



# A Systematic Screen Reveals a Diverse Collection of Medications That Induce Antifungal Resistance in *Candida* Species

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**ABSTRACT** The increasing incidence of and high mortality rates associated with invasive fungal infections (IFIs) impose an enormous clinical, social, and economic burden on humankind. In addition to microbiological resistance to existing antifungal drugs, the large number of unexplained treatment failures is a serious concern. Due to the extremely limited therapeutic options available, it is critical to identify and understand the various causes of treatment failure if patient outcomes are to improve. In this study, we examined one potential source of treatment failure: antagonistic drug interactions. Using a simple screen, we systematically identified currently approved medications that undermine the antifungal activity of three major antifungal drugs—fluconazole, caspofungin, and amphotericin B—on four prevalent human fungal pathogens—*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*. This revealed that a diverse collection of structurally distinct drugs exhibit antagonistic interactions with fluconazole. Several antagonistic agents selected for follow-up studies induce azole resistance through a mechanism that depends on Tac1p/Pdr1p zinc-cluster transcription factors, which activate the expression of drug efflux pumps belonging to the ABC-type transporter family. Few antagonistic interactions were identified with caspofungin or amphotericin B, possibly reflecting their cell surface mode of action that should not be affected by drug efflux mechanisms. Given that patients at greatest risk of IFIs usually receive a multitude of drugs to treat various underlying conditions, these studies suggest that chemically inducible azole resistance may be much more common and important than previously realized.

**KEYWORDS** *Candida*, Cdr1p, Tac1p, Upc2p, amphotericin B, antagonism, antifungal, azoles, echinocandins, resistance

Invasive fungal infections (IFIs) are estimated to kill 1.5 million people each year (1), with mortality rates remaining disturbingly high, despite the availability of three major classes of antifungal drug (1). Millions more suffer debilitating infections of the mucosal membranes or destructive subcutaneous mycoses. Worryingly, the population of susceptible individuals and the incidences of both mucosal and systemic mycoses continue to rise due to the widespread use of broad-spectrum antibacterial agents, cancer chemotherapies, and the immune suppressive regimes necessary for organ transplantation, as well as the ongoing HIV and type 2 diabetes pandemics (2–6).

The azoles remain the most important and widely used class of antifungal treatment, acting through the inhibition of ergosterol biosynthesis. The polyenes, such as amphotericin B, damage the plasma membrane through direct interactions with ergosterol (7), while the members of the newest class of antifungals, the echinocandins,

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disrupt cell wall biosynthesis through inhibition of  $\beta$ 1,3 glucan synthase (8). The increasing incidence of acquired fungal resistance, as well as the emergence of species with intrinsic resistance to each of these agents (9), undoubtedly contributes to the high frequency of treatment failure for IFIs. However, as many as two-thirds of clinical treatment failures occur in the absence of microbiological drug resistance (10–12). A variety of factors, such as inadequate drug distribution to the site of infection or the severity of a patient's immune dysfunction, have been proposed to contribute to this phenomenon—however, little evidence is available in support of these arguments.

One factor that has been largely overlooked is the influence of other medications taken by the patient on the fungal pathogen itself. This is especially pertinent when one considers the fundamental similarity of fungal cells to those of their mammalian hosts. As eukaryotic organisms, fungi and mammals share similar cell biology and highly conserved signaling pathways. Accordingly, drugs designed to induce a physiological or biochemical response in humans may stimulate a similar response within fungal cells. These similarities have been previously exploited as the basis to identify existing drugs with antifungal activity or that enhance the activity of currently approved antifungals (13–16). However, there has not been a reciprocal effort to systematically identify pharmacotherapies that diminish the efficacy of antifungal drugs. Moreover, the influence of each coadministered medication on the clinical effectiveness of antifungal therapy remains largely unknown. This is important to consider given that the number of individuals within the population receiving more than one medication has grown dramatically in recent years (17, 18), and those at greatest risk of developing life-threatening fungal infections usually receive a multitude of drugs to treat a variety of underlying conditions (19). Recently, we screened a small collection of 129 oncology-related drugs and identified several that decreased the *in vitro* antifungal activity of the azoles on three of the most prevalent human fungal pathogens: *Candida albicans*, *Candida glabrata*, and *Aspergillus fumigatus* (20). Others have also noted individual drugs that oppose or antagonize the antifungal activity of the azoles (21, 22). The purpose of this study was to conduct a more comprehensive and systematic investigation of antifungal antagonism using a broader cross-section of approved medications in combination with all three antifungal drug classes. These screens revealed that a multitude of approved medications can induce antifungal resistance in *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*. Finally, we have established one of the major mechanisms by which many of these antagonistic compounds exert their effect.

## RESULTS

**A systematic screen reveals that a variety of approved medications antagonize the antifungal activity of fluconazole.** To identify approved medications that undermine the efficacy of currently approved antifungal drugs, we conducted a simple screen to identify those which enhance fungal growth in the presence of inhibitory concentrations of fluconazole, caspofungin, or amphotericin B. The screens were conducted using the Prestwick collection of 1,280 compounds, ~95% of which are off-patent drugs approved by the FDA or other agencies, and *C. albicans* SC5314 (23), *C. glabrata* ATCC 2001 (24), *C. parapsilosis* CDC317 (25), or *C. tropicalis* MYA3404 (26). The MIC of each antifungal drug was determined for each strain using the standard CLSI broth microdilution protocol (see Table S1 in the supplemental material) (27). The antagonism screens were then set up in 96-well plates with RPMI medium containing approximately 2 $\times$  to 8 $\times$  the MIC of fluconazole, caspofungin, or amphotericin B and 10<sup>3</sup> cells/well—conditions similar to those established by the CLSI. To ensure that the majority of drugs in the collection were provided at or above pharmacologically relevant concentrations, each library compound was added to a final concentration of 5  $\mu$ M. Control wells contained either solvent alone (minus drug control) or the antifungal agent alone. After 24 h of incubation at 35°C, growth was quantified as optical density at 600 nm (OD<sub>600</sub>). Compounds that restored fungal growth in the presence of the antifungal drug (to  $\geq$ 50% of the level seen with the untreated control) were called hits.

