to the rising population of immunocompromised patients who have had HIV/AIDS, cancer, or organ transplants.^{1–3} Infectious fungal diseases are associated with organ transplant, and their prevention, diagnosis, and management are essential for improved outcome in transplant patients.^{4–9} Although *Candida albicans* and *Aspergillus* spp. are solely to blame for causing life-threatening diseases such as candidiasis and aspergillosis in critically ill patients, the incidence of infections due to non-*albicans* *Candida* strains such as *C. glabrata* and *C. parapsilosis* are also increasing simultaneously, thereby complicating antifungal therapy.^{10–15} Despite the availability of an expanding list of families of antifungal drugs such as azoles (e.g., fluconazole (FLC) and voriconazole (VOR)), polyenes (e.g., amphotericin B (AmB)), echinocandins (e.g., caspofungin (CAS)), and allylamines (e.g., terbinafine), the current antifungal reservoir is far from perfect to meet the necessity of treating a wide array of fungal diseases.¹⁶ Besides issues with efficacy, additional challenges encountered with the current antifungal agents include emerging resistance, significant side effects, toxicity, and drug–drug interactions.^{17–23} As resistance to the currently available antifungal agents is emerging in many of these fungal species, there is a need for developing novel antifungals.

It has previously been demonstrated that the introduction of long alkyl chains on aminoglycoside antibiotics can provide compounds with strong antifungal activity.^{24–26} We also recently reported that bisbenzimidazoles, which have been extensively studied in the past for their antimicrobial,^{27,28} anticancer,^{29,30} and DNA sequence recognition properties,^{31–33} can act as antifungal agents.³⁴ It was also shown that aminoglycoside–fluoroquinolone hybrids (e.g., neomycin B (NEO)–ciprofloxacin (CIP)) perform better as antibacterial agents (better activity; they were found to be more potent inhibitors than CIP in supercoiling assays with DNA gyrase, relaxation assays with TopoIV, and in *in vitro* transcription/translation assays with an *E. coli* S30 extract system) than the parent unlinked drugs used individually or in a 1:1 mixture.³⁵ Inspired by these findings, we postulated that covalently conjugating benzimidazoles to aminoglycosides via an alkyl chain could potentially lead to better antifungal agents than their respective individual components.

We previously reported the preparation of mono- and bisbenzimidazoles conjugated to the aminoglycoside NEO to study their effect on DNA and RNA binding.^{36–39} These NEO–benzimidazole conjugates linked via thiourea and triazole linkages showed remarkable stabilization of DNA duplexes compared to the individual parent compounds NEO and benzimidazole. These NEO–bisbenzimidazole conjugates displayed linker length-dependent selectivity in RNA versus DNA binding studies.⁴⁰ On the other hand, NEO–mono-benzimidazole conjugates exhibited linker-dependent stabilization of the HIV–TAR RNA duplex.⁴¹ With these NEO–benzimidazoles conjugates in hand, we decided to now explore their effect on antifungal activity and how the linkers between these molecules can be correlated to their activity.

Herein, we report the antifungal activity of six NEO–monobenzimidazole derivatives (**1–6**) and four NEO–bisbenzimidazole derivatives (**7–10**). We evaluate the antifungal activity of these compounds against a variety of *Candida albicans*, non-*albicans Candida*, and *Aspergillus* strains by *in vitro* minimum inhibitory concentration (MIC) determination as well as by time-kill studies. We also explore their cytotoxicity as well as their hemolytic activity against mammalian cell lines and mouse erythrocytes, respectively. Finally, we investigate the potential mechanism(s) of action of selected hybrids.

RESULTS AND DISCUSSION

In Vitro Antifungal Susceptibility Testing

The antifungal activity (minimum inhibitory concentration (MIC)) of the NEO–monobenzimidazole derivatives **1–6** and NEO–bisbenzimidazole derivatives **7–10** was first evaluated against a panel of seven *Candida albicans* strains (**A–G**), three non-*albicans Candida* (**H–J**), and three *Aspergillus* strains (**K–M**) using a concentration range of 0.03–31.3 $\mu\text{g/mL}$ (Table 1). The synthesis of these six NEO–monobenzimidazole derivatives (**1–6**), four NEO–bisbenzimidazole derivatives (**7–10**), and the bisbenzimidazole intermediate **11** were previously reported (Figure 1).^{39,42} Commercially available antifungal agents such as AmB, CAS, FLC, and VOR were used as positive controls for comparison. For compounds **1–10** as well as for the reference drugs AmB and CAS, we reported MIC-0 values, which correspond to no visible growth of the 13 fungal strains tested. We reported MIC-2 values (i.e., 50% growth inhibition) for FLC and VOR against all fungal strains tested with the exception of strain **A** by VOR. We define the antifungal activity as excellent (1.95 $\mu\text{g/mL}$), good (3.9–7.8 $\mu\text{g/mL}$), or poor (15.6 $\mu\text{g/mL}$) based on MIC values.

By a rapid survey of the MIC data presented in Table 1, we could conclude that, in general, the NEO–monobenzimidazole derivatives **1–6** exhibited poor antifungal activity against all *C. albicans* (**A–G**) and *Aspergillus* (**K–M**) strains tested, with the exception of compound **5**, which showed good activity (7.8 $\mu\text{g/mL}$) against *C. albicans* strains **A** and **B**. Similarly, compounds **3**, **4**, and **6** did not show activity against the three non-*albicans Candida* strains (**H–J**) tested. However, compounds **1**, **2**, and **5** displayed good activity (3.9–7.8 $\mu\text{g/mL}$) against these three non-*albicans Candida* strains (**H–J**), and compound **2** even showed excellent activity (1.95 $\mu\text{g/mL}$) against *C. parapsilosis* ATCC 22019 (strain **J**). When investigating the NEO–bisbenzimidazole derivatives **7–10**, we found compound **7**, without

