In vitro activities of the novel investigational tetrazoles VT-1161 and VT-1598 compared to the triazole antifungals against azole-resistant strains and clinical isolates of *Candida albicans*

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ABSTRACT

The fungal Cyp51-specific inhibitors VT-1161 and VT-1598 have emerged as promising new therapies to combat fungal infections, including *Candida* spp. To evaluate their *in vitro* activities compared to other azoles, minimum inhibitory concentrations (MICs) were determined by CLSI method for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, and posaconazole against 68 *C. albicans* clinical isolates well-characterized for azole resistance mechanisms and mutant strains representing individual azole resistance mechanisms. VT-1161 and VT-1598 demonstrated potent activity (geometric mean MICs ≤0.15 μg/mL) against predominantly fluconazole-resistant (≥8 μg/mL) isolates. However, five of 68 isolates exhibited MICs greater than six dilutions (>2 μg/mL) to both tetrazoles compared to fluconazole-susceptible isolates. Four of these isolates likewise exhibited high MICs beyond the upper limit of the assay for all triazoles tested. A premature stop codon in *ERG3* likely explained the high-level resistance in one isolate. VT-1598 was effective against strains with hyperactive Tac1, Mrr1, and Upc2 transcription factors and against most *ERG11* mutant strains. VT-1161 MICs were elevated compared to the control strain SC5314 for hyperactive Tac1 strains and two strains with Erg11 substitutions (Y132F and Y132F&K143R), but showed activity against hyperactive Mrr1 and Upc2 strains. While mutations affecting Erg3 activity appear to greatly reduce susceptibility to VT-1161 and VT-1598, the elevated MICs of both tetrazoles for four isolates could not be explained by known azole resistance mechanisms, suggesting the presence of undescribed resistance mechanisms to triazole- and tetrazole-based sterol demethylase inhibitors.

INTRODUCTION

Candida albicans is a dimorphic yeast and opportunistic pathogen that is known to cause a wide range of infections in healthy and immunocompromised patients. In the United States, *C. albicans* is the leading *Candida* species identified in oropharyngeal and vulvovaginal infections, where recurrent infections remain problematic (1-5). In more serious systemic disease such as bloodstream infections (BSI), *Candida* species collectively are the fourth-leading cause of nosocomial BSI in the United States (6). Moreover, resistance to currently available antifungal agents continues to be a problem, particularly given the relatively limited armamentarium against fungal infections (7-11). In particular, azole antifungal resistance in *Candida* spp. threatens to diminish the efficacy of arguably the most widely used antifungal drug class (12). Appropriate clinical use of available drugs on the market and eventual expansion of the antifungal arsenal is therefore paramount to safeguarding its effectiveness.

Azole antifungal resistance in *C. albicans* can be attributed to multiple mechanisms. First, efflux pump overexpression, such as the ATP-binding cassette (ABC) transporters Cdr1 and Cdr2 as well as the major facilitator transporter Mdr1, prevents drug accumulation within the yeast cell (13-16). Second, increased production of the azole target 14α-lanosterol demethylase (CYP51) can attenuate the inhibitory effects of the azoles drug class (17-19). Increases in efflux pump and drug target production is often the result of gain-of-function mutations in zinc cluster transcription factors (ZCFs) (Tac1 for *CDR1* and *CDR2*, Mrr1 for *MDR1*, Upc2 for *ERG11*) that regulate their gene expression, though polyploidy of chromosomes in the yeast genome can also result in increased expression of the genes encoding these azole-resistance determinants. Third, mutations in *ERG11* can confer azole resistance through alteration of the drug target (20-23). Lastly, alternative sterol biosynthesis as a result

of changes within the ergosterol biosynthetic pathway allows some *C. albicans* isolates to circumvent the effects of azole inhibition altogether (24-27).

VT-1161 and VT-1598 are novel tetrazole antifungal agents with high specificity for fungal CYP51 compared to human CYP enzymes (28-30), and thus may have improved adverse effect and drug-drug interaction profiles due to lesser off-target inhibition. In this study, we compare the *in vitro* activity of the novel tetrazoles VT-1161 and VT-1598 to the current triazole antifungals fluconazole, voriconazole, itraconazole, and posaconazole against a collection of clinical isolates and laboratory strains with known resistance mechanisms.

RESULTS

***In vitro* activity of VT-1161 and VT-1598 against fluconazole-susceptible and fluconazole-resistant clinical isolates.** VT-1161 and VT-1598 showed potent *in vitro* activity against 68 previously described clinical isolates of *C. albicans*, the majority (57 of 68) of which were fluconazole-resistant (MIC ≥8 μg/mL) and possessed multiple known azole resistance mechanisms (Table S1) (19). Both VT-1161 and VT-1598 had lower MIC_{50} values (0.06 and 0.125 μg/mL, respectively), and VT-1598 had a lower MIC_{90} value (0.25 μg/mL) when compared to the other tested azole antifungals (Table 1). VT-1161 and VT-1598 MICs were ≤0.015 μg/mL against the 11 fluconazole-susceptible isolates within the collection, and the VT-1598 MICs were 0.03 μg/mL against 33% (19 of 57) of the fluconazole-resistant clinical isolates. This suggests that some fluconazole-resistance mechanisms do not affect the *in vitro* potency of VT-1598. Posaconazole also demonstrated activity against many, but not all, of the same fluconazole-resistant isolates, as posaconazole MICs were within a two-fold increase (1-dilution difference) to those of the fluconazole-susceptible isolates for 15 of the fluconazole-resistant

isolates. Using this same metric, VT-1161 maintained *in vitro* potency against 8 fluconazole-resistant clinical isolates, which was comparable to that of voriconazole (6 isolates) and greater than that of itraconazole (2 isolates). Overall, VT-1598 and VT-1161 thus appear to have additional activity against several fluconazole-resistant isolates, and in this respect are at least comparable to commercially available triazoles.

VT-1598 MICs were elevated at least four-fold (≥0.06 μg/mL, range 0.06 to >8 μg/mL) against 38 fluconazole-resistant isolates compared to its activity against the fluconazole-susceptible isolates. VT-1161 MICs were elevated at least four-fold (≥0.06 μg/mL, range 0.06 to >8 μg/mL) against 49 fluconazole-resistant isolates. Five clinical isolates displayed highly elevated VT-1598 and VT-1161 MICs (range 4 to >8 μg/mL) and also high fluconazole, voriconazole, itraconazole, and posaconazole MICs. Sequencing and/or relative quantitation of mRNA expression of known resistance genes revealed that four of these isolates overexpressed *CDR1* relative to the *CDR1* mRNA levels of fluconazole-susceptible clinical isolates (19). The fifth isolate contained a premature stop codon in *ERG3*, resulting in truncation of the protein after Gly130, which likely explains its significantly elevated resistance not only to VT-1161 and VT-1598, but also to all other tested azole antifungals.

