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Design and Synthesis of a Class of Compounds That Inhibit the Growth of Fungi Which Cause Invasive Infections

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aminohydrazone derivative
antifungal compounds
Candida species
high-throughput screening
invasive fungal infections

The compound 1-[(*E*)-[4-(3',4'-dichlorobenxyloxy)phenyl methylidene]amino]-guanidine belongs to a novel class of antifungals and its fungicidal activities against *Candida albicans* are equivalent or superior to those of conventional drugs on a weight-per-volume basis. In addition, the newly synthesized compound inhibited the growth of *Candida auris* NCPF 8985, a multidrug-resistant strain of *Candida* species.

Invasive fungal infections are growing causes of morbidity and mortality in immunocompromised patients. However, only one antifungal drug class has been developed in the last 30^^years, extremely limiting current therapeutic options. To address unmet medical needs, we performed high-throughput screening of 9600 chemical compounds and identified an aminohydrazone derivative as a novel and potent antifungal compound. We then designed and synthesized a series of aminohydrazone derivatives, and demonstrated that 1-[(*E*)-[4-(3',4'-dichlorobenxyloxy)phenyl methylidene]amino]-guanidine had the most potent inhibitory activity and exhibited a broad spectrum of antifungal activities against *Candida* species (including multidrug resistant *C. auris*), *Aspergillus* species, *Cryptococcus neoformans*, and *Rhizopus oryzae*. Against *C. albicans*, the leading cause of *Candida* infections, the compound had fungicidal activity for planktonic cells at 8^^μg $mL^{<M->1}$ (25^^μM) and anti-biofilm activity at 34^^μg $mL^{<M->1}$ (100^^μM). This study provides new insights for the development of a new drug class for the treatment of invasive fungal infections which are often refractory to conventional therapies.

Introduction

Invasive fungal infections are life-threatening, especially in immunocompromised patients. The increased incidence of invasive fungal infections is correlated with the expansion of HIV infections that suppress the immune system and the development of advanced immunosuppressive interventions, such as chemotherapeutic treatments and hematologic and solid organ transplantation.[1] Invasive fungal infections can kill an estimated 1.5 million people globally each year. In fact, their mortality rate is greater than that of malaria and is equivalent to that of tuberculosis or HIV.[1b,2] Bloodstream fungal infections, mostly caused by *Candida* species, lead to a variety of complications, including infective endocarditis and endophthalmitis.[3] By global estimates, ~750000 cases of invasive candidiasis occur annually, and the mortality rate is 46--75% among overall infected populations and approximately 40% in patients receiving antifungal therapies.[1b,4] Aspergillosis caused by *Aspergillus* species is one of the major pulmonary fungal infections. Recent global estimates have reported that ~3000000 cases of chronic pulmonary aspergillosis and >250000–300000 cases of invasive aspergillosis occur each year.[1,5] An estimated 450000 deaths occur due to chronic pulmonary aspergillosis annually and almost 100% of patients with invasive aspergillosis die if left untreated.[1b,5] The major fungal infection of the central nervous system is cryptococcal meningitis, which is typically found in patients with HIV/AIDS (~223000 cases annually) and is responsible for 15% of AIDS-related deaths.[1d] The unacceptably high mortality rates associated with invasive fungal infections represent the major limitations of current antifungal treatments.

The polyene amphotericin B was identified in the 1950s as the first antifungal drug. Since then, only a few classes of antifungal agents have been developed, such as fluoropyrimidine analogs, azoles, and echinocandins.[6] Echinocandins were most recently approved for clinical use in the 2000s; thereafter, a new class of antifungal drugs has not been introduced to the market.[6--7] The currently used antifungal drugs fail to exhibit potent fungicidal activities. Instead, they exert toxic effects on human cells as a high level of similarity exists between the pathogen and human protein targets.[7] Because of the limited availability of antifungal drugs, the emergence of drug-resistant isolates, particularly for azoles and echinocandins, has

become a critical issue in clinical settings.[8] Such knowledge points to the urgent and unmet need for the development of new antifungal drugs.

