AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

An Azole-Tolerant Endosomal Trafficking Mutant of *Candida albicans* Is Susceptible to Azole Treatment in a Mouse Model of Vaginal Candidiasis

Brian M. Peters,[a,b] Arturo Luna-Tapia,[a] Hélène Tournu,[a] Jeffrey M. Rybak,[a] P. David Rogers,[a] Glen E. Palmer[a]

Department of Clinical Pharmacy, College of Pharmacy, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA[a]; Department of Oral and Craniofacial Biology, School of Dentistry, LSU Health Sciences Center, New Orleans, Louisiana, USA[b]

ABSTRACT We recently reported that a *Candida albicans* endosomal trafficking mutant continues to grow after treatment with the azole antifungals. Herein, we report that the *vps21*Δ/Δ mutant does not have a survival advantage over wild-type isolates after fluconazole treatment in a mouse model of vaginal candidiasis. Furthermore, loss of *VPS21* does not synergize with established mechanisms of azole resistance, such as overexpression of efflux pumps or of Erg11p, the target enzyme of the azoles. In summary, although loss of *VPS21* function enhances *C. albicans* survival after azole treatment *in vitro*, it does not seem to affect azole susceptibility *in vivo*.

KEYWORDS azoles, *Candida albicans*, resistance, tolerance, vaginal candidiasis, Vps21p

The azole antifungals inhibit lanosterol demethylase (Erg11p), an enzyme required for the synthesis of the membrane lipid ergosterol. This leads to accumulation of the Erg11p substrate lanosterol, a portion of which is converted into the abnormal sterol 14α-methylergosta-8,24(28)-dien-3β,6α-diol by C-5 sterol desaturase (Erg3p). The intracellular depletion of ergosterol together with the accumulation of the toxic diol are thought to cause plasma membrane dysfunction and ultimately the inhibition of fungal growth. Several well-described mechanisms contribute to azole resistance in *Candida albicans*, including increased expression of the target enzyme Erg11p and of drug efflux pumps such as Cdr1p (1), Cdr2p (2), and Mdr1p (3), which expel azoles from the fungal cell. However, a combination of these mechanisms is usually necessary to confer a significant increase in azole resistance (4). Furthermore, the resistance of many *C. albicans* isolates is not fully accounted for by these established mechanisms (5).

We recently reported that a *C. albicans vps21*Δ/Δ mutant, deficient in membrane trafficking through the late endosomal prevacuolar compartment (PVC), can continue growing in the presence of the azole antifungal drugs despite depletion of cellular ergosterol (6). This phenotype resembles an exaggerated form of the trailing growth phenomenon, which can be observed in ~18% of *C. albicans* clinical isolates with the standard CLSI broth microdilution antifungal susceptibility testing protocol (7, 8). The growth of trailing isolates is inhibited by the azoles, and thus they appear susceptible after 24 h of incubation. However, significant growth can be observed in the same cultures after 48 h of incubation. This can make these isolates appear to be azole resistant (9, 10), although they are not typically associated with antifungal treatment failure, and are therefore considered to be azole susceptible (11). Furthermore, all of the few trailing clinical isolates of *C. albicans* tested to date were susceptible

Received 13 January 2017 **Returned for modification** 5 March 2017 **Accepted** 22 March 2017

Accepted manuscript posted online 27 March 2017

Citation Peters BM, Luna-Tapia A, Tournu H, Rybak JM, Rogers PD, Palmer GE. 2017. An azole-tolerant endosomal trafficking mutant of *Candida albicans* is susceptible to azole treatment in a mouse model of vaginal candidiasis. Antimicrob Agents Chemother 61:e00084-17. https://doi.org/10.1128/AAC.00084-17.

Address correspondence to Glen E. Palmer, gpalmer5@uthsc.edu.

B.M.P. and A.L.-T. contributed equally to this article.