an oxygen atom in its linker, to be inactive against all fungal strains tested. However, when examining the MIC values for compounds **8** and **9**, with an oxygen atom in their linkers, we observed that these compounds showed excellent antifungal activity against 12 out of the 13 fungal strains tested (Note: these compounds did not display activity against *A. flavus* (strain **K**)). Compound **10** was also found to display good to excellent activity against the same 12 strains and not to be active against strain **K**. More importantly, compounds **8–10** exhibited either comparable or, in most of the cases, enhanced antifungal activity against all fungal strains tested when compared to the control drugs AmB, CAS, FLC, and VOR. These observations point to the importance of the ether bridge in the linkers of molecules **8–10** for conferring antifungal activity. To confirm the benefit of covalently attaching the bisbenzimidazole moiety to NEO, we next tested the antifungal activity of these two components (NEO and compound **11**, a bisbenzimidazole containing the optimal alkyl length in an ether linkage) individually and in a combination by using a checkerboard assay against *C. albicans* ATCC 10231 (strain **A**) and *C. albicans* ATCC MYA-1237 (strain **F**) (Table 2). Neither NEO nor compound **11** showed antifungal activity in these assays, confirming the necessity of the conjugation for activity. Overall, from these data, we established the following structure–activity relationship (SAR) for the NEO–monobenzimidazole derivatives **1–6** and NEO–bisbenzimidazole derivatives **7–10**. An oxygen atom in the linker connecting NEO and a monobenzimidazole (compound **3**) results in an inactive antifungal. However, the presence of an oxygen atom in the linker is not detrimental when NEO is connected to a bisbenzimidazole (compounds **8–10**), but its absence results in a compound devoid of activity (compound **7**). Changes in the linker length in the NEO–bisbenzimidazole compounds **8–10** are well tolerated and do not greatly affect antifungal activity, while those in the NEO–monobenzimidazole compounds **4–6** play an important role in dictating activity, with compounds **4** and **6** being inactive and compound **5** displaying good activity.

For the most active compounds in general, **8–10**, we also determined the minimum fungicidal concentration (MFC) values against yeast strains **A–J**. In all cases, the cell content from the 2× MIC well plated on potato dextrose agar (PDA) plates yielded 3 colonies, suggesting that the MFC values correspond to the 2× MIC values of compounds **8–10** (Table 3). FLC was also used as a control, but the majority of the yeast strains exhibited trailing growth effect at the tested concentrations. Thus, MFC values were not determined for FLC.

Effect of Serum on Antifungal Activity

Serum has been shown to have a direct impact on *in vitro* efficacy of some known antifungals (CAS and AmB) by reducing their activity against fungi, probably as a result of drug–serum binding.⁴³ The serum binding ability of these drugs is problematic as it compromises treatment of fungal diseases in humans. In this study, we examined the influence of 10% fetal bovine serum (FBS) on the antifungal activity of compounds **8–10** against three fungal strains: *C. albicans* ATCC 10231 (strain **A**), *C. parapsilosis* ATCC 22019 (strain **J**), and *A. nidulans* ATCC 38163 (strain **L**) (Table 4). The data collected demonstrated that the antifungal activity of compounds **8–10** was not affected by addition of FBS, suggesting that these molecules have little to no affinity for FBS, and therefore would

probably exist in a free and active form *in vivo* during antifungal therapy. The low binding affinity of the standard drug control VOR to serum that we observed was consistent with previously published data,⁴³ confirming the validity of our assay.

Time-Kill Assays

To determine if the NEO-bisbenzimidazoles are fungistatic or fungicidal, we performed time-kill assays over 24 h periods by using compound **8** as a model against *C. albicans* ATCC 10231 (strain **A**) (Figure 2). We found compound **8** to be fungicidal at a 1× MIC value against strain **A**. The control FDA-approved drug VOR displayed fungistatic activity against strain **A** at 1× and 2× MIC values. The fungicidal activity displayed by compound **8** compared to VOR points to its superiority as an antifungal agent.

Biofilm Assays

A biofilm is defined as a community of microorganisms that can attach to a surface by encasing themselves with the self-produced extracellular matrices.⁴⁴ *Candida* species are yeasts that are known to form biofilms. Biofilms complicate treatment of candidiasis as they increase resistance to various antifungal drugs.⁴⁵ This warrants the need for development of novel antifungal agents to prevent and treat biofilm-associated candidiasis. After analyzing the MIC values of NEO-bisbenzimidazole conjugates **1–10** against fungi (Table 1), we selected the three best compounds (**8–10**) to further evaluate their activity against sessile (biofilm) forms of two fungal strains, *C. albicans* ATCC 64124 (strain **B**) and *C. albicans* ATCC MYA-2876 (strain **C**) by the XTT reduction assay. The antifungal MICs of compounds **8–10** for sessile cells (SMIC₅₀ and SMIC₈₀) are shown in Table 5 (and the plates themselves are provided in Figure S1). The results showed that SMIC₅₀ and SMIC₈₀ values for compounds **8–10** against biofilms of *C. albicans* ATCC 64124 (strain **B**) were 3.9 and 7.8 µg/mL, respectively, with the exception of the SMIC₈₀ value for compound **10** against strain **B**, which was 15.6 µg/mL. Similarly, SMIC₅₀ and SMIC₈₀ values for compounds **8–10** against biofilms of *C. albicans* ATCC MYA-2876 (strain **C**) were 7.8 and 15.6 µg/mL, respectively. For the reference drug VOR, we observed SMIC₅₀ and SMIC₈₀ values to be 15.6 and 31.3 µg/mL against *C. albicans* ATCC 64124 (strain **B**) biofilms. Likewise, we found SMIC₅₀ and SMIC₈₀ values of 7.8 and 31.3 µg/mL against *C. albicans* ATCC MYA-2876 (strain **C**) biofilms. In brief, we observed a 1 to 2-fold increase in SMIC₅₀ values or a 4-fold increase in SMIC₈₀ values for compounds **8–10** when compared to the planktonic MIC values of *C. albicans* ATCC 64124 (strain **B**). Likewise, we observed either a 2- to 4-fold increase in SMIC₅₀ values or a 4-to 8-fold increase in SMIC₈₀ values for compounds **8–10** compared to the planktonic MIC values of *C. albicans* ATCC MYA-2876 (strain **C**). It is very encouraging to observe that our compounds **8–10** exhibited a remarkable inhibitory effect against *C. albicans* (strains **B** and **C**) biofilms, which is either superior or comparable to the control drug VOR.