In this study, a high-throughput screening of a chemical compound library was conducted to identify a new class of antifungal compounds for the development of novel modalities to treat invasive fungal infections.

Results and Discussion

Screening of antifungal compounds

The compound library was composed of 9600 drug-like and lead-like compounds, which were selected as a core library from the University of Tokyo chemical library consisting of more than 220000 compounds. Several lead-like compounds have already been identified from this core library, including those with antibacterial activity, antitrypanosoma activity, and antibiofilm effects against methicillin-resistant *Staphylococcus aureus*.[9]

We first screened the chemical library to identify compounds that could inhibit the growth of *Candida albicans* and/or *Aspergillus fumigatus*, known as the major etiologic agents of invasive fungal infections. Growth inhibitory effects were evaluated based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI),[10] in which fungal growth is scored as 0, 1, 2, 3, or 4 by visual observation, with scores 0, 1, and 2 indicating 50% inhibition or greater, as shown in Figure^^1<figr1>.

From a chemical library consisting of 9,600 compounds, 43 and 8 compounds exhibited scores of 2 or less at a concentration of 10^^μM after 24^^h and 48^^h for *C. albicans* and *A. fumigatus*, respectively. Because three compounds were identical in structure, the total number of hit compounds was 48 (compounds **1** to **48**) in the first screening (data not shown).

In the second screening, the reproducibility of the assay was evaluated by using replication panels comprised of *C. albicans* and *A. fumigates.* As shown in Supplementary Table^^1<tabr1>, 31 compounds had scores of 2 or less at a concentration of 10^^μM or 20^^μM at 24^^h or 48^^h. Subsequently, we determined the antifungal activities of the primary hit compounds against the other five major *Candida* species (*C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*), one *Cryptococcus* strain

(*Cryptococcus neoformans*), two additional major *Aspergillus* strains (*A. flavus* and *A. niger*), and one major *Mucor* strain (*Rhizopus oryzae*) (Supplementary Table^^S1). Based on their antifungal activities, backbone structures, and antifungal spectra, compounds **24**, **29**, and **30** were selected (Scheme^^1<schr1>).

However, it has been previously reported that compound **30** with methyl 5-benzoil benzimidazole-2-carbamat and compound **24** derivatives with 1-alkylamino-2-(4-adamantylphenoxy)ethane structures have antifungal activity.[11] In addition, we failed to find compound **24** analogs that exhibited more potent antifungal activities than **24**. We, therefore, focused on the design of a series of derivatives of **29**, 1-[(*E*)-[4-(3',4'-dichlorobenzyloxy) phenyl methylidene]amino]-guanidine.

As shown in the Chemistry subsection of the Experimental Section, below, we synthesized compounds **49--64** (Scheme^^2<schr2/u>) and evaluated their 50% inhibitory concentration (IC_{50}) and IC_{90} values against the major pathogenic fungi (Table^^1<xtabr1>). Compounds **29**, **49**, **58**, **59**, **61**, and **64** contained an aminoguanidine group on their side chains and exhibited antifungal activities. Compound **49** had the most potent antifungal activities and the broadest antifungal spectra among the derivatives, with IC_{50} values of 12.5^^μM (4^^μg $mL^{<M->1}$) against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. neoformans*, 25^^μM (8^^μg $mL^{<M->1}$) against *C. guilliermondii*, *A. fumigatus*, *A. niger*, and *R. oryzae*, and 50^^μM (17^^μg $mL^{<M->1}$) against *A flavus*. Similarly, the IC_{90} values were 12.5^^μM (4^^μg $mL^{<M->1}$) against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. neoformans*, 25^^μM (8^^μg $mL^{<M->1}$) against *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *A. fumigatus*, *A. niger*, and *R. oryzae*, and >100^^μM (34^^μg $mL^{<M->1}$) against *A. flavus*. The antifungal activity of compound **49** was increased by replacing the 2,4-dichlorobenzene group with a 3,4-dichlorobenzene group based on the activity of compound **28**. However, the amino-guanidine derivatives **59--61** with or without a substituted group, such as 4-fluoro, 3-fluoro-4-chloro, or 2-fluoro-4-bromorobenzene, failed to elicit antifungal activity. Compound **61** with a phenoxy group also exhibited poor antifungal activity. We, therefore, concluded that the 2,4-dichlorobenzene ring of the amino-guanidine group plays an

