University of Tennessee Health Science Center
UTHSC Digital Commons

Theses and Dissertations (ETD)

College of Graduate Health Sciences

5-2019

An Investigation into Clinically Relevant Determinants of Azole Resistance in *Candida albicans*

Andrew T. Nishimoto
University of Tennessee Health Science Center

Follow this and additional works at: <https://dc.uthsc.edu/dissertations>

Part of the [Medicine and Health Sciences Commons](#), and the [Molecular Biology Commons](#)

Recommended Citation

Nishimoto, Andrew T. (0000-0003-1244-4656), "An Investigation into Clinically Relevant Determinants of Azole Resistance in *Candida albicans*" (2019). *Theses and Dissertations (ETD)*. Paper 493.
<http://dx.doi.org/10.21007/etd.cghs.2019.0486>.

This Dissertation is brought to you for free and open access by the College of Graduate Health Sciences at UTHSC Digital Commons. It has been accepted for inclusion in Theses and Dissertations (ETD) by an authorized administrator of UTHSC Digital Commons. For more information, please contact jwelch30@uthsc.edu.

An Investigation into Clinically Relevant Determinants of Azole Resistance in *Candida albicans*

Abstract

"*Candida albicans* is a commensal organism commonly colonizing the human gut and skin. As an opportunistic pathogen, it can cause persistent and serious infections in individuals with compromised immune systems, including the very young and elderly. Moreover, *C. albicans* can cause a wide spectrum of diseases ranging from superficial mucosal infections to life-threatening invasions of the organs and bloodstream. *Candida* species are the most common cause of invasive fungal disease, which is associated with high mortality and imposes a heavy toll on the healthcare system. Over the last 30 years, the azole antifungals have been a mainstay of antifungal therapy, being effective in a wide variety of fungal infections and serving as the primary oral treatment option. However, increased use, inappropriate dosing, and prolonged treatments have given rise to azole-resistant *Candida albicans* and other *Candida* species. Resistance in *C. albicans* results from a combination of different mechanisms. Increased expression of the efflux pump encoding genes *CDR1*, *CDR2*, and *MDR1* as well as increased expression of *ERG11*, encoding the azole target (14 α -lanosterol demethylase, also known as CYP51) are all primary mechanisms of azole resistance that arise in azole-resistant clinical isolates. These changes are known to be mediated through gain-of-function mutations in the genes of a fungal-specific transcription factor family known as the zinc cluster transcription factors. Furthermore, genetic changes in the ergosterol biosynthesis genes, *ERG11* and *ERG3*, encoding a C-5 sterol desaturase, also contribute to clinical azole resistance in *C. albicans*. The interplay of these mechanisms can result in azole-resistance, treatment failure, and ultimately, poorer outcomes in patients. Therefore, to improve healthcare outcomes, understanding resistance development and the mechanisms that drive them in *C. albicans* is crucial. Within a collection of predominantly fluconazole-resistant clinical isolates of *C. albicans*, our lab had previously characterized most known mechanisms of azole resistance present in each isolate. Increased *CDR1* expression in isolates lacking *TAC1* gain-of-function mutations coupled with recent literature suggesting a role of the Mrr2 zinc cluster transcription factor in azole resistance lead us to sequence and test mutations in the *MRR2* gene in across this collection. By placing mutant *MRR2* alleles in azole-susceptible backgrounds, we hoped to measure the contribution of *MRR2* mutations to azole resistance through changes in *CDR1* expression and fluconazole minimum inhibitory concentrations (MICs). Counter to what has been recorded in the literature, we found no evidence that mutations in *MRR2* impact either *CDR1* expression or azole susceptibility in *C. albicans*. This is a novel finding correcting a previous mistaken paradigm of a clinically relevant mechanism driving resistance in *C. albicans*. Next we more closely examined the role of *ERG11* mutations found in clinical isolates. Though the contributory effects of *ERG11* mutations to azole resistance had been quantified, the specific biochemical impact of these mutations on enzyme function and ligand-binding interaction have only recently come to light. Here we introduced additional CaCYP51 amino acid substitution mutants (D278N and Y132H) in *C. albicans*" "vi" "and tested our entire collection of CaCYP51 mutant strains to determine their in vitro azole susceptibilities in the context of these findings. In general, we observed differences in the fluconazole and voriconazole MICs between CaCYP51 amino acid substitutions. In contrast, MICs to itraconazole showed a small, fairly consistent increase in MIC across tested CaCYP51 strains and MICs to posaconazole did not increase at all over the wild type except for the G448E substitution, suggesting posaconazole possesses the best in vitro activity against these CaCYP51 mutants. Overall, we also revealed that not all *ERG11* mutations confer azole resistance through decreased binding interactions with the target and the azole drug, suggesting that CaCYP51 amino acid substitutions may instead interact with other associated proteins to confer resistance. Furthermore, it was discovered that many *ERG11* mutations from clinical isolates result in low catalytic turnover of the enzyme, which is crucial to normal rates of ergosterol production in a healthy cell. Though preliminary results of growth in CaCYP51 mutant strains does not support attenuated fitness in competitive assay, the findings here prove that some clinical *ERG11*