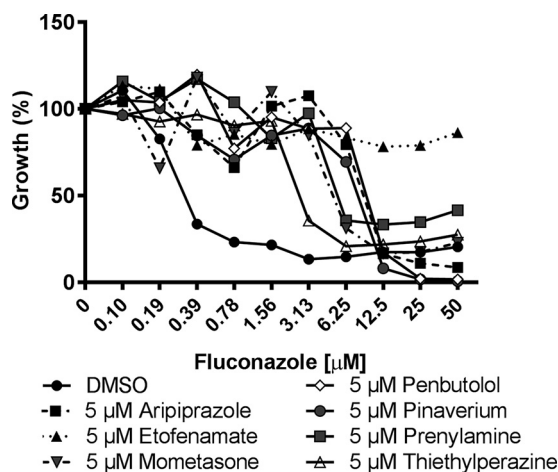
**TABLE 1** Agents from the Prestwick collection that suppress the antifungal activity of fluconazole on *Candida albicans* or *Candida glabrata*<sup>a</sup>

Screen	Compound	Growth restoration (% of untreated control)
<i>C. albicans</i> , 5 $\mu$ M fluconazole	Untreated	100
	Fluconazole alone	0
	Fendiline hydrochloride	132
	Methiothepin maleate	122
	Hexetidine	115
	Prenylamine lactate	83
	Mometasone furoate	121
	Etofenamate	102
	Dimethisoquin hydrochloride	58
	Pinaverium bromide	131
	Halofantrine hydrochloride	119
	Penbutolol sulfate	109
	Thiethylperazine dimalate	70
	Toltrazuril	63
Aripiprazole	122	
<i>C. glabrata</i> , 100 $\mu$ M fluconazole	Untreated	100
	Fluconazole alone	0
	Parthenolide	61
	Estramustine	58

<sup>a</sup>The Prestwick collection, containing a variety of FDA-approved drugs, was screened with each compound at a final concentration of 5  $\mu$ M to identify those which restore the growth of either *C. albicans* or *C. glabrata* in the presence of inhibitory concentrations of fluconazole. Hits were defined as compounds that restored fungal growth to at least 50% of level seen with the untreated control wells.

Thirteen compounds were identified that promoted *C. albicans* growth in the presence of 5  $\mu$ M fluconazole (~8-fold MIC) (Table 1). These hits encompass a wide array of therapeutic classes and do not appear to share significant structural or functional similarities. Seven compounds were selected for confirmation and follow-up analysis, based primarily on the prevalence of their clinical use and the likelihood of coadministration with fluconazole. This confirmed that the addition of any of the 7 compounds at a concentration of 5  $\mu$ M resulted in an increase in the concentration of fluconazole required to inhibit *C. albicans* growth. The MIC of fluconazole increased approximately 8-fold in the presence of the antiemetic thiethylperazine and >128-fold in the presence of the nonsteroidal anti-inflammatory drug (NSAID) etofenamate (Fig. 1). The other 5 compounds, including the antipsychotic aripiprazole, the beta blocker penbutolol, and the corticosteroid mometasone, induced intermediate (16-fold to 32-fold) levels of resistance. Full checkerboard assays revealed that 5 of the 7 compounds tested antagonized fluconazole's antifungal activity at concentrations of <1  $\mu$ M (Fig. 2). Additional checkerboard assays revealed that all 7 compounds antagonized the antifungal activity of both voriconazole and itraconazole to similar extents (see Fig. S1 and S2 in the supplemental material), confirming that these interactions are not specific for fluconazole. Interestingly, when the same screen was conducted with 1  $\mu$ M fluconazole (~2-fold MIC), a total of 88 hits were found to restore *C. albicans* growth (Table S2). This suggests that a much larger number of approved medications may have a subtler effect on *C. albicans* susceptibility to fluconazole. The same screen was performed with *C. parapsilosis* and 25  $\mu$ M fluconazole (~4-fold MIC) as well as with *C. tropicalis* and 1.6  $\mu$ M fluconazole (~4-fold MIC), yielding 29 and 98 hits, respectively (Table S3 and Table S4). While the list of fluconazole antagonists identified was unique for each species, significant overlap was also observed (Fig. 3).

We next conducted the same screen with *C. glabrata* in the presence of 100  $\mu$ M fluconazole (~4-fold MIC). Just two compounds were identified that restored growth to at least half the level observed in the untreated control—the steroidal estrogen and alkylating antineoplastic agent estramustine and parthenolide, which exhibits a variety of biological activities. Dose-response experiments confirmed that both compounds



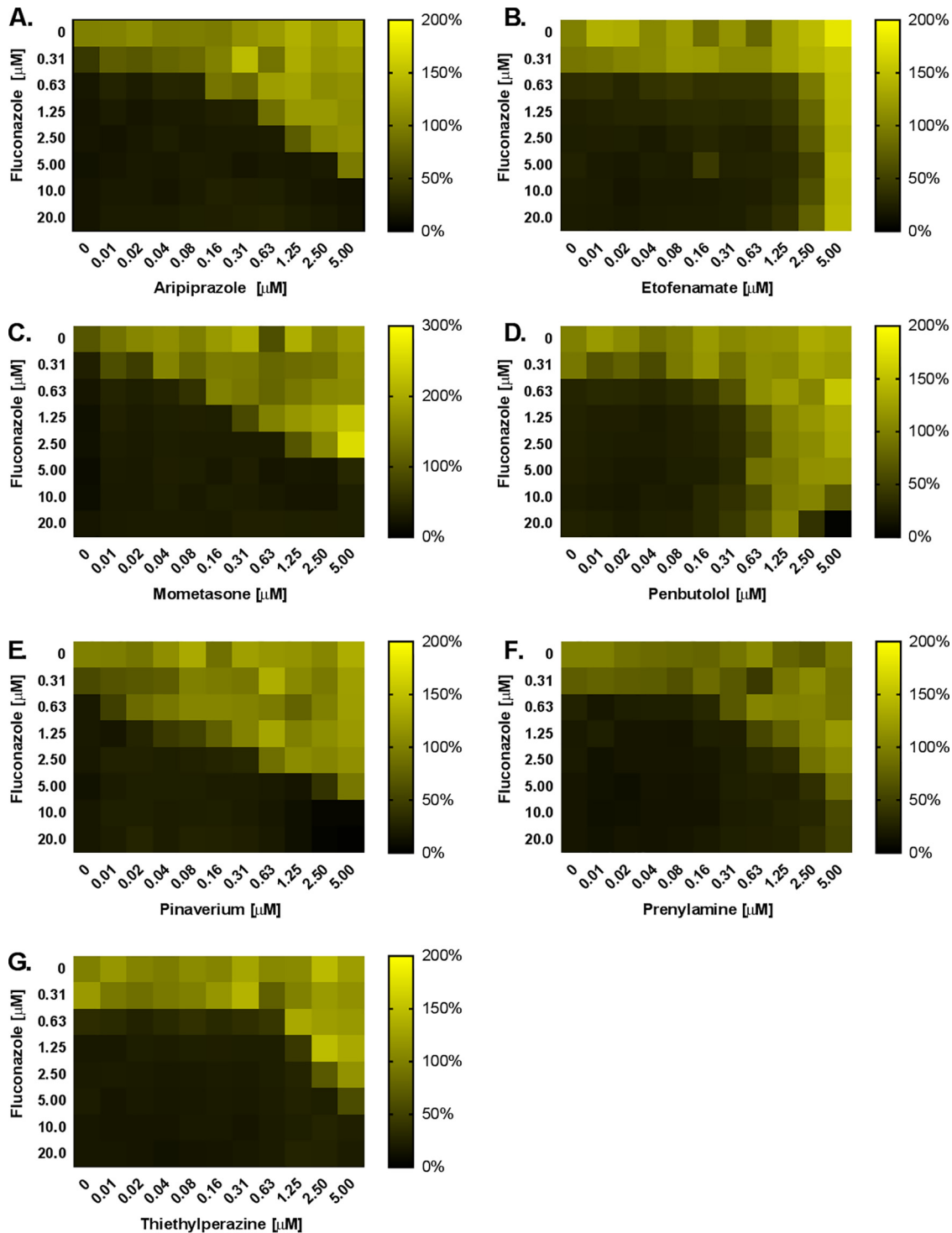
**FIG 1** A variety of approved medications substantially diminish *Candida albicans* susceptibility to fluconazole. *C. albicans* (strain SC5314) susceptibility to fluconazole was tested in the absence (DMSO vehicle alone) or presence of 5 μM of the indicated compounds in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as OD<sub>600</sub> after 24 h of incubation at 35°C, and data are presented as percent growth relative to the drug-free control. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

reduce fluconazole's antifungal activity on *C. glabrata*, increasing the MIC by ~8-fold (Fig. 4).