Cytotoxicity Assay

Being eukaryotic cells, fungi share most of the cellular components and biochemical features with their mammalian cell counterparts. Consequently, the drugs that are designed to target fungi could potentially also cause side effects on mammalian cells. To determine

the selectivity of our NEO–mono/bisbenzimidazole derivatives toward fungal cells, we tested compounds **2**, **5**, and **7–10** for their toxicity against two mammalian cell lines, A549 (Figure 3A) and BEAS-2B (Figure 3B). As a comparator, we also used the clinical antifungal agent Amb. In general, we did not observe toxicity by NEO–monobenzimidazoles derivatives **2** and **5** against A549 and BEAS-2B. Therefore, compounds **2** and **5** could be used for treating non-*albicans Candida* infections as no mammalian toxicity is observed at concentrations that are at least 2- to 16-fold higher than their respective antifungal MIC values against non-*albicans Candida* strains **H**, **I**, and **J** (Table 1). For the NEO–bisbenzimidazole derivatives, no toxicity was observed for compounds **7–10** at a concentration of 31.3 $\mu\text{g/mL}$ against A549 (Figure 3A). When tested against BEAS-2B at 31.3 $\mu\text{g/mL}$, compounds **7** and **10** were also found to be nontoxic. By a quick glance at the bar graph (at 31.3 $\mu\text{g/mL}$) presented in Figure 3B, one could come to the erroneous conclusion that compounds **8** and **9** are too toxic to be useful. However, it is important to note that the antifungal MIC values for these compounds are very low (in general $<1.95 \mu\text{g/mL}$ against most strains tested). Therefore, the fact that compounds **8** and **9** are nontoxic at 15.6 $\mu\text{g/mL}$, which is $\sim 10\times$ higher than the antifungal MIC values of these molecules, is highly promising. Importantly, our best overall compounds **8–10** also showed either a superior or comparable toxicity profile to that of the reference antifungal drug Amb. These results further confirmed that compounds **8–10** show promise as potential candidates for the development of antifungal agents. Cytotoxicity (EC_{50}) values of compounds **8–10** against A549 and BEAS-2B cell lines are presented in Table S1.

After determining the EC_{50} values of compounds **8–10** against mammalian cell lines, we calculated the selectivity index (SI) values of these compounds against all the fungal strains (Table S2). The SI for a compound is defined as the ratio of its EC_{50} value against a mammalian cell line (e.g., A549 or BEAS-2B) to its MIC value against a specific fungal strain. Compounds **8–10** were highly selective against *C. albicans* and non-*albicans Candida* with SI values ranging from 8 to 260. However, against *Aspergillus* strains, compounds **8–10** were less specific with SI values ranging from 0.8 to 16. These SI values for the compounds suggest better a safety profile for treating infections caused by *C. albicans* and non-*albicans Candida*. In addition to SI values, we also calculated LogP values (Table S2) for compounds **8–10**. The LogP values were found to be ideal, and the presence of the aminoglycoside NEO on these hybrids rendered them highly water-soluble.

Hemolysis Assay

Since compounds **8–10** showed potent antifungal activities and limited toxicity, we further investigated their hemolytic activity against mouse red blood cells (mRBCs) to determine the selectivity of our compounds **8–10** toward fungal cells. Overall, compounds **8–10** displayed little to no hemolysis of mRBCs at least at up to 15.6 $\mu\text{g/mL}$ (Figure 4 and Table S3). Compound **8** lysed 49% of mRBCs at 31.3 $\mu\text{g/mL}$, a concentration that is 8- to 64-fold higher than its antifungal MIC values (Table 1). Likewise, at 62.5 $\mu\text{g/mL}$, compounds **9** and **10** only lysed 35% and 18% of mRBCs, respectively. The lack of hemolytic activity of compounds **8–10** combined with fact that the FDA-approved VOR (standard drug control) caused 83% and 100% lysis of mRBCs at 31.3 and 62.5 $\mu\text{g/mL}$, respectively, is very

exciting. The 50 percent hemolytic concentration (HC₅₀) values of compounds **8–10** are presented in Table S4.

Sterol Composition

As a benzimidazole derivative, EMC120B12, has been shown to inhibit ergosterol formation,⁴⁶ we decided to verify if our compounds **8–10** could also inhibit the fungal ergosterol biosynthetic pathway. To investigate the mechanism of action of NEO–bisbenzimidazoles, we selected one of the best compounds, **8**, and evaluated its effect on sterol composition of *C. albicans* ATCC 10231 (strain **A**) at the sub-MIC levels of 0.975 $\mu\text{g/mL}$, by using gas chromatography– mass spectrometry (GC-MS) (Figure 5). VOR and no drug control were used as a positive and a negative control, respectively. On the basis of our sterol profile results (Figure 5E), we observed close to complete accumulation of ergosterol in strain **A** in the absence of azole as well as in the presence of compound **8**, suggesting that the ergosterol biosynthesis in strain **A** was fully functional in both of the cases. However, with VOR-treated (0.125 $\mu\text{g/mL}$) cells, we saw a reduction in the amount of ergosterol (64.93%) with a concomitant increase in the amount of lanosterol (18.49%), eburicol (2.14%), and the fungistatic metabolite 14 α -methyl ergosta-8,24,(28)-dien-3 β ,6 α -diol (10.05%), as well as unknown sterols **5** (2.79%) and **6** (1.56%). Unlike VOR and EMC120B12, compound **8** appears not to inhibit or disrupt the ergosterol biosynthetic pathway in fungi, suggesting a different antifungal mode of action.