To gain additional insight on the determinants that could confer decreased susceptibility to VT-1161 and VT-1598 in the clinical isolates, a point-biserial correlation between the $\log_2$-fold increase in VT-1598 and VT-1161 MICs and the mRNA expression levels of *CDR1*, *MDR1*, and *ERG11* in the clinical isolates was performed. The $\log_2$-fold increase in MICs was compared to the baseline MIC measurement for VT-1598 and VT-1161 against fluconazole-susceptible isolates (MIC ≤ 0.015) and expression levels of either *CDR1*, *MDR1*, and *ERG11* were measured via RT-qPCR in a previous study (19). The majority of fluconazole-resistant clinical isolates

exhibited increased *CDR1* expression, however there was no significant correlation between *CDR1* expression and VT-1598 resistance ($p = 0.287$). In contrast, higher levels of *CDR1* expression did positively correlate with increasing VT-1161 MICs ($p < 0.01$). Similarly, while there was no relationship between *MDR1* expression and VT-1598 MIC ($p = 0.105$), there was a slight positive correlation between *MDR1* expression and increased VT-1161 MIC ($p < 0.05$). No significant correlation was established with either drug and *ERG11* expression ($p = 0.512$ and $p = 0.355$ for VT-1598 and VT-1161, respectively).

VT-1598 and VT-1161 MICs against the clinical isolates were plotted directly against those of fluconazole, voriconazole, itraconazole, and posaconazole to visualize relative susceptibility differences (Figure 1). As previously noted, both VT-1161 and VT-1598 retained potency against several fluconazole-resistant isolates, and all isolates with reduced VT-1598 or VT-1161 potency were resistant to fluconazole. By comparison, VT-1161 MICs were disproportionately higher against some clinical isolates compared to those of the other tested azoles. One voriconazole-susceptible isolate (0.125 μg/mL) that contained a K143R *ERG11* mutation and exhibited increased *CDR1* expression had an 8-fold increase in the MIC of VT-1161 compared to that observed against fluconazole-susceptible isolates (≤0.015 μg/mL). In addition, a single itraconazole-susceptible isolate (0.125 μg/mL) demonstrated a 32-fold increase in the VT-1161 MIC (0.5 μg/mL). This isolate contained three Erg11 amino acid substitutions (F126L, Y132F, H283R), but lacked other obvious azole resistance mechanisms. Against isolates with low posaconazole MICs (range ≤0.03 to 0.25 μg/mL), seven displayed ≥16-fold increases in VT-1161 MIC over fluconazole-susceptible isolates. Among these seven isolates, all contained various *ERG11* mutations, four overexpressed *CDR1* by at least two-fold, one

overexpressed both *ERG11* and *CDR1*, and one overexpressed *MDR1*. In contrast, there were no strong outliers for VT-1598 MICs when compared against those of the triazoles.

***In vitro* activity of VT-1161 and VT-1598 against strains with known azole resistance mechanisms.** To identify determinants of VT-1161 and VT-1598 resistance, we evaluated the influence of specific azole-resistance mechanisms on VT-1161 and VT-1598 MICs when placed in the fluconazole-susceptible isolate SC5314 (Figure 2). Increased *CDR1* and *CDR2* expression through artificial activation of the *TAC1* gene increased the VT-1161 MICs more than eight-fold compared to the susceptible parent strain. This increase in VT-1161 MIC was diminished, but not completely abolished, when the *CDR1* gene was deleted, suggesting that overexpression of *CDR1* as well as other Tac1 target genes (most likely *CDR2*) was responsible for the decreased susceptibility to VT-1161. On the other hand, Tac1 activation did not result in reduced susceptibility to VT-1598, as opposed to its effect on VT-1161, fluconazole and voriconazole resistance, which increased between four- and 16-fold compared to that of the parental strain SC5314. Posaconazole and itraconazole MICs were only slightly elevated (two-fold) by the hyperactive Tac1. Thus, it appears that while drug efflux via Cdr1 plays a role in VT-1161 resistance, Tac1 activation and the approximately 10-fold increase in *CDR1* expression are not sufficient to alter MICs of VT-1598.

While a hyperactive Mrr1 did not result in increased resistance to VT-1598, it caused a four-fold increase in the MIC of VT-1161. This increase was abolished upon *MDR1* deletion, suggesting that the Mdr1 transporter is involved in VT-1161 resistance. Fluconazole and voriconazole were the only tested azole drugs against the *MDR1*-overexpressing strain that showed a greater than two-fold increase in MIC (32-fold and four-fold, respectively) over

SC5314. By comparison, itraconazole showed a minimal two-fold increase (1-dilution difference) in MIC, and posaconazole MICs were not affected by *MDR1* overexpression. Strangely, there was a 4-fold increase in posaconazole MIC when *MDR1* was deleted in the hyperactive Mrr1 strain. However, this is consistent with variability observed for posaconazole MICs in other published strains and fluconazole-susceptible clinical isolates (31).

Upregulated expression of *ERG11* via artificial activation of the Upc2 transcription factor also did not affect VT-1161 or VT-1598 MICs. However, despite an approximate 4- to 8-fold increase in *ERG11* expression (data not shown), the activated Upc2 strain failed to demonstrate a relevant change in voriconazole, posaconazole, and itraconazole MICs. Surprisingly, this strain also exhibited no change in fluconazole MIC as has previously been reported. We therefore decided to also test azole susceptibilities in a strain homozygous for the G648D amino acid substitution, previously shown to be the strongest clinically-derived gain-of-function mutation in Upc2 (19, 32). Strains SCUPC2R14A and –B overexpressed *ERG11* relative to the parent strain SC5314 by 6.4-fold (previously published) and 4.5-fold (unpublished data), respectively (data not shown) (19). A modest 2-fold increase in fluconazole MIC in these two strains was observed compared to SC5314, whereas no changes were observed for the MICs of any of the other antifungal agents.

To compare the effects of different alterations in the azole target enzyme on the susceptibility of *C. albicans* to VT-1161 and VT-1598, twelve single Erg11 amino acid substitutions and four double substitutions in Erg11 were tested (Table S2, Figure 3). The Y132F single substitution caused a substantial (16-fold) increase inVT-1161 MIC. Surprisingly, the double amino acid substitutions Y132F&K143R and Y132F&F145L had a lesser impact on VT-1161 MIC (eight-fold and four-fold increase, respectively) than the single Y132F

substitution alone. Other amino acid substitutions did not have an appreciable effect on VT-1161 MICs, and none of the tested *ERG11* mutants showed greater than a two-fold increase in the MIC of VT-1598. The F145L and S405F single mutants and the double substitution mutants D278N&G464S and Y132F&F145L showed a slight two-fold increase in the VT-1598 MIC compared to that against SC5314.

DISCUSSION

VT-1598 has previously demonstrated a broad spectrum of activity *in vitro* against yeasts such as *Candida* and *Cryptococcus* spp., moulds including *Aspergillus* spp. and endemic fungi (33), and has shown improved survival and reduced fungal burden in murine models of CNS coccidioidomycosis (34) and cryptococcosis (35). Pertinent to the present study, VT-1598 has also recently shown potent *in vitro* and *in vivo* antifungal activity against fluconazole-sensitive and -resistant *Candida* spp. isolated from chronic mucocutaneous candidiasis patients (36). Structurally, while the tetrazole moiety has lower affinity for interaction with the heme iron of CYP51, other structural modifications have made the drug more fungal-specific. For example, a critical H-bond between VT-1598 and the CYP51 enzyme of many fungi likely confers its broad activity (37). This greater selectivity may decrease the potential for undesirable adverse effects and drug interactions that occur with the triazoles through inhibition of human cytochrome P-450 enzymes.