Similar antagonism screens were performed with the reference strains of each species in the presence of either 4-fold the MIC of caspofungin or 4-fold the MIC of amphotericin B. Just one compound—the topical antifungal amorolfine—restored *C. albicans* growth (to ≥50% that of the minus drug control) in the presence of caspofungin, while none restored growth of *C. parapsilosis*, *C. tropicalis*, or *C. glabrata* (Table 2). Two compounds—the antipsychotic haloperidol and ifenprodil—were identified as partially restoring the growth of *C. albicans* in the presence of amphotericin B (Table 2), but again, none restored the growth of *C. parapsilosis*, *C. tropicalis*, or *C. glabrata*. Dose-response experiments confirmed that amorolfine and haloperidol modestly increased the concentrations of caspofungin and amphotericin B, respectively, required to inhibit the growth of *C. albicans* (Fig. 5).

**Zinc-cluster transcription factors facilitate azole-antagonistic interactions in both *C. albicans* and *C. glabrata*.** In an effort to define the mechanisms underlying the antagonistic interactions observed with fluconazole, dose-response experiments were performed with a panel of *C. albicans* gene deletion strains lacking known mediators of antifungal resistance. This included three zinc-cluster transcription factors: Mrr1p, which activates expression of the major facilitator protein encoded by *MDR1* (28); Tac1p, which activates the transcription of the *CDR1* and *CDR2* genes encoding ABC family multidrug transporters (29); and Upc2p, which activates the transcription of several genes in the ergosterol biosynthetic pathway (30). An *mrr1Δ/Δ* mutant exhibited the same patterns of fluconazole antagonism as those previously described for the wild-type strain, with all seven of the compounds tested (data not shown), indicating it has little or no relevant role. However, with the *tac1Δ/Δ* mutant, fluconazole antagonism was largely or completely lost for 6 of the 7 compounds. This indicates that the observed antagonism is dependent on Tac1p-mediated responses (Fig. 6 and Table 3). Only etofenamate was able to antagonize the activity of fluconazoles upon the *tac1Δ/Δ* mutant, suggesting that it acts through a distinct, Tac1p-independent mechanism. However, upon being tested with the *upc2Δ/Δ* mutant, the antagonism was completely abolished, indicating that etofenamate antagonizes fluconazole activity in a Upc2p-dependent manner (Fig. 6 and Table 3).

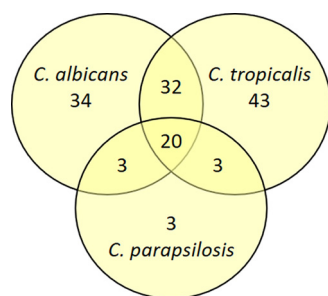
To confirm that the Tac1p-dependent antagonistic interactions occur through activation of *CDR1* and *CDR2* transcription, wild-type *C. albicans* was grown with aripipra-



**FIG 2** Several antagonistic compounds elevate *Candida albicans* fluconazole resistance at submicromolar concentrations. Checkerboard assays were performed in RPMI medium with *C. albicans* (strain SC5314) across a range of fluconazole doses and of (A) aripiprazole, (B) etofenamate, (C) mometasone, (D) penbutolol, (E) pinaverium, (F) prenylamine, and (G) thiethylperazine. After 24 h of incubation at 35°C, the growth in each well was quantified as OD<sub>600</sub>, normalized to the untreated control well, and data are presented as heat maps. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

zole or etofenamate alone or in combination with fluconazole and the relative abundances of each gene's transcripts were compared.

Treatment with 5  $\mu\text{M}$  aripiprazole, either alone or in the presence of fluconazole, resulted in a dramatic induction of both *CDR1* and *CDR2*, consistent with Tac1p



**FIG 3** There is significant overlap in the azole antagonistic compounds from the *C. albicans*, *C. parapsilosis*, and *C. tropicalis* screens. The number of common and distinct compounds identified as hits from the fluconazole screens with these three species at 1, 25, and 1.6  $\mu\text{M}$ , respectively, is illustrated in the Venn diagram.

activation. As expected, treatment with 5  $\mu\text{M}$  etofenamate, which induces fluconazole resistance in a Tac1p-independent manner, did not elevate the levels of either transcript (Fig. 7).

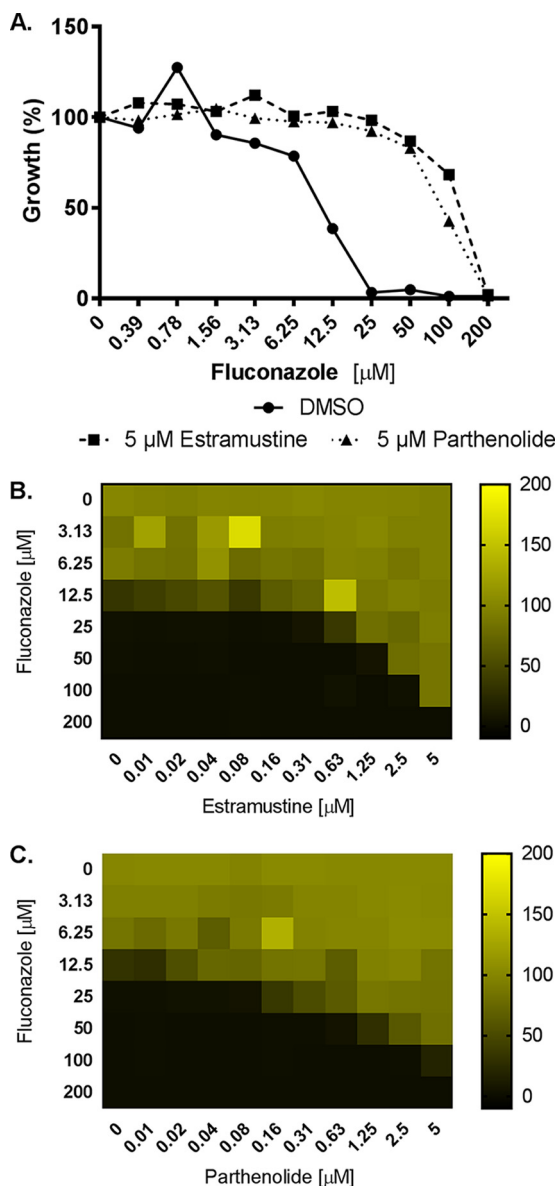
In *C. glabrata*, Pdr1p—the functional paralog of Tac1p—activates the transcription of *PDR5*, a gene encoding an ABC type multidrug transporter that is a major driver of azole resistance in this species (31). Fluconazole dose-response experiments were performed with a *C. glabrata pdr1* $\Delta$  mutant in the presence or absence of both parthenolide or estramustine. Antagonism was significantly reduced compared to the levels seen with the wild-type strain for both compounds (Fig. 8 and Table 4), indicating that the Pdr1p transcription factor is required. In contrast, both estramustine and parthenolide antagonized the effect of fluconazole's antifungal activity on a *C. glabrata upc2a* $\Delta$  mutant, indicating that these interactions were not facilitated by the Upc2a transcription factor.