important role in the antifungal activity of compound **49**. Thus, it is most likely that the substitution group is responsible for the high antifungal activity of **49**.

We also determined the antifungal activity of **49** against the multidrug-resistant *C. auris* strain NCPF 8985, which is highly resistant to echinocandins (micafungin and caspofungin), azoles (fluconazole, itraconazole, and voriconazole), and flucytosine (Table^^2<tabr2/or>). The IC_{50} of **49** against *C. auris* was 25^^μM, which corresponded to 8^^μg $mL^{<M->1}$. Although the IC_{50} value of **49** was higher than that of amphotericin B, compound 49 was much more potent than many other antifungal drugs that are currently used in clinical practice.

The antifungal activities of compounds **51--58** were significantly lower than those of **49** (Table^^1<xtabr1>). As **51--58** contained modified aminoguanidyl groups in their structures, the results indicate that the aminoguanidyl group played a pivotal role in the antifungal activity of the aminoguanidine-containing compounds. We proceeded to perform an in-depth examination of the antifungal properties of **49**.

Evaluation of the fungicidal activity of compound 49^in the time-kill assay

As the overestimation of the fungicidal effect of **49** in the time-kill assay was a concern, we first evaluated the effect of the antifungal carryover of drugs. When 30 μL of the fungal samples containing 4 μg $mL^{<M->1}$ amphotericin B was plated onto potato-dextrose-agar (PDA) plates, fungal growth was not inhibited. In contrast, explicit inhibition of fungal growth was observed for **49** at concentrations of 2 μg $mL^{<M->1}$, 4 μg $mL^{<M->1}$, and 8 μg $mL^{<M->1}$. We thus diluted the fungal suspensions 1:10 to yield concentrations of 0.2 μg $mL^{<M->1}$, 0.4 μg $mL^{<M->1}$, and 0.8 μg $mL^{<M->1}$. When 100 μL of the diluted samples was plated onto PDA plates, no antifungal carryover was observed. The fungal samples were, therefore, diluted 1:10 and plated onto PDA plates for the time-kill assay.

A time-kill assay was performed to evaluate the fungicidal activity of **49** against *C. albicans*. The time-kill kinetics of compound **49** at concentrations of 2 μg $mL^{<M->1}$, 4 μg $mL^{<M->1}$, and 8 μg $mL^{<M->1}$ were compared to those of amphotericin B at concentrations of 2^^μg $mL^{<M->1}$ and 4^^μg $mL^{<M->1}$. As shown in Figure^^2<figr2>, both **49** and amphotericin

B exhibited concentration- and time-dependent fungicidal activity. Both **49** and amphotericin B exhibited fungicidal activity (<99.9% reduction in the number of viable cells relative to the initial fungal concentration) at a concentration of 4 μg mL$^{<M->1}$. The reference drug amphotericin B is the most potent fungicidal drug among the currently available antifungals in clinical use. The fungicidal effect of **49** was also high on a weight-per-volume (w/v) basis.

Evaluation of anti-biofilm activity of compound 49

We examined the anti-biofilm activity of **49**, amphotericin B, and fluconazole, using *C. albicans* biofilms. Compound **49**, amphotericin B, and fluconazole were serially diluted 2-fold from 135 μg mL$^{<M->1}$ (400^^μM), 100 μg mL$^{<M->1}$, and 1024 μg mL$^{<M->1}$, respectively. The maximum final concentrations of dimethyl sulfoxide (DMSO) in the assay systems were 4% for **49** and fluconazole and 1% for amphotericin B, where no anti-biofilm effect was observed.