Our study supports the previous finding that VT-1598 has potent activity against *C. albicans* isolates. Overall, VT-1598 displayed the lowest MIC_{50} and MIC_{90} values compared to fluconazole, voriconazole, posaconazole, itraconazole, and VT-1161 against the clinical isolates tested. More importantly, VT-1598 MICs often remained unchanged from its baseline

measurement against SC5314 and other fluconazole-susceptible clinical isolates even against isolates containing known resistance mechanisms, indicating that this tetrazole may retain activity against isolates that are normally less susceptible to other azole antifungals. This included multiple fluconazole-resistant isolates with various combinations of *CDR1*, *MDR1* and *ERG11* expression increases and mutations in the *ERG11* gene.

Interestingly, when tested against laboratory strains containing individual azole resistance mechanisms, VT-1598 MICs changed relatively little. Traditional azole resistance mechanisms such as efflux pump overexpression (Cdr1 and Mdr1) and overexpression of the azole target (Erg11) did not alter VT-1598 MICs within the concentration ranges tested here. While it is possible that testing lower concentrations might reveal differences in MIC, the clinical relevance at such low concentrations is questionable. Our current finding suggests that these mechanisms individually are not sufficient to confer resistance to VT-1598. Previously, the Tyr132 and Lys143 substitutions in Erg11 were reported to have the strongest individual effects on fluconazole and voriconazole MICs (20). The combination substitutions Y132F&K143R and Y132F&F145L, which have been shown to have some of the strongest increases in fluconazole and voriconazole MIC, respectively, did not appreciably change the MIC of VT-1598. Against VT-1161, both these double substitutions showed less of an effect than the single amino acid substitution Y132F. The K143R substitution is thought to alter the H-bond strength of the heme ring propionates of the *C. albicans* Erg11 protein, which may possibly interfere with the coordination of the azole ring nitrogen and the iron of the CYP51 heme group, and the F145L amino acid substitution is located on the Erg11 proximal surface, allowing possible interactions with NADPH-cytochrome P450 reductase (20, 38). Based on the crystal structure of the *C. albicans* CYP51 enzyme complexed with VT-1161, the Y132F substitution is thought to

altogether abolish one of six H-bonds between VT-1161 and the protoporphyrin IX propionates on Erg11 (38). It is possible that the Y132F substitution is more important to VT-1161 resistance than either K143R or F145L and that combination mutations might interfere with the primary Y132F substitution, thus leading to the differences in the observed VT-1161 MICs.

Against a collection of predominantly fluconazole-resistant clinical isolates, VT-1161 showed good activity, though its individual MIC_{50} and MIC_{90} were higher compared to VT-1598. In contrast to VT-1598, the potency of VT-1161 appeared to be more affected by the presence of *CDR1* and *MDR1* overexpression and *ERG11* mutations. This was supported by the significant positive correlation established between *CDR1*/*MDR1* expression and VT-1161 MIC in *C. albicans* clinical isolates. Additionally, susceptibility testing in strains containing individual mechanisms of azole resistance, wherein the *CDR1*-overexpressing strains SCTAC1GAD1A and –B and the *MDR1*-overexpressing strains SCMRR1GAD1A and -B demonstrated increased VT-1161 MICs, further supports Mrr1 and Tac1 as mediators of resistance to VT-1161, at least in part through increased production of the transporters Mdr1 and Cdr1, respectively. The recent work by Monk *et al*. also demonstrated that both the Cdr1 and Mdr1 efflux pumps reduced the effectiveness of VT-1161, and activity against Cdr1- and Mdr1-overexpressing isolates could be restored via the Cdr1- and Mdr1-specific inhibitors RC21v3 and MCC1189, respectively (39). The Erg11 amino acid substitutions Y132F, Y132F&K143R, and Y132F&F145L also resulted in shifts in VT-1161 MIC and may contribute to VT-1161 resistance. However, VT-1161 retained activity against a number of isolates with known azole resistance mechanisms. The tested *ERG11* mutant strains containing the Y132H, K143R, F145L, E266D, D278N, S405F, G448E, F449V, G450E, G464S, and D466E single substitutions and the D278N&G464S, and G450E&I483V double substitutions showed little change in VT-

1161 MICs compared to the susceptible parent strain SC5314. Thus, VT-1161 has potential to be a future treatment option of azole-resistant *C. albicans* infections or recurrent infections previously treated with older members of the azole class.

Within the five clinical isolates that displayed greatly reduced susceptibility to VT-1161, VT-1598, and the other commercially available triazoles tested, one isolate contained an early stop codon at Trp131 within the *ERG3* gene, which encodes for sterol Δ5,6-desaturase and is critical for ergosterol biosynthesis in *C. albicans*. It has been previously reported that mutations in *ERG3* can result in azole resistance and alternative sterol biosynthesis by avoidance of accumulation of toxic sterol intermediates through defective desaturase enzyme (26, 27, 40). The inhibition of the azole target 14α-lanosterol demethylase causes accumulation of the toxic sterol intermediate, 14α-methylergosta-8,24(28)-dien-3β,6α-diol, which is thought to be the source of the fungistatic effect seen with azole antifungal class (24, 25, 41). However, dysfunctional Erg3 results in alternative sterol usage and an inability to produce this toxic intermediate. Therefore, mechanisms that result in a non-functional Erg3 might render an isolate resistant to the azole antifungal drug class, as is seen in the case of the isolate containing the premature stop codon in *ERG3*.

In summary, the *in vitro* activity of VT-1161 and VT-1598 against azole-resistant *C. albicans* clinical isolates and strains with known azole resistance mechanisms suggests that they may prove useful against resistant *C. albicans* infections. Furthermore, given their low and relatively unchanged MICs against many azole-resistant strains, it is possible that VT-1161 and VT-1598 may fill some of the gaps in coverage against azole-resistant isolates. This, in combination with the potential for favorable safety and drug interaction profiles, makes VT-1161 and VT-1598 attractive options as alternative therapies for azole-resistant *C. albicans* infections.

However, the presence of strongly resistant isolates, such as the five clinical isolates with greatly increased MICs to all azoles tested here, suggests the existence of azole-resistance determinants that can provide obstacles to the successful utilization of all azoles, including these new tetrazoles. Further investigation should be undertaken to identify the mechanism(s) responsible for resistance to these agents.

MATERIALS AND METHODS

Isolate and strain growth conditions. Sixty-eight clinical *C. albicans* isolates were obtained from the University of Iowa. *C. albicans* isolates and strains were cultured from -80˚C freezer stock (40% glycerol in YPD media) onto YPD-agar plates overnight at 30˚C. Colonies from YPD-agar plates were then either streaked onto Sabouraud Dextrose agar for azole susceptibility testing or grown in liquid YPD media and incubated overnight at 30˚C for preparation of genomic DNA.