Reactive Oxygen Species Assay

As we found that our NEO–bisbenzimidazole derivatives did not inhibit the ergosterol biosynthetic pathway in fungi, we next turned our attention to see if these compounds could be capable of inducing reactive oxygen species (ROS) in yeast cells. Certain antifungals (e.g., CAS) have been shown to kill *C. albicans* by inducing ROS.⁴⁷ Similarly, we have also previously demonstrated that benzimidazoles analogues are capable of inducing ROS in yeast cells.³⁴ On the basis of this knowledge, in this study, we explored the ability of compound **8** to induce ROS in *C. albicans* ATCC 10231 (strain **A**) by using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye.⁴⁸ All eukaryotic cells are known to produce a basal amount of ROS as a byproduct during cellular metabolism. Any excess accumulation of ROS in cells is detrimental. When ROS is in excess, cells activate their antioxidant functions to neutralize them. We did not observe induction of ROS in *C. albicans* ATCC 10231 (strain **A**) when yeast cells were treated with 0.5 $\times$ MIC of compound **8**. However, when treated at 1 $\times$ and 2 $\times$ MIC values of compound **8**, we saw an increase in the production of ROS in cells in a dose-dependent manner (Figure 6). Likewise, the control drug CAS also displayed a dose-dependent effect in the production of ROS when used at 0.5 $\times$, 1 $\times$, and 2 $\times$ of its MIC value. No ROS production was observed for untreated yeast cells. It is likely that an excessive amount of ROS accumulation may cause oxidative stress in yeast cells treated with compound **8**, which would result in cells undergoing apoptosis. However, it is not very clear that the induction of ROS by compound **8** is the primary cause of cell death. It is also possible that this NEO–bisbenzimidazole may act like the traditional benzimidazoles (e.g., nodoconazole) by inhibiting fungal microtubules assembly by affecting the fungal cell division,⁴⁹ which is outside of the scope of this study.

CONCLUSION

In summary, we determined the antifungal activity of six NEO–monobenzimidazole (**1–6**) and four NEO–bisbenzimidazole derivatives (**7–10**) against a panel of *Candida albicans*, non-*albicans Candida*, and *Aspergillus* strains. In this study, we demonstrated the broad-spectrum antifungal activity of NEO–bisbenzimidazole compounds **8–10** against yeasts and filamentous fungi. The antifungal efficacy of compounds **8–10** against yeast was not affected by the presence of FBS. More importantly, these compounds also displayed good activity against *C. albicans* biofilms. Besides, they were found to not be hemolytic to mRBCs and they displayed little to no toxicity to mammalian cells. Finally, our preliminary work on the mechanism of action showed that the antifungal activity of compound **8** might be due to induction of ROS but not interference with the fungal ergosterol biosynthetic pathway. In sum, the NEO–bisbenzimidazoles compounds **8–10** appear attractive as new antifungal candidates that could possibly help treat fungal infections in humans. In the future, characterization in mammalian models of infection will be warranted to validate the efficacy of these compounds. These experiments are out of scope for the current study.

EXPERIMENTAL SECTION

Materials

We previously published the synthesis and characterization of compounds **1–11** tested herein.^{39,41,42} The identity of the compounds **1–11** was determined by ¹H and ¹³C NMR as well as by mass spectrometry. The purity of compounds **1–6** was herein confirmed to be 96% by RP-HPLC (Figures S2–S7). RP-HPLC for compounds **1–6** was performed on an Agilent Technologies 1260 Infinity HPLC system by using the following general method 1: Flow rate = 0.8 mL/min; λ = 254 nm; column = Vydac 238DE54 C18, 250 × 4.6 mm, 120 Å, 5 μ m; eluents: A = H₂O + 0.1% TFA, B = MeCN; gradient profile: 1% B for 20 min, increasing from 1% B to 100% B over 5 min, holding at 100% B for 2 min, decreasing from 100% B to 1% B over 3 min. Prior to each injection, the HPLC column was equilibrated for 15 min with 1% B. The purity of compounds **7–11** was previously reported to be >95% by RP-HPLC.^{39,42} In previous publications,^{34,39,41,42} we also referred to these molecules as **1** (DPA 116), **2** (DPA 118), **3** (DPA 119), **4** (DPA 120), **5** (DPA 121), **6** (DPA 122), **7** (DPA 167), **8** (DPA 168), **9** (DPA 169), **10** (DPA 170), and **11** (DPA 153 or SGT 249). All the chemicals used in this study for synthesis or testing (e.g., neomycin B (NEO) that we used as a control) were purchased from Sigma-Aldrich (St. Louis, MO) or AK Scientific (Union City, CA) and used without any further purification. All purchased compounds were all 95% as per the suppliers.

Antifungal Agents

A 5 mg/mL stock solution of compounds **1–11** was prepared in sterile Milli-Q H₂O and stored at –20 °C. The antifungal agents amphotericin B (AmB), fluconazole (FLC), and voriconazole (VOR) were obtained from AK Scientific Inc. (Mountain View, CA, USA). The antifungal agent caspofungin (CAS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AmB, FLC, VOR, and CAS were dissolved in DMSO at final concentrations of 5 mg/mL and were stored at –20 °C.

Organisms and Culture Conditions

Candida albicans ATCC 10231 (**A**), *C. albicans* ATCC 64124 (**B**), and *C. albicans* ATCC MYA-2876 (**C**) were kindly provided by Dr. Jon Y. Takemoto (Utah State University, Logan, UT, USA). *C. albicans* ATCC MYA-90819 (**D**), *C. albicans* ATCC MYA-2310 (**E**), *C. albicans* ATCC MYA-1237 (**F**), *C. albicans* ATCC MYA-1003 (**G**), *Candida glabrata* ATCC 2001 (**H**), *Candida krusei* ATCC 6258 (**I**), *Candida parapsilosis* ATCC 22019 (**J**), *Aspergillus flavus* ATCC MYA-3631 (**K**), and *Aspergillus terreus* ATCC MYA-3633 (**M**) were obtained from the American Type Culture Collection (Manassas, VA, USA). *Aspergillus nidulans* ATCC 38163 (**L**) was received from Dr. Jon S. Thorson (University of Kentucky, Lexington, KY, USA). Filamentous fungi and yeasts were cultivated at 35 °C in RPMI 1640 medium (with L-glutamine, without sodium bicarbonate, Sigma-Aldrich) buffered to a pH of 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich).