mutations result in diminished enzyme function." We also sequenced the collection of clinical isolates and discovered an A351V Erg3 amino acid substitution in our azole-resistant isolates and predominantly in those with multiple ERG11 mutations. This suggested a possible connection between CaCYP51 mutants and amino acid substitutions in Erg3. As the proteins encoded for by the ERG11 and ERG3 genes are involved in the same ergosterol biosynthesis pathway, defects in Erg11 enzyme function might be expected to impact accumulation of substrates of Erg3, specifically, precursors of the toxic sterol metabolite 14 α -methylergosta- 8,24(28)-dien-3 β , 6 α -diol. By testing ERG11 mutant strains with and without the ERG3A351V allele in growth competition experiments, we hoped to observe a conferred fitness benefit by the ERG3 mutation. Interestingly, we were unable to generate one of our selected ERG11 mutants with the poorest catalytic turnover in the absence of the A351V amino acid change in Erg3. Future investigation by other lab members is needed to determine if ERG3 mutations can indirectly influence azole susceptibility through permissive mutation." "Lastly, we tested susceptibility of our clinical collection to the new tetrazole antifungals VT-1161 and VT-1598, which have been reported to exhibit potent activity against azole-resistant *C. albicans* and a host of other fungal species. We additionally investigated determinants of resistance to the two new agents by obtaining susceptibilities to *C. albicans* strains containing individual known mechanisms of azole resistance. While susceptibility to VT-1161 was reduced when CDR1 and MDR1 were overexpressed, VT-1598 seemed unaffected by any tested resistance mechanism. Importantly, both retained activity against a significant portion of mutant ERG11 strains. VT-1598 MICs were not affected by any single mechanism of resistance. However, screening of our azole-resistant clinical isolates identified five isolates with greatly elevated MICs to all tested agents. While one of these isolates possesses an ERG3 nonsense mutation that likely explains its pan-azole resistant profile, the other four isolates do not uniquely overexpress known resistance genes or possess known gene mutations that might explain their resistance. This finding suggests that there are determinants of azole resistance that are as yet undiscovered in *C. albicans*."

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Pharmaceutical Sciences

Research Advisor

P. David Rogers, Pharm.D., Ph.D.

Keywords

Azole, *Candida albicans*, ERG11, ERG3, MRR2, Resistance

Subject Categories

Medicine and Health Sciences | Molecular Biology

**An Investigation into Clinically Relevant Determinants of Azole Resistance in
*Candida albicans***

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Andrew T. Nishimoto
May 2019

Chapter 2 and Chapter 5 © 2019 by American Society for Microbiology
All other material © 2019 by Andrew T. Nishimoto
All rights reserved.

DEDICATION

To Mom, Dad, and Sushma.

ACKNOWLEDGEMENTS

I do acknowledge that there are simply too many people to acknowledge. To my mentor, collaborators, colleagues, labmates, and friends. To the fungal pathogens group, the department, the administration, the college and the university. Ten years is far too short a time to spend among such excellent people.

“I don’t know half of you half as well as I should I like, and I like less than half of you half as well as you deserve.” - Bilbo Baggins

J.R.R. Tolkien, *The Fellowship of the Ring*, 1954

Finally, I would like to thank Dr. Daniel Diekema from the University of Iowa for generously sending us the clinical isolates of *C. albicans* used in Chapters 2, 3, 4, and 5. All work was supported by NIH grant R01 AI058145 to P.D.R.

Thank you, everyone.

ABSTRACT

Candida albicans is a commensal organism commonly colonizing the human gut and skin. As an opportunistic pathogen, it can cause persistent and serious infections in individuals with compromised immune systems, including the very young and elderly. Moreover, *C. albicans* can cause a wide spectrum of diseases ranging from superficial mucosal infections to life-threatening invasions of the organs and bloodstream. *Candida* species are the most common cause of invasive fungal disease, which is associated with high mortality and imposes a heavy toll on the healthcare system. Over the last 30 years, the azole antifungals have been a mainstay of antifungal therapy, being effective in a wide variety of fungal infections and serving as the primary oral treatment option. However, increased use, inappropriate dosing, and prolonged treatments have given rise to azole-resistant *Candida albicans* and other *Candida* species.

Resistance in *C. albicans* results from a combination of different mechanisms. Increased expression of the efflux pump encoding genes *CDR1*, *CDR2*, and *MDR1* as well as increased expression of *ERG11*, encoding the azole target (14 α -lanosterol demethylase, also known as CYP51) are all primary mechanisms of azole resistance that arise in azole-resistant clinical isolates. These changes are known to be mediated through gain-of-function mutations in the genes of a fungal-specific transcription factor family known as the zinc cluster transcription factors. Furthermore, genetic changes in the ergosterol biosynthesis genes, *ERG11* and *ERG3*, encoding a C-5 sterol desaturase, also contribute to clinical azole resistance in *C. albicans*. The interplay of these mechanisms can result in azole-resistance, treatment failure, and ultimately, poorer outcomes in patients. Therefore, to improve healthcare outcomes, understanding resistance development and the mechanisms that drive them in *C. albicans* is crucial.

Within a collection of predominantly fluconazole-resistant clinical isolates of *C. albicans*, our lab had previously characterized most known mechanisms of azole resistance present in each isolate. Increased *CDR1* expression in isolates lacking *TAC1* gain-of-function mutations coupled with recent literature suggesting a role of the Mrr2 zinc cluster transcription factor in azole resistance lead us to sequence and test mutations in the *MRR2* gene in across this collection. By placing mutant *MRR2* alleles in azole-susceptible backgrounds, we hoped to measure the contribution of *MRR2* mutations to azole resistance through changes in *CDR1* expression and fluconazole minimum inhibitory concentrations (MICs). Counter to what has been recorded in the literature, we found no evidence that mutations in *MRR2* impact either *CDR1* expression or azole susceptibility in *C. albicans*. This is a novel finding correcting a previous mistaken paradigm of a clinically relevant mechanism driving resistance in *C. albicans*.

