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Flucytosine Antagonism of Azole Activity versus *Candida glabrata*: Role of Transcription Factor Pdr1 and Multidrug Transporter Cdr1

Zoë Steier,[a] John-Paul Vermitsky,[b] Geoffrey Toner,[b] Scott E. Gygax,[b] Thomas Edlind,[a] Santosh Katiyar[a]

Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, Pennsylvania, USA[a]; Femeris Women's Health Research Center, Medical Diagnostic Laboratories, Hamilton, New Jersey, USA[b]

Infections with the opportunistic yeast *Candida glabrata* have increased dramatically in recent years. Antifungal therapy of yeast infections commonly employs azoles, such as fluconazole (FLC), but *C. glabrata* frequently develops resistance to these inhibitors of ergosterol biosynthesis. The pyrimidine analog flucytosine (5-fluorocytosine [5FC]) is highly active versus *C. glabrata* but is now rarely used clinically due to similar concerns over resistance and, a related concern, the toxicity associated with high doses used to counter resistance. Azole-5FC combination therapy would potentially address these concerns; however, previous studies suggest that 5FC may antagonize azole activity versus *C. glabrata*. Here, we report that 5FC at subinhibitory concentrations antagonized the activity of FLC 4- to 16-fold versus 8 of 8 *C. glabrata* isolates tested. 5FC antagonized the activity of other azoles similarly but had only indifferent effects in combination with unrelated antifungals. Since azole resistance in *C. glabrata* results from transcription factor Pdr1-dependent upregulation of the multidrug transporter gene *CDR1*, we reasoned that 5FC antagonism might be similarly mediated. Indeed, 5FC-FLC antagonism was abrogated in *pdr1*Δ and *cdr1*Δ strains. In further support of this hypothesis, 5FC exposure induced *CDR1* expression 6-fold, and this upregulation was Pdr1 dependent. In contrast to azoles, 5FC is not a Cdr1 substrate and so its activation of Pdr1 was unexpected. We observed, however, that 5FC exposure readily induced petite mutants, which exhibit Pdr1-dependent *CDR1* upregulation. Thus, mitochondrial dysfunction resulting in Pdr1 activation is the likely basis for 5FC antagonism of azole activity versus *C. glabrata*.

Candida albicans and related yeasts are typically present in low numbers among the normal mucosal flora, but antibiotic exposure or various immunologic abnormalities can lead to its overgrowth, resulting in oral or vaginal thrush. Immunocompromised individuals are also at risk for life-threatening invasive candidiasis; indeed, *Candida* species are the fourth-most-common cause of nosocomial bloodstream infection (1, 2). These infections are generally treated with oral or intravenous fluconazole (FLC), a triazole that, like the imidazoles that preceded it, inhibits the heme-dependent enzyme sterol 14α-demethylase in the ergosterol biosynthesis pathway. Additionally, more-recently introduced triazoles include itraconazole, voriconazole, and posaconazole.

In recent years, *Candida glabrata* has emerged as the second-most-common agent of candidiasis, trailing only *C. albicans* (2). Its emergence parallels the introduction and wide-spread clinical use of triazoles. In contrast to *C. albicans*, which typically exhibits an FLC MIC of ≤0.5 μg/ml, *C. glabrata* exhibits intrinsically low FLC susceptibility, with MICs of 8 to 16 μg/ml (3). Furthermore, under selective pressure, *C. glabrata* readily mutates to FLC resistance, with MICs of ≥64 μg/ml. Nearly all such mutants characterized to date have gain-of-function mutations in transcription factor Pdr1 that result in upregulated expression of multidrug transporter genes, particularly *CDR1* (4–7). In light of *C. glabrata*'s poor response to azoles, echinocandins are now recommended as first-line agents for *C. glabrata* infection (8). However, echinocandins must be administered intravenously, as is also the case with amphotericin B, which greatly restricts and complicates their use.

The pyrimidine analog 5-fluorocytosine (flucytosine [5FC]) can be administered orally or intravenously and exhibits high activity versus *C. glabrata*, with MICs of ≤0.06 μg/ml (9). However, 5FC is now rarely used clinically, primarily due to concerns over resistance associated with monotherapy and, a related concern, toxicity associated with the high doses often employed to overcome resistance (10). 5FC resistance in *C. glabrata* has been associated with loss-of-function mutations in cytosine permease (Fcy2L), cytosine deaminase (Fcy1), or uracil phosphoribosyltransferase (Fur1) (11).

In theory, 5FC-azole combination therapy represents a promising alternative to 5FC or FLC monotherapy of *C. glabrata* infection, since it would reduce the likelihood of resistance. Combinations are typically additive in their activity, or in some cases synergistic, providing further rationale for their use. For example, the combination 5FC-amphotericin B was reported to be synergistic versus *C. glabrata* (12). Since uptake is a major determinant of 5FC susceptibility (as evidenced by the resistance conferred by cytosine permease mutation), a likely explanation for this synergism is increased membrane permeability in amphotericin B-treated cells (13).

Conversely, antifungal combinations may be antagonistic. Indeed, 5FC has been reported to antagonize the activity of three different azoles versus *C. glabrata*, including FLC and the imidazoles miconazole and ketoconazole (12, 14, 15). This antagonism argues against the clinical use of 5FC-azole combination therapy.

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Address correspondence to Santosh Katiyar, santosh.katiyar@drexelmed.edu.
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On the other hand, only additive activity was reported for 5FC in combination with voriconazole and itraconazole (12, 16). The basis for these different results is unclear.

We begin here by showing that 5FC antagonized the activity of all four triazoles versus *C. glabrata*, although the converse was not observed; i.e., triazoles did not antagonize 5FC activity. Since 5FC and azoles have distinct mechanisms of both action and resistance, elucidating the mechanism behind 5FC-azole antagonism was of considerable interest. Here, we demonstrate that 5FC activity, as expected, was not affected by *pdr1* and *cdr1* mutations, which strongly affect azole susceptibility. On the other hand, 5FC-FLC antagonism was dependent on both of these genes. Consistent with this, 5FC treatment induced Pdr1-dependent *CDR1* upregulation. This is further consistent with our finding that 5FC treatment of *C. glabrata* induced petite mutants with dysfunctional mitochondria at high frequency. These petite mutants, similar to those induced by ethidium bromide (6, 17, 18), exhibit Pdr1-dependent *CDR1* upregulation and, hence, reduced FLC susceptibility, revealing the likely mechanism behind 5FC-azole antagonism.

MATERIALS AND METHODS

Media, strains, and drugs. The media used were RPMI (RPMI 1640 [Sigma-Aldrich], 0.165 M MOPS [morpholinepropanesulfonic acid] [pH 7.0], 2% dextrose) and YPD (1% yeast extract, 2% peptone, 2% dextrose). *C. glabrata* strains 66032 and 38326 were obtained from the American Type Culture Collection (Manassus, VA); all other strains were clinical isolates from diverse sources. Disruptants with *pdr1*Δ, *cdr1*Δ, and *ade2*Δ mutations in strain backgrounds 66032, BG2, and 200989 were previously described (5, 19). 5FC (Sigma-Aldrich), FLC (Pfizer), voriconazole (Pfizer), itraconazole (Jannsen), posaconazole (Merck), amphotericin B (Sigma-Aldrich), caspofungin (Merck), and terbinafine (Novartis) were dissolved in 50 or 100% dimethyl sulfoxide (diluted to ≤0.5% in all experiments) and stored at −20°C until use.

Broth microdilution assays. Log-phase cultures in the media indicated below were diluted to 3×10^3 cells/ml and aliquoted to 6 tubes. 5FC was added to final concentrations of 0, 0.002, 0.004, 0.008, 0.016, and 0.032 μg/ml. Aliquots (200 μl in row A and 100 μl in row B through H) were distributed to wells of a 96-well plate. Antifungal (e.g., FLC) was added to well A at various concentrations and serially 2-fold diluted into wells B through G; well H served as control. Plates were incubated at 35°C, and the absorbance at 630 nm was read at 24 h. The MIC was defined as the concentration inhibiting growth by ≥80% relative to the growth of the FLC-free control.

RNA analysis. Log-phase cultures in YPD (treated with 0.3 μg/ml 5FC for 0, 2, or 4 h) were aliquoted to microcentrifuge tubes, and the cells (5×10^7) pelleted and resuspended in 300 μl of 10 mM Tris, 10 mM EDTA, and 0.5% SDS. RNA was extracted by using hot SDS-phenol followed by ethanol precipitation as described previously (5), suspended in 50 μl nuclease-free water, and treated with RQ1 DNase (Promega) as recommended by the manufacturer. RNA (diluted to 50 ng/μl) was analyzed by quantitative reverse transcription (qRT)-PCR as described previously (20), using one-step qRT-PCR on the Stratagene Mx3000P QPCR system (Stratagene). Assays (25 μl) in triplicate contained 125 ng RNA, 0.6 μM each primer (CDR1F and CDR1R or ACT1F and ACT1R), 0.2 μM FAM-labeled probe, 12.5 μl Quanta one-step master mix (2×) (Quanta Biosciences), 6.5 μl water, and 0.5 μl Quanta Escript one-step reverse transcriptase. The PCR conditions consisted of an initial incubation at 50°C for 10 min and then 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 20 s and annealing/extension at 51°C for 1 min. Fluorescence acquisition was performed at the end of each cycle immediately following the annealing/extension step. Negative controls substituted water for RNA. *CDR1* expression levels were normalized to *ACT1* expression. The cycle threshold (C_T) value of *ACT1* was subtracted from that of *CDR1* to obtain a ΔC_T value, and expression relative to the wild-type expression was expressed as $2^{-\Delta\Delta CT}$.

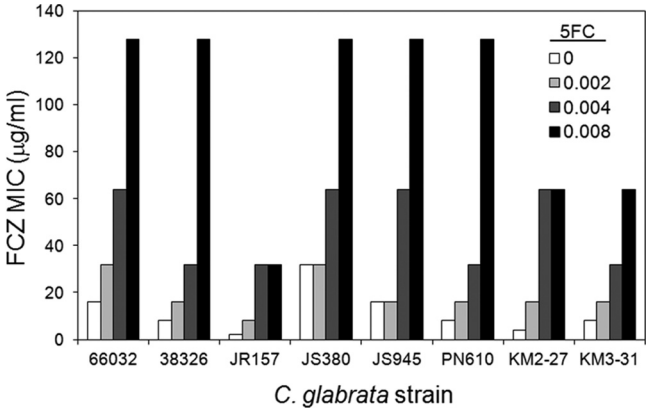

FIG 1 5FC antagonizes FLC activity versus 8 of 8 *C. glabrata* strains from diverse sources. Broth microdilution assays in RPMI medium were used to determine FLC MICs in the presence of the indicated concentrations of 5FC (μg/ml).

5FC induction and characterization of petite mutants. YPD plates containing 1 or 8 μg/ml 5FC were spread with 1×10^7 cells of strains 66032 or BG2 and incubated for 3 days. Regions with no visible colonies were streaked for isolation on drug-free YPD, yielding a mixture of petite and normal-sized colonies. To assess mitochondrial function, ca. 1,000 cells of representative colonies in 3 μl were spotted in parallel on YPD and YP-glycerol (substituting 3% glycerol for the 2% dextrose) and the plates incubated for 2 to 3 days. Loss of mitochondrial DNA was assessed by PCR using *COX1* primers 5′-AGCAACAATTTATGGAGGTTCT and 5′-GAATGAACAGCTGGTGGTGA (and ACT1F-ACT1R as a positive control), equivalent amounts of DNA (125 ng) prepared from log-phase cultures by bead beating and phenol-chloroform extraction, and 20 cycles of amplification with annealing at 56°C; products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

To determine the frequency of petite induction, equivalent log-phase cultures (1×10^6 cells/ml) of strain 200989 *ade2*Δ (19) were incubated with 0, 1, or 5 μg/ml 5FC in YPD for 24 h at 35°C with aeration. Cultures were then equivalently diluted, spread on YPD plates, and incubated for an additional 3 days before red (wild-type mitochondria) and white (petite) colonies were counted.

RESULTS

5FC specifically antagonizes azole activity versus *C. glabrata*. The interaction of FLC and 5FC was evaluated using checkerboard broth microdilution assays in RPMI medium with eight *C. glabrata* strains from diverse sources. The FLC MICs ranged from 2 to 32 μg/ml (median, 8 μg/ml). The 5FC MIC was 0.016 μg/ml for all strains. As shown in Fig. 1, in the presence of subinhibitory concentrations of 5FC, the FLC MIC increased in all eight strains. Specifically, in the presence of 0.008 μg/ml 5FC, the FLC MICs increased 4- to 16-fold (median, 8-fold). Conversely, subinhibitory concentrations of FLC had little or no effect (≤2-fold) on the 5FC MIC for all eight strains (data not shown).

Further studies were conducted with representative strains. In YPD medium, 5FC exhibits substantially reduced activity compared to its activity in RPMI, presumably due to competition with endogenous cytosine. Nevertheless, FLC activity versus the two strains tested was similarly antagonized 4- to 16-fold at subinhibitory 5FC concentrations (0.5 to 2 μg/ml). Also, 5FC antagonism of azole activity versus the three strains tested extended to vori-

TABLE 1 5FC antagonism of FLC activity is *PDR1* and *CDR1* dependent

Strain background	Genotype	Medium	MIC (μg/ml) of:	
			FLC	FLC + 5FC[a]
66032	Wild type	RPMI	16	128
	*pdr1*Δ		8	8
	*cdr1*Δ		4	8
66032	Wild type	YPD	16	128
	*pdr1*Δ		4	4
	*cdr1*Δ		2	4
BG2	Wild type	YPD	8	128
	*pdr1*Δ		4	8
	*cdr1*Δ		2	4

[a] 5FC concentrations were subinhibitory at 0.008 and 0.5 μg/ml in RPMI and YPD media, respectively.

TABLE 2 Pdr1-dependent *CDR1* upregulation following 5FC treatment and in petite mutants[a]

Strain description	5FC treatment time (h)	*CDR1* expression (fold change ± SD)
Wild type	0	1
	2	4.0 ± 1.7
	4	6.4 ± 2.7
*pdr1*Δ	0	0.11 ± 0.03
	2	0.13 ± 0.04
	4	0.09 ± 0.08
Petite	0	14 ± 5.3
	2	16 ± 3.8
	4	8.4 ± 5.1
Petite *pdr1*Δ	0	0.76 ± 0.85
	2	0.36 ± 0.28
	4	0.53 ± 0.36

[a] Log-phase cultures in YPD were treated with 0.3 μg/ml 5FC for 0, 2, or 4 h as indicated, and then RNA was extracted and analyzed for *CDR1* expression (normalized to *ACT1* and to results for untreated wild-type parent 66032).

conazole (4- to 16-fold), itraconazole (2- to 8-fold), and posaconazole (2- to 4-fold). On the other hand, 5FC had additive or indifferent effects on the activity of the structurally distinct antifungals amphotericin B, caspofungin, and terbinafine (0.5-, 1-, and 1-fold changes in MIC, respectively).

5FC antagonism of FLC activity is mediated by Pdr1 and Cdr1. To explore the basis for this 5FC-azole antagonism, we first tested mutants demonstrating resistance to one of these antifungals for cross-resistance to the other. We recently characterized 5FC-resistance-conferring mutations in *C. glabrata* (11). Representative mutants and their wild-type parent strain 66032 were tested for FLC susceptibility, and identical MICs were obtained (16 μg/ml). Similarly, 5FC activity was unaffected by *CDR1* or *PDR1* mutations known to modulate azole susceptibility (4, 5). Specifically, *cdr1*Δ and *pdr1*Δ disruptants are azole hypersusceptible (MICs of ≤8 μg/ml), while a *pdr1-F15* gain-of-function mutant is azole resistant (MIC of >128 μg/ml); all three have unaltered 5FC susceptibility (0.016 μg/ml in RPMI) compared to that of the wild-type parent 66032. These results are consistent with a previous report that 5FC does not compete with FLC for transport (21).

Conversely, 5FC antagonism of FLC activity was Cdr1 and Pdr1 dependent. Specifically, the 8- to 16-fold increase in FLC MICs in the presence of subinhibitory 5FC observed for the parent strain was decreased to ≤2-fold in its *pdr1*Δ and *cdr1*Δ derivatives (Table 1). This effect was reproduced in both RPMI and YPD medium and in two different strain backgrounds.

5FC antagonism correlates with Pdr1-dependent induction of *CDR1* expression. Previous studies in our laboratory demonstrated that diverse compounds are capable of upregulating the expression of *C. albicans* multidrug transporter genes, resulting in antagonism of azole activity versus this yeast (22). We hypothesized that 5FC antagonism of azole activity versus *C. glabrata* is mediated by an analogous mechanism; specifically, activation of Pdr1 leading to *CDR1* upregulation. To test this, *CDR1* expression was compared in log-phase YPD cultures not treated or treated for 2 and 4 h with subinhibitory (0.3 μg/ml) 5FC. Indeed, 5FC treatment resulted in 6.4-fold *CDR1* upregulation in wild-type strain 66032 (Table 2). In its *pdr1*Δ derivative, *CDR1* expression was negligible both in the absence and presence of 5FC.

5FC exposure induces petite mutants. Since 5FC is not a substrate for Cdr1 transport, it was not obvious how 5FC treatment induced *CDR1* expression, as shown above. During our studies of *C. glabrata* 5FC resistance (11), however, we noted that many of the cells representing background growth on 5FC-containing selection plates formed small colonies following transfer to drug-free plates. Their size suggested a petite (dysfunctional mitochondria) phenotype, which was confirmed by replica plating on medium containing fermentative (dextrose) or respiratory (glycerol) carbon sources (Fig. 2A). Furthermore, loss of mitochondrial DNA was demonstrated in a PCR assay targeting *COX1* (*CaglfMp04*) (Fig. 2B). These observations are consistent with studies published in 1976 of 5FC-induced petite formation in *Saccharomyces cerevisiae*, a close relative of *C. glabrata* (23).

To confirm and quantitate this observation, we extended to *C. glabrata* a method developed in *S. cerevisiae* in which the red pigmentation associated with *ade2* mutation is lost upon petite formation (24). A *C. glabrata ade2*Δ strain similarly forms red colonies (19), with a 0.4% (3 of 780) background frequency of readily distinguished white colonies following 24 h of incubation in drug-

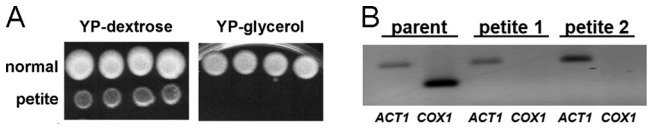

FIG 2 *C. glabrata* petite colonies induced by 5FC exposure have mitochondrial defects indicated by lack of growth on respiratory carbon source glycerol and absence of mitochondrially encoded *COX1* DNA. YPD plates containing 1 or 8 μg/ml 5FC were spread with 1×10^7 cells of strains 66032 or BG2 and incubated for 3 days. Regions with no visible colonies were streaked for isolation on drug-free YPD, yielding an apparent mixture of petite and normal-sized colonies. (A) Approximately 1,000 cells of representative colonies in 3 μl were spotted in parallel on YP-dextrose (YPD) and YP-glycerol (substituting 3% glycerol for the 2% dextrose), and the plates incubated for 2 to 3 days before being photographed. (B) DNA was prepared from two representative petite colonies and their wild-type 66032 parent and used as the template in PCRs with *ACT1* and *COX1* primers (nuclear and mitochondrially encoded, respectively). Products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

free YPD (see Fig. S1 in the supplemental material). This frequency increased to 8% (65 of 775) and 24% (185 of 765) (see Fig. S1) following incubation in 1 and 5 μg/ml 5FC, respectively, concentrations which had no detectable effect on growth under these conditions. Representative white colonies from these plates were confirmed as petites by using the replica plating assay.

5FC-induced petites exhibit *CDR1* upregulation and reduced azole susceptibility. It was previously shown that ethidium bromide-induced petite mutants of both *C. glabrata* and *S. cerevisiae* exhibit upregulated expression of the multidrug transporter gene *CDR1* or, respectively, its ortholog *PDR5* (6, 17, 18, 25). Furthermore, this upregulation is dependent on Pdr1 or, respectively, its paralog Pdr3. Similarly, a representative 5FC-induced petite mutant of *C. glabrata* exhibited pronounced *CDR1* upregulation which was fully Pdr1 dependent (Table 2).

Consistent with *CDR1* upregulation, broth microdilution assays in YPD medium of two representative 5FC-induced petite mutants of strain 66032 demonstrated 8- to 16-fold reduced susceptibilities to FLC, voriconazole, and posaconazole (MICs of 128, 4, and 4 μg/ml, respectively) but wild-type susceptibilities to amphotericin B, caspofungin, and 5FC itself (MICs of 0.5, 0.03, and 4 μg/ml, respectively). An equivalent susceptibility profile was obtained for the Pdr1 gain-of-function mutant F15, tested in parallel.

DISCUSSION

C. glabrata emerged in the azole era as a major cause of life-threatening fungal infections, potentially as a direct consequence of its intrinsically low susceptibility and high capacity for acquired resistance to this class of antifungals. *C. glabrata* is susceptible to echinocandins and amphotericin B; however, these agents require intravenous administration. Orally administered 5FC represents an attractive alternative to azoles, although its use has diminished in recent years due to concerns regarding resistance and the related concern of toxicity associated with the high doses often used to counter resistance. 5FC-azole combination therapy would in theory reduce the likelihood of resistance to either agent, but previous studies observed, albeit inconsistently, that 5FC-azole combinations are antagonistic in *C. glabrata* (12, 14, 15, 16). Here, we confirmed 5FC antagonism of azole activity and, furthermore, identified its likely mechanism.

5FC at a subinhibitory concentration antagonized FLC activity 4- to 16-fold versus all eight *C. glabrata* strains tested. It similarly antagonized the activity of other triazoles but had only indifferent effects on amphotericin B, caspofungin, and terbinafine activity. Since azole resistance in *C. glabrata* results from transcription factor Pdr1-dependent upregulation of multidrug transporter gene *CDR1*, we reasoned that 5FC antagonism might be similarly mediated. Indeed, 5FC-FLC antagonism was abrogated in *pdr1*Δ and *cdr1*Δ strains. In further support of this hypothesis, 5FC exposure induced *CDR1* expression 6-fold, and this upregulation was Pdr1 dependent. In contrast to azoles, 5FC is not a Cdr1 substrate and so its activation of Pdr1 was unexpected. We observed, however, that 5FC exposure readily induced petite mutants, which exhibit Pdr1-dependent *CDR1* upregulation. Thus, mitochondrial dysfunction resulting in Pdr1 activation is the likely basis for 5FC antagonism of azole activity versus *C. glabrata*.

Oliver and Williamson reported in 1976 that 5FC induced petite mutants of *S. cerevisiae* with high efficiency (23), and so its ability to do the same in the closely related yeast *C. glabrata* is, in retrospect, not unexpected. These authors further argued that the effect was mediated by 5-fluoro-modified RNA, rather than inhibition of dTMP synthesis. Regardless, these observations suggest that 5FC, as is the case with petite-phenotype-inducing ethidium bromide, preferentially inhibits mitochondrial versus nuclear replication. In support of this, PCR analysis of two 5FC-induced petite mutants showed loss of mitochondrially encoded *COX1*. It would be of interest to see whether specific inhibitors of mitochondrial function other than replication (e.g., respiration) similarly activate Pdr1 to lead to *CDR1* upregulation. We anticipate that this will not be the case, since studies in *S. cerevisiae* have shown that a respiratory-deficient *atp2*Δ mutant did not exhibit *PDR5* upregulation or multidrug resistance (25).

How mitochondrial dysfunction activates *C. glabrata* Pdr1 also remains to be clarified, although studies in *S. cerevisiae* suggest a potential mechanism. In *S. cerevisiae* petite mutants, *PDR5* upregulation is mediated at least in part by the retrograde (mitochondrion-to-nucleus) signaling components Rtg1, a transcription factor, and Rtg2, a component of the SLIK histone acetyltransferase complex (25, 26). Disruption of the *C. glabrata* ortholog of *RTG2* conferred modestly increased FLC susceptibility in a wild-type background (27); its effects in a petite background warrant examination.

In contrast to *C. glabrata*, for its distant relative *C. albicans*, several studies have reported that 5FC-azole combinations are additive or even synergistic (13, 14). This could reflect a reduced effect of 5FC on mitochondrial function in this yeast or, perhaps more likely, differences in retrograde signaling. Indeed, BLASTP analysis (unpublished data) revealed that *C. albicans* and related yeasts in the CTG clade (species in which CTG is translated as serine rather than leucine) lack an *RTG2* homolog, in contrast to all other ascomycetes.

In conclusion, both microbiological and, now, molecular analyses argue against the clinical use of 5FC-azole combinations in the treatment of *C. glabrata* infection. On the other hand, these data provide further impetus for the development of Pdr1 inhibitors, since these would reverse not only azole resistance but also 5FC-azole antagonism in this challenging pathogen.

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