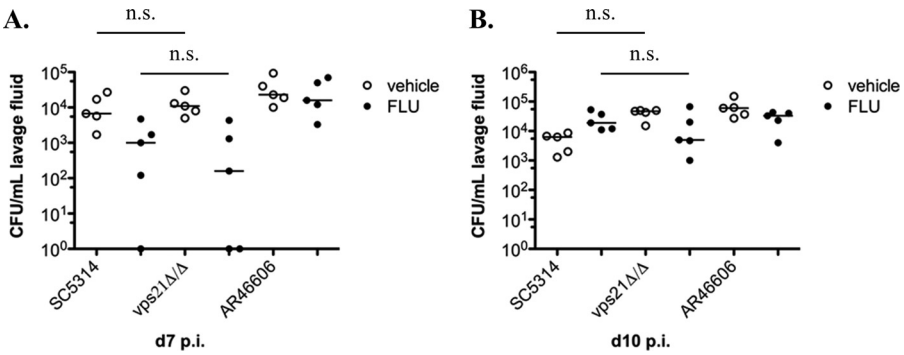

FIG 1 The *C. albicans vps21*Δ/Δ mutant is susceptible to azole treatment in a mouse model of vaginal candidiasis. Three groups of 10 estrogen-treated female C57BL/6 mice were inoculated intravaginally with a wild-type strain (SC5314), a *vps21*Δ/Δ mutant strain, or an azole-resistant clinical isolate of *C. albicans* (AR46606). Each group was then split into two treatment groups of 5 animals. Four days postinoculation, one group was treated with fluconazole (FLU) 25 μg/ml and the second with vehicle (phosphate-buffered saline) alone by oral gavage. Levels of fungal colonization in each group were then compared at 7 (A) and 10 (B) days p.i., by quantifying CFU from vaginal lavage fluid. Pairwise comparisons were performed using one-way analysis of variance (ANOVA) with the Kruskal-Wallis test between groups infected with either strain, with and without fluconazole treatment. The results were not significant at $P > 0.05$.

to azole treatment in a murine model of disseminated candidiasis (9, 10). Nonetheless, this pattern of growth suggests that trailing isolates are better able to tolerate the consequences of Erg11p inhibition by the azoles, at least under some *in vitro* conditions. The trailing phenotype is especially pronounced in the *C. albicans vps21*Δ/Δ mutant, in that significant growth can be observed in the presence of elevated azole concentrations, even at the 24-h time point. The purpose of this study was to determine whether loss of *VPS21* function in *C. albicans* is sufficient to confer a survival advantage following azole treatment *in vivo*. We also determined whether the mutant's phenotype can synergize with well-established mechanisms known to confer azole resistance (see above).

We previously reported that the *vps21*Δ/Δ mutant had reduced virulence in a murine model of disseminated candidiasis compared with a wild-type control. However, preliminary studies revealed that the mutant colonized to levels similar to those of the wild-type control in a mouse model of vaginal infection. We therefore compared the susceptibility of the *vps21*Δ/Δ mutant to fluconazole with that of the wild-type control strain using this model. Estrogen-treated C57BL/6 mice (Charles River) were inoculated intravaginally with 5×10^6 *C. albicans* yeast cells of each strain, then divided into two groups of five mice. Four days postinoculation (p.i.), one group was treated with fluconazole 25 mg/kg by oral gavage and the second group with vehicle (phosphate-buffered saline) alone. Vaginal lavage was then carried out at 3 and 6 days after treatment, and fungal burden was quantified as CFU. As expected, the mutant and wild-type strains colonized to similar levels in the absence of fluconazole, confirming that the mutant has no significant deficiency in colonization of the mouse vagina (Fig. 1). Furthermore, fluconazole treatment decreased mutant and wild-type colonization to similar extents, suggesting that the mutant has no obvious survival advantage and is fluconazole susceptible, as previously observed in the mouse model of disseminated candidiasis. At day 10 p.i., colonization by the wild-type strain and the *vps21*Δ/Δ mutant had recovered (Fig. 1B). In contrast, colonization levels of the azole-resistant vaginal isolate AR46606 were unaffected by fluconazole, with similar CFU counts obtained in the treatment and vehicle groups.