**Induced and genetically encoded mechanisms can act in concert to elevate azole resistance.** Azole resistance in *Candida* is usually a multifactorial trait, with multiple genetically encoded mechanisms required to confer clinically relevant levels of azole resistance (32, 33). We therefore determined if the increase in fluconazole MIC induced by the aforementioned medications would be able to further elevate resistance in strains possessing a single, genetically encoded mechanism of resistance. We first compared the effects of the different drugs on a strain harboring an activated allele of *UPC2* (34). As expected, the *UPC2<sup>G648D</sup>* allele elevated the MIC of fluconazole by 8-fold compared to the isogenic control (Fig. 9). The addition of any of the 7 azole-antagonistic compounds tested further elevated the MIC of the *UPC2<sup>G648D</sup>* strain (Fig. 9), demonstrating that they can make an independent and additive contribution to azole resistance. A similar pattern of results was observed with a strain harboring an activated allele of *MRR1* (35). However, as expected, the effects of the six Tac1p-dependent antagonistic drugs were much less pronounced in a strain harboring an activated allele of *TAC1* (Fig. 9). These data are again consistent with these six compounds acting through activation of the Tac1p transcription factor.

## DISCUSSION

Reports have suggested that as many as one-third of all patients with disseminated *Candida* infections fail to respond to antifungal therapy (10, 36–39). Both intrinsic and acquired mechanisms of antifungal drug resistance are known to reduce the chances of successful treatment of IFIs (11). However, on the basis of *in vitro* susceptibility testing, a substantial proportion of treatment failures occur in patients infected with antifungal-sensitive isolates. In addition, many infections involving apparently drug-resistant fungi do in fact respond to treatment (40). In fact, data from clinical trials indicate response rates of 60% to 81% with fluconazole for patients with disseminated *Candida* infections caused by isolates with *in vitro* MICs of  $\leq 8 \mu\text{g/ml}$ , i.e., that are deemed susceptible according to current clinical breakpoints (10). Several host-related





**FIG 4** Estramustine and parthenolide elevate *Candida glabrata* fluconazole resistance. (A) The susceptibility of *C. glabrata* (strain ATCC 2001) to fluconazole was tested in the absence (DMSO vehicle alone) or presence of 5  $\mu\text{M}$  estramustine or parthenolide in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as OD<sub>600</sub> after 24 h of incubation at 35°C, and data are presented as percent growth relative to the drug-free control. (B and C) Checkerboard assays were performed in RPMI medium with *C. glabrata* (ATCC 2001) across a range of fluconazole doses and of estramustine (B) and parthenolide (C). After 24 h of incubation at 35°C, the growth in each well was quantified as OD<sub>600</sub>, normalized to the untreated control well, and data are presented as heat maps. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

factors have been proposed to explain the discordance between the results of *in vitro* susceptibility tests and patient outcomes (10).

First, any factor that impacts an antifungal drug pharmacokinetics, including the efficiency of drug distribution and peak concentrations at the site of infection as well as the duration over which supratherapeutic concentrations are maintained, is likely to be a critical determinant of a patient’s response to therapy (41, 42). Certainly, subtherapeutic concentrations of the antifungal drug at the site of infection could explain unexpected treatment failures in those afflicted by a susceptible fungus. To some extent, these factors may be controlled through dosing schedules and potentially the route of antifungal administration. Nonetheless, some tissues pose specific challenges

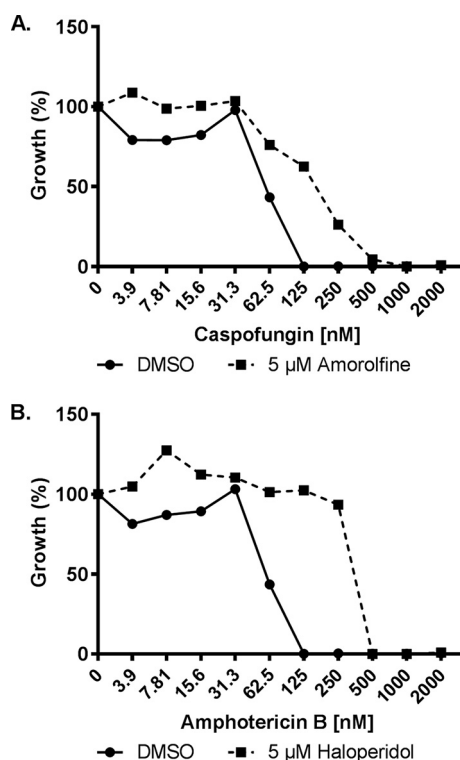
**TABLE 2** Agents from the Prestwick collection that suppress the antifungal activity of caspofungin or amphotericin B on *Candida albicans*<sup>a</sup>

Screen	Compound	Growth restoration (% of untreated control)
0.5 $\mu$ M caspofungin	Untreated	100
	Caspofungin alone	0
	Amorolfine	58
0.5 $\mu$ M amphotericin B	Untreated	100
	Fluconazole alone	0
	Haloperidol	52
	Ifenprodil tartrate	63

<sup>a</sup>The Prestwick collection, containing a variety of FDA-approved drugs was screened with each compound at a final concentration of 5  $\mu$ M, to identify those which restore the growth of *C. albicans* in the presence of inhibitory concentrations of caspofungin or amphotericin B. Hits were defined as compounds that restore fungal growth to at least 50% of the untreated control wells.