It has been reported that amphotericin B has potent antibiofilm activity, but fluconazole does not.[12] As expected, more than a 1000-fold increase in fluconazole's sessile MIC (SMIC) was observed compared to its planktonic MIC (PMIC) (Table^^3<tabr3>). The $PMIC_{50}$ and $PMIC_{90}$ of **49** were 4 μg mL$^{<M->1}$ and 8 μg mL$^{<M->1}$, respectively. Both the $SMIC_{50}$ and $SMIC_{80}$ of **49** were 34 μg mL$^{<M->1}$, indicating that **49** had antibiofilm activity to a certain extent. The fold changes between the PMICs and SMICs in compound **49** were similar to those in amphotericin B.

Cellular cytotoxicity of compound 49

Finally, the cytotoxicity of **49** against A549, a human lung adenocarcinoma cell line, was determined. Two-fold serial dilutions were prepared with starting concentrations of 34 μg mL$^{<M->1}$ (100^^μM) for compounds **49**, **29**, **58**, **61**, and **62**, and 100 μg mL$^{<M->1}$ for amphotericin B. A549 cells were incubated in the presence of the compound dilutions for 4^^days. As shown in Figure^^3<figr3>, the IC_{50} value of **49** was 8 μg mL$^{<M->1}$ (25^^μM). The cellular cytotoxicity of DMSO alone was negligible at the concentrations tested. For reference, the cellular cytotoxicity of amphotericin B was determined and the IC_{50} was greater

than 50 μg mL$^{<M->1}$, indicating that compound **49** was more toxic to the human cell line than amphotericin B.

Because of a paucity of clinically available antifungal drugs, it is imperative to explore compounds that can be used for the treatment of patients with invasive fungal infections, especially for patients infected with drug-resistant fungi. In the present study, we designed and synthesized **49**, 1-[(*E*)-[4-(3',4'-dichlorobenxyloxy)phenyl methylidene]amino]-guanidine. This compound belongs to a novel class of antifungals and the fungicidal activity of **49** against *C. albicans* was similar to that of amphotericin B on a weight-per-volume basis. In addition, compound **49** had a broad antifungal spectrum exhibiting antifungal activities against the main causative pathogens of major fungal infections. However, as **49** failed to exhibit a synergistic effect with conventional drugs (data not shown), it is worthwhile to synthesize its derivatives to derive more biologically potent compounds. In terms of anti-biofilm activity, **49** inhibited biofilm formation by exhibiting potent activity against *C. albicans* biofilm cells that were resistant to azole antifungals. Nonetheless, the anti-biofilm activity of amphotericin B was superior to that of **49**.

Because the cellular cytotoxicity of **49** was observed at a concentration of 8 μg mL$^{<M->1}$ (25^^μM), a value close to 2 x MIC, we attempted to reduce the cytotoxicity by modifying its structure. Derivatives of **49** and **62**, including an aminobenzimidamide analog, and **58** (a guanidine derivative), however, failed to exhibit reduced cytotoxicity (Figure^^3<xfigr3>). Based on these results, it is difficult to improve cellular cytotoxicity while retaining the core structure of aminoguanidine. Nonetheless, this is the first report showing that aminoguanidine-containing compounds display antifungal activity. Although we did not explore the mechanisms of compound **49** in this study, aminoguanidine derivatives, by nature, are expected to be protonated at physiological pHs and the cationic molecular structure is known to form complexes with phospholipid head groups by bidentate hydrogen-bonded ion pairing. Their affinity for phospholipids is thus most likely to be responsible for the interaction of the compounds with cell membranes, leading to high cell penetration ability and subsequent antifungal activity.[13]

Conclusions

New antifungal drug classes are needed urgently because the morbidity and mortality of invasive fungal infections remain unacceptably high. Herein, we designed and synthesized the aminohydrazone derivative **49** which displayed broad antifungal spectra and potent antifungal activities even against multidrug resistant *C. auris*. In addition, **49** had antibiofilm activity against *C. albicans*. However, **49** exhibited cytotoxicity to human A549 cells. Although future studies are needed to increase its antifungal activity and reduce its cytotoxicity, the present study provides fundamental information that can facilitate the development of new antifungal agents.