In Vitro Antifungal Susceptibility Testing

The MIC values of compounds **1–10** against yeast cells were determined in 96-well plates as described in the CLSI document M27-A3 with minor modifications.⁵⁰ A single colony was used to inoculate 5 mL of yeast extract peptone dextrose broth (YPD) and incubated overnight with shaking at 200 rpm at 35 °C. The overnight culture was further diluted to achieve $2\text{--}4 \times 10^3$ CFU/mL in RPMI 1640 medium by measuring the optical density of cells at 600 nm. In the meantime, 2-fold serial dilutions of compounds **1–10**, AmB, CAS, FLC, and VOR were prepared in RPMI 1640 to yield twice the final concentration required for testing followed by the addition of 100 μ L of cell suspension to each well of the 96-well plates. This resulted in final concentrations of 0.06–31.3 μ g/mL for compounds **1–10**, 0.48–31.3 μ g/mL for AmB, 0.03–31.3 μ g/mL for CAS, 0.975–62.5 μ g/mL for FLC, and 0.03–31.3 μ g/mL for VOR. The growth control (no drugs) and negative control (no cells) were also added in the same MIC assay for comparison and incubated at 35 °C for 48 h. Each test was performed in duplicate. The final concentration of DMSO was ensured to be <1% in all experiments. The MIC values for compounds **1–10**, AmB, and CAS were defined as the minimum drug concentration that yielded optically clear well or MIC-0. For FLC and VOR, we used MIC-2 values, which are defined as the minimum drug concentration that yielded at least 50% growth inhibition with respect to the growth control as these compounds show trailing growth effects. One exception for VOR was that against *C. albicans* ATCC 10231 (strain **A**) for which we reported MIC-0 (optically clear well). These MIC values are presented in Table 1.

Similarly, the MIC values of compounds **1–10** and all control drugs against filamentous fungi (strains **K**, **L**, and **M**) were determined as previously described in CLSI document M38-A2.⁵¹ Spores were harvested from sporulating cultures growing in potato dextrose agar (PDA) by filtration through sterile glass wool and counted by using a hemocytometer to obtain the desired inoculum size. 2-fold serial dilutions of compounds **1–10**, CAS, and VOR were made in sterile 96-well microplates in the range of 0.03–31.3 μ g/mL, except for AmB (0.48–31.3 μ g/mL) and FLC (0.975–62.5 μ g/mL), using RPMI medium, and spore suspensions were added to make a final concentration of $1\text{--}5 \times 10^5$ CFU/mL. The plates were incubated at 35 °C for 72 h. The MIC values of compounds **1–10**, azoles, AmB, and

CAS against filamentous fungi were based on complete growth inhibition with respect to the growth control, also referred to as MIC-0. Each test was performed in duplicate. These MIC values are also presented in Table 1.

The minimal fungicidal concentration (MFC) values for compounds **8–10** were also determined against yeast cells as previously described with minor modification.⁵² Briefly, MIC assays for compounds **8–10** were performed against yeast cells as described above. After 48 h of incubation, 20 μ L aliquots from 1 $\times$ MIC, 2 $\times$ MIC, and 4 $\times$ MIC wells were homogenized with a micropipette and the cell contents were spread on PDA plates, which were incubated for 24–48 h at 35 °C for colony counts. The MFC was defined as the lowest drug concentration from which 3 colonies were visible on the PDA plates.⁵² Each test was performed in duplicate. The MFC values are shown in Table 3.

After seeing promising antifungal activity for some of the NEO–bisbenzimidazole conjugates (compounds **8–10**), we wondered if noncovalently attached compound **11** and NEO could also show synergy when used in a 1:1 mixture. To test this hypothesis, we did a checkerboard assay using compound **11** and NEO against two strains of *C. albicans* (strains **A** and **F**), as previously described. NEO was serially diluted (2-fold dilutions) in the 96-well plates, while compound **11** was double-diluted in tubes outside of the 96-well plates and then later added into the plates using a multichannel pipet. The concentration of NEO varied horizontally while that of compound **11** varied vertically. The appropriate range of concentrations for each compound (0.25–32 μ g/mL for NEO and 0.5–32 μ g/mL for **11**) was determined on the basis of their corresponding MIC values against each fungal strain. The inoculum sizes for yeast cells were the same as in the MIC experiments described in Time-Kill Assays. The 96-well plates were incubated at 35 °C for 48 h for yeasts before visual inspection for growth. The observed MIC values for NEO and compound **11** alone as well as the MIC values for the two compounds in combo were then used to calculate the fractional inhibitory concentration index (FICI). The interaction would be defined as synergistic if the FICI was ≤ 0.5 , indifferent if >0.5 to 4, and antagonistic if >4 . The MIC and FICI values for these combination studies are presented in Table 2.

Determination of *in Vitro* Serum MIC Values

On the basis of the antifungal efficacy of compounds **1–10** against fungal strains as shown in Table 1, we selected three potent compounds, **8–10**, and determined their MIC values against three fungal strains, *C. albicans* ATCC 10231 (strain **A**), *C. parapsilosis* ATCC 22019 (strain **J**), and *A. nidulans* ATCC 38163 (strain **L**), in the presence or absence of 10% fetal bovine serum (FBS) in a manner similar to that described in Time-Kill Assays. VOR served as a reference drug control; the medium without drugs was used as untreated cell control, and the medium alone was the blank control. The plates were then incubated at 35 °C for 48 h, and MIC end points were determined on the basis of complete inhibition of growth (MIC-0) with respect to growth control. These serum MIC values are presented in Table 4.

Time-Kill Assays

A representative time-kill study was performed by selecting one of the best compounds, **8**, against a representative strain, *C. albicans* ATCC 10231 (strain **A**), as described previously.⁵³ An overnight culture of the yeast cell was used to inoculate 5 mL of RPMI 1640 broth (2×10^5 CFU/mL) and was incubated at 37 °C in the presence of 0.5× and 1× of the MIC of compound **8** and 1× and 2× of the MIC of VOR. Sterility control and no drug control were also included for the same sets of experiments. At 0, 3, 6, 12, and 24 h intervals, 100 μ L aliquots were removed from each solution and serially diluted in sterile ddH₂O. 100 μ L of each dilution was spread onto potato dextrose agar (PDA) plates and incubated at 35 °C. Colony counts were determined after 48 h of incubation. The experiment was performed in duplicate. The time-kill curves are presented in Figure 2.