Construction of C. *albicans* strains. Table 2 lists the constructed strains used in this study. Ten single *ERG11* mutations and four double mutations were selected from previous studies (20). Two additional strains expressing the Y132H and D278N *ERG11* gene mutations were created in a previous study utilizing the *SAT* flipper cassette (42, 43). Briefly, to create the mutant strain SCERG11R1S1C1, *ERG11* gene fragments were generated by primers pairs Ca*ERG11*_1F with CaERG11SOE-3R_Y132H and CaERG11SOE-2F_Y132H with CaERG11_4R using SC5314 template genomic DNA (Table S3). Short-overlapping extension PCR was used to fuse the resulting *ERG11* gene fragments using nested primers CaERG11-AF_(ApaI) and CaERG11-BR_(XhoI). For mutant strain SCERG11R3S3C1, *ERG11* gene

297 fragments were generated by primers pairs Ca*ERG11*_1F with CaERG11SOE-6R using
298 template genomic DNA from clinical isolate 43 and CaERG11SOE-5F with CaERG11_4R
299 using SC5314 genomic DNA. The resulting fragments were again fused into a single fragment
300 containing either the D278N-containing mutant *ERG11* open reading frame (ORF) using nested
301 primers CaERG11-AF_(ApaI) and CaERG11-BR_(XhoI). In constructing the plasmids used in
302 the transformation of both strains, the *ERG11* 3' flanking sequence was amplified from SC5314
303 genomic DNA and primers CaERG11_C-F' and CaERG11_D-R'. The inserts 3' of the *ERG11*
304 ORF were digested with restriction enzymes NotI and SacII, and cloned into the pSFS2-derived
305 plasmid pBSS2 previously described by Vasicek *et al*. containing the *SAT1* flipper cassette
306 from Reuβ et al. (42, 44) to create plasmid pERG11CD. The *ERG11* ORF-containing
307 fragments with either the Y132H or D278N mutations were digested using restriction enzymes
308 ApaI and XhoI, gel excised, and cloned into the plasmid pERG11CD to generate plasmids
309 pERG11A1 and pERG11A3. Plasmids were digested with restriction enzymes ApaI and SacII
310 and used to transform strain SC5314 by electroporation to generate heterozygous *ERG11*
311 mutants. Recycling of the nourseothricin selection marker through 24 hours of growth in YPD
312 and repeat transformation of the resultant strains generated the homozygous *ERG11* allele
313 replacements SCERG11R1S1C1 and SCERG11R3S3C1, confirmed via Southern hybridization
314 and Sanger sequencing. The artificially-activated Tac1, Mrr1, and Upc2 mutants used in this
315 study as well as SCΔ*cdr1*TAC1GAD1A and -B, containing the artificially activated *TAC1*
316 allele in a *cdr1*Δbackground, were described in a previous study (45). Strains
317 SCΔ*mdr1*MRR1GAD1A and -B were constructed by introducing the artificially activated
318 *MRR1* allele from plasmid pMRR1-GAD1 (32) into the *mdr1*Δ mutants SCMDR1M4A and -B
319 (46), respectively.

320

321 ***ERG11* amplification and sequencing.** Table S3 lists the primers used for *ERG11*
322 amplification and sequence verification. The *ERG11* ORF of each isolate was PCR amplified
323 from genomic DNA using primers Ca*ERG11*_F_Amp and Ca*ERG11*_R_Amp. PCR products
324 were purified using the QIAquick® PCR Purification Kit (Qiagen) and product was sequenced
325 on an ABI 3130XL genetic analyzer using sequencing primers. Sequencing was accomplished in
326 duplicate in independently grown isolates.

327

328 **Relative gene expression by real-time PCR.** Expression levels of the genes *CDR1*, *MDR1*, and
329 *ERG11* in clinical *C. albicans* isolates were measured in a previous study, and *CDR1*, *MDR1*,
330 and *ERG11* expression of laboratory strains were measured similarly using previously described
331 methods (19). Briefly, first-strand cDNA was generated from 1 μg of extracted RNA for each
332 strain using the SuperScript® VILO™ Master Mix (Invitrogen) reaction kit. Quantitative PCRs
333 were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in
334 technical and biological triplicate for each sample. SYBR green PCR master mix (Applied
335 Biosystems) was used for amplification detection of candidate genes against the *CaACT1*
336 normalizing gene. Calculation of the relative quantitation values of *CDR1*, *ERG11*, and *MDR1*
337 gene expression was accomplished using the StepOne Software v2.3 (Applied Biosystems).
338 Primers used in the amplification of genes measured via qPCR are listed in Table S3.

339

340 **Susceptibility testing.** Minimum inhibitory concentrations (MIC) of VT-1161, VT-1598,
341 fluconazole, voriconazole, posaconazole, and itraconazole were measured using broth
342 microdilution methods in accordance with the Clinical Laboratory and Standards Institute (47,

48). 96-well microtiter plates containing RPMI-1640 media (0.165M MOPS, with L-glutamine, without sodium bicarbonate, pH 7.0) were used to incubate strains across serially-diluted concentrations of the each azole. 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. MICs were visually read at 24 hours post-incubation at 35˚ C as the minimum concentration required to reduce growth of cells by approximately 50% or greater compared to drug-free control wells. MICs were performed in duplicate for clinical isolates, *ERG11* mutant strains and laboratory strains SCTAC1GAD1A and -B, SCΔ*cdr1*TAC1GAD1A and -B, SCMRR1GAD1A and -B, SCΔ*mdr1*MRR1GAD1A and -B and SCUPC2GAD1A and -B. When reporting MICs for strains and isolates, the higher of the MICs was used in this analysis (Table S1, Table S2), though 98% (592/606) of MIC duplicate readings were identical or within a single dilution of each other. The geometric mean MIC (GM MIC), MIC_{50}, and MIC_{90} was reported for clinical *C. albicans* isolates for each triazole and tetrazole agent used in this study. The MIC_{50} and MIC_{90} values reported for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, and posaconazole were defined as the minimum drug concentrations required to inhibit 50% and 90% of the clinical *C. albicans* isolates tested, respectively.

Statistical Analysis. A point-biserial correlation or phi coefficient was used for all continuous and dichotomous variables, respectively, to identify possible predictors of azole resistance. For all statistical tests, a *p*-value less than 0.05 was considered significant. Statistical calculations were performed using IBM® SPSS© analytical software, version 23.