Supporting Information Summary

Experimental section (including materials and methods for high throughput screening, antifungal susceptibility testing, time-kill kinetic assay, anti-biofilm assay and cytotoxicity assay), antifungal activity of 48 hit conpounds, detailed information on the synthesis of conpounds **49--64** and NMR spectra are available in the supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

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Table^^1 IC_{50} and IC_{90} values of a series of aminohydrazone derivatives and reference drugs against *Candida* species, *Cryptococcus neoformans*, *Aspergillus* species, and *Rhizopus oryzae*.[a]<w=3>

	C. albicans		*C. glabrata*		*C. parapsilosis*		*C. tropicalis*		*C. krusei*		*C. guilliermondii*	
Compound	IC_{50}(μM)	IC_{90}(μM)	IC_{50}(μM)	IC_{90}(μM)	IC_{50}(μM)	IC_{90}(μM)	IC_{50}(μM)	IC_{90}(μM)	IC_{50}(μM)	IC_{90}(μM)	IC_{50}(μM)	IC_{90}(μM)
29	25	50	25	25	25	100	25	25	25	25	100	>100
49	12.5	12.5	12.5	12.5	12.5	25	12.5	25	12.5	12.5	25	25
50	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
51	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
52	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
53	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
54	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	100	>100
55	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
56	>100	>100	>100	>100	50	>100	>100	>100	100	100	>100	>100
57	>100	>100	50	100	>100	>100	>100	>100	100	>100	50	>100
58	25	50	25	50	25	50	25	25	12.5	12.5	100	100
59	50	50	50	100	50	100	50	50	50	100	100	100
60	50	100	100	>100	100	>100	100	>100	100	100	>100	>100
61	50	50	50	50	50	50	25	50	50	50	100	>100
62	50	>100	50	50	100	>100	50	50	50	50	>100	>100
63	100	100	100	>100	100	>100	100	100	50	100	100	>100
64	25	25	100	>100	25	25	12.5	12.5	25	25	25	50

FLCZ (μg mL$^{<M->1}$)	<0.125	16	2	8	2	8	1	0.5	1	1	2	4
AMB (μg mL$^{<M->1}$)	0.5	0.5	0.125	0.5	0.125	0.5	0.5	0.5	0.5	1	0.125	0.5

	C. neoformans		*A. fumigatus*		*A. flavus*		*A. niger*		*R. oryzae*			
Compound	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)		
29	50	50	>100	>100	>100	>100	>100	>100	>100	>100		
49	12.5	12.5	25	25	50	>100	25	25	25	25		
50	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
51	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
52	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
53	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
54	100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
55	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
56	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100		
57	100	100	100	>100	>100	>100	>100	>100	100	100		
58	25	50	50	50	>100	>100	>100	>100	>100	>100		
59	50	50	>100	>100	>100	>100	>100	>100	>100	>100		
60	100	100	>100	>100	>100	>100	>100	>100	>100	>100		

61	25	50	>100	>100	>100	>100	>100	>100	>100	>100
62	100	100	>100	>100	>100	>100	>100	>100	>100	>100
63	100	100	>100	>100	>100	>100	>100	>100	>100	>100
64	50	50	>100	>100	>100	>100	>100	>100	>100	>100
FLCZ(μg $mL^{<M->1}$)	0.5	0.5	-	-	-	-	-	-	-	-
AMB(μg $mL^{<M->1}$)	1	4	0.5	1	1	1	0.25	0.5	0.125	0.25

[a] Data are representative of three independent experiments

Table^^2 IC_{50} value of compound 49 and reference drugs against *Candida auris* NCPF 8985.[a]

	IC_{50}(μg $mL^{<M->1}$)							
	Compound **49**	MCFG	CPFG	FLCZ	ITCZ	VRCZ	5FC	AMB
C. auris	8	>16	>16	>64	>8	>8	>64	0.5

[a] MCFG, micafungin; CPFG, caspofungin; FLCZ, fluconazole; ITCZ, itraconazole; VRCZ, voriconazole; 5FC, 5-flucytosine; and AMB, amphotericin B. Data are representative of two independent experiments.