Although alone it was insufficient to affect azole susceptibility *in vivo*, we next explored whether the enhanced growth of the *vps21*Δ/Δ mutant observed *in vitro* in the presence of the azoles can synergize with mechanisms known to confer azole resistance. To test this, we overexpressed three well-characterized azole efflux pumps, i.e., *MDR1*, *CDR1*, and *CDR2*, in *vps21*Δ/Δ mutant and control strains and compared each strain's susceptibility to fluconazole. Overexpression of the major facilitator protein

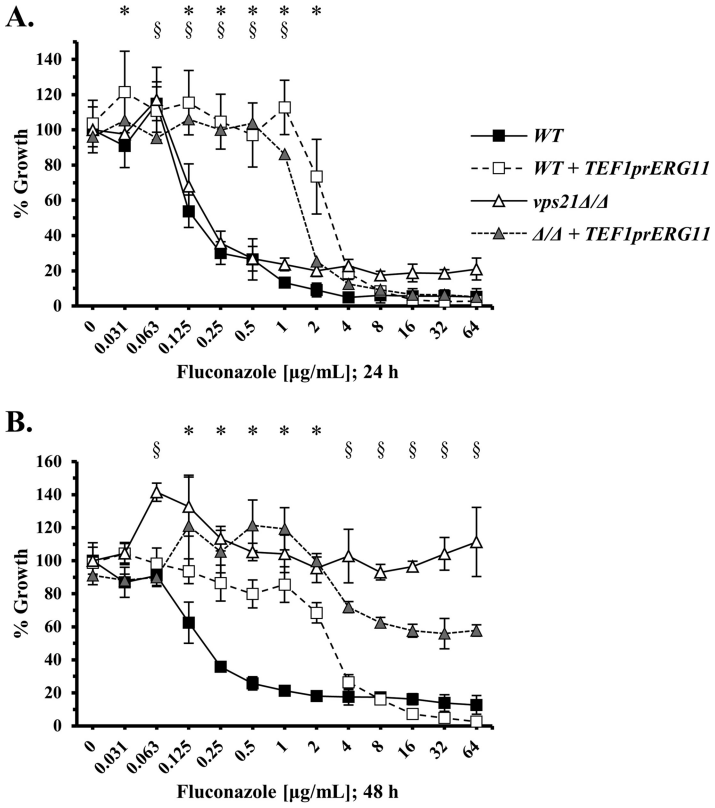

FIG 2 Overexpression of *ERG11* in the *C. albicans vps21*Δ/Δ mutant does not result in synergistic azole resistance. The *ERG11* open reading frame was expressed from the *TEF1* promoter in CAI4 (wild-type strain [WT]) and the *vps21*Δ/Δ mutant. The susceptibilities of the *ERG11*-overexpressing and control strains (vector alone) to fluconazole were then evaluated using the standard CLSI broth microdilution protocol. After 24 (A) and 48 (B) h incubation, growth was measured as optical density at 600 nm (OD_{600}) and expressed as a percentage of the growth in the minus-drug (dimethyl sulfoxide-alone) control wells of the vector-alone control strains. The means ± SD of three biological replicates are shown. Growth of each *ERG11*-overexpressing strain was compared with the vector-alone control for each drug concentration using two-way ANOVA with Tukey's test. *, $P < 0.05$ for WT versus WT + *TEF1prERG11*. §, $P < 0.05$ for *vps21*Δ/Δ versus *vps21*Δ/Δ + *TEF1prERG11*.

coding gene *MDR1* or the ABC transporter coding *CDR1* and *CDR2* modestly reduced fluconazole susceptibility in mutant and control strains to a similar extent (~2-fold for *MDR1* and *CDR2*; ~4- to ~8-fold for *CDR1*) (see Fig. 1 in the supplemental material). This suggests that the *vps21*Δ/Δ mutant's azole-tolerant phenotype does not synergize with azole efflux pump overexpression.

Overexpression of *ERG11*, encoding the target enzyme of the azoles lanosterol demethylase, also reduced azole susceptibility of mutant and wild-type strains to a similar extent (~8-fold) (Fig. 2A and B). In addition, overexpression of a gain-of-function *UPC2*^{G648D} allele, encoding a transcription factor that enhances expression of the ergosterol biosynthetic genes (12) and known to confer partial azole resistance, reduced azole susceptibility of wild-type and *vps21*Δ/Δ mutant strains to a similar extent (~4-fold) (data not shown).