Next we more closely examined the role of *ERG11* mutations found in clinical isolates. Though the contributory effects of *ERG11* mutations to azole resistance had been quantified, the specific biochemical impact of these mutations on enzyme function and ligand-binding interaction have only recently come to light. Here we introduced additional CaCYP51 amino acid substitution mutants (D278N and Y132H) in *C. albicans*

and tested our entire collection of CaCYP51 mutant strains to determine their *in vitro* azole susceptibilities in the context of these findings. In general, we observed differences in the fluconazole and voriconazole MICs between CaCYP51 amino acid substitutions. In contrast, MICs to itraconazole showed a small, fairly consistent increase in MIC across tested CaCYP51 strains and MICs to posaconazole did not increase at all over the wild type except for the G448E substitution, suggesting posaconazole possesses the best *in vitro* activity against these CaCYP51 mutants. Overall, we also revealed that not all *ERG11* mutations confer azole resistance through decreased binding interactions with the target and the azole drug, suggesting that CaCYP51 amino acid substitutions may instead interact with other associated proteins to confer resistance. Furthermore, it was discovered that many *ERG11* mutations from clinical isolates result in low catalytic turnover of the enzyme, which is crucial to normal rates of ergosterol production in a healthy cell. Though preliminary results of growth in CaCYP51 mutant strains does not support attenuated fitness in competitive assay, the findings here prove that some clinical *ERG11* mutations result in diminished enzyme function.

We also sequenced the collection of clinical isolates and discovered an A351V Erg3 amino acid substitution in our azole-resistant isolates and predominantly in those with multiple *ERG11* mutations. This suggested a possible connection between CaCYP51 mutants and amino acid substitutions in Erg3. As the proteins encoded for by the *ERG11* and *ERG3* genes are involved in the same ergosterol biosynthesis pathway, defects in Erg11 enzyme function might be expected to impact accumulation of substrates of Erg3, specifically, precursors of the toxic sterol metabolite 14 α -methylergosta-8,24(28)-dien-3 β , 6 α -diol. By testing *ERG11* mutant strains with and without the *ERG3*^{A351V} allele in growth competition experiments, we hoped to observe a conferred fitness benefit by the *ERG3* mutation. Interestingly, we were unable to generate one of our selected *ERG11* mutants with the poorest catalytic turnover in the absence of the A351V amino acid change in Erg3. Future investigation by other lab members is needed to determine if *ERG3* mutations can indirectly influence azole susceptibility through permissive mutation.

Lastly, we tested susceptibility of our clinical collection to the new tetrazole antifungals VT-1161 and VT-1598, which have been reported to exhibit potent activity against azole-resistant *C. albicans* and a host of other fungal species. We additionally investigated determinants of resistance to the two new agents by obtaining susceptibilities to *C. albicans* strains containing individual known mechanisms of azole resistance. While susceptibility to VT-1161 was reduced when *CDR1* and *MDR1* were overexpressed, VT-1598 seemed unaffected by any tested resistance mechanism. Importantly, both retained activity against a significant portion of mutant *ERG11* strains. VT-1598 MICs were not affected by any single mechanism of resistance. However, screening of our azole-resistant clinical isolates identified five isolates with greatly elevated MICs to all tested agents. While one of these isolates possesses an *ERG3* nonsense mutation that likely explains its pan-azole resistant profile, the other four isolates do not uniquely overexpress known resistance genes or possess known gene mutations that might explain their resistance. This finding suggests that there are determinants of azole resistance that are as yet undiscovered in *C. albicans*.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
General Background	1
Biology of <i>Candida albicans</i>	2
Epidemiology of Azole Resistance in <i>C. albicans</i>	3
Current Antifungal Treatment of <i>C. albicans</i> Infections	5
Azole Resistance Mechanisms in <i>C. albicans</i>	6
Efflux pumps	6
Cdr1 and Cdr2	6
Mdr1	7
Upc2 and increased <i>ERG11</i> expression	9
Other zinc cluster transcription factors	10
Mutations in <i>ERG11</i>	10
Mutations in <i>ERG3</i> and alternative sterol biosynthesis	11
Aneuploidy and loss of heterozygosity	12
Azole import	13
Azole tolerance	13
Future Directions	14
Hypothesis and Objectives	15
 CHAPTER 2. THE CONTRIBUTION OF CLINICALLY-DERIVED MUTATIONS IN THE GENE ENCODING THE ZINC CLUSTER TRANSCRIPTION FACTOR MRR2 TO FLUCONAZOLE ANTIFUNGAL RESISTANCE AND <i>CDR1</i> EXPRESSION IN <i>CANDIDA ALBICANS</i>	 19
Introduction	19
Results	20
SNPs in <i>MRR2</i> among fluconazole-resistant clinical isolates of <i>C. albicans</i>	20
Fluconazole susceptibility in <i>MRR2</i> mutant strains	21
<i>MRR2</i> mutant strains do not overexpress <i>CDR1</i> when expressed from the <i>MRR2</i> locus	21
Amino acid substitutions S466L, A468G, S469T, and T470N do not influence fluconazole MICs or <i>CDR1</i> expression	24
Activated Mrr2 does not appear to regulate <i>MRR2</i> expression	27
Overexpression of wildtype or mutant <i>MRR2</i> in <i>C. albicans</i> strain does not affect fluconazole susceptibility or <i>CDR1</i> expression	27
Discussion	27
Materials and Methods	30
Strains and growth conditions	30
Plasmid construction	30
<i>MRR2</i> amplification and sequencing	31
<i>Candida albicans</i> strain construction	31
Azole susceptibility testing	34
Quantitative real-time reverse transcription PCR	37
Nucleotide sequence accession numbers	37