Biofilm Assays

The effect of compounds **8–10** was previously examined on preformed *C. albicans* ATCC MYA-2876 (strain **C**) and *C. albicans* ATCC 64124 (strain **B**) biofilm.^{50,54} A quantitative measurement of the metabolic activity of biofilm was acquired by using a XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay.⁵⁴ Briefly, *C. albicans* ATCC MYA-2876 (strain **C**) and *C. albicans* ATCC 64124 (strain **B**) were grown overnight in YPD broth at 35 °C. The culture was then diluted to an OD₆₀₀ of 0.12 (equivalent to 1×10^6 CFU/mL), and 100 μ L was placed into each well of a 96-well plate. The cells were incubated at 37 °C for 24 h for growth. The following day, the spent medium containing planktonic cells was aspirated and each well was carefully washed three times with sterile phosphate buffer saline (PBS). RPMI 1640 medium containing a 2-fold serial dilution of compounds **8–10** (0.24–125 μ g/mL) was added to the selected wells containing the biofilm. VOR (0.06–31.3 μ g/mL) served as a reference drug control; the medium without drug was used as untreated cell control, and the medium alone was the blank control. The plates were then incubated at 37 °C statically for an additional 24 h and then washed with sterile PBS. 100 μ L of a XTT (0.5 mg/mL)/menadione (1 μ M) solution was added to each well. The plates were covered with aluminum foil and incubated for 2 h at 37 °C. Then, 80 μ L of the colored supernatant from each well was transferred to a new 96-well plate, and the absorption was recorded at 490 nm. The percent metabolic activity of the formed biofilm at various drug concentration combinations was calculated by dividing the metabolic activity of biofilm formed for that well by that of the biofilm formed in the growth control well (in the absence of any drug). For these experiments, we determined the sessile MIC values, SMIC₅₀ and SMIC₈₀, which are defined as the drug concentration required to inhibit the metabolic activity of biofilm by 50% and 80%, respectively, as compared to the growth control. The assay for each combination was performed in duplicate. The data for the biofilm assays are presented in Table 5 and Figure S1.

In Vitro Cytotoxicity Assays

Cytotoxicity assays were performed as previously described.⁵⁵ The human lung carcinoma epithelial cells A549 and the normal human bronchial epithelial cells BEAS-2B were grown in F12-K and DMEM media containing 10% fetal bovine serum (FBS) and 1% antibiotics, respectively. The confluent cells were then trypsinized with 0.05% trypsin–0.53 mM EDTA,

centrifuged (1200 rpm) at room temperature, and resuspended in fresh medium (F12-K or DMEM). The cells were seeded into 96-well microtiter plates at a density of 3000 cells/well and were grown overnight. The following day, the media were replaced by 100 μ L of fresh culture media containing serially diluted compounds **2**, **5**, **7**, and **8–10**, as well as a reference compound, AmB (at final concentrations of 0.24–31.3 μ g/mL), or no drug control. The cells were incubated for an additional 24 h at 37 °C with 5% CO₂ in a humidified incubator. Each well was treated with 10 μ L (25 μ g/mL) of resazurin sodium salt (Sigma-Aldrich) for 3–6 h to determine cell viability. Metabolically active cells can convert the blue nonfluorescent dye resazurin to the pink and highly fluorescent dye resorufin, which can be detected at λ_{560} excitation and λ_{590} emission wavelengths by using a SpectraMax M5 plate reader. Triton X-100 (0.5%, v/v) gave complete loss of cell viability and was used as a positive control. The percent cell survival was calculated as (test value/control value) $\times$ 100, where control value represents cells + resazurin – drug, and test value represents cells + resazurin + drug. These data are presented in Figure 3. The cytotoxicity (EC₅₀) values of compounds **8–10** against A549 and BEAS-2B cell lines are presented in Table S1. The selectivity index (SI) was also calculated for compounds **8–10** to assess the cell selectivity of our compounds against fungal pathogens using the formula as SI = EC₅₀/MIC (Table S2). Note: The EC₅₀ values against both the A549 and BEAS-2B mammalian cell lines were utilized to calculate SI values (Table S2).

Hemolytic Activity Assays

Hemolytic activity was determined by using previously described methods with minor modifications.^{25,26} Murine red blood cells (mRBCs) (1 mL) were suspended in 3 mL of PBS and centrifuged at 1000 rpm for 5 min to obtain the mRBCs. The mRBCs were washed four times in PBS and resuspended in the same buffer to a final concentration of 10⁷ erythrocytes/mL. 2-fold serial dilution of compounds **8–10** were prepared in Eppendorf tubes containing 100 μ L of PBS buffer, and 100 μ L of mRBC suspension was added to achieve a final concentration of compounds ranging from 0.48 to 62.5 μ g/mL and 5 $\times$ 10⁶ erythrocytes/mL of mRBCs. The tubes were incubated at 37 °C for 60 min. Tubes with PBS buffer (200 μ L) and Triton X-100 (1% v/v, 2 μ L) served as negative (blank) and positive controls, respectively. The percentage of hemolysis was calculated using the following equation: % hemolysis = [(absorbance of sample) – (absorbance of blank)] $\times$ 100/ (absorbance of positive control). These data are presented in Figure 4 and Table S3. The 50 percent emolytic concentration (HC₅₀) values of compounds **8–10** are displayed in Table S4.

Determination of Sterol Composition in *C. albicans* Pre- and Post-Treatment

A single colony of *C. albicans* ATCC 10231 (strain **A**) was picked from a fresh culture plate to inoculate 3 mL of YPD broth, which was then incubated at 35 °C for ~18 h with continuous agitation (180 rpm). The overnight yeast culture was used to inoculate RPMI 1640 medium (15 mL), and the final inoculum concentration was adjusted to 1 $\times$ 10⁶ CFU/mL (~OD₆₀₀ = 0.12) using a spectrophotometric method. Afterward, the yeast cells were treated with compound **8** (1.95 μ g/mL) or VOR (0.12 μ g/mL) at their sub-MIC values. An equivalent amount of DMSO without drug (untreated control) was also prepared. The cells were harvested by centrifugation (5000 rpm) for 10 min at room temperature, and the cell pellets were saponified at 80 °C for 2 h with 3 mL of MeOH, 2 mL of pyrogallol

dissolved in MeOH (0.5%, wt/v) (CAS # 87-66-1, Sigma-Aldrich Chemical Co., St. Louis, Mo.), and 2 mL of potassium hydroxide (60%, wt/v). The nonsaponifiable sterols were then extracted three times with 5 mL of heptane. The extracts were evaporated under a stream of nitrogen to dryness and resuspended in 500 μ L of heptane. The sterol suspension was then transferred to a GC-MS vial and derivatized with 250 μ L of N-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS # 24589-78-4, Sigma-Aldrich Chemical Co., St. Louis, Mo.) at 70 °C for 20 min. GC-MS analyses were performed on an Agilent 7890A gas chromatograph with splitless injection, coupled to an Agilent 5970C inert XL mass spectrometer with a triple-axis detector and an Agilent 19091S-433 capillary column (30 m $\times$ 250 μ m). The oven temperature was programmed to hold at 70 °C for 2 min and then ramped to 270 °C at a rate of 20 °C/min. Helium (10 psi) was used as the carrier gas; the electron ionization energy was 70 eV, and the inlet temperature was 250 °C. Identification of sterols was achieved using the NIST (the National Institute of Standards and Technology) reference database. These data are presented in Figure 5.

