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MECHANISMS OF RESISTANCE

# Impact of the Major *Candida glabrata* Triazole Resistance Determinants on the Activity of the Novel Investigational Tetrazoles VT-1598 and VT-1161

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**ABSTRACT** VT-1161 and VT-1598 are promising investigational tetrazole antifungals that have shown *in vitro* and *in vivo* activity against *Candida* and other fungi. *Candida glabrata* is a problematic opportunistic pathogen that is associated with high mortality in invasive infection, as well as both intrinsic and rapidly acquired antifungal resistance. The MICs of VT-1161 and VT-1598 were determined by CLSI methodology to evaluate their *in vitro* activities against clinical *C. glabrata* isolates and strains containing individual deletions of the zinc cluster transcription factor genes *PDR1* and *UPC2A* as well as the efflux transporter genes *CDR1*, *PDH1*, and *SNQ2*. Overall, both tetrazoles demonstrated relative activities comparable to those of the tested triazole antifungals against clinical *C. glabrata* isolates (MIC range, 0.25 to 2 mg/liter and 0.5 to 2 μg/ml for VT-1161 and VT-1598, respectively). Deletion of the *PDR1* gene in fluconazole-resistant matched clinical isolate SM3 abolished the decreased susceptibility phenotype completely for both VT-1161 and VT-1598, similarly to the triazoles. *UPC2A* deletion also increased susceptibility to both triazoles and tetrazoles but to a lesser extent than *PDR1* deletion. Of the three major transporter genes regulated by Pdr1, *CDR1* deletion resulted in the largest MIC reductions for all agents tested, while *PDH1* and *SNQ2* deletion individually impacted MICs very little. Overall, both VT-1161 and VT-1598 have comparable activities to those of the available triazoles, and decreased susceptibility to these tetrazoles in *C. glabrata* is driven by many of the same known resistance mechanisms.

**KEYWORDS** *Candida glabrata*, antifungal resistance, azole antifungals, tetrazole

*Candida* spp. are the fourth most commonly reported organism identified in health care-associated infections in the United States (1). *Candida glabrata* is the most common non-*albicans Candida* species in the United States and Northern Europe (2–5). Importantly, *C. glabrata* has recently seen the largest proportional increase in frequency in the United States, with estimates of mortality upwards of 50% in cases of nosocomial candidemia (6–9). While current guidelines now recommend the echinocandins as first-line agents in serious infections, such as candidemia, the azole antifungals remain essential frontline agents for treating many invasive *Candida* infections (10). However, *C. glabrata*, unlike *Candida albicans*, demonstrates inherently low susceptibility to fluconazole and can rapidly acquire resistance (6, 11, 12). Azole resistance in *C. glabrata* is primarily driven by Pdr1-dependent overexpression of the ATP-binding cassette (ABC) transporters Cdr1, Pdh1, and Snq2 (13–15). In addition, activating mutations in the zinc cluster transcription factor (ZCF) gene *PDR1* leads to constitutive overexpression of all three transporters and is largely responsible for the azole resistance seen in *C. glabrata* (16–18).

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**TABLE 1** CLSI MIC values for selected *C. glabrata* clinical isolates

| Isolate | MIC ($\mu$g/ml) for: | | | | | |
|---|---|---|---|---|---|---|
| | FLC | ITC | POS | VRC | VT-1161 | VT-1598 |
| CG26 | 64 | 2 | 1 | 2 | 2 | 1 |
| CG27 | 64 | 2 | 1 | 2 | 2 | 1 |
| CG28 | 64 | 2 | 1 | 2 | 2 | 1 |
| CG29 | 64 | 2 | 1 | 4 | 2 | 2 |
| CG30 | 32 | 1 | 1 | 2 | 0.25 | 0.5 |
| CG31 | 32 | 1 | 1 | 1 | 0.5 | 1 |
| CG32 | 64 | 2 | 1 | 1 | 2 | 1 |
| CG33 | 32 | 2 | 1 | 1 | 2 | 1 |
| CG34 | 64 | 2 | 1 | 1 | 2 | 1 |
| CG35 | >64 | 4 | 2 | 2 | 2 | 2 |