Table^^3 Comparison of planktonic and sessile sensitivities of *Candida albicans* biofilms to the newly synthesized compound **49** and reference drugs, fluconazole (FLCZ) and amphotericin B (AMB).[a]

	Planktonic MIC (μg $mL^{<M->1}$)		Sessile MIC (μg $mL^{<M->1}$)	
	$PMIC_{50}$	$PMIC_{80}$	$SMIC_{50}$	$SMIC_{80}$

49	4	8	34	34
FLCZ	0.125	1	>1024	>1024
AMB	0.25	0.5	0.4	3.1

[a] The XTT assay was performed in triplicate. Representative data of two independent experiments are shown.

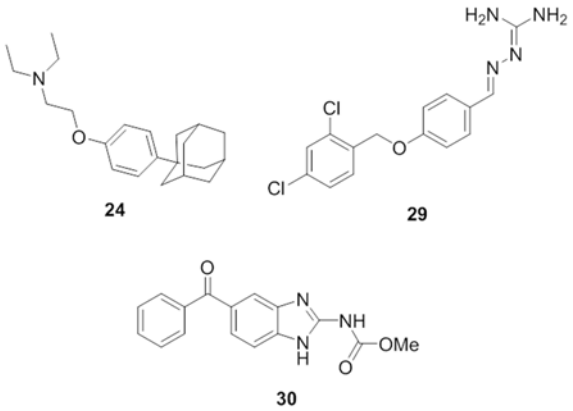
Scheme^^1 Chemical structures of the primary hit compounds **24**, **29**, and **30**.

Scheme^^2 Chemical structures of Compound **29** and the synthesized compounds **49--64**.

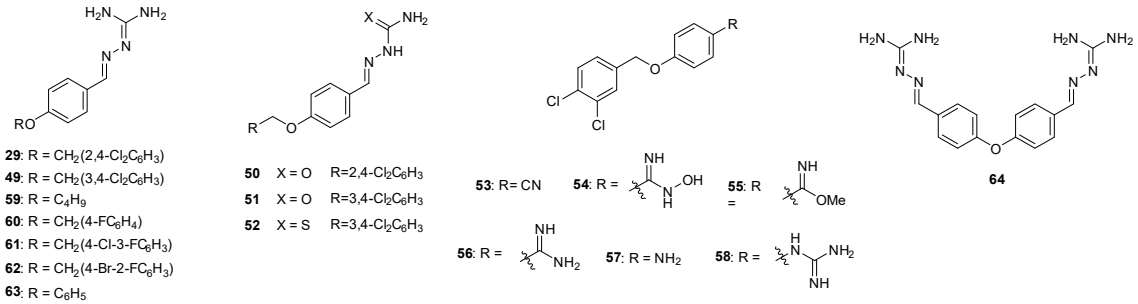
Figure^^1 Evaluation of antifungal activity by visual observation. Inhibition of fungal growth was determined as follows: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity.

Figure^^2 Time-kill curves of **49** and amphotericin B (AMB) against *Candida albicans* SC5314. Fungicidal activity of **49** and AMB was determined by time-kill assay. Limit of quantitation was 50 CFU $mL^{<M->1}$.<+>NOTE: The assay was performed in triplicate. Data are representative of two independent experiments.

Figure^^3 Cytotoxicity of compound 49 to the human cell line A549 in comparison with compound 29, other derivatives of 29, and amphotericin B (AMB).<+>NOTE: The ATP-based luminescence assay was performed in triplicate. Data are representative of two independent experiments.