To test potential interactions between Vps21p-mediated azole tolerance and the resistance mechanism conferred by loss of Erg3p activity, we made a *vps21*Δ/Δ *erg3*Δ/Δ double mutant. However, as both *erg3*Δ/Δ and *vps21*Δ/Δ *erg3*Δ/Δ strains were apparently insensitive to fluconazole at all concentrations tested, no meaningful interactions were discernable (data not shown). To further examine possible interactions between these two mechanisms, we overexpressed *ERG3* from the powerful *TEF1* promoter in wild-type and *vps21*Δ/Δ strains. Interestingly, overexpression of *ERG3* substantially diminished trailing growth of the *vps21*Δ/Δ mutant (see Fig. 2 in the supplemental material). However, *ERG3* overexpression also eradicated the small amount of trailing

growth observed for the wild-type control strain. As such, while our results indicate that *ERG3* expression levels do generally impact trailing growth in *C. albicans*, no specific interaction was observed in the *vps21*Δ/Δ mutant.

In conclusion, the mechanisms that confer enhanced survival of the *vps21*Δ/Δ endosomal trafficking mutant in the presence of fluconazole *in vitro* do not confer a survival advantage *in vivo*. Moreover, they do not seem to synergize with well-defined molecular mechanisms known to confer azole resistance. Our results suggest that loss of Vps21p function is unlikely to provide *C. albicans* with a clinically relevant means to survive azole treatment *in vivo*. Nevertheless, the exaggerated trailing growth observed in this mutant highlights the need for caution in interpreting the results of the standard CLSI azole susceptibility assays.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00084-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under awards R01AI099080 to G.E.P., R01AI058145 to P.D.R., and K22AI110541 to B.M.P.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank Aaron Mitchell (Carnegie Mellon University) and William Fonzi (Georgetown University) for providing strains and plasmids that were used in this study.

REFERENCES

1. White TC. 1997. Increased mRNA levels of *ERG16*, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother 41:1482–1487.
2. Sanglard D, Ischer F, Monod M, Bille J. 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology 143:405–416. https://doi.org/10.1099/00221287-143-2-405.
3. Goldway M, Teff D, Schmidt R, Oppenheim AB, Koltin Y. 1995. Multidrug resistance in *Candida albicans*: disruption of the BENr gene. Antimicrob Agents Chemother 39:422–426. https://doi.org/10.1128/AAC.39.2.422.
4. Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D, Patterson TF. 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 45:2676–2684. https://doi.org/10.1128/AAC.45.10.2676-2684.2001.
5. White TC, Holleman S, Dy F, Mirels LF, Stevens DA. 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob Agents Chemother 46:1704–1713. https://doi.org/10.1128/AAC.46.6.1704-1713.2002.
6. Luna-Tapia A, Kerns ME, Eberle KE, Jursic BS, Palmer GE. 2015. Trafficking through the late endosome significantly impacts *Candida albicans* tolerance of the azole antifungals. Antimicrob Agents Chemother 59:2410–2420. https://doi.org/10.1128/AAC.04239-14.
7. Arthington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajjeh RA, Harrison LH, Sofair AN, Warnock aDW. 2002. Comparison of visual and spectrophotometric methods of broth microdilution MIC end point determination and evaluation of a sterol quantitation method for *in vitro* susceptibility testing of fluconazole and itraconazole against trailing and nontrailing *Candida* isolates. Antimicrob Agents Chemother 46:2477–2481. https://doi.org/10.1128/AAC.46.8.2477-2481.2002.
8. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
9. Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinel-Ingroff A, Anaissie EJ. 1998. Optimizing the correlation between results of testing *in vitro* and therapeutic outcome *in vivo* for fluconazole by testing critical isolates in a murine model of invasive candidiasis. Antimicrob Agents Chemother 42:129–134.
10. Arthington-Skaggs BA, Warnock DW, Morrison CJ. 2000. Quantitation of *Candida albicans* ergosterol content improves the correlation between *in vitro* antifungal susceptibility test results and *in vivo* outcome. Antimicrob Agents Chemother 44:2081–2085. https://doi.org/10.1128/AAC.44.8.2081-2085.2000.
11. Revankar SG, Kirkpatrick WR, McAtee RK, Fothergill AW, Redding SW, Rinaldi MG, Patterson TF. 1998. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards Method. J Clin Microbiol 36:153–156.
12. Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhauser J, Rogers PD. 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. Eukaryot Cell 7:1180–1190. https://doi.org/10.1128/EC.00103-08.