CHAPTER 3. MUTATIONS IN <i>ERG11</i> HAVE VARYING CONTRIBUTIONS TO AZOLE RESISTANCE, <i>ERG11</i> PROTEIN FUNCTION, AND COMPETITIVE GROWTH FITNESS OF THE CELL	38
Introduction.....	38
Results.....	43
Discussion.....	46
Methods	50
Construction of <i>C. albicans</i> CYP51 mutant strains	50
Susceptibility testing of <i>C. albicans</i> CYP51 mutant strains	52
Competitive growth assay.....	52
 CHAPTER 4. THE ROLE OF <i>ERG3</i> MUTATIONS TO AZOLE RESISTANCE AND <i>ERG11</i> MUTABILITY IN <i>C. ALBICANS</i>.....	53
Introduction.....	53
Results.....	54
Frequency of Erg11 and Erg3 amino acid substitutions in <i>C. albicans</i> clinical isolates.....	54
Sterol content in clinical isolates	59
Allelic replacement of <i>ERG3</i>	59
The A351V amino acid substitution in Erg3 does not directly affect <i>in vitro</i> fluconazole susceptibility.....	59
A homozygous <i>ERG11</i> ^{Y132H,K143T} mutant is conditionally constructed in combination with <i>ERG3</i> ^{A351V}	66
A homozygous <i>ERG11</i> ^{G307S} mutant cannot be constructed.	66
Discussion.....	68
Materials and Methods.....	71
Strains and growth conditions.....	71
Plasmid construction.....	71
<i>ERG3</i> and <i>ERG11</i> amplification and sequencing	73
<i>C. albicans</i> strain construction.....	73
Azole susceptibility testing	74
 CHAPTER 5. <i>IN VITRO</i> ACTIVITIES OF THE NOVEL INVESTIGATIONAL TETRAZOLE VT-1161 AND VT-1598 COMPARED TO THE TRIAZOLE ANTIFUNGALS AGAINST AZOLE-RESISTANCE STRAINS AND CLINICAL ISOLATES OF <i>CANDIDA ALBICANS</i>	75
Introduction.....	75
Results.....	76
<i>In vitro</i> activity of VT-1161 and VT-1598 against fluconazole-susceptible and fluconazole-resistant clinical isolates.....	76
<i>In vitro</i> activity of VT-1161 and VT-1598 against strains with known azole resistance mechanisms	78
Discussion.....	85
Materials and Methods.....	87
Isolate and strain growth conditions	87

Construction of <i>C. albicans</i> strains	87
<i>ERG11</i> amplification and sequencing	89
Relative gene expression by real-time PCR	89
Susceptibility testing	90
Statistical analysis	90
Transparency Declaration	90
CHAPTER 6. CONCLUSIONS.....	91
Introduction.....	91
Summary and Conclusions of Chapter 2	91
Summary and Conclusions of Chapter 3	92
Summary and Conclusions of Chapter 4	93
Summary and Conclusions of Chapter 5	94
A Final Word	94
LIST OF REFERENCES	95
APPENDIX A. AMINO ACID SUBSTITUTIONS IN MRR2 CLINICAL C. ALBICANS ISOLATES	118
APPENDIX B. SUPPLEMENTARY DATA TABLES TO CHAPTER 5.....	120
VITA.....	127

LIST OF TABLES

Table 2-1.	Mrr2 amino acid substitutions found in azole-resistant clinical isolates expressing <i>CDR1</i>	22
Table 2-2.	Fluconazole susceptibilities for MRR2 mutant strains	23
Table 2-3.	Strains used in Chapter 2	25
Table 2-4.	Primers used in this study	32
Table 3-1.	Yield and relative velocities of CaCYP51 proteins	41
Table 3-2.	Residual CYP51 activity in the presence of 4 μ M triazole antifungal	42
Table 3-3.	Triazole antifungal IC ₅₀ values obtained with CaCYP51 proteins	44
Table 3-4.	CLSI MICs(μ g mL ⁻¹) for fluconazole, voriconazole, itraconazole, and posaconazole against strains containing CaCYP51 amino acid substitutions.....	45
Table 3-5.	Strains used in Chapter 3	51
Table 4-1.	Fluconazole MIC, ergosterol content, and mutations in <i>ERG11</i> and <i>ERG3</i> in clinical isolates of <i>C. albicans</i>	57
Table 4-2.	Percent total cell membrane sterol content composition in <i>C. albicans</i> clinical isolates	60
Table 4-3.	24-hour fluconazole MICs for <i>C. albicans</i> strains in Chapter 4	67
Table 4-4.	Primers used in Chapter 4.....	72
Table 5-1.	Geometric mean MICs, MIC ₅₀ , MIC ₉₀ , and ranges (μ g/mL) for tested compounds against 68 clinical isolates of <i>C. albicans</i>	77
Table 5-2.	Constructed strains used in Chapter 5.....	88
Table A-1.	Amino acid substitutions in Mrr2 clinical <i>C. albicans</i> isolates	118
Table B-1.	Minimum inhibitory concentration values for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, posaconazole, and known azole resistance mechanisms in clinical <i>C. albicans</i> isolates	120
Table B-2.	MICs for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, and posaconazole against <i>ERG11</i> mutant strains	125
Table B-3.	Primers used in Chapter 5	126

LIST OF FIGURES

Figure 1-1. Expression levels of <i>CDR1</i> , <i>CDR2</i> , <i>MDR1</i> , and <i>ERG11</i> in 63 fluconazole-resistant clinical isolates	16
Figure 2-1. Fold-change in expression levels of <i>MRR2</i> mutant strains compared to SC5314	26
Figure 2-2. Fold-change in <i>CDR1</i> and <i>MRR2</i> expression compared to SC5314.....	28
Figure 2-3. Representative schematic of <i>MRR2</i> mutant strain construction in <i>C. albicans</i>	35
Figure 3-1. Reported locations of CaCYP51 amino acid substitutions in <i>C. albicans</i>	40
Figure 3-2. Competitive growth fitness testing of Erg11 mutants.....	49
Figure 4-1. Normal ergosterol biosynthesis pathway inhibition by the azole antifungals in <i>C. albicans</i>	55
Figure 4-2. Ergosterol biosynthesis pathway inhibition by the azole antifungals in <i>C. albicans</i> with defective C-5 sterol desaturase	56
Figure 4-3. Normal ergosterol biosynthesis pathway in <i>C. albicans</i>	64
Figure 4-4. Alternative sterol biosynthesis pathway in <i>C. albicans</i> cells with defective C-5 sterol desaturase.....	65
Figure 4-5. Proposed sterol biosynthesis pathway in <i>C. albicans</i> cells with <i>ERG11</i> mutations resulting in slow Erg11 enzyme function	69
Figure 5-1. Comparison of the MICs of VT-1161 and VT-1598 against the MICs of triazoles	79
Figure 5-2. MICs of tested azole compounds against strains with individual known azole resistance mechanisms	81
Figure 5-3. Relative fold-change compared to SC5314 in MIC of various azole antifungal agents against strains containing single and double <i>ERG11</i> mutations	84