The novel tetrazole antifungal agents VT-1161 and VT-1598 have been developed to possess high specificity for fungal CYP51 compared to human CYP enzymes (19–21). Specifically, by replacing the three-nitrogen-containing triazole ring with a tetrazole moiety, the affinity for the heme group iron in CYP enzymes, and therefore of nonselective CYP inhibition, is reduced, which may potentially translate into improved drug adverse effect profiles and lower potential for drug-drug interactions. In particular, VT-1161 had previously shown to potently bind fungal CYP51 without binding the human equivalent of the fungal target enzyme, whereas VT-1598 boasts a broader spectrum of antifungal activity (20, 22). Both agents have previously been shown to be effective against *Candida* strains, including azole-resistant strains, *in vitro* and *in vivo* (23–27).

SM1 and SM3 are a matched pair of clinical isolates taken from the same patient before and during treatment with fluconazole, respectively (28). While SM1 is considered fluconazole susceptible dose dependent (≤32 $\mu$g/ml), SM3 is a fluconazole-resistant isolate possessing an activating mutation in *PDR1*, leading to constitutive overexpression of the ABC transporters Cdr1, Pdh1, and Snq2. Previously, the individual contributions of these transporters to resistance against commonly available azole antifungals were reported (29). Upc2A is a transcription factor that regulates genes of the ergosterol biosynthesis pathway and has been shown to be essential for fluconazole resistance in *C. glabrata*. The relative importance, however, of the ABC transporter genes and the azole resistance-related genes *PDR1* and *UPC2A* have not yet been determined for VT-1161 and VT-1598. In this study, we investigate the *in vitro* activities of these two tetrazoles against a collection of *C. glabrata* clinical isolates and compared them to those of fluconazole, itraconazole, posaconazole, and voriconazole. We also examine the activities of VT-1161 and VT-1598 against the matched clinical isolates SM1 and SM3 in addition to their derivative strains lacking the azole resistance genes *PDR1*, *CDR1*, *PDH1*, *SNQ2*, and *UPC2A*.

## RESULTS

***In vitro* activity of VT-1161 and VT-1598 against clinical isolates of *C. glabrata*.** Susceptibility testing was performed against a mix of fluconazole-susceptible dose-dependent and fluconazole-resistant *C. glabrata* clinical isolates. Based on 24-h CLSI broth microdilution MICs (Table 1), 3 of 10 clinical isolates of *C. glabrata* were classified as susceptible dose dependent (≤32 $\mu$g/ml), and the remaining seven isolates were considered resistant (≥64 $\mu$g/ml) to fluconazole. Against voriconazole, all isolates were equal to or above the epidemiological cutoff value of 0.5 $\mu$g/ml (30). Overall, there was little variation in the MIC distribution of the clinical isolates for each drug tested. Only the fluconazole-susceptible dose-dependent isolate CG30 showed a decreased MIC to VT-1161 (0.25 $\mu$g/ml) and VT-1598 (0.5 $\mu$g/ml) compared to those of the other clinical isolates. All clinical isolates displayed elevated MICs to each agent tested compared to that of the susceptible dose-dependent isolate SM1 (Fig. 1).

***In vitro* activity of VT-1161 and VT-1598 against the matched pair of clinical isolates SM1 and SM3 and derivative strains.** Table 2 shows the MICs of fluconazole,

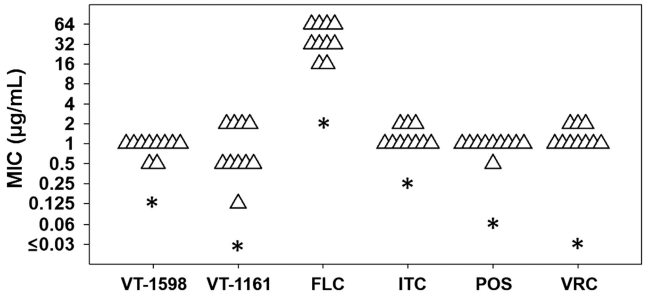


**FIG 1** Susceptibility testing for novel tetrazoles, VT-1598 and VT-1161, compared to that for a panel of triazole antifungals. Each triangle represents an isolate from a collection of fluconazole-resistant clinical isolates. The asterisks represent the fluconazole-susceptible dose-dependent clinical isolate SM1. FLC, fluconazole; ITC, itraconazole; POS, posaconazole; VRC, voriconazole.

itraconazole, posaconazole, voriconazole, VT-1161, and VT-1598 against SM1 and SM3. Not surprisingly, the fluconazole MIC against the resistant isolate SM3 (64 $\mu$g/ml) was 32-fold higher than that of the matched susceptible dose-dependent isolate SM1 (2 $\mu$g/ml) (see Fig. S1 in the supplemental material). For itraconazole, posaconazole, and voriconazole, the MICs against isolate SM3 were 8-, 32-, and $\geq$64-fold higher than their respective MICs against SM1. By comparison, the VT-1161 MIC was 32-fold higher against SM3 than that against SM1, while the VT-1598 MIC against SM3 was only 8-fold higher than the MIC against SM1.

To determine the effects of the zinc cluster transcription factor Pdr1 on tetrazole susceptibility in these isolates, we obtained MICs for the previously reported strains SM1Δ*pdr1* and SM3Δ*pdr1*, in which the *PDR1* open reading frame (ORF) was deleted (31). As shown in Fig. 1, fluconazole resistance was completely abolished in SM3 when *PDR1* was deleted. Moreover, deletion of *PDR1* resulted in identical MICs against both SM1 and SM3 backgrounds for all agents tested. The MICs of VT-1161, posaconazole, and voriconazole against both SM1Δ*pdr1* and SM3Δ*pdr1* dropped to the lowest tested concentrations ($\leq$0.015, $\leq$0.03, and $\leq$0.03 $\mu$g/ml, respectively). The MICs of VT-1598, fluconazole, and itraconazole against strains SM1Δ*pdr1* and SM3Δ*pdr1* decreased 2-fold compared to MICs against SM1.

To elucidate the mechanisms by which *PDR1* deletion increased susceptibility to VT-1161, VT-1598, and the triazoles, we tested these agents against individual deletions of the Pdr1-regulated ABC transporter genes *CDR1*, *PDH1*, and *SNQ2* in an SM1 isolate derivative, SM1RPDR1(SM3), in which the native *PDR1* open reading frame (ORF) was replaced with the *PDR1* ORF from SM3 (Fig. 2). Deletion of *CDR1* had the greatest effect on increasing triazole and tetrazole susceptibility in SM1RPDR1(SM3), in which the MIC

**TABLE 2** Twenty-four-hour CLSI MIC values for *C. glabrata* strains

| | MIC ($\mu$g/ml) for: | | | | | |
|---|---|---|---|---|---|---|
| **Strain** | **FLC** | **ITC** | **POS** | **VRC** | **VT-1161** | **VT-1598** |
| SM1 | 2 | 0.25 | 0.06 | $\leq$0.03 | 0.03 | 0.125 |
| SM3 | 64 | 2 | 2 | 2 | 1 | 1 |
| SM1PDR1(SM3) | 64 | 2 | 1 | 2 | 1 | 1 |
| SM1Δ*pdr1* | 1 | 0.125 | $\leq$0.03 | $\leq$0.03 | $\leq$0.015 | 0.06 |
| SM3Δ*pdr1* | 1 | 0.125 | $\leq$0.03 | $\leq$0.03 | $\leq$0.015 | 0.06 |
| SM1Δ*upc2A* | 1 | 0.25 | 0.125 | 0.06 | $\leq$0.015 | $\leq$0.015 |
| SM3Δ*upc2A* | 4 | 0.5 | 0.125 | 0.06 | 0.125 | 0.125 |
| S1RPS3CDR1M2A | 8 | 0.5 | 0.125 | 0.06 | 0.06 | 0.25 |
| S1RPS3PDH1M2A | 64 | 2 | 1 | 2 | 1 | 1 |
| S1RPS3SNQ2M2A | 64 | 2 | 1 | 2 | 1 | 1 |
| S1RPS3CAPDH1M2A | 2 | 0.125 | $\leq$0.03 | $\leq$0.03 | 0.03 | 0.125 |
| S1RPS3CASNQ2M2A | 4 | 0.5 | 0.06 | 0.06 | 0.06 | 0.125 |
| S1RPS3SAPDH1M2A | 64 | 2 | 1 | 1 | 1 | 1 |
| S1RPS3CAPASNQ2M2A | 0.25 | 0.125 | $\leq$0.03 | $\leq$0.03 | $\leq$0.015 | $\leq$0.015 |

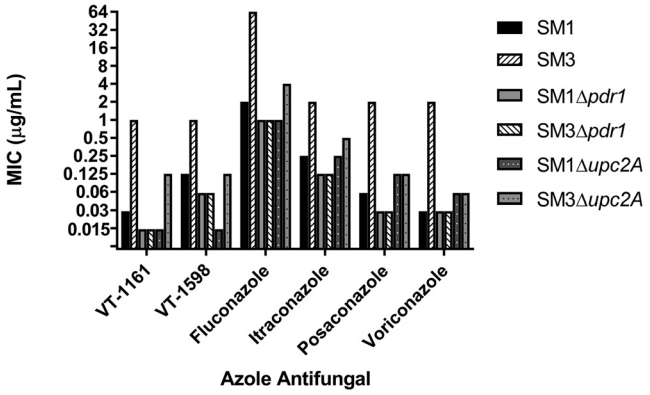


**FIG 2** Twenty-four-hour CLSI MIC comparison of strains SM1Δ*pdr1*, SM3Δ*pdr1*, SM1Δ*upc2A*, and SM3Δ*upc2A* with the matched clinical isolates SM1 and SM3 for VT-1161, VT-1598, fluconazole, itraconazole, posaconazole, and voriconazole.

decreased by 32-fold for voriconazole, 16-fold for VT-1161, 8-fold for fluconazole, posaconazole, and VT-1598, and 4-fold for itraconazole (Fig. 3). In contrast, individual deletion of *PDH1* or *SNQ2* showed no decrease in MICs against SM1RPDR1(SM3) for any antifungal agent.

Tetrazole and triazole susceptibilities were also obtained against the different combinations of ABC transporter gene deletions, as well as a strain lacking all three transporter genes. In total, we tested four additional strains containing the double deletions of *CDR1* and *PDH1* (S1RPS3CBPDH1M2A), *CDR1* and *SNQ2* (S1RPS3CASNQ2M2A), *PDH1* and *SNQ2* (S1RPS3SAPDH1M2A), and the triple null mutant for *CDR1*, *PDH1*, and *SNQ2* (S1RPS3CAPASNQ2M2A). Consistent with the susceptibilities of the individual transporter deletions, Cdr1 appears to be the major ABC transporter contributing to decreased azole susceptibility, as the deletion of *PDH1* or *SNQ2* in combination with the *CDR1* deletion reduced the MIC between 4- and 64-fold for the various triazoles and between 8- and 32-fold for the tetrazoles (see Fig. S2 in the supplemental material). By comparison, strain S1RPS3SAPDH1M2A, which lacks only the *PDH1* and *SNQ2* genes, exhibited a 2-fold reduction in MIC compared to that of SM1RPDR1(SM3) only for voriconazole. The deletion of both *PDH1* and *SNQ2* did not alter the MIC for SM1RPDR1(SM3) against VT-1161, VT-1598, fluconazole, itraconazole, or posaconazole. Removing all three transporter genes resulted in MICs equal to or less than those for the *PDR1* deletion of SM1 and SM3. For VT-1598 and fluconazole, the MIC decreased 4-fold between SM3Δ*pdr1* (0.06 and 1 μg/ml, respectively) and the triple null Δ*cdr1*/Δ*pdh1*/Δ*snq2* mutant (≤0.015 and 0.25 μg/ml, respectively). No

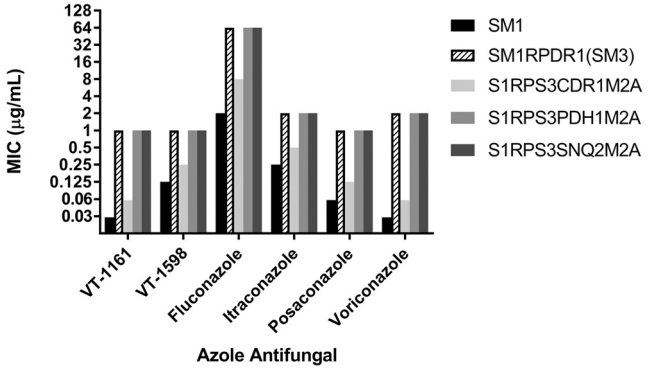


**FIG 3** Twenty-four-hour CLSI MIC comparison of the Δ*cdr1* deletion strain S1RPS3CDR1M2A, the Δ*pdh1* deletion strain S1RPS3PDH1M2A, and the Δ*snq2* deletion strain S1RPS3SNQ2M2A in background SM1RPDR1(SM3) with SM1 and SM1RPDR1(SM3) for VT-1161, VT-1598, fluconazole, itraconazole, posaconazole, and voriconazole. Strain SM1RPDR1(SM3) is an SM1 derivative in which the *PDR1* ORF of SM1 was replaced with the *PDR1* ORF of SM3.

difference in MIC was observed between the triple mutant and SM3Δ*pdr1* for VT-1161, itraconazole, posaconazole, or voriconazole. However, the VT-1161, posaconazole, and voriconazole MICs for SM3Δ*pdr1* were already at the lowest concentration tested, so it is unknown if deletion of *CDR1*, *PDH1*, and *SNQ2* would further increase susceptibility to these antifungals.

In addition to Pdr1 and the regulated ABC transporters, we also wanted to test the effects of the sterol-regulating transcription factor Upc2 on tetrazole susceptibility. Similar to the Δ*pdr1* deletion in SM1 and SM3, the deletion of *UPC2A* also resulted in reduced MICs for most of the triazoles and tetrazoles tested (Fig. 1). Compared to those of the fluconazole-resistant isolate SM3, the SM3Δ*upc2A* MIC decreased 8-fold against VT-1598, VT-1161, and itraconazole; 16-fold against fluconazole and posaconazole; and 32-fold against voriconazole. Deleting *UPC2A* in SM1 did not show as great a reduction in MIC compared to that of the parent for all azoles tested. In SM1Δ*upc2A*, the VT-1598 MIC decreased 4-fold and fluconazole and voriconazole MICs decreased 2-fold compared to that of the parent strain SM1. The itraconazole MICs did not change at all between SM1 and SM1Δ*upc2A*, and the MIC actually showed a small 2-fold increase in the SM1Δ*upc2A* strain compared to that of SM1.

## DISCUSSION

In *C. glabrata*, the vast majority of reported fluconazole resistance originates from activating mutations in the zinc cluster transcription factor Pdr1, which leads to constitutive upregulated expression of the ABC transporters Cdr1, Pdh1, and Snq2, all of which have been shown to play a role in fluconazole resistance against the species (13–15, 17, 32–34). Based on CLSI breakpoints, SM1 is susceptible dose dependent to fluconazole (≤32 μg/ml) while SM3 is resistant (≥64 μg/ml). Similarly, SM1, but not SM3, is below the most recent epidemiological cutoff values for voriconazole (≥1 μg/ml). As a result of an L946S amino acid substitution in the zinc cluster transcription factor Pdr1 of SM3, the isolate overexpresses efflux pumps Cdr1, Pdh1, and Snq2 relative to SM1, which lacks any activating mutation in *PDR1* (31).

As we might expect, SM3—and the *PDR1*$^{SM3}$-containing strain SM1RPDR1(SM3)—was less susceptible than SM1 to every agent tested, including VT-1161 and VT-1598. Due to differences in agent-specific clinical breakpoints and epidemiological cutoff values, direct comparisons of MIC values are not useful between different antifungal agents. Instead, we compared the relative change in activity between the less susceptible SM3 and SM1 for each of the tested triazoles and tetrazoles by examining the fold increase in the MIC of SM3 over SM1. For example, for voriconazole, there was a sizeable difference (≥64-fold) between its MIC against SM1 (≤0.03 mg/liter) and that against SM3 (2 mg/liter). This relative difference was markedly less for both VT-1598 and itraconazole, both of which only showed an 8-fold increase in SM3 MICs compared to SM1 MICs. Fluconazole, posaconazole, and VT-1161 both exhibited 32-fold increases in SM3 MICs compared to those of SM1. In this regard, the tetrazole compounds were comparable to many of the clinically available azoles tested here against the SM3 resistance phenotype. Similarly, the relative activity of VT-1161 and VT-1598 against the ten *C. glabrata* clinical isolates, all of which overexpress *CDR1* (data not shown), was comparable to that of the other tested triazoles. The increased MICs seen with all agents for SM3 and the clinical isolates suggest cross-resistance between the triazole and the newer tetrazole antifungals.

It had been previously shown that when comparing the relative contributions of Cdr1, Pdh1, and Snq2 to azole resistance in *C. glabrata*, deletion of *CDR1* had the largest impact on Pdr1-mediated azole resistance (29). This also held true for the tetrazoles VT-1161 and VT-1598. While the single deletion of *CDR1* in SM1RPDR1(SM3) lowered MICs by 4- to 32-fold compared to those of the wildtype for each antifungal tested, deletion of either *PDH1* or *SNQ2* resulted in little to no change (≤2-fold decrease) in MIC. Thus, for VT-1161 and VT-1598, as well as the other triazoles, Cdr1 is the key contributor of antifungal resistance in *C. glabrata*. This is supported by the fact that in SM1RPDR1(SM3) strains with combined deletions of two of the three ABC transporter

genes, the Δ*cdr1*/Δ*pdh1* and Δ*cdr1*/Δ*snq2* strains showed large decreases in MIC compared to that of the Δ*pdh1*/Δ*snq2* double deletion strain, which retained the *CDR1* gene and the resistance phenotype.

The zinc cluster transcription factors *PDR1* and *UPC2A* are also important in the potential development of resistance to VT-1161 and VT-1598 in *C. glabrata*. Earlier reports demonstrated that *UPC2A* is necessary for retaining a high-level triazole-resistant phenotype in *C. glabrata* and that deletion of *UPC2A* resulted in decreased cellular ergosterol content as well as reduced MICs to fluconazole, itraconazole, ketoconazole, and voriconazole (35). Deleting either the *PDR1* gene or the *UPC2A* gene in SM3 and SM1 appeared to reduce the MICs against the tetrazoles compared to those of the wildtype isolates. Thus, *PDR1* is important for reduced susceptibility to the tetrazoles VT-1161 and VT-1598, as has been established with the clinically available triazoles (17). Likewise, our results suggest that *UPC2A* contributes to susceptibility to the tetrazole antifungals as it does with the triazole antifungals. It should, however, be noted that increased *ERG11* and activating mutations in *UPC2A* are not known phenomena driving azole resistance in clinical isolates of *C. glabrata* (18, 36).

In a randomized, double-blind phase 2 clinical trial evaluating oral VT-1161 in treating recurrent vulvovaginal candidiasis in 215 women, VT-1161 was found to be efficacious and safe compared to placebo with recurrence rates of between 0 and 7% through 48 weeks (24). VT-1598 has been effective in murine infection models with *Candida*, *Coccidioides*, and *Cryptococcus* species (23, 37, 38). However, reduced susceptibility has been observed to both VT-1161 and VT-1598 in clinical isolates of *C. albicans*, and this reduced *in vitro* susceptibility seems to be at least partly due to increased expression of the *C. albicans* ABC transporter Cdr1 (25, 39). This parallels our results in *C. glabrata*, where Cdr1 and its regulator Pdr1 appear to play an important role in reduced susceptibility to both VT-1161 and VT-1598, more so than either Pdh1 or Snq2.

Overall, the *in vitro* activities of VT-1161 and VT-1598 against *C. glabrata* are comparable to those of many of the commercially available triazole antifungals. This in combination with the potential for an improved adverse effect profile suggests that VT-1161 and VT-1598 could be useful in cases of *C. glabrata* infection where azole treatment is appropriate. However, our findings suggest that VT-1161 and VT-1598 would not be desirable alternatives in triazole-resistant *C. glabrata* infections, given that they seem to be substrates for the same efflux pumps involved in triazole resistance. Both tetrazoles appear to be affected by the same mechanisms driving resistance to the commonly used triazoles, though further study with VT-1161 and VT-1598 is warranted to determine cutoff values and susceptibility breakpoints relevant to clinical outcomes.

## MATERIALS AND METHODS

**Strains and growth medium.** All constructed strains used in this study are listed in Table S1 in the supplemental material. The clinical isolates and the *PDR1* replacement strains have been described previously (28, 31). All strains were stored as frozen stocks at −80°C with 40% glycerol. Strains were routinely grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) broth at 30°C in a shaking incubator except as indicated for specific experimental conditions. Clinical isolates were obtained from Daniel Diekema at the University of Iowa repository.

**Construction of *C. glabrata* strain SM3Δ*pdr1*.** For strain SM3Δ*pdr1*, disruption of the *PDR1* gene was performed via the previously described *SAT1* flipper method (31, 40). Briefly, flanking sequences proximal and distal to the *PDR1* ORF were PCR amplified and cloned into the pSFS2-derived plasmid vector pBSS2 containing the *SAT1* nourseothricin selection marker and FLP recombinase. Plasmids were chemically transformed using a lithium acetate method previously described (29). Successful transformants were screened on YPD agar plates containing 200 mg/liter nourseothricin, and genomic integration was confirmed through Southern blot hybridization. Subsequent induction of the FLP recombinase was achieved through growth of the correct integrants in YPD medium for 24 h to recycle the *SAT1* flipper cassette.

**Susceptibility testing.** MICs of VT-1161, VT-1598, fluconazole, voriconazole, posaconazole, and itraconazole were measured using broth microdilution methods as described by the Clinical and Laboratory Standards Institute (41, 42). Briefly, 96-well microtiter plates containing RPMI 1640 medium (0.165 M morpholinepropanesulfonic acid [MOPS] with L-glutamine and without sodium bicarbonate, pH 7.0) with serially diluted concentrations of each azole were used to incubate strains. Drug concentrations ranged from 0.015 to 8 μg/ml for VT-1161 and VT-1598; 0.125 to 64 μg/ml for fluconazole; and 0.03 to 16 μg/ml for voriconazole, posaconazole, and itraconazole. *C. glabrata* strains and isolates were incubated at 35°C, and MIC values were determined after 24 h and interpreted as the minimum concentration

required to reduce cell growth by approximately 50% or greater compared to drug-free control wells. MICs were performed in duplicate for clinical isolates. The large majority if duplicates (264/288 or 92%) were identical or within a single dilution of each other. If the duplicate readings were different, the higher of the MICs was used in this analysis.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01304-19.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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