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EXPERIMENTAL THERAPEUTICS

In Vitro Activity of a Novel Antifungal Compound, MYC-053, against Clinically Significant Antifungal-Resistant Strains of *Candida glabrata*, *Candida auris*, *Cryptococcus neoformans*, and *Pneumocystis* spp.

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ABSTRACT An urgent need exists for new antifungal compounds to treat fungal infections in immunocompromised patients. The aim of the current study was to investigate the potency of a novel antifungal compound, MYC-053, against the emerging yeast and yeast-like pathogens *Candida glabrata*, *Candida auris*, *Cryptococcus neoformans*, and *Pneumocystis* species. MYC-053 was equally effective against the susceptible control strains, clinical isolates, and resistant strains, with MICs of 0.125 to 4.0 μg/ml. Notably, unlike other antifungals such as azoles, polyenes, and echinocandins, MYC-053 was effective against *Pneumocystis* isolates, therefore being the only synthetic antifungal that may potentially be used against *Pneumocystis* spp., *Candida* spp., and *Cryptococcus* spp. MYC-053 was highly effective against preformed 48-h-old *C. glabrata* and *C. neoformans* biofilms, with minimal biofilm eradication concentrations equal to 1 to 4 times the MIC. Together, these data indicated that MYC-053 may be developed into a promising antifungal agent for the treatment and prevention of invasive fungal infections caused by yeasts and yeast-like fungi.

KEYWORDS *Candida glabrata*, *Pneumocystis*, antifungal resistant

In the last decade, invasive fungal infections caused by non-*albicans Candida* species and other less-common emerging yeasts, such as *Cryptococcus* spp., have become the leading cause of mortality in immunocompromised individuals (1–4).

Thus, *Candida glabrata* has emerged as the most common non-*albicans Candida* causative agent of invasive fungal infection, including the cases of hospital-acquired bloodstream infections in the United States in patients with an aberrant immune response (5–9).

Antifungal resistance among fungi causing invasive fungal infections represents a clinical challenge due to the limited classes of antimycotics available (polyenes, azoles, and echinocandins) (10). The spread of multidrug-resistant strains of *C. glabrata* in the United States, i.e., those displaying resistance to at least two classes of antifungal drugs, is consistently associated with increased mortality, as described in recent studies (11–13). Notably, resistance to echinocandins is also increasing among *C. glabrata* isolates, with reported resistance rates of 3% to 12% in different countries (14, 15).

Another global health care concern is the emerging multidrug-resistant pathogenic species *Candida auris* (16, 17). Unlike most other *Candida* spp., this fungus is commonly transmitted within health care facilities (18–20). Moreover, the drug resistance rate of *C. auris* exceeds that of *C. glabrata*, with over 41% of isolates reportedly resistant to at least two antifungal classes (18).

Cryptococcus neoformans is another opportunistic pathogen and an etiologic agent

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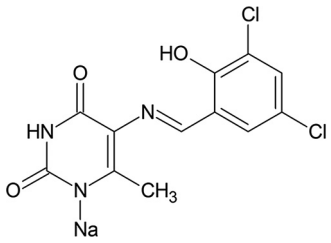

FIG 1 Chemical structure of MYC-053.

of cryptococcosis, a life-threatening infection in immunocompromised hosts (4). Although the rates of cryptococcosis have dropped substantially since the development of highly active antiretroviral therapy, the mortality of HIV patients associated with cryptococcal meningitis remains high. One of the causes of treatment failure is the emergence of azole-resistant and -heteroresistant mutants (21–23).

Pneumocystis species also affect immunocompromised hosts causing pneumocystis pneumonia (PcP) that, according to the Centers for Disease Control and Prevention, affects over 9% of hospitalized HIV patients in the United States (24) (http://www.cdc.gov/fungal/diseases/pneumocystis-pneumonia/statistics.html). *Pneumocystis* spp., originally classified as protozoa, are now classified as fungi but are not susceptible to antifungal drugs, being treated with sulfamethoxazole and pentamidine with a high mortality rate, from 5% to 40% (25, 26).

In this paper, we describe the fungicidal activity of a novel antifungal compound, MYC-053 {sodium 5-[1-(3,5-dichloro-2-hydroxyphenyl)methylideneamino]-6-methyl-1,2,3,4-tetrahydro-2,4-pyrimidinedionate} (Fig. 1), which is not related to any existing classes of antifungal agents and was investigated against planktonic and biofilm-forming *Candida* spp., *Cryptococcus* spp., and *Pneumocystis* spp. (27–31).

RESULTS

***In vitro* antifungal activity of MYC-053 against *C. glabrata*, *C. auris*, and *C. neoformans*.** The efficacy of MYC-053 against a panel of 20 *C. glabrata* strains (including seven fluconazole [FLC]-resistant and four caspofungin [CAS]-resistant strains), five *C. auris* strains, and 18 *C. neoformans* strains (including 12 FLC-resistant strains) was determined by the broth microdilution method (Table 1). MIC values of MYC-053 for *C. glabrata* strains varied from 1 to 4 μg/ml, while if applying a less restrictive endpoint criterion of 50% inhibitory concentration (IC_{50}), values were in the low μg/ml range (0.125 to 0.5 μg/ml). *C. auris* and *C. neoformans* strains were also sensitive to this compound, with MICs of 1 to 4 μg/ml. Notably, the antifungal activity of MYC-053 against the susceptible strains, including the control *C. glabrata* ATCC 90030 and *C. neoformans* ATCC 90112 strains, was lower than that of FLC but higher than that of CAS. In contrast, while certain resistant clinical isolates exhibited reduced susceptibility to FLC and CAS, they were highly sensitive to the same concentrations of MYC-053 as the control strains (Table 1).

***In vitro* antifungal activity of MYC-053 against *Pneumocystis carinii* and *Pneumocystis murina*.** The responses of *Pneumocystis carinii* and *Pneumocystis murina* to MYC-053 were evaluated by a cytotoxicity assay based on ATP-driven bioluminescence (32). The results, expressed as the IC_{50} after 24, 48, and 72 h of exposure to the drug, were assigned activity ranks based on the degree of reduction of ATP compared to the untreated controls (33, 34) (Table 2). The exposure of *P. carinii* to 1 μg/ml MYC-053 for 72 h resulted in a level of ATP reduction that was slightly lower than that of pentamidine and can be considered moderate activity. However, the increase in MYC-053 concentration to 10 μg/ml resulted in smaller amounts of ATP pools than with 1 μg/ml pentamidine. The inhibitory effect of MYC-053 against *P. murina* was higher than that against *P. carinii*. In this assay, MYC-053 demonstrated activity against *P. murina* comparable to that with pentamidine, with over 94.4% reduction of the ATP pool

TABLE 1 Susceptibility and MIC and IC_{50} values of MYC-053 and other antifungal agents against *C. glabrata* and *C. auris*

				IC_{50} and MIC data by drug (μg/ml)[b]					
		Susceptibility to[a]:		MYC-053		FLC		CAS	
Fungal species	Isolate no.	FLC	CAS	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
C. glabrata	ATCC 90030	S	S	0.5	4	1	4	0.25	1
	CG1	I	NA	0.5	4	64	–	–	–
	CG2	S	NA	0.5	4	0.5	–	–	–
	CG3	I	NA	0.25	4	64	–	–	–
	CG4	S	NA	0.5	2	4	–	–	–
	CG5	S	NA	0.5	2	2	–	–	–
	CG6	I	NA	0.5	2	32	–	–	–
	CG7	I	NA	0.125	2	64	–	–	–
	CG8	I	NA	0.5	4	32	–	–	–
	CG9	I	NA	0.5	2	64	–	–	–
	CG10	R	NA	0.5	4	>64	–	–	–
	MR-V32	R	S	0.25	2	>64	>64	0.25	0.5
	MR-V35	R	R	0.5	4	>64	>64	4	4
	MR-V51	R	I	0.5	2	>64	>64	0.5	2
	MR-V16	R	R	0.125	1	>64	>64	4	8
	MR-V18	R	I	0.5	2	>64	>64	0.5	2
	MR-V19	R	R	0.5	4	>64	>64	2	2
	SS-V120	I	I	0.5	2	8	32	0.5	1
	SS-V114	S	S	0.25	2	2	8	0.125	0.25
	SS-V10	S	R	0.25	2	2	4	2	2
C. auris	CAU1	S	NA	1	4	2	–	–	–
	CAU2	S	NA	4	4	0.5	–	–	–
	CAU3	R	NA	4	4	>64	–	–	–
	V-2016-1	R	R	2	4	>64	>64	2	2
	V-2016-2	R	I	1	4	>64	>64	0.5	2
C. neoformans	ATCC 90030	S	NA	1	2	1	4	–	–
	CN1	R	NA	1	2	8	–	–	–
	CN2	S	NA	1	2	1	–	–	–
	CN3	R	NA	1	1	4	–	–	–
	CN4	R	NA	1	2	64	–	–	–
	CN5	R	NA	2	4	4	–	–	–
	CN6	R	NA	2	2	64	–	–	–
	CN7	R	NA	2	4	4	–	–	–
	CN8	S	NA	2	4	2	–	–	–
	CN9	S	NA	2	2	2	–	–	–
	CN10	S	NA	1	2	1	–	–	–
	RR-94	R	NA	0.5	2	64	>64	–	–
	RR-112	R	NA	2	2	8	>64	–	–
	RR-1025	R	NA	0.5	1	64	>64	–	–
	HR-30	R	NA	2	2	32	>64	–	–
	HR-02	R	NA	1	1	2	8	–	–
	SS-18	R	NA	2	4	2	>64	–	–
	SS-10	S	NA	1	1	1	8	–	–

[a]*Candida* spp. were considered susceptible (S) to FLC at an MIC of ≤8 μg/ml, intermediate (I) at an MIC of 8 to 64 μg/ml, and resistant (R) at an MIC of ≥64 μg/ml (50–52). *Candida* spp. were considered susceptible to CAS at an MIC of ≤0.25 μg/ml, intermediate at an MIC of 0.5 μg/ml, and resistant at an MIC of ≥1 μg/ml (35). Only potential breakpoints for FLC against *C. neoformans* were used, as follows: susceptible, ≤2 mg/liter; resistant, >2 mg/liter. NA, not available.
[b]–, not tested.

following 72 h of exposure, which is considered to indicate marked activity on the efficacy scale (34). Overall, MYC-053 effectively reduced the ATP content of both *Pneumocystis* species at microgram levels. The following IC_{50} values were calculated over 3 days of *P. carinii* exposure to MYC-053: 3.90 μg/ml at 24 h, 2.56 μg/ml at 48 h, and 1.61 μg/ml at 72 h. Against *P. murina*, the IC_{50} values were 3.30 μg/ml at 24 h, 1.50 μg/ml at 48 h, and 0.165 μg/ml at 72 h.

Activity of MYC-053 against *C. glabrata* and *C. neoformans* biofilms. The antibiofilm effects of MYC-053, FLC, and CAS on preformed 48-h-old *C. glabrata* biofilms and the effects of MYC-053 and FLC on cryptococcal biofilms were evaluated (Table 3). Preformed biofilms were exposed to drugs provided at concentrations equal to 1 to 64

TABLE 2 IC_{50} values for MYC-053 for *P. carinii* and *P. murina* following different exposure times in the ATP assay

Drug, concn (μg/ml)	% reduction in ATP/media control[a]		
	24	48	72
P. carinii			
Ampicillin, 10	7.84	1.51	0
Pentamidine, 1	81.14	86.58	86,57
MYC-053, 50	96.81	97.61	99.21
MYC-053, 10	68.26	90.29	95.58
MYC-053, 1	14.95	11.08	26.77
MYC-053, 0.1	0	3.20	13.42
IC_{50}	3.90 $\pm$ 2.0 μg/ml	2.56 $\pm$ 0.57 μg/ml	1.61 $\pm$ 1.72 μg/ml
P. murina			
Ampicillin, 10	2.86	0.26	0
Pentamidine, 1	92.07	97.70	98.12
MYC-053, 50	97.84	98.89	98.77
MYC-053, 10	76.56	98.51	97.92
MYC-053, 1	1.082	42.11	94.42
MYC-053, 0.1	0	0	27.82
IC_{50}	3.30 $\pm$ 0.19 μg/ml	1.50 $\pm$ 0.13 μg/ml	0.165 $\pm$ 0.06 μg/ml

[a]Results represent the means from the 3 experiments which each contained three technical replicates.

times their MICs. MYC-053 significantly reduced the CFU of preformed biofilms of both *C. glabrata* and *C. neoformans* after 24 h of incubation, starting at a concentration of 1$\times$ the MIC. MYC-053 at a concentration of 1$\times$ the MIC decreased the number of viable fungi in all strains by more than 50%; this value was recorded as the 50% minimum biofilm eradication concentration ($MBEC_{50}$). Moreover, MYC-053 was the only drug that showed $MBEC_{90}$ values equal to 1 to 4 times its MIC. In the assay, higher relative concentrations of FLC and CAS were required to kill yeasts in preformed biofilms than concentrations of MYC-053. The $MBEC_{50}$ and $MBEC_{90}$ values of FLC and CAS against the tested preformed *C. glabrata* biofilms were equal to 4 to 64 times and 1 to 32 times their MICs, respectively. Similar data with high relative $MBEC_{50}$ and $MBEC_{90}$ of FLC required were obtained against *C. neoformans* biofilms (Fig. 2). CAS efficacy was not

TABLE 3 Susceptibility of 48-h-old *C. glabrata* biofilms to MYC-053, FLC, and CAS, expressed as multiples of MIC values

Fungal species	Isolate no.	MBEC data by drug (μg/ml)[a]					
		MYC-053		FLC		CAS[b]	
		$MBEC_{50}$	$MBEC_{90}$	$MBEC_{50}$	$MBEC_{90}$	$MBEC_{50}$	$MBEC_{90}$
C. glabrata	ATCC 90030	1	2	4	4	1	4
	MR-V32	1	2	4	>64	4	16
	MR-V35	1	1	8	>64	8	4
	MR-V51	1	2	4	>64	2	2
	MR-V16	1	2	4	>64	4	32
	MR-V18	1	4	32	>64	2	16
	MR-V19	1	2	16	32	4	32
	SS-V120	1	1	8	8	2	4
	SS-V114	1	4	4	32	4	32
	SS-V10	1	1	4	16	16	32
C. neoformans	ATCC 90030	1	2	2	16	–	–
	RR-94	1	4	4	>64	–	–
	RR-112	1	4	32	>64	–	–
	RR-1025	1	1	8	32	–	–
	HR-30	1	1	16	64	–	–
	HR-02	1	2	64	>64	–	–
	SS-18	1	2	8	16	–	–
	SS-10	1	1	4	16	–	–

[a]Results represent the means from 3 experiments, which each contained three technical replicates.
[b]–, not tested.

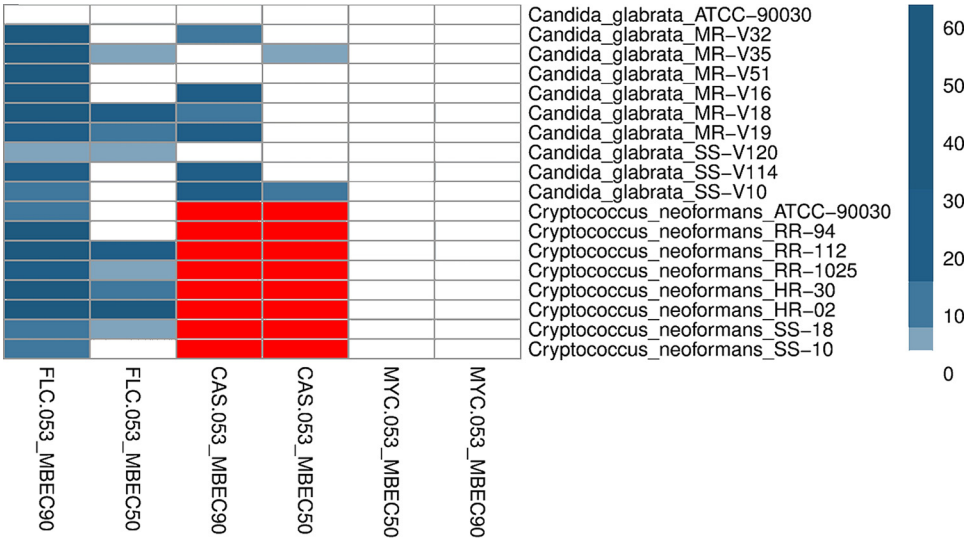

FIG 2 Heatmap and cluster analysis of MYC-053, FLC, and CAS against 48-h-old *C. glabrata* and *C. neoformans* biofilms expressed as $MBEC_{50}$ or $MBEC_{90}$, in multiples of MIC. The MIC values were ordered by hierarchical clustering using the Euclidean distance method and are represented by a heatmap, with the intensity indicated by a color code (dark blue, MIC; light blue, 8× the MIC; white, ≥64 times the MIC). *C. glabrata* strains that were not tested in this assay and *C. neoformans* strains that were not tested against CAS are highlighted in red.

tested against *C. neoformans* biofilms in this assay, as this microorganism is known to be resistant both *in vitro* and *in vivo* to echinocandins.

DISCUSSION

In the current study, we described a novel antifungal drug candidate, MYC-053, which exhibited a high level of antimicrobial activity against *C. glabrata*, *C. auris*, *C. neoformans*, and *Pneumocystis* spp. *in vitro* that are well-known causes of morbidity in immunocompromised patients, being characterized by growing antibiotic resistance (35–42).

Importantly, the MIC experiment revealed that MYC-053 exerted a pronounced cidal effect against resistant fungal isolates at concentrations identical to the ones killing susceptible control fungal strains. These data correspond well with the notion that MYC-053 is a representative of a novel chemical class of antifungal agents; it is not relevant to the existing antifungal agents whose use is frequently characterized by cross-resistance (43). Notably, MYC-053 was effective against *C. auris*, which is often multidrug resistant (44). Although we have only tested the activity of MYC-053 against five *C. auris* strains, low standard error of the mean (SEM) values in the assay suggested high precision of the measurements, allowing us to determine the mean IC_{50} as 1 μg/ml and MIC as 4 μg/ml. Despite the fact that the MIC values of MYC-053 against *C. auris* were higher than those against *C. glabrata*, these values were nonetheless promising given the low susceptibility of certain tested strains to FLC and CAS, with MIC values over 64 μg/ml for these antifungals.

MYC-053 was also effective against *C. neoformans*, with MIC values starting at 1.0 μg/ml. These concentrations were dramatically different from the FLC MIC values. Although we did not test the sensitivity of *C. neoformans* strains against other azoles, it is known that this fungus is commonly cross-resistant to other antifungal agents of this class, including voriconazole (45, 46). Therefore, we propose that MYC-053 might be effective against other non-azole-resistant strains of *C. neoformans*.

This investigation also revealed that MYC-053 was effective against *Pneumocystis* spp., other yeast-like pathogens that are challenging to treat in immunocompromised patients. The anti-*Pneumocystis* activity of MYC-053 was promising since, despite being originally classed as protozoa, *Pneumocystis* spp. are now classified as fungi and continue to be generally treated with antibacterial and antiprotozoan medications (32,

47). The determination of ATP levels for the assessment of MYC-053 activity against *Pneumocystis* spp. constitutes a highly sensitive assay enabling a reduction in the number of tested organisms (33). The activity of MYC-053 was considered marked and was comparable to the activity of pentamidine against *P. murina* at the 72-h time point. To the best of our knowledge, MYC-053 is the first new synthetic compound that can be potentially used against *Pneumocystis* spp., *Candida* spp., and *Cryptococcus* spp.

We also revealed that MYC-053 was highly effective against 48-h-old preformed fungal biofilms. At a concentration equal to the MIC, MYC-053 caused a 50% reduction in the viable cell counts in all studied fungal biofilms. The $MBEC_{90}$ values of MYC-053 were equal to 1 to 4 times the MIC values. Notably, the $MIC/MBEC_{50/90}$ ratios of MYC-053 were significantly lower than those of the control antifungals FLC and CAS. In summary, MYC-053 was equally effective against sessile and planktonic nonresistant organisms and multiresistant clinical isolates.

Taken together, the results of the current study on the efficacy of MYC-053 against certain yeasts and yeast-like pathogens, including ones in a biofilm state, indicate the possibility of developing MYC-053 further into an antifungal drug candidate; however, it requires more *in vivo* research.

MATERIALS AND METHODS

Test substance and antimicrobials. MYC-053 was synthesized by TGV-Therapeutics, Inc. (Wilmington, DE); FLC, CAS, and pentamidine were purchased from Sigma-Aldrich (St. Louis, MO) (Fig. 1).

Fungal strains. Forty-four fungal species were used in this study. *C. glabrata* CG1, CG2, CG3, CG4, CG5, CG6, CG7, CG8, CG9, and CG10, *C. auris* CAU1, CAU2, and CAU3, and *C. neoformans* CN1, CN2, CN3, CN4, CN5, CN6, CN7, CN8, CN9, and CN10 were obtained from the Fungus Testing Laboratory at the University of Texas Health Science Center (San Antonio, TX). *C. glabrata* MR-V32, MR-V35, MR-V51, MR-V16, MR-V18, MR-V19 SS-V120, SS-V114, and SS-V10, *C. auris* V-2016-1 and V-2016-2, and *C. neoformans* RR-94, RR-112, RR-1025, HR-30, HR-02, SS-18, and SS-10 were provided by V. Tetz (Human Microbiology Institute) from a private collection. *P. carinii* and *P. murina* were obtained from Melanie Cushion's laboratory at the University of Cincinnati (Cincinnati, OH). The control strains were *C. glabrata* ATCC 90030 and *C. neoformans* ATCC 90112 (ATCC, Rockville, MD, USA). *C. glabrata* and *C. auris* isolates were subcultured on Sabouraud dextrose agar before testing (Oxoid Ltd., Basingstoke, UK).

***In vitro* antifungal susceptibility testing.** Microdilution broth susceptibility testing was performed in duplicate according to the CLSI M27-A3 method in RPMI 1640 growth medium (Sigma-Aldrich) to determine the MIC values (48). Standard inoculum for yeast testing was 2.5×10^3 CFU/ml. FLC and CAS were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), whereas MYC-053 was dissolved in sterile water. The IC_{50} was defined as the lowest concentration of a drug that at which 50% growth inhibition was observed compared to the growth control. MIC was defined as the lowest concentration of the drug that resulted in no visual growth after 24 h of incubation at 35°C. Fungal isolates were categorized as susceptible, intermediate, or resistant according to the susceptibility breakpoints for antifungals based on CLSI criteria (49–52). The MIC experiments were performed in triplicate.

***In vitro P. carinii* and *P. murina* ATP assays.** MYC-053 was diluted directly in the culture medium (0.1, 1, 10, and 50 μg/ml). The culture medium was RPMI 1640 containing 20% horse serum, 1% minimum essential medium (MEM)-vitamin solution, 1% MEM-nonessential amino acids (NEAA), 200 U/ml penicillin, and 0.2 mg/ml streptomycin (Sigma-Aldrich). The medium alone and medium containing 10 μg/ml ampicillin (Sigma-Aldrich) were the negative controls. Medium supplemented with 1 μg/ml pentamidine isethionate was the positive control. Cryopreserved and characterized *P. carinii* strains isolated from rat lung tissue and *P. murina* strains isolated from mouse lung tissue were distributed into triplicate wells of 48-well plates (final volume, 500 μl; final concentrations, 5×10^7 nuclei/ml for *P. carinii* and 5×10^6 nuclei/ml for *P. murina*). The controls and diluted compounds were added to the cultures and incubated at 35°C under 5% CO_2. After 24, 48, and 72 h, 10% of the well volume was removed, and ATP content was determined using the ATP-Lite luciferin-luciferase assay (PerkinElmer, Waltham, MA). The ATP-associated luminescence was determined using a spectrophotometer (POLARstar Optima; BMG-Labtech, Germany). Each sample was examined microscopically on the final day of the assay to rule out the presence of bacteria. A quench control assay to determine compound interference in the luciferin/luciferase reaction was negative at all tested concentrations. Background luminescence was subtracted, and triplicate well readings were averaged. For each time point, the percent reduction in ATP content in all groups was calculated as follows: [ATP medium control − (ATP experimental/ATP medium control)] × 100. The IC_{50} was calculated using the INSTAT linear regression program (GraphPad Software, Inc., San Diego, CA). Each test was performed in triplicate.

Effect of MYC-053 on preformed fungal biofilms. A standardized *C. glabrata* or *C. neoformans* culture inoculum (200 μl; 5×10^5 CFU/ml) in RPMI 1640 was added to each well of a 96-well round-bottom polystyrene tissue culture microtiter plate (Sarstedt, Nümbrecht, Germany) (48, 53). Following 48 h of incubation at 35°C, biofilm samples were washed twice with phosphate-buffered saline to remove nonadherent cells and then exposed for 24 h to 200 μl of RPMI 1640 containing MYC-053, FLC, or CAS at concentrations equal to 1, 2, 4, 8, 16, 32, and 64 times their MICs. Untreated biofilms were used as negative controls. The number of viable fungi in the biofilm was determined by estimating the CFU

number. Briefly, to estimate the CFU number, following exposure, well contents were aspirated to prevent antimicrobial carryover, and each well was washed three times with sterile deionized water. Biofilms were scraped thoroughly, with a particular attention to well edges (27). The well contents were aspirated and placed in 2 ml of isotonic phosphate buffer (0.15 M; pH 7.2), the total fungal CFU number was determined by serial dilution and plating on Sabouraud dextrose agar (SDA), and the culture was incubated for 24 h at 35°C. Data were $\log_{10}$ transformed and were compared with the data for untreated biofilms. The MBEC values of drugs were defined as the concentrations of drug that killed 50% ($MBEC_{50}$) or 90% ($MBEC_{90}$) of yeasts in preformed 48-h-old biofilms. All assays included three replicates and were repeated in three independent experiments.

Statistical analysis. The Mann-Whitney *U* test was used to evaluate the differences between antifungal-treated and control samples. Differences at a *P* value of <0.05 were considered significant. The nonparametric paired Wilcoxon signed-rank test was employed to analyze the pre- and postchallenge differences, and a *P* value of <0.05 was considered significant.

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