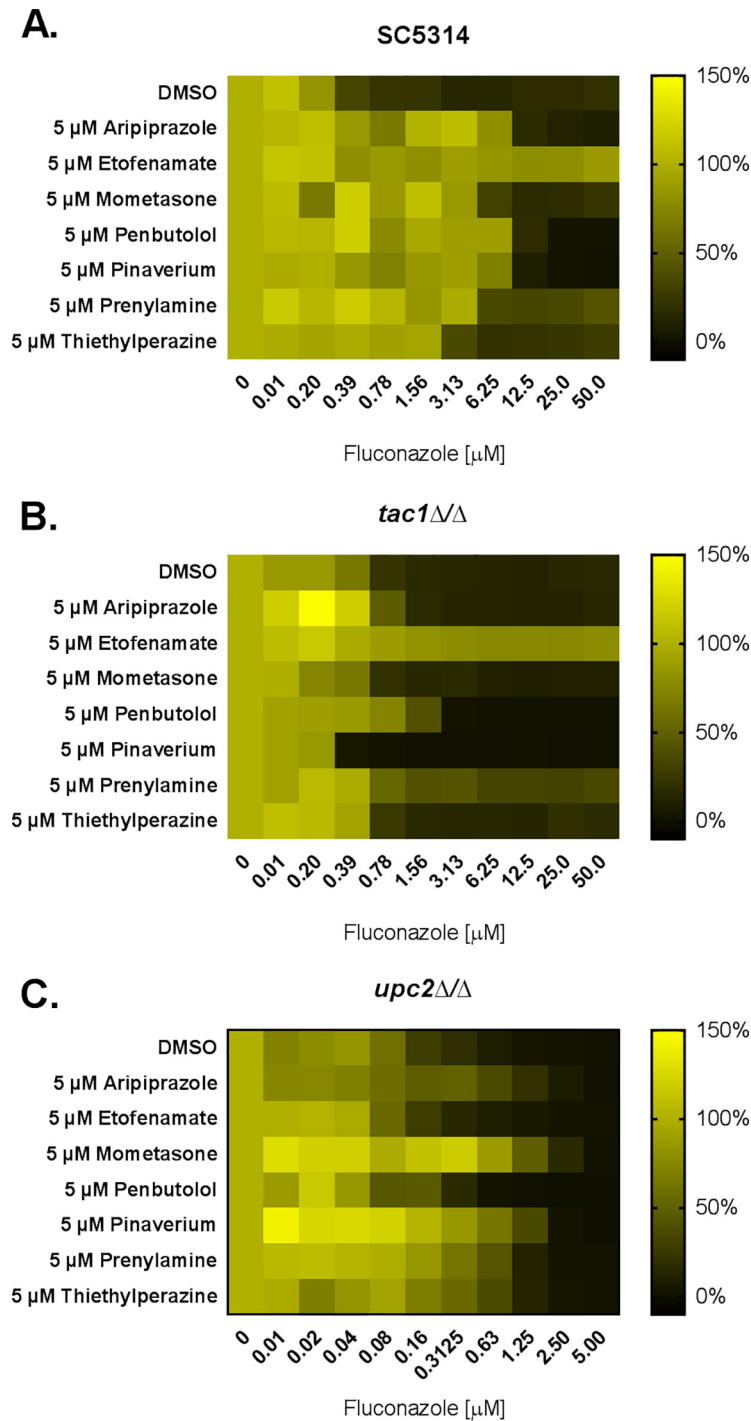
for a given class of antimicrobial, and pathology induced by the invading microbe (e.g., necrosis) may also restrict drug penetration to foci of infection. Additionally, the host's genetics, age, physiological status (e.g., renal or hepatic function), underlying comorbidities, and drug-drug interactions may directly or indirectly impinge upon the pharmacokinetics of an antimicrobial drug and thus, ultimately, its clinical efficacy.

The severity of patient immunosuppression may also have an impact upon the response to antifungal therapy. Patients experiencing severe immunosuppression may be less likely to respond to treatment, especially with fungistatic drugs such as the azoles, and the response may depend in part upon host immune system-mediated clearance. However, it has been difficult to find unequivocal evidence in support of this



**FIG 5** Amorolfine and haloperidol reduce *Candida albicans* susceptibility to caspofungin and amphotericin B, respectively. *C. albicans* (strain SC5314) susceptibility to caspofungin (A) or amphotericin B (B) were compared in the absence (DMSO vehicle alone) or presence of 5  $\mu$ M amorolfine (A) and haloperidol (B) in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as OD<sub>600</sub> after 24 h of incubation at 35°C and is presented as percent growth relative to the drug-free control. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.





**FIG 6** The Tac1p transcription factor is a major mediator of azole-antagonistic drug interactions. The susceptibility of wild-type (SC5314) (A), *tac1Δ/Δ* (B), and *upc2Δ/Δ* (C) *C. albicans* strains to fluconazole was tested in the absence (DMSO vehicle alone) or presence of 5  $\mu\text{M}$  of the indicated compounds in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as  $\text{OD}_{600}$  after 24 h of incubation at 35°C and expressed as a percentage of growth in the drug-free control, and data are presented as heat maps. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

argument since immunosuppressed mice and immunocompetent mice with a disseminated *C. albicans* infection respond similarly to azole treatment (43). Other factors that might account for variable patient outcomes relate to the size of the infectious dose and/or the time elapsed before an appropriate antifungal is administered. Intuitively, a

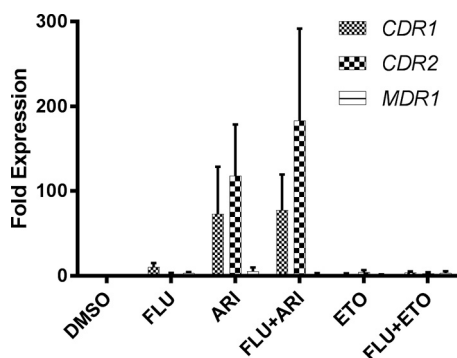
**TABLE 3** Relative levels of fluconazole resistance induced in *Candida albicans* by selected antagonistic compounds<sup>a</sup>

Compound	Fluconazole resistance		
	Strain SC5314	Strain <i>tacΔ/Δ</i>	Strain <i>upc2Δ/Δ</i>
DMSO	1×	1×	1×
Aripiprazole (5 μM)	32×	1×	>4×
Etofenamate (5 μM)	>256×	>128×	1×
Mometasone (5 μM)	16×	1×	>4×
Penbutolol (5 μM)	32×	4×	>2×
Pinaverium (5 μM)	32×	1/2×	>4×
Prenylamine (5 μM)	16×	1×	>2×
Thiethylperazine (5 μM)	8×	1×	>2×