Scheme 1. Chemical structures of the primary hit compounds **24**, **29**, and **30**.



Scheme 2. Chemical structures of Compound **29** and the synthesized compounds **49**-**64**.

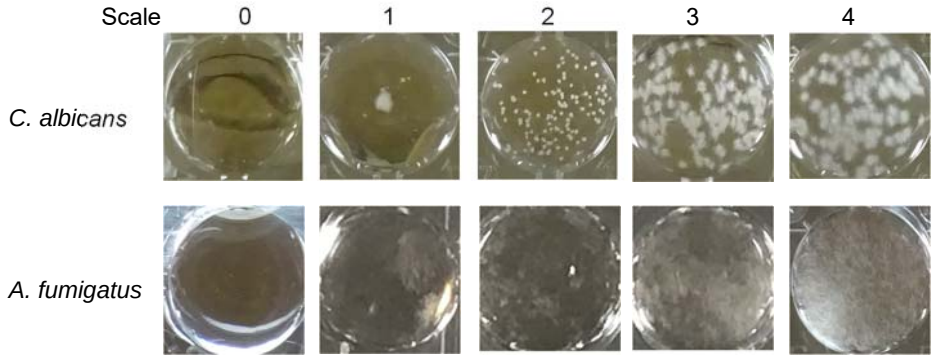


Figure 1. Evaluation of antifungal activity by visual observation. Inhibition of fungal growth was determined as follows: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity.

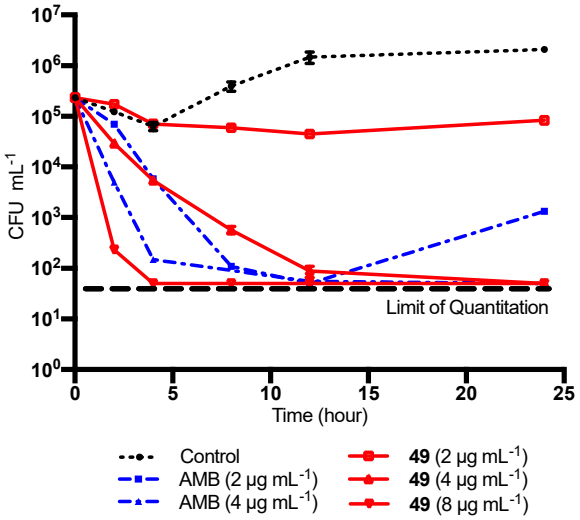


Figure 2. Time-kill curves of **49** and amphotericin B (AMB) against *Candida albicans* SC5314. Fungicidal activity of **49** and AMB was determined by time-kill assay. Limit of quantitation was 50 CFU mL^{-1}.
NOTE: The assay was performed in triplicate. Data are representative of two independent experiments.

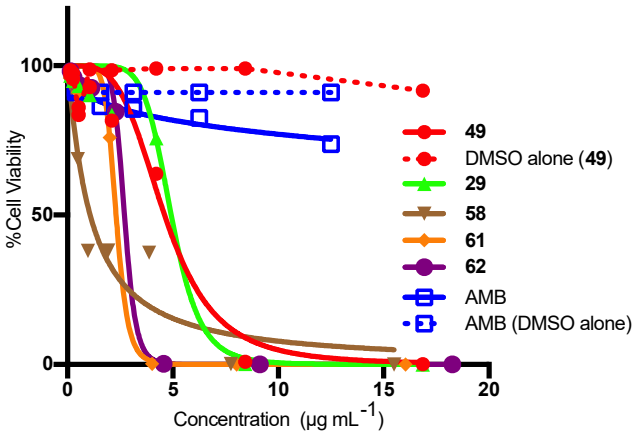


Figure 3. Cytotoxicity of compound **49** to the human cell line A549 in comparison with compound **29**, other derivatives of **29**, and amphotericin B (AMB).
NOTE: The ATP-based luminescence assay was performed in triplicate. Data are representative of two independent experiments.