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REFERENCES

1. Sobel JD. 2007. Vulvovaginal candidosis. Lancet 369:1961-71.
2. Goncalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. 2016. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. Crit Rev Microbiol 42:905-27.
3. Berberi A, Noujeim Z, Aoun G. 2015. Epidemiology of Oropharyngeal Candidiasis in Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome Patients and CD4+ Counts. J Int Oral Health 7:20-3.
4. Sangeorzan JA, Bradley SF, He X, Zarins LT, Ridenour GL, Tiballi RN, Kauffman CA. 1994. Epidemiology of oral candidiasis in HIV-infected patients: colonization, infection, treatment, and emergence of fluconazole resistance. Am J Med 97:339-46.
5. Askinyte D, Matulionyte R, Rimkevicius A. 2015. Oral manifestations of HIV disease: A review. Stomatologija 17:21-8.
6. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39:309-17.
7. Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. 2011. Candida bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009. Antimicrob Agents Chemother 55:561-6.
8. Solomon SL, Oliver KB. 2014. Antibiotic resistance threats in the United States: stepping back from the brink. Am Fam Physician 89:938-41.
9. Rex JH, Rinaldi MG, Pfaller MA. 1995. Resistance of Candida species to fluconazole. Antimicrob Agents Chemother 39:1-8.
10. Ruhnke M, Eigler A, Tennagen I, Geiseler B, Engelmann E, Trautmann M. 1994. Emergence of fluconazole-resistant strains of Candida albicans in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J Clin Microbiol 32:2092-8.
11. Bhattacharya S, Sobel JD, White TC. 2016. A Combination Fluorescence Assay Demonstrates Increased Efflux Pump Activity as a Resistance Mechanism in Azole-Resistant Vaginal Candida albicans Isolates. Antimicrob Agents Chemother 60:5858-66.
12. Kontoyiannis DP. 2017. Antifungal Resistance: An Emerging Reality and A Global Challenge. J Infect Dis 216:S431-S435.
13. Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A, Berman J, Bille J, Sanglard D. 2006. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics 172:2139-56.
14. Tsao S, Rahkhoodaee F, Raymond M. 2009. Relative contributions of the Candida albicans ABC transporters Cdr1p and Cdr2p to clinical azole resistance. Antimicrob Agents Chemother 53:1344-52.
15. Morschhauser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rogers PD. 2007. The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS Pathog 3:e164.
16. Manoharlal R, Gaur NA, Panwar SL, Morschhauser J, Prasad R. 2008. Transcriptional activation and increased mRNA stability contribute to overexpression of CDR1 in azole-resistant Candida albicans. Antimicrob Agents Chemother 52:1481-92.

17. MacPherson S, Akache B, Weber S, De Deken X, Raymond M, Turcotte B. 2005. Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother 49:1745-52.
18. Silver PM, Oliver BG, White TC. 2004. Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell 3:1391-7.
19. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhauser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryot Cell 11:1289-99.
20. Flowers SA, Colon B, Whaley SG, Schuler MA, Rogers PD. 2015. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob Agents Chemother 59:450-60.
21. Sanglard D, Ischer F, Koymans L, Bille J. 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother 42:241-53.
22. Kelly SL, Lamb DC, Kelly DE. 1999. Y132H substitution in Candida albicans sterol 14alpha-demethylase confers fluconazole resistance by preventing binding to haem. FEMS Microbiol Lett 180:171-5.
23. Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. 1999. The G464S amino acid substitution in Candida albicans sterol 14alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem Biophys Res Commun 262:174-9.
24. Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U, Einsele H. 1997. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett 400:80-2.
25. Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE. 1995. Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem Biophys Res Commun 207:910-5.
26. Martel CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Rolley N, Kelly DE, Kelly SL. 2010. Identification and characterization of four azole-resistant erg3 mutants of Candida albicans. Antimicrob Agents Chemother 54:4527-33.
27. Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. 2012. Amino acid substitutions in the Candida albicans sterol Delta5,6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. J Antimicrob Chemother 67:2131-8.
28. Hoekstra WJ, Garvey EP, Moore WR, Rafferty SW, Yates CM, Schotzinger RJ. 2014. Design and optimization of highly-selective fungal CYP51 inhibitors. Bioorg Med Chem Lett 24:3455-8.
29. Warrilow AG, Hull CM, Parker JE, Garvey EP, Hoekstra WJ, Moore WR, Schotzinger RJ, Kelly DE, Kelly SL. 2014. The clinical candidate VT-1161 is a highly potent inhibitor of Candida albicans CYP51 but fails to bind the human enzyme. Antimicrob Agents Chemother 58:7121-7.
30. Yates CM, Garvey EP, Shaver SR, Schotzinger RJ, Hoekstra WJ. 2017. Design and optimization of highly-selective, broad spectrum fungal CYP51 inhibitors. Bioorg Med Chem Lett 27:3243-3248.

31. Sanglard D, Coste AT. 2016. Activity of Isavuconazole and Other Azoles against Candida Clinical Isolates and Yeast Model Systems with Known Azole Resistance Mechanisms. Antimicrob Agents Chemother 60:229-38.
32. Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhauser J. 2010. An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in Candida albicans. Antimicrob Agents Chemother 54:353-9.
33. Wiederhold NP, Patterson HP, Tran BH, Yates CM, Schotzinger RJ, Garvey EP. 2018. Fungal-specific Cyp51 inhibitor VT-1598 demonstrates in vitro activity against Candida and Cryptococcus species, endemic fungi, including Coccidioides species, Aspergillus species and Rhizopus arrhizus. J Antimicrob Chemother 73:404-408.
34. Wiederhold NP, Shubitz LF, Najvar LK, Jaramillo R, Olivo M, Catano G, Trinh HT, Yates CM, Schotzinger RJ, Garvey EP, Patterson TF. 2018. The Novel Fungal Cyp51 Inhibitor VT-1598 Is Efficacious in Experimental Models of Central Nervous System Coccidioidomycosis Caused by Coccidioides posadasii and Coccidioides immitis. Antimicrob Agents Chemother 62.
35. Garvey EP, Sharp AD, Warn PA, Yates CM, Schotzinger RJ. 2018. The novel fungal CYP51 inhibitor VT-1598 is efficacious alone and in combination with liposomal amphotericin B in a murine model of cryptococcal meningitis. J Antimicrob Chemother 73:2815-2822.
36. Break TJ, Desai JV, Healey KR, Natarajan M, Ferre EMN, Henderson C, Zelazny A, Siebenlist U, Yates CM, Cohen OJ, Schotzinger RJ, Perlin DS, Garvey EP, Lionakis MS. 2018. VT-1598 inhibits the in vitro growth of mucosal Candida strains and protects against fluconazole-susceptible and -resistant oral candidiasis in IL-17 signalling-deficient mice. J Antimicrob Chemother 73:2089-2094.
37. Hargrove TY, Garvey EP, Hoekstra WJ, Yates CM, Wawrzak Z, Rachakonda G, Villalta F, Lepesheva GI. 2017. Crystal Structure of the New Investigational Drug Candidate VT-1598 in Complex with Aspergillus fumigatus Sterol 14alpha-Demethylase Provides Insights into Its Broad-Spectrum Antifungal Activity. Antimicrob Agents Chemother 61.
38. Hargrove TY, Friggeri L, Wawrzak Z, Qi A, Hoekstra WJ, Schotzinger RJ, York JD, Guengerich FP, Lepesheva GI. 2017. Structural analyses of Candida albicans sterol 14alpha-demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. J Biol Chem 292:6728-6743.
39. Monk BC, Keniya MV, Sabherwal M, Wilson RK, Graham DO, Hassan HF, Chen D, Tyndall JDA. 2019. Azole Resistance Reduces Susceptibility to the Tetrazole Antifungal VT-1161. Antimicrob Agents Chemother 63.
40. Vale-Silva LA, Coste AT, Ischer F, Parker JE, Kelly SL, Pinto E, Sanglard D. 2012. Azole resistance by loss of function of the sterol Delta(5),(6)-desaturase gene (ERG3) in Candida albicans does not necessarily decrease virulence. Antimicrob Agents Chemother 56:1960-8.
41. Watson PF, Rose ME, Ellis SW, England H, Kelly SL. 1989. Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem Biophys Res Commun 164:1170-5.
42. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene 341:119-27.