ROS Assay

The cell permeable dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to measure ROS production in the fungal cells upon treatment with our compounds. At first, cellular esterases hydrolyze DCFH-DA to the nonfluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which can further be oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by the intracellular ROS. Five mL of RPMI 1640 medium was inoculated using a single colony of *C. albicans* ATCC 10231 (strain A) in a Falcon tube and grown overnight at 35 °C at 200 rpm. The overnight culture was diluted by adding 200 μ L of yeast cells to 800 μ L of the same medium. The cell suspension (100 μ L) was then added to the RPMI 1640 medium containing no drug (negative control), compound 8, or CAS at their 0.5 $\times$, 1 $\times$, and 2 $\times$ respective MIC values and treated for 1 h at 35 °C. Cells were centrifuged and washed twice with PBS (pH 7.2) buffer. Cells were resuspended in the same buffer and incubated with DCFH-DA (20 μ g/mL) for 30 min in the dark. Afterward, cells were centrifuged and washed with PBS buffer to remove excess DCFH-DA. Glass slides with 10–15 μ L of each mixture were prepared and observed in bright field and fluorescence modes (FITC filter set, excitation and emission wavelengths of 488 and 512 nm, respectively) using a Zeiss Axovert 200 M fluorescence microscope. These data are presented in Figure 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AmB amphotericin B

CAS	caspofungin
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
FBS	fetal bovine serum
FICI	fractional inhibitory concentration index
FLC	fluconazole
MFC	minimum fungicidal concentration
MIC	minimum inhibitory concentration
mRBCs	mouse red blood cells
NEO	neomycin B
PDA	potato dextrose agar
ROS	reactive oxygen species
SAR	structure-activity relationship
VOR	voriconazole

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Figure 1.
Structures of NEO-monobenzimidazoles **1–6** and NEO-bisbenzimidazole **7–10** used in this study along with that of bisbenzimidazole derivative **11** used for the combination study.

Figure 2.

Representative time-kill studies of NEO-bisbenzimidazole compound **8** against *C. albicans* ATCC 10231 (strain A). Cultures were exposed to compound **8** at 0.5× (○) and 1× (▼) MICs, VOR at 1× (△) and 2× (■) MICs, and no drug control (●).

Figure 3. Mammalian cell cytotoxicity of NEO-mono/bisbenzimidazole conjugates against (A) A549 and (B) BEAS-2B cell lines.

Figure 4.

3D bar graph depicting the dose-dependent hemolytic activity of NEO-bisbenzimidazoles conjugates **8–10** against mRBCs. mRBCs were treated and incubated for 1 h at 37 °C with compounds **8–10** and VOR at concentrations ranging from 0.48 to 62.5 $\mu\text{g/mL}$. Triton X-100 (1% v/v) was used as a positive control (100% hemolysis, not shown).

A. Simplified ergosterol biosynthetic pathway and products resulting from inhibition of *ERG11*: **E. Sterol composition of untreated as well as VOR- and cpd 8-treated *C. albicans* ATCC 10231:**

Sterol	Sterol composition (%)		
	No drug	VOR	Cpd 8
Lanosterol (1)	ND	18.49	ND
Ergosterol (2)	100	64.93	98.49
Eburicol (3)	ND	2.14	ND
14 α -methyl ergosta-8,24(28)-dien-3 β ,6 α -diol (4)	ND	10.05	1.50
Unknown sterol (5)	ND	2.79	ND
Unknown sterol (6)	ND	1.56	ND

ND = not detected

Figure 5.

(A) A simplified ergosterol biosynthetic pathway and products resulting from inhibition of *ERG11*. (B–D) GC-MS chromatograms of the sterols extracted from untreated and antifungal-treated *C. albicans* ATCC 10231 (strain A), which was treated with DMSO (no drug) as a control (panel B) or VOR as a positive control (panel C) at 0.125 $\mu\text{g/mL}$, and compound **8** (panel D) at 1.95 $\mu\text{g/mL}$. The peaks are for lanosterol (**1**), ergosterol (**2**), eburicol (**3**), 14 α -methyl ergosta-8,24(28)-dien-3 β ,6 α -diol (**4**), and unknown sterols (**5** and **6**). (E) A table summarizing the percentage of each sterol from panels B–D.

Figure 6.

Effect of NEO-bisbenzimidazole **8** and CAS on intracellular ROS production by *C. albicans* ATCC 10231 (strain A). Yeast cells were treated with no drug (negative control), compound **8**, or CAS (positive control) at their 0.5 $\times$, 1 $\times$, and 2 $\times$ respective MIC values for 1 h at 35 °C. After staining with DCFH-DA (40 μ g/mL), the samples were analyzed using a Zeiss Axiovert 200 M fluorescence microscope.