LIST OF ABBREVIATIONS

Ca	<i>Candida albicans</i>
CLSI	Clinical Laboratory and Standards Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CYP	cytochrome P450
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GM	geometric mean
GOF	gain-of-function
LOF	loss-of-function
LOH	loss-of-heterozygosity
MIC	minimum inhibitory concentration
PCR	polymerase chain reaction
qPCR	real time quantitative polymerase chain reaction
spp.	species
ZCF	zinc cluster transcription factor

CHAPTER 1. INTRODUCTION

General Background

Manifestations of *Candida* infections range from superficial mucosal and dermal infections such as oral thrush and vaginal yeast infections to disseminated bloodstream infections and are associated with high mortality rates (upwards of >40%) (1-5). *Candida* infections require extreme attention in immunocompromised individuals, such as AIDS patients, transplant recipients, patients undergoing chemotherapy or immunosuppression therapies, in which they cause life-threatening invasive infections (6-8). Studies have suggested that healthy individuals with implanted medical devices, those who experienced major trauma, and patients requiring extended stays in intensive care units are at equal risk of acquiring *Candida* infection (9). Global estimates suggest that invasive candidiasis occur in ~700,000 cases which clearly exceeds ~250,000 cases of invasive aspergillosis (10).

In the USA, *Candida* spp. remain the fourth leading cause of nosocomial bloodstream infections and the number three cause of bloodstream infections in the intensive care unit (ICU) (11). *Candida* spp. are also the third leading cause of central-line-associated bloodstream infections and the second leading cause of catheter-associated urinary tract infections in the U.S. (12, 13). Of all the *Candida* species, *C. albicans* dominates almost all patient groups and disease manifestations in terms of incidence followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (14). It is pertinent to mention here that there has been considerable change in species distribution, dependent upon geographical location and patient population, with a decrease in the proportion of *C. albicans* and an increase in *C. glabrata*, *C. parapsilosis*, and other non-*albicans* *Candida* species (14). *C. glabrata* is the second most common species in the United States and northwestern Europe in the non-outbreak setting (15, 16). However, *C. parapsilosis* and/or *C. tropicalis* are much more frequently encountered than *C. glabrata* in Latin America, Southern Europe, India and Pakistan (17-23). Of the five major *Candida* spp., *C. krusei* is the least common, and it is most often found among patients with underlying haematological malignancies with prior antifungal exposure (24, 25). Another worrisome multidrug resistant species that is emerging globally is *C. auris* (26).

C. albicans is a member of native human microflora and as a commensal organism colonizes the gastrointestinal (GI) tract, reproductive tract, oral cavity, and skin of most humans (27). Failure to identify a possible environmental reservoir for *C. albicans* suggests that this species is exquisitely adapted to healthy mammalian hosts. However, this benign commensal colonization can become pathogenic due to shifts in pH and oxygen levels, alterations in host microbiota (e.g. from antibiotic usage), or changes in the host immune response (e.g. during stress, infection by another microbe, or immunosuppressant therapy) (28). Rapid increase in number of *Candida* infections may be attributed to availability of modern medical treatments including excessive usage of broad-spectrum antibiotics, anticancer therapy, solid organ transplantation, presence of

indwelling catheters, lack of awareness among multiple medical specialties, and poor performance of routine microbiological tests.

Fluconazole is the first line of antifungal drug available for an effective treatment for invasive candidiasis and is the recommended treatment for most infections (29). While antifungal resistance in *C. albicans* is uncommon relative to species like *C. glabrata*, gradual use of fluconazole for long term prophylaxis or treatment may lead to selective pressure resulting in emergence of secondary resistance in otherwise susceptible strains/species. Overall, fluconazole resistance (MIC ≥ 8 $\mu\text{g/ml}$) in *C. albicans* ranges between 0% and 3% (30-34). Although overall azole resistance of naïve *C. albicans* isolates is rare, a steady increase in reports of azole resistance, resulting in therapeutic failures, has been a matter of serious clinical concern. In the current review, we aim to address the magnitude of azole-resistance in *C. albicans* and the underlying resistance mechanism implicated in the development of azole resistance and highlight the therapeutic concerns.

Biology of *Candida albicans*

Candida albicans possess key virulence traits that not only differentiate it from other *Candida* species but also help it in establishing itself as a successful pathogen. It secretes a number of proteases, phospholipases, esterases and lipases in the context of invasive infection that helps in promoting degradation of host connective tissue, cleavage of host immune defence proteins thereby leading to nutrition acquisition, invasion and evasion of pathogen from host innate immune response (35, 36).

C. albicans can adapt to use alternative carbon sources due to its ability to successfully thrive in heterogenous carbon microenvironment in the host. The versatility of metabolic elasticity of this species helps in morphogenic transition between unicellular yeast cells, pseudohyphae, and hyphae thereby promoting invasion (37-41). This is in contrast to *C. glabrata*, which has the intrinsic inability to form hyphae and does not require this morphogenic change in order to establish invasive disease. *C. albicans* has the ability for cell wall remodeling, white opaque switching and can form highly structured biofilms composed of multiple cell types (i.e. round yeast-form cells; oval pseudohyphal cells; and elongated, cylindrical hyphal cells) encased in an extracellular matrix (42-46). Due to their effective adherence and invasion in endothelial and epithelial cells they can easily disseminate into the bloodstream. *C. albicans* can invade host epithelial cells either through forced hyphal-mediated invasion or by induced endocytosis into endothelial and epithelial cells (47-50). It is pertinent to mention here that all *Candida* species exhibit different antifungal susceptibility patterns depending upon their site of isolation, geographical location, and prior antifungal exposure. *C. albicans* and *C. parapsilosis* are most frequently susceptible to all antifungals available. On the other hand, *C. tropicalis* might exhibit fluconazole resistance while maintaining susceptible profile to amphotericin B and echinocandins. Interestingly, *C. glabrata* tends to display high azole MICs while retaining amphotericin B and echinocandin susceptibility. *C.*

lusitaniae, on the other hand, is resistant to Amphotericin B while the emerging fungal threat *C. auris* is multidrug resistant.