43. Warrilow AG, Nishimoto AT, Parker JE, Price CL, Flowers SA, Kelly DE, Rogers PD, Kelly SL. 2019. The Evolution of Azole Resistance of in Candida albicans Sterol 14α-Demethylase (CYP51) through Incremental Amino Acid Substitutions. Antimicrob Agents Chemother.
44. Vasicek EM, Berkow EL, Bruno VM, Mitchell AP, Wiederhold NP, Barker KS, Rogers PD. 2014. Disruption of the transcriptional regulator Cas5 results in enhanced killing of Candida albicans by Fluconazole. Antimicrob Agents Chemother 58:6807-18.
45. Schillig R, Morschhauser J. 2013. Analysis of a fungus-specific transcription factor family, the Candida albicans zinc cluster proteins, by artificial activation. Mol Microbiol 89:1003-17.
46. Schubert S, Barker KS, Znaidi S, Schneider S, Dierolf F, Dunkel N, Aid M, Boucher G, Rogers PD, Raymond M, Morschhauser J. 2011. Regulation of efflux pump expression and drug resistance by the transcription factors Mrr1, Upc2, and Cap1 in Candida albicans. Antimicrob Agents Chemother 55:2212-23.
47. Clinical and Laboratory Standards Institute. 2008. Reference method for broth microdilution antifungal susceptibility testing of yeasts; approved standard, 3rd ed. CLSI document M27-A3, Wayne, PA.
48. Clinical and Laboratory Standards Institute. 2012. Reference method for broth microdilution antifungal susceptibility testing of yeasts; fourth informational supplement. CLSI document M27-S4, Wayne, PA.
49. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Diekema DJ. 2011. Wild-type MIC distributions and epidemiological cutoff values for posaconazole and voriconazole and Candida spp. as determined by 24-hour CLSI broth microdilution. J Clin Microbiol 49:630-7.

536 **Table 1.** Geometric mean MICs, MIC_{50}, MIC_{90}, and ranges (µg/mL) for tested compounds against 68 clinical isolates of

Drug	GM MIC	MIC_{50}	MIC_{90}	Range
VT-1161	0.15	0.125	1	≤0.015 - >8
VT-1598	0.05	0.06	0.25	≤0.015 - >8
Fluconazole[a]	20.2	32	>64	≤0.125 - >64
Voriconazole[b]	0.32	0.5	2	≤0.03 - >16
Itraconazole[c]	0.31	0.25	1	≤0.03 - >16
Posaconazole[c,d]	0.2	0.5	2	≤0.03 - >16

[a]CLSI clinical breakpoints: resistant ≥8 µg/mL, susceptible dose-dependent 4 µg/mL, susceptible ≤2 µg/mL (48)
[b]CLSI clinical breakpoints: resistant ≥1 µg/mL, intermediate 0.25 – 0.5 µg/mL, susceptible ≤0.125 µg/mL (48)
[c]n = 66 clinical isolates for itraconazole and posaconazole
[d]CLSI epidemiological cutoff value = 0.06 µg/mL (49)

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538 **Table 2.** Constructed strains used in this study.

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Strains[a]	**Genotype**	**Source or reference**
SC5314	*ERG11-1/ERG11-2*	ATCC
Constructed laboratory strains[a]		
20E1II1B1	*ERG11*^{Y132F}*::FRT / ERG11*^{Y132F}*::FRT*	Flowers *et al*., 2015
SC*ERG11*R1S1C1	*ERG11*^{Y132H}*::FRT / ERG11*^{Y132H}*::FRT*	Warrilow *et al.*, 2019
10B1A3A	*ERG11*^{K143R}*::FRT / ERG11*^{K143R}*::FRT*	Flowers *et al*., 2015
2B1A51A	*ERG11*^{F145L}*::FRT / ERG11*^{F145L}*::FRT*	Flowers *et al*., 2015
29NA29A23A	*ERG1*^{E266D}*::FRT / ERG11*^{E266D}*::FRT*	Flowers *et al*., 2015
SC*ERG11*R3S3C1	*ERG11*^{D278N}*::FRT / ERG11*^{D278N}*::FRT*	Warrilow *et al.*, 2019
21C1M1B1	*ERG11*^{S405F}*::FRT / ERG11*^{S405F}*::FRT*	Flowers *et al*., 2015
20NA11A57A	*ERG11*^{G448E}*::FRT / ERG11*^{G448E}*::FRT*	Flowers *et al*., 2015
7A5A5A	*ERG11*^{F449V}*::FRT / ERG11*^{F449V}*::FRT*	Flowers *et al*., 2015
15A3A108A	*ERG11*^{G450E}*::FRT / ERG11*^{G450E}*::FRT*	Flowers *et al*., 2015
19A1A1C1	*ERG11*^{G464S}*::FRT / ERG11*^{G464S}*::FRT*	Flowers *et al*., 2015
22B12A58A	*ERG11*^{D466E}*::FRT / ERG11*^{D466E}*::FRT*	Flowers *et al*., 2015
9A14A21A	*ERG11*^{Y132F,K143R}*::FRT / ERG11*^{Y132F,K143R}*::FRT*	Flowers *et al*., 2015
27A5A33A	*ERG11*^{Y132F,F145L}*::FRT/ ERG11*^{Y132F,F145L}*::FRT*	Flowers *et al*., 2015
13A5A57A	*ERG11*^{D278N,G464S}*::FRT / ERG11*^{D278N,G464S}*::FRT*	Flowers *et al*., 2015
8A4A1A	*ERG11*^{G450E,I483V}*::FRT / ERG11*$^{G450E\ I483V}$*::FRT*	Flowers *et al*., 2015
SCTAC1GAD1A and -B	*ADH1/adh1::*P$_{ADH1}$*-TAC1-GAL4AD-3xHA-caSAT1*	Schillig *et al.*, 2013
SCΔ*cdr1*TAC1GAD1A and -B	*ADH1/adh1::*P$_{ADH1}$*-TAC1-GAL4AD-3xHA-caSAT1 cdr1Δ::FRT/cdr1Δ::FRT*	Schillig *et al.*, 2013
SCMRR1GAD1B and -B	*ADH1/adh1::*P$_{ADH1}$*-MRR1-GAL4AD-3xHA-caSAT1*	Schillig *et al*., 2013
SCΔ*mdr1*MRR1GAD1A and -B	*ADH1/adh1::*P$_{ADH1}$*-MRR1-GAL4AD-3xHA-caSAT1 mdr1Δ::FRT/mdr1Δ::FRT*	This study
SCUPC2GAD1A and -B	*ADH1/adh1::*P$_{ADH1}$*-UPC2-GAL4AD-3xHA-caSAT1*	Schillig *et al*., 2013
SCUPC2R14A and -B	*UPC2*^{G648D}*::FRT / UPC2*^{G648D}*::FRT*	Heilmann *et al*., 2010

[a]All laboratory strains have SC5314 as background.