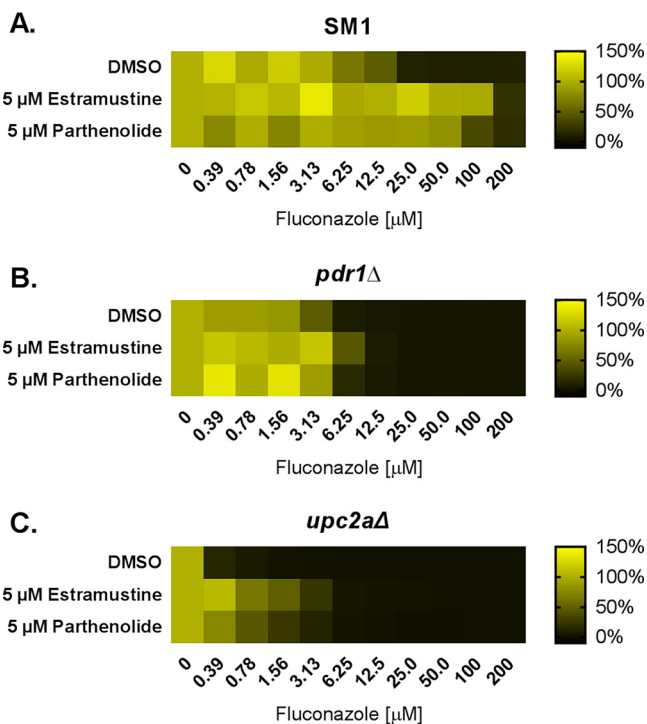
<sup>a</sup>Fluconazole susceptibility assays were performed using a modified version of the CLSI broth microdilution method for the indicated strains in the presence and absence of the seven indicated compounds at a final concentration of 5 μM. After 24 h of incubation, growth was quantified as OD<sub>600</sub>, and fluconazole MICs determined as the lowest concentration resulting in a 50% decrease in growth. The fluconazole MIC occurring with each drug treatment was then expressed relative to the fluconazole-alone control (DMSO).

more severe infection with a heavier fungal burden would be expected to have a reduced chance of therapeutic success. Similarly, more-advanced infections that have already caused significant damage to the host may also be associated with worse therapeutic outcomes. Certainly, rapid diagnosis, identification of the etiological agent, and administration of an appropriate antifungal agent can dramatically reduce the mortality rates of IFIs. Thus, while standardized antifungal susceptibility tests can be a useful predictive tool to facilitate selection of the antimicrobial agent with the greatest chance of therapeutic success, there are clearly a variety of additional factors that determine the likelihood of an effective intervention.

The results of this study have highlighted another factor that could potentially have a substantive impact upon a patient's response to antifungal therapy and ultimately help explain poor outcomes—the other medications that a patient receives. While there are a number of previous reports of antifungal antagonism, these individual interactions were usually found inadvertently and/or were restricted to testing of specific drug combinations (22, 44, 45). Notably, Henry et al. tested a small set of 16 anti-infective agents and found that both sulfadiazine and albendazole antagonized the *in vitro* activity of fluconazole on *C. albicans* to a modest degree (21). As such, the current study and our previous one (20) appear to be the only comprehensive and systematic efforts to identify this type of interaction using multiple classes of antifungal drug and with multiple pathogenic species. Our most striking finding is that antago-



**FIG 7** Aripiprazole induces transcription of the *CDR1* and *CDR2* genes in *C. albicans*. SC5314 was grown in YPD medium buffered with MOPS and adjusted to pH 7 in the presence of the indicated concentrations of drugs or of DMSO vehicle alone before RNA was isolated, and the relative abundance of the *CDR1*, *CDR2*, and *MDR1* transcripts were determined by qRT-PCR analysis. The relative abundances of these transcripts in each sample were normalized to that of the *ACT1* transcript and expressed relative to the level seen with the DMSO control (fold expression). Flu, fluconazole; Ari, aripiprazole; Eto, etofenamate. Mean and standard deviations of results from three separate experiments, each performed with three technical replicates, are shown.



**FIG 8** The Pdr1p transcription factor facilitates azole-antagonistic drug interactions in *Candida glabrata*. The susceptibility of wild-type (SM1) (A), *pdr1Δ* (B), and *upc2aΔ* (C) strains of *C. glabrata* to fluconazole was tested in the absence (DMSO vehicle alone) or presence of 5 μM of the indicated compounds in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as OD<sub>600</sub> after 24 h of incubation at 35°C, expressed as a percentage of growth in the drug-free control, and data are presented as heat maps. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

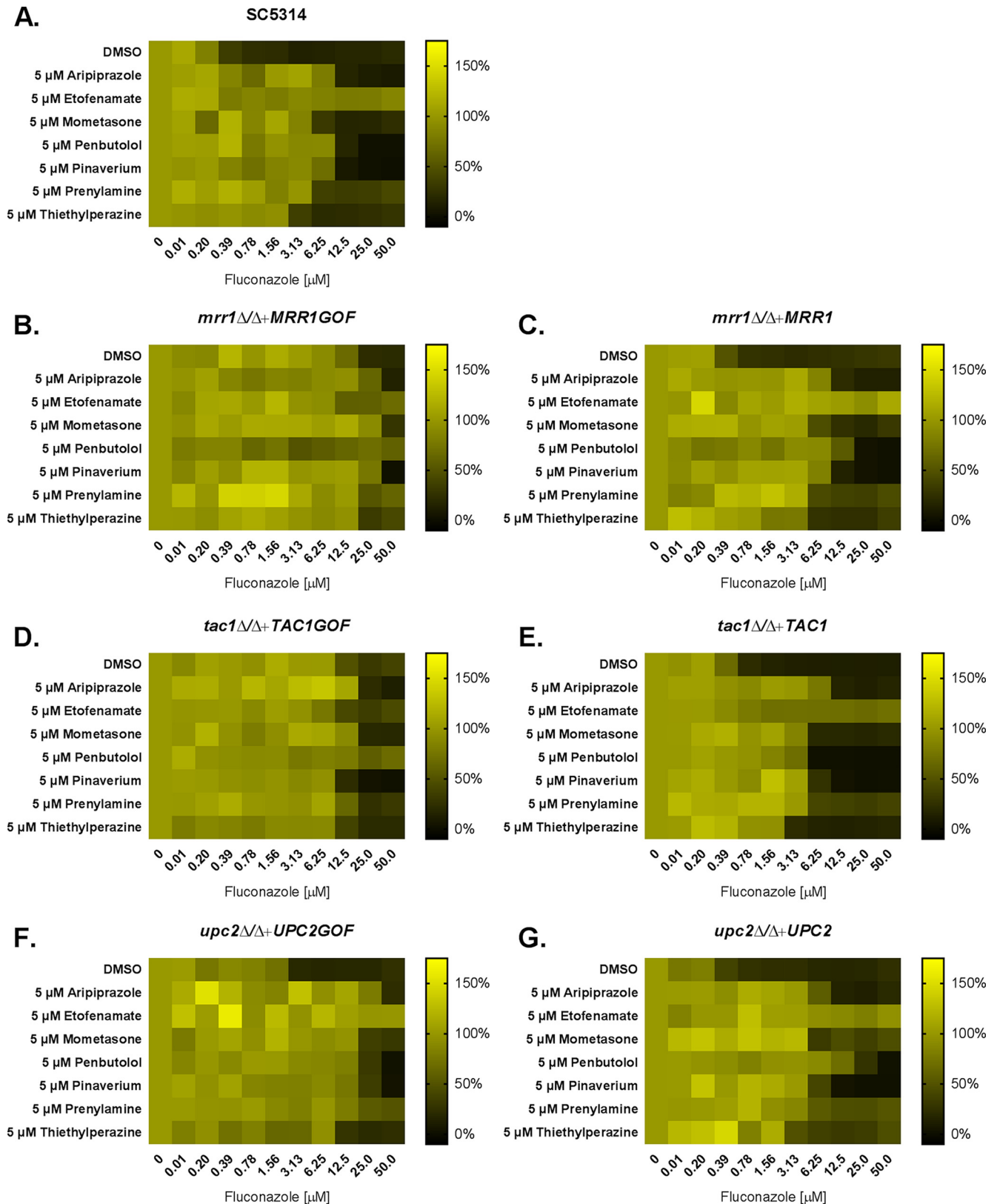
nistic interactions are much more common than previously appreciated. A staggering 139 of the 1,280 compounds in the Prestwick collection were found to induce fluconazole resistance in at least one of the four *Candida* species tested. Given that the collection used in this study is far from a complete representation of the current pharmacopeia, we anticipate that a more comprehensive screen of all approved medications would reveal an even larger number of antagonistic interactions. Thus, on the basis of the prevalence of this phenomenon, it is highly likely that one or more of the antagonistic interactions reported here are clinically relevant and reduce the efficacy of antifungal therapy.