MIC Values (in $\mu\text{g/mL}$) Determined for Compounds 1–10 and for Four Control Antifungal Agents (AmB, CAS, FLC, and VOR) against Various Yeast Strains and Filamentous Fungi^a

cpd #	yeast strains										filamentous fungi			
	A	B	C	D	E	F	G	H	I	J	K	L	M	
1	15.6	15.6	15.6	15.6	15.6	15.6	15.6	7.8	7.8	3.9	>31.3	>31.3	31.3	
2	15.6	>31.3	31.3	>31.3	>31.3	>31.3	31.3	3.9	3.9	1.95	>31.3	31.3	31.3	
3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	
4	31.3	31.3	>31.3	31.3	>31.3	>31.3	>31.3	31.3	31.3	31.3	>31.3	>31.3	31.3	
5	7.8	7.8	15.6	15.6	15.6	31.3	15.6	7.8	7.8	3.9	>31.3	31.3	31.3	
6	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3	>31.3	31.3	>31.3	31.3	31.3	
7	15.6	31.3	31.3	31.3	31.3	31.3	15.6	31.3	31.3	15.6	>31.3	31.3	31.3	
8	1.95	1.95	1.95	1.95	1.95	1.95	1.95	0.975	0.975	0.12	>31.3	1.95	1.95	
9	1.95	1.95	1.95	1.95	1.95	1.95	1.95	0.975	1.95	0.12	>31.3	3.9	1.95	
10	1.95	3.9	3.9	3.9	3.9	1.95	3.9	1.95	1.95	0.48	>31.3	1.95	1.95	
AmB	3.9	3.9	1.95	0.975	1.95	3.9	3.9	1.95	3.9	1.95	15.6	15.6	3.9	
CAS	0.975	0.24	0.06	0.12	0.12	0.24	0.48	0.06	0.48	1.95	>31.3	>31.3	>31.3	
FLC	62.5	>125	15.6	>125	>125	62.5	62.5	>31.3	>31.3	1.95	62.5	62.5	62.5	
VOR	0.24	3.9	1.95	1.95	0.975	7.8	1.95	0.06	0.12	0.03	0.24	0.12	0.12	

^aYeast strains: **A** = *C. albicans* ATCC 10231, **B** = *C. albicans* ATCC 64124, **C** = *C. albicans* ATCC 90819(R), **E** = *C. albicans* ATCC MYA-2310(S), **F** = *C. albicans* ATCC MYA-1237(R), **G** = *C. albicans* ATCC MYA-1003(R), **H** = *C. glabrata* ATCC 2001, **I** = *C. krusei* ATCC 6258, and **J** = *C. parapsilosis* ATCC 22019. NOTE: Here, the (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to itraconazole (ITC) and FLC. Filamentous fungi: **K** = *Aspergillus flavus* ATCC MYA-3631, **L** = *Aspergillus nidulans* ATCC 38163, and **M** = *A. terreus* ATCC MYA-3633. Known antifungal agents: AmB = amphotericin B, CAS = caspofungin, FLC = fluconazole, and VOR = voriconazole. For yeast strains, MIC-0 values are reported for compounds 1–10 as well as AmB and CAS, whereas MIC-2 values are reported for azoles. For filamentous fungi, MIC-0 values are reported for all compounds.

Table 2
In Vitro Susceptibility of Two Yeast Strains to NEO and Compound 11 Alone and in Combination^a

yeast ATCC strains	MICs of drugs ($\mu\text{g/mL}$)					FICI	interpretation
	alone		in combination				
	NEO	11	NEO	11			
<i>C. albicans</i> ATCC 10231 (A)	>32	>32	>32	>32	>32	2	IND
<i>C. albicans</i> ATCC MYA-1237 (F)	>32	>32	>32	>32	>32	2	IND

^aFICI = fractional inhibitory concentration index. Note: IND indicates indifferent (FICI > 0.5–4).

Table 3

Minimal Fungicidal Concentration (MFC) Values (in $\mu\text{g/mL}$) Determined for Compounds 8–10 against Various Yeast Strains

strain	8	9	10
<i>C. albicans</i> ATCC 10231 (A)	3.9	3.9	3.9
<i>C. albicans</i> ATCC 64124 (B)	3.9	3.9	7.8
<i>C. albicans</i> ATCC MYA-2876(S) (C)	3.9	3.9	7.8
<i>C. albicans</i> ATCC 90819(R) (D)	3.9	3.9	7.8
<i>C. albicans</i> ATCC MYA-2310(S) (E)	3.9	3.9	7.8
<i>C. albicans</i> ATCC MYA-1237(R) (F)	3.9	3.9	3.9
<i>C. albicans</i> ATCC MYA-1003(R) (G)	3.9	3.9	7.8
<i>C. glabrata</i> ATCC 2001 (H)	1.95	1.95	3.9
<i>C. krusei</i> ATCC 6258 (I)	1.95	3.9	3.9
<i>C. parapsilosis</i> ATCC 22019 (J)	0.24	0.24	0.975

Table 4

MIC Values (in $\mu\text{g/mL}$) Determined for Compounds 8–10 and for One Control Antifungal Agent VOR against Various Yeast Strains and Filamentous Fungi

cpd #	yeast strains				filamentous fungi		
	<i>Candida albicans</i> ATCC 10231 (A) FBS)	<i>Candida albicans</i> ATCC 10231 (A) (+10% FBS)	<i>Candida parapsilosis</i> ATCC 22019 (J) FBS)	<i>Candida parapsilosis</i> ATCC 22019 (J) (+10% FBS)	<i>Aspergillus nidulans</i> ATCC 38163 (L) FBS)	<i>Aspergillus nidulans</i> ATCC 38163 (L) (no FBS)	<i>Aspergillus nidulans</i> ATCC 38163 (L) (+10% FBS)
8	1.95	1.95	0.12	0.24	1.95		1.95
9	1.95	1.95	0.12	0.24	3.9		3.9
10	1.95	3.9	0.48	0.975	1.95		1.95
VOR	0.48	0.48	0.015	0.015	0.12		0.24

Table 5Antibiofilm Activity of Compounds 8–10 against Two Strains of *C. albicans* by the XTT Assay^a

cpd #	<i>C. albicans</i> ATCC 64124 (B)		<i>C. albicans</i> ATCC MYA-2876 (C)	
	SMIC ₅₀ (μg/mL)	SMIC ₈₀ (μg/mL)	SMIC ₅₀ (μg/mL)	SMIC ₈₀ (μg/mL)
8	3.9	7.8	7.8	15.6
9	3.9	7.8	7.8	15.6
10	3.9	15.6	7.8	15.6
VOR	15.6	31.3	7.8	31.3

^aSMIC₅₀ = sessile minimum inhibitory concentration that reduced the metabolic activity of biofilm by 50%. SMIC₈₀ = sessile minimum inhibitory concentration that reduced the metabolic activity of biofilm by 80%.