Epidemiology of Azole Resistance in *C. albicans*

In vitro susceptibility testing of yeasts and moulds helps in not only selecting the most clinically active antifungal agent but also in detecting resistance rates. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods are reference methods to detect *in vitro* resistance of yeasts and moulds against various antifungal agents. Resistance can be classified as microbiologic or clinical. Microbiologic resistance is defined by the situation when concentration of antifungal agent required to inhibit the growth of the pathogen is higher than the range seen for wild-type strains due to presence of an acquired or innate mutational resistance mechanism. Clinical resistance, on the other hand, is denominated by therapeutic failure in spite of administering high doses of the antifungal agent.

Emergence of azole resistance in *Candida* species, especially those originating from patients on prolonged azole therapy, have become a worldwide problem thereby stressing the need for antifungal stewardship. Decreased *in vitro* susceptibility of *C. albicans* to azoles such as miconazole, econazole, and clotrimazole has been recognized since the late 1970s (51, 52). Horsburgh and Kirkpatrick in 1983 reported two of 21 patients with chronic mucocutaneous candidiasis on ketoconazole treatment suffering clinical relapses associated with *C. albicans* possessing high ketoconazole MICs (53). Similarly, high ketoconazole MICs were subsequently reported by Warnock et al. and Smith et al. in similar cases (54, 55). These relatively ketoconazole-resistant isolates demonstrated decreased *in vitro* susceptibility to miconazole, itraconazole, fluconazole, and tioconazole. There have also been reports of mucosal candidiasis due to *C. albicans* with reduced *in vitro* and *in vivo* responsiveness to fluconazole, ketoconazole, and clotrimazole in HIV-infected patients (56, 57). Evans et al. reported three cases of treatment failure in invasive mycoses due to fluconazole resistant *C. albicans* (58).

Most of the data related to the epidemiology of antifungal susceptibility profile of *Candida* spp. originates mainly from studies carried out in the USA and Europe. Cleveland et al., in 2012 showed an azole resistance rate of about 2.3% in *C. albicans* bloodstream isolates (59). The SENTRY surveillance data from 2008–2009 investigated 1239 bloodstream *Candida* isolates from 79 medical centers throughout the world and reported variation in *Candida* spp. distribution and antifungal resistance rates (32). *Candida albicans* (n=620, 50%) was the most common species isolated in all age groups followed by *C. glabrata* and *C. parapsilosis* (n=215, 17.4%), and *C. tropicalis* (n=122, 9.8%). None of the *C. albicans* isolates were resistant to any of the antifungal tested in this study. Interestingly, a recent SENTRY surveillance data analyzing 1514 *Candida* spp. collected from Europe (41.0%), the Asia Pacific (24.5%), North America (23.5%), and Latin America (11.0%) during 2013 revealed the similar pattern of *Candida* species isolation (15). However, azole antifungal resistance of about 0.4% and 0.3% in *C.*

albicans (n=712) was noted against fluconazole and voriconazole, respectively. Another large population-based surveillance program, the ARTEMIS DISK Global Antifungal Surveillance Study was carried out from 1997 to 2007, analyzing 256,882 isolates of *Candida* spp. collected and tested at 142 study centers throughout the world (30). Of all 31 different *Candida* species isolated, *C. albicans* (65.3%) was the most common followed by *C. glabrata* (10.2% to 11.7%), *C. tropicalis*, (5.4% to 8.0%), and *C. parapsilosis* (4.8% to 5.6%). Of 128,625 isolates of *C. albicans* tested, 1.4% were resistant to fluconazole. Of 1782 fluconazole-resistant *C. albicans* strains, 63.6% were cross resistant to voriconazole (30). A surveillance study was conducted in Denmark to evaluate the species distribution and *in vitro* antifungal susceptibility profile against fluconazole, itraconazole, amphotericin B and caspofungin in 303 episodes of fungaemia (60). Here again, *C. albicans* was the predominant species isolated followed by *C. glabrata*, *C. tropicalis* and *C. parapsilosis*. Of all the *C. albicans* tested, 3% (n=6) were resistant to fluconazole. In another Danish study, a 6-year nationwide study of fungemia from 2004-2009 reported fluconazole (MIC of >4 µg/ml) resistance rate of about 0.6% (7/1183 isolates) (61).

A recent study from Spain assessed the incidence and prevalence of antifungal resistance in bloodstream *Candida* infections (62). Interestingly, in a collection of 766 *Candida* strains, *C. albicans* (45.4%) was the predominant species followed by *C. parapsilosis* (24.9%), *C. glabrata* (13.4%), *C. tropicalis* (7.7%), *C. krusei* (2%), and other *Candida* species (6.5%). Of all *C. albicans* strains tested for antifungal susceptibility, 1% was resistant to fluconazole. A multi-center study from Belgium during 2013-2014 demonstrated eleven different *Candida* species, with *C. albicans* being the most prevalent (50.4 %), followed by *C. glabrata* (27.3 %), *C. parapsilosis sensu lato* (9.8 %), and *C. tropicalis* (5.6 %) (63). About 3.9% (n=7) of *C. albicans* (n=179) isolates were found to be fluconazole resistant here. A twenty-two year Norwegian national surveillance of candidaemia found 98.8% *C. albicans* isolates to be susceptible to fluconazole (64). Only 0.25% of *C. albicans* isolates were fluconazole resistant during 2004-2012.