The results of this study have provided several additional interesting insights, each of which has potentially important clinical implications. First, the compounds identified as antifungal antagonists were not obviously enriched for any one mechanistic class or indication and no common chemical features were apparent. Given the often complex and patient-specific drug regimens provided to those with IFIs, as well as the species

**TABLE 4** Relative levels of fluconazole resistance induced in *Candida glabrata* by selected antagonistic compounds<sup>a</sup>

Compound	Strain SM1	Strain <i>pdr1Δ</i>
DMSO	1×	1×
Estramustine (5 μM)	8×	2×
Parthenolide (5 μM)	8×	1×

<sup>a</sup>Fluconazole susceptibility assays were performed using a modified version of the CLSI broth microdilution method for the indicated strains in the presence and absence of the two indicated compounds at a final concentration of 5 μM. After 24 h of incubation, growth was quantified as OD<sub>600</sub>, and fluconazole MICs determined as the lowest concentration resulting in a 50% decrease in growth. The fluconazole MIC occurring with each drug treatment was then expressed relative to the fluconazole-alone control (DMSO).



**FIG 9** Drug-induced and genetically encoded mechanisms can act in an additive manner to confer azole resistance in *Candida albicans*. The susceptibility of wild-type *C. albicans* (SC5314) (A), of strains harboring activated (gain of function—GOF) alleles of *MRR1* (B), *TAC1* (D), or *UPC2* (F), or of isogenic control strains (C, E, and G) to fluconazole was tested in the absence (DMSO vehicle alone) or presence of 5  $\mu$ M of the indicated compounds in RPMI medium according to the CLSI broth microdilution protocol. Growth was quantified as OD<sub>600</sub> after 24 h of incubation at 35°C, expressed as a percentage of growth in the drug-free control, and presented as heat maps. Data represent averages of results from technical duplicates from a single experiment representative of two biological replicates.

specificity of the antagonistic medications, it would likely be extremely difficult to detect the negative consequences of such interactions through clinical observation alone. Second, notably fewer drugs were identified that antagonized either caspofungin or amphotericin B activity compared to fluconazole. This could reflect the fact that both amphotericin and caspofungin are fungicidal against *Candida* species, whereas the azoles are only fungistatic. The static nature of fluconazole's activity may afford a greater opportunity for antagonistic compounds to induce protective physiological responses in the fungus than is the case for antifungals with a rapidly cidal mode of action. In our straightforward screening protocol, the antifungal drug was essentially coadministered with the test compound. A greater number of antagonistic agents might have been detected if the fungi had been precultured with each library compound before either amphotericin or caspofungin was introduced, as this might have provided an opportunity to induce or activate protective responses that oppose these agents. Perhaps a more relevant explanation for the different frequencies with which antagonistic compounds were identified with the three classes of antifungal drug relates to the cellular location of their respective targets. Lanosterol demethylase (Erg11p) is anchored to the endoplasmic reticulum within the cell, whereas both ergosterol and  $\beta$ -(1,3)-D-glucan synthase, targets of amphotericin and caspofungin, respectively, are partially exposed at the cell surface. Furthermore, the echinocandins are believed to inhibit  $\beta$ -(1,3)-D-glucan synthase through binding to an extracellular domain (46). As such, in contrast to the azoles, neither amphotericin B nor caspofungin needs to enter the cell to engage their target. Either way, the echinocandins are not substrates of the same drug efflux pumps that confer azole resistance (47). This is significant based on our finding that the majority of the fluconazole-antagonistic compounds act through inducing the expression of drug efflux pumps that transport the antifungal out of the cell. These mechanisms are not likely to affect sensitivity to antifungals that act at the cell surface, as they would not remove these agents from their site of action. As such, we might expect antifungals which act upon intracellular targets to be more prone to antagonistic drug interactions than those acting upon cell surface targets.

Traditionally, antifungal resistance has been considered to result from either the selection of intrinsically resistant species or the acquisition of mutations that confer resistance in a previously susceptible isolate. In both instances, antifungal resistance is a genetically encoded trait that is stably inherited from one generation to the next. Several genetic mechanisms can confer azole resistance to *Candida* spp., including those which alter the expression or function of enzymes in the ergosterol biosynthesis pathway. However, the most common involve mutations that result in increased expression of the efflux pumps that actively transport the azoles out of the cytoplasm (48, 49). In *C. albicans*, gain-of-function mutations in the zinc-cluster transcription factors Mrr1p and Tac1p trigger the transcription of *MDR1* and of *CDR1* and *CDR2*, respectively, each of which encode azole efflux pumps. The results of this and previous studies (21) challenge the perception of antifungal susceptibility as a relatively fixed and genetically encoded trait and suggest that the incidence of inducible antifungal resistance may have been grossly underestimated. This may help to explain the often-poor correlation between the results of *in vitro* antifungal susceptibility testing and patient outcomes. In the simplest case, a patient receiving medications that sensitize an invading fungal pathogen to an antifungal is likely to be more responsive to therapy than a patient receiving an antagonistic medication. Thus, all medications received by the patient could potentially have a pronounced impact upon the chances of therapeutic success. It is interesting to note that many of the antagonistic drugs act through the same mechanisms as those previously described as genetically encoded determinants, i.e., zinc-cluster transcription factors. However, this does not rule out the possibility that other antagonistic compounds may act through unrelated or distinct mechanisms.

Our results are consistent with a previous study by Thakur et al. that demonstrated that *C. glabrata* Pdr1p plays a direct role in sensing compounds through a xenobiotic

binding domain, which leads to the activation of the transcription factor and the upregulation of a major efflux pump (50). That there were relatively few azole antagonistic hits common to *C. glabrata* and the other *Candida* species is somewhat surprising, especially given the significant overlap of the other three species. This suggests that there may be divergence in the specific xenobiotic detection mechanisms and/or associated substrate specificities responsible for the induction of the multidrug resistance pumps. Either way, our findings leave several unanswered questions. How can such a diverse group of xenobiotics be sensed by a single pathway? Does Tac1p/Pdr1p directly interact with and sense the antagonistic drugs, or are other ancillary proteins required? Additionally, do variants exist in genes encoding the xenobiotic-sensing proteins that alter sensitivity or substrate specificity and hence select for the medications that induce resistance for a given fungal species?

While the results described above are interesting from a biological perspective, the fundamentally important question remains—do the antifungal antagonistic interactions observed *in vitro* occur within patients, and if so, are they sufficient to affect clinical efficacy or outcomes? Since a single concentration of 5  $\mu\text{M}$  was selected for screening purposes, it is unlikely that all of the compounds identified through our screens would be antagonistic at pharmacologically relevant concentrations or under physiological conditions. However, checkerboard assays revealed that four of the seven antagonistic compounds selected for follow-up analysis were able to elevate *C. albicans* fluconazole resistance at, or close to, pharmacologically relevant concentrations. For example, plasma concentrations of  $\sim 1 \mu\text{M}$  aripiprazole, a widely used antipsychotic and mood stabilizer, can be achieved in patients, a concentration sufficient to increase the fluconazole MIC of *C. albicans* by 4-fold to 8-fold. Thus, it seems highly likely that at least a subset of the described antagonistic drugs would affect susceptibility in patients and, by extension, the chances for a successful intervention.

While drug-drug interactions have been a serious concern from the perspective of patient toxicity, the effects of most medications on fungal physiology and antifungal susceptibility have not been considered to date. A more comprehensive assessment of all approved medications with a broader variety of infectious fungi is needed to determine the full extent of this phenomenon. Furthermore, the potential clinical implications of antifungal antagonism should be thoroughly investigated using animal models of invasive fungal disease, as well as careful analysis of clinical outcomes from large patient cohorts that are appropriately controlled for and stratified according to their underlying risk factors.

## MATERIALS AND METHODS

**Growth conditions.** Strains were routinely grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. The RPMI 1640 medium (Sigma-Aldrich) used for antifungal susceptibility testing as well as for the antagonism screens was prepared according to the CLSI M27-A3 document (51) and was buffered with morpholinepropanesulfonic acid (MOPS), with pH adjusted to 7.0 using NaOH and HCl.

**Fungal strains.** *C. albicans* strain SC5314 (23), *C. parapsilosis* strain CDC317 (25), and *C. tropicalis* strain MYA-3404 (26) and isolate Ct8 and *C. glabrata* strain ATCC 2001 (24) were used throughout this study. Mutant strains used to determine the mechanism(s) of these interactions are listed in Table S5 in the supplemental material.

**Compounds.** Stock solutions of fluconazole, itraconazole, voriconazole, amphotericin B, and caspofungin (Sigma-Aldrich) and each hit compound (Selleckchem and Prestwick) were prepared at 10 mM in dimethyl sulfoxide (DMSO) and diluted as needed in the same solvent. The Prestwick library was purchased from Prestwick Chemical, with all 1,280 compounds dissolved in DMSO at 10 mM in 96-well plates. Each was further diluted in DMSO to 1 mM, and 1- $\mu\text{l}$  volumes were dispensed into the flat-bottomed 96-well polystyrene plates that were used for the chemical screens.

**Antifungal susceptibility testing.** Antifungal susceptibility testing was performed using the broth microdilution method essentially as described in CLSI document M27-A3 (51), with minor modifications. RPMI 1640 medium (Sigma-Aldrich) was prepared according to the CLSI document. Each antifungal drug (fluconazole, caspofungin, or amphotericin B) was diluted in DMSO to a final 200 $\times$  concentration before dilution in buffered RPMI medium at a final 2 $\times$  concentration. *Candida* spp. were grown overnight in YPD at 30°C and resuspended at  $1 \times 10^4$  cells/ml in RPMI medium (pH 7.0). Finally, 100  $\mu\text{l}$  of the RPMI medium containing a final 2 $\times$  drug concentration was dispensed into the wells of a flat-bottomed polystyrene 96-well plate, and 100  $\mu\text{l}$  of the cell suspension was added to each well. The final concentration of DMSO was 0.5% for all treatments, with drug-free control wells having DMSO alone. Plates were incubated without shaking for 24 h at 35°C, and then the content of each well was carefully resuspended by



pipetting up and down using a multichannel pipette. Fungal growth in each well was then quantified by measuring OD<sub>600</sub> using a Cytation 5 plate reader (Bio-Tek Instruments, Inc.), and the results were expressed as a percentage of growth in the drug-free control well.

To test the extent of antifungal antagonism of hit compounds identified from the screen, antifungal susceptibility assays were performed as described above but in the absence or presence of the hit compound at a final concentration of 5  $\mu$ M.

**Antifungal antagonism screens.** The wells of the 96-well flat-bottom assay plates were seeded with 1  $\mu$ l of the 1 mM stock solutions of each library compound in DMSO or with DMSO alone (control). *Candida* spp. were grown overnight in YPD medium at 30°C, and the cells were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), and resuspended at  $\sim 5 \times 10^3$  cells/ml in RPMI medium supplemented with the indicated concentration of fluconazole, amphotericin B, or caspofungin. A 199- $\mu$ l volume of the cell-plus-antifungal drug suspension was then dispensed into each well of the drugged 96-well plates. Control wells had antifungal drug but no test/library compound (antifungal alone) or had neither the antifungal nor the library compound (minus drug/untreated control). The final concentration of DMSO was 0.55% in all wells, with each library compound supplied at a final concentration of 5  $\mu$ M. After 24 h of incubation at 35°C, the fungal cells in each well were resuspended using a multichannel pipette and growth was measured as OD<sub>600</sub>. Wells with visible precipitation were excluded from analysis.

**RNA isolation and reverse transcription-quantitative PCR (qRT-PCR) analysis.** *C. albicans* strain SC5314 was grown in YPD medium at 30°C overnight before being subcultured into fresh YPD medium supplemented with the indicated concentrations of fluconazole, aripiprazole, and/or etofenamate or with DMSO (minus drug control) to an OD<sub>600</sub> of 0.1, incubated at 30°C with shaking for 6 h, harvested by centrifugation, and frozen at  $-80^\circ\text{C}$  on three separate days. Total cellular RNA was extracted using the hot phenol method (52). cDNA was synthesized from total RNA using a Verso cDNA synthesis kit (Thermo Scientific), in accordance with the manufacturer's instructions, and was used as the template for amplification of *ACT1*, *MDR1*, *CDR1*, or *CDR2* transcripts using Maxima SYBR green PCR master mix (ThermoScientific) in technical triplicate. Gene-specific primers were designed using the PrimerQuest Tool from Integrated DNA Technologies and are listed in Table S6. The threshold cycle ( $2^{-\Delta\Delta\text{CT}}$ ) method was used to calculate changes in gene expression among the strains (53).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00054-19>.

**SUPPLEMENTAL FILE 1**, PDF file, 2.7 MB.

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