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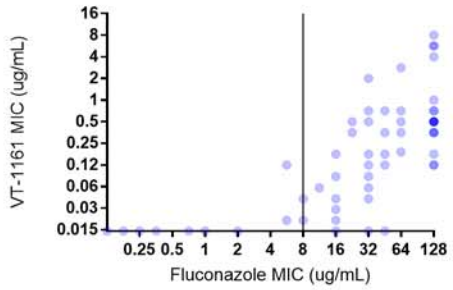
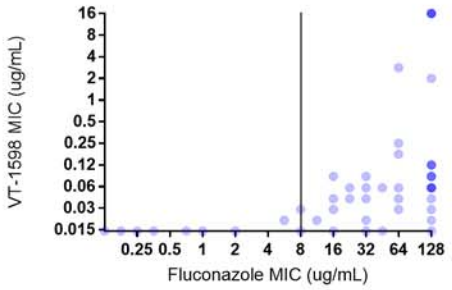
Figure 1. Comparison of the MICs of VT-1161 and VT-1598 against the MICs of (a) fluconazole, (b) voriconazole, (c) itraconazole, and (d) posaconazole in a collection of *C. albicans* clinical isolates. Plotted points represent the MICs of clinical isolates, with darker points representative of multiple, superimposed points. Concentration of points to the lower right of each plot represent favorable activity (low MICs relative to susceptible isolates) for VT-1161 or VT-1598 versus the comparator azole. Conversely, points concentrated to the top left of each plot represent isolates with high MICs of VT-1161 and VT-1598 relative to the comparator azole. Solid vertical lines represent the resistant clinical break point for fluconazole and voriconazole, while dotted vertical lines represent the epidemiological cutoff values for posaconazole.

a

MICs of VT-1161 versus fluconazole in clinical *C. albicans* isolates

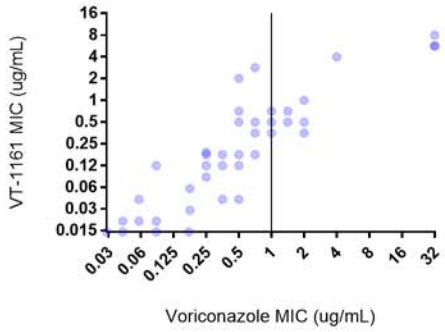


MICs of VT-1598 versus fluconazole in clinical *C. albicans* isolates

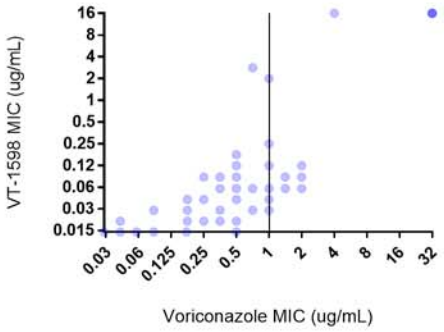


b

MICs of VT-1161 versus voriconazole in clinical *C. albicans* isolates

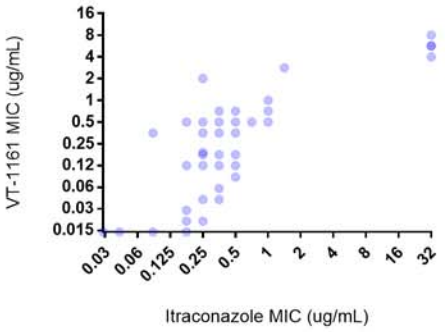


MICs of VT-1598 versus voriconazole in clinical *C. albicans* isolates

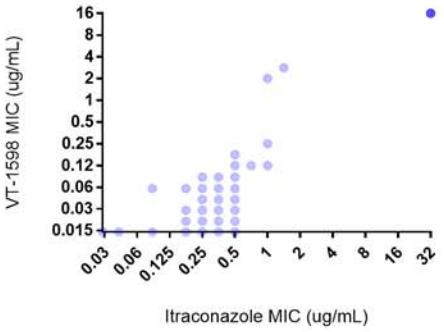


c

MICs of VT-1161 versus itraconazole in clinical *C. albicans* isolates

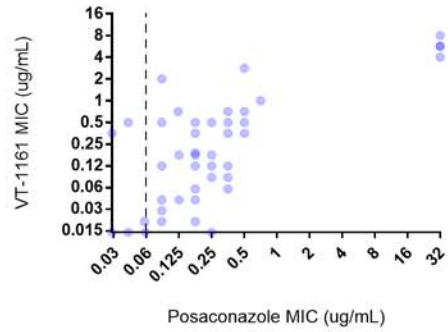


MICs of VT-1598 versus itraconazole in clinical *C. albicans* isolates



d

MICs of VT-1161 versus posaconazole in clinical *C. albicans* isolates



MICs of VT-1598 versus posaconazole in clinical *C. albicans* isolates

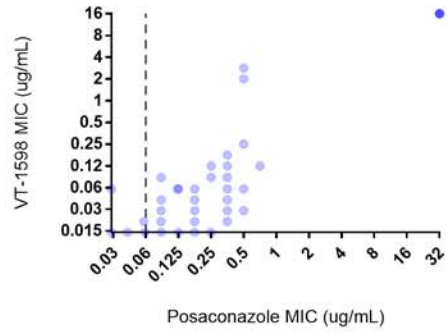
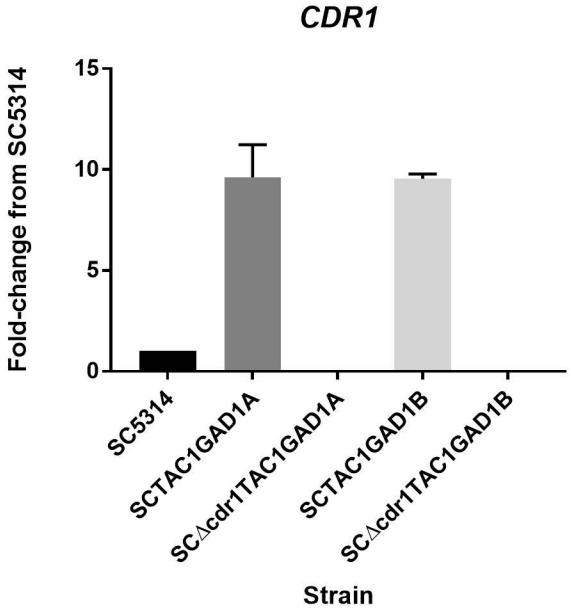


Figure 2. MICs of tested azole compounds against strains with individual known azole resistance mechanisms. Tested strains include those containing the artificially-activated transcription factors Tac1 and Mrr1 in strains SCTAC1GAD1A and –B and SCMRR1GAD1A and -B, respectively, as well as Δ*cdr1* derivatives of SCTAC1GAD1A (SCΔ*cdr1*TAC1GAD1A and -B), Δ*mdr1* derivatives of SCMRR1GAD1A and -B (SCΔ*mdr1*MRR1GAD1A and -B), and SCUPC2R14A and –B containing the G648D gain-of-function mutation in *UPC2*. The MICs for the strains with artificially activated Tac1, Mrr1, and for the *UPC2*^{G648D} gain-of-function mutation are displayed as the highest MIC value of both independently created A- and B- strains for each respective transcription factor. The relative fold-change in expression compared to the parent strain SC5314 of (a) *CDR1* for SCTAC1GAD1A and B and (b) *MDR1* for SCMRR1GAD1A and -B is shown on the left of the figure. The antifungal MICs of the *UPC2*^{G648D} homozygous strains SCUPC2R14A and -B is shown in (c).

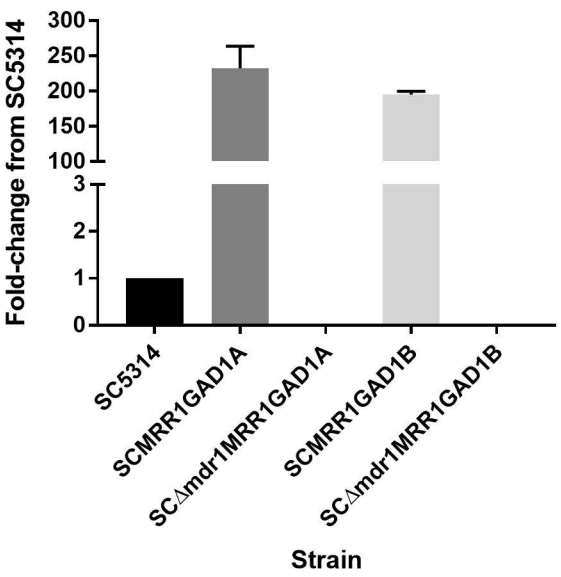
A



MICs (μg/mL)

	SC5314	SCTAC1GAD1	SCΔcdr1 TAC1GAD1
VT-1598	≤ 0.015	≤ 0.015	≤ 0.015
VT-1161	≤ 0.015	0.125	0.06
Fluconazole	0.5	8	2
Voriconazole	≤ 0.03	0.125	≤ 0.03
Itraconazole	0.125	0.25	0.125
Posaconazole	≤ 0.03	0.06	0.06

B



MICs (μg/mL)

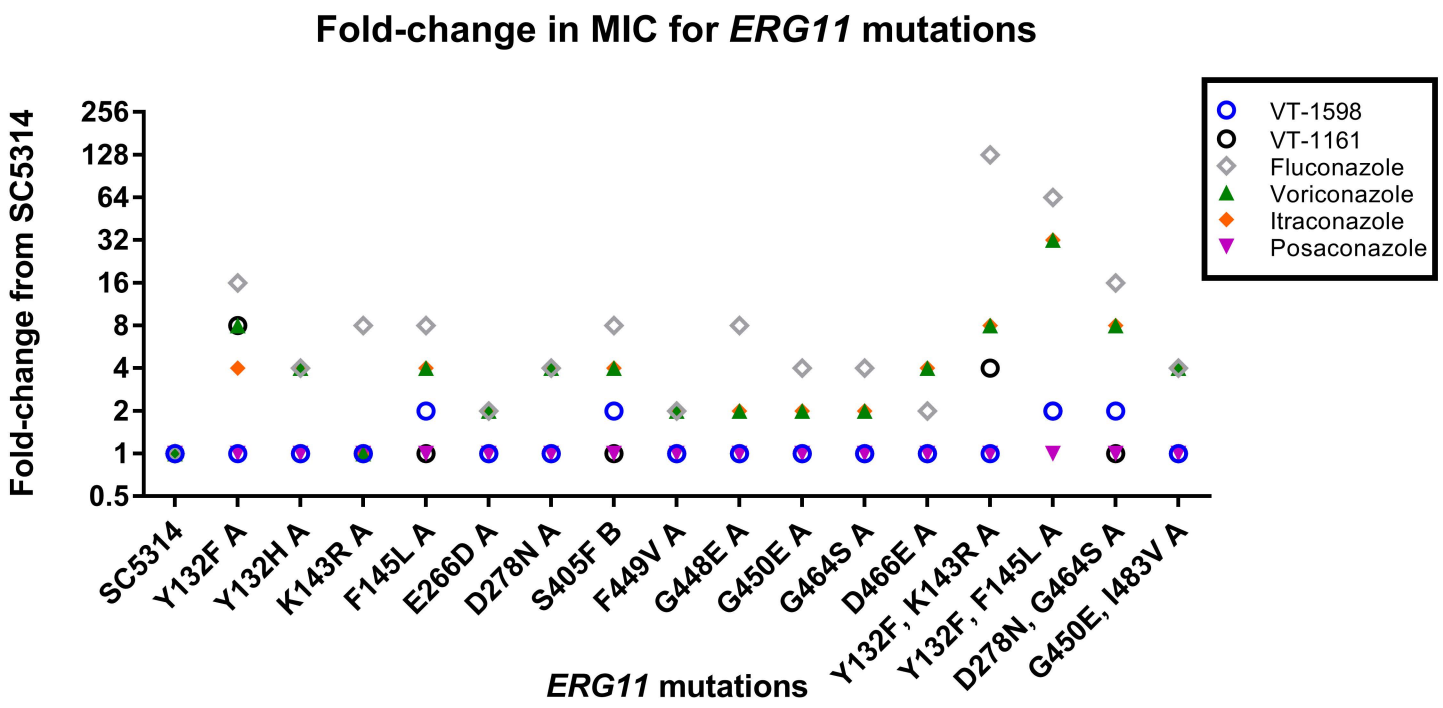
	SC5314	SCMRR1GAD1	SCΔmdr1 MRR1GAD1
VT-1598	≤ 0.015	≤ 0.015	≤ 0.015
VT-1161	≤ 0.015	0.06	≤ 0.015
Fluconazole	0.5	16	8
Voriconazole	≤ 0.03	0.125	0.06
Itraconazole	0.125	0.25	0.125
Posaconazole	≤ 0.03	≤ 0.03	0.125

C

MICs (μg/mL)

	SC5314	SCUPC2R14
VT-1598	< 0.015	< 0.015
VT-1161	< 0.015	< 0.015
Fluconazole	0.25	1
Voriconazole	< 0.03	< 0.03
Itraconazole	0.125	0.125
Posaconazole	< 0.03	< 0.03

Figure 3. Relative fold-change compared to SC5314 in MIC of various azole antifungal agents against strains containing single and double *ERG11* mutations. Open blue circles represent VT-1598 MICs, while open black circles represent VT-1161. Open grey diamonds represent fluconazole. Solid green triangles represent voriconazole. Solid orange diamonds represents itraconazole, and solid inverted purple triangles represent posaconazole.


Fold-change in MIC for ERG11 mutations
Fold-change from SC5314
256
128
64
32
16
8
4
2
1
0.5
VT-1598
VT-1161
Fluconazole
Voriconazole
Itraconazole
Posaconazole
SC5314
Y132F A
Y132H A
K143R A
F145L A
E266D A
D278N A
S405F B
F449V A
G448E A
G450E A
G464S A
D466E A
Y132F, K143R A
Y132F, F145L A
D278N, G464S A
G450E, I483V A
ERG11 mutations