Chapman et al., from Australia reported two (0.9%) isolates of *C. albicans* that were resistant to fluconazole, cross-resistant to voriconazole and had non-wild type MICs of itraconazole (>16 mg/L) and posaconazole (>8 mg/L) (65). A national South African surveillance for candidemia from 2009-2010 revealed 98% *C. albicans* isolates to be azole susceptible. However, a single fluconazole-resistant *C. albicans* isolate exhibited cross resistance to voriconazole (66). In a 2015 study conducted in ICU centers across India, 5.2% of *C. albicans* strains sourced from ICU-acquired candidemias were identified as fluconazole-resistant (67).

Overall, the global data suggests that *C. albicans* remains the predominant infective organism identified in *Candida*-based infections. While reported resistance rates vary from study to study, collected surveillance data suggests that fluconazole-resistance and azole resistance rates in general for *C. albicans* remains relatively low. However, superficial mucosal *C. albicans* infections pose a considerable threat in immunocompromised populations, and the high morbidity and mortality associated with

serious invasive *Candida* infections continues to burden our healthcare systems (68-70). As such we must not overlook *C. albicans* as a serious antimicrobial resistance threat.

Current Antifungal Treatment of *C. albicans* Infections

Currently, antifungal therapy revolves around three main classes of antifungal drugs: the azole antifungals, echinocandins, and polyene antifungals such as amphotericin B. The antimetabolite pyrimidine analog flucytosine is also used in treatment of certain invasive *Candida* infections and cryptococcal meningitis, but is limited to combination therapies with amphotericin B (29). The azole antifungals are a current mainstay of therapy for treatment and prophylaxis of both superficial and invasive candidiasis. Through inhibition of the *ERG11*-encoded protein 14 α -lanosterol demethylase, the production of ergosterol normally required for membrane integrity is halted. In *C. albicans*, the inability to demethylate lanosterol via Erg11 shunts sterol biosynthesis production to other alternate biosynthesis pathways, resulting in the accumulation of 14 α -methyleergosta-8,24(28)-dien-3 β ,6 α -diol (71). The accumulation of this compound is toxic to the cell and results in the fungistatic effect seen with the azole antifungals (72).

While there are dozens of azole antifungal drugs sold commercially worldwide, ranging from research use to topical antifungal use in humans to agriculture, only a select few triazoles are typically used for treating invasive candidiasis in the United States. While recent updates to clinical practice guidelines now recommend the echinocandin class of antifungals in many invasive *Candida* infections, fluconazole remains a viable first-line alternative in many cases, such as in non-neutropenic patients with *Candida* bloodstream infection (29). Voriconazole is another triazole considered as alternative or step-down therapy in many instances where fluconazole is also used, and the triazoles itraconazole and posaconazole have niche uses in invasive candidiasis, such as alternatives for oropharyngeal or esophageal candidiasis in fluconazole-refractory disease. Additionally, as currently no orally available echinocandin exists, fluconazole and the other triazoles are important for outpatient and step-down therapies for prophylaxis and treatment. The newly approved triazole prodrug isavuconazonium sulfate, while effective against *Candida*, including azole-resistant *Candida* species, does not currently have a place in treatment of invasive candidiasis and is instead reserved for the serious mould infections like aspergillosis and mucormycosis (73, 74).

Notably, a new subclass of azole antifungal compounds are in the drug pipeline. The tetrazole compounds, VT-1129, VT-1161 and VT-1598, so named because of an additional nitrogen on the five-membered azole ring that defines the class, are currently awaiting or undergoing clinical trials to treat a wide variety of fungal infections (75, 76). VT-1161, more specifically, is currently undergoing clinical trials as a treatment for recurrent vaginal candidiasis and may be a plausible future therapy against azole-resistant *C. albicans*.

There are many barriers to effective treatment with the azole antifungals in invasive candidiasis. Improper dosing and inadequate drug delivery to the source of

infection are obvious clinical faux pas to avoid, however, *C. albicans* and other *Candida* species have a variety of methods to survive even proper treatment practices. The next section details the specifics of the known azole resistance mechanisms in *Candida albicans*.

Azole Resistance Mechanisms in *C. albicans*

Efflux pumps

Cdr1 and Cdr2

Like many infecting organisms, *C. albicans* utilizes an efflux system to transport drugs extracellularly, thereby reducing intracellular drug concentrations. As such the overexpression of azole-targeting efflux pumps is a key mechanism driving azole resistance in *C. albicans*. The ATP-binding cassette (ABC) transporters Cdr1 and Cdr2 have both been well-documented as drivers of azole resistance and were first implicated in clinical azole resistance in isolates in HIV-infected patients with oropharyngeal candidiasis (77-79). In two separate studies, it was found that isolates of *C. albicans* taken from HIV-infected individuals had increased mRNA levels of *CDR1*, which has been shown to confer resistance to the azole antifungals (79-81).

Importantly, the zinc-cluster transcription factor Tac1 was discovered to regulate the expression of both *CDR1* and *CDR2* and that a codominant mutation in the putative C-terminal activation domain conferred hyperactivity of the Tac1 transcription factor, leading to constitutive increased expression of *CDR1* and *CDR2* (82, 83). In a comparison of azole-susceptible versus azole-resistant matched isolates, constitutive high expression of these transporters was suggested to be a result of both increased transcription and increased mRNA stability, the latter of which is tied to mRNA hyperadenylation and loss-of-heterozygosity at the poly(A) polymerase 1 locus (84, 85). Since then, multiple gain-of-function mutations in Tac1 leading to increased expression of the Cdr1 and Cdr2 efflux transporters have been described (83, 86-88).

Additionally, it was shown that increased *CDR1* expression could be induced in agents such as estradiol or fluphenazine (89). While it was previously shown that *CDR1* expression could be induced in response to fluconazole in a Cdr1-GFP fusion protein construct, a more recent study found that no increase in *CDR1* or *CDR2* expression as measured by RT-qPCR could be observed in the presence of fluconazole in a *C. albicans* strain with 6His3Flag-tagged Tac1 (90, 91). Both the constitutive and transient expression of *CDR1* and *CDR2* genes seems to be regulated via interactions at multiple cis-acting elements within the *CDR1* and *CDR2* promoters (92). The presence of drug-responsive elements (DRE) within each gene's respective promoter regions, for example, has been shown to contribute to the induced and constitutive expression of *CDR1* and *CDR2*, while a separate basal expression element (BEE) contributes to *CDR1* baseline expression (82, 89, 93).

In a genome-wide location analysis of Tac1 binding sites using ChIP-chip, Tac1 was shown to bind to the DRE of both *CDR1* and *CDR2* under nonactivating conditions, indicating that post-binding mechanisms such as repressor interaction loss or coactivator recruitment may occur during Tac1 activation (93). More recently, components of the tail module of the Mediator complex have been identified as required for Tac1-activated *CDR1* expression. In a study examining the coactivator requirements for hyperactive Tac1 regulation, Liu and Myers described decreased fluconazole MICs in *TAC1* gain-of-function mutant strains lacking either Med3 or Med15, both of which are part of the Mediator tail module (91). Furthermore, not only was Tac1-driven transcription of *CDR1* decreased in gain-of-function Tac1 mutant strains lacking either Med3 or Med15, but deletion of *MED3* also diminished xenobiotic-induced expression levels of *CDR1*, *CDR2*, and *RTA3* with fluphenazine and estradiol. ChIP assays revealed that occupancy of the Mediator tail module at the *CDR1* DRE increases during Tac1 hyperactivation, as well as with *TAC1* overexpression, however higher occupancy at the DRE did not result in increased fluconazole MICs, unless Tac1 was activated. Importantly, the authors also delineated a C-terminal region of Tac1 (amino acids 865 to 981), so called the transcription activation domain (TAD), whose interaction with an as yet uncharacterized inhibitory middle domain, may be crucial to Tac1 hyperactivation, and have posited that gain-of-function mutations in Tac1 may work to antagonize the normally inhibitory interaction between these two regions (91).

Mdr1

Mdr1, part of the major facilitator superfamily of transporters, has also been shown to play a role in fluconazole and voriconazole resistance, though it does not effect large MIC changes with other azole antifungals such as itraconazole, isavuconazole, and posaconazole (94-96). The gene encoding the Mdr1 efflux pump in *C. albicans* was originally discovered by its ability to confer resistance to several structurally-unrelated compounds such as the anti-mitotic drug benomyl, methotrexate, cycloheximide, sulfometuron methyl, 4-nitroquinoline-*N*-oxide, and bentriazoles in *Saccharomyces cerevisiae* expressing the *C. albicans MDR1* gene, which at the time was known as *BENr* (97, 98). It was not until serial isolates taken from HIV-infected patients with oropharyngeal candidiasis were analyzed for *ERG11* and *MDR1* mRNA expression levels was a link established between the *MDR1* gene and fluconazole resistance (99).

A study assessing sequential isolates of *C. albicans* in patients with recurrent oropharyngeal candidiasis treated with fluconazole found that increased *MDR1* mRNA, as well as increased *CDR1/2* and *ERG11* gene expression, correlated with increasing fluconazole drug resistance, indicating that *MDR1* plays a role in the multifaceted development of resistance in *C. albicans* (100). When the *MDR1* gene was disrupted in two fluconazole-resistant clinical *C. albicans* isolates, susceptibility increased to fluconazole compared to the resistant isolates with intact *MDR1*, demonstrating a direct relationship between *MDR1* and fluconazole resistance in clinical isolates (101).

Importantly, matched pairs of fluconazole-susceptible and fluconazole-resistant isolates were shown to lack major polymorphic differences in the nucleotide sequence of their respective *MDR1* promoter regions, despite high expression of *MDR1* mRNA in the fluconazole-resistance isolate (102). Subsequent expression of *GFP* from the *MDR1* promoter in the fluconazole-resistant isolate but not the fluconazole-susceptible isolate indicated that *MDR1* expression was likely guided by a *trans*-regulatory factor. Induction of *MDR1* mRNA expression through benomyl and *tert*-butyl hydrogen peroxide identified several *cis*-acting elements in the *MDR1* promoter involved in induction with these agents, though fluconazole itself failed to induce the *MDR1* promoter (103).

It was not until more recently in 2007 that the zinc-cluster transcription factor Mrr1 was discovered to regulate the Mdr1 efflux pump. Transcriptional profiling of three *MDR1*-overexpressing clinical isolates via DNA microarray analysis revealed 21 genes coordinately upregulated with *MDR1* and seven genes downregulated in all three isolates (104). Among these, the then putative zinc cluster transcription factor Zcf36, later known as the multidrug resistance regulator Mrr1, was hypothesized to control *MDR1* expression. Deletion of *MRR1* in *MDR1*-overexpressing isolates F5 and G5 managed to abolish *MDR1* promoter activity in a P_{MDR1} -*GFP* reporter fusion, implicating Mrr1 in the constitutive expression of *MDR1* in these fluconazole-resistant clinical isolates. Interestingly, deletion of *MRR1* in each *MDR1*-overexpressing isolate did not yield identical MICs to strains lacking *MDR1* in the same background. Deletion of *MDR1* alone in both cases, resulted in a higher MIC in the resistant clinical isolate background compared to deletion of its transcriptional regulator Mrr1, providing evidence that Mrr1 may regulate other determinants of fluconazole resistance apart from Mdr1.

Similar to the zinc cluster transcription factor encoding gene *TAC1* in *CDR1*- and *CDR2*-overexpressing isolates, mutated *MRR1* alleles were present in the *MDR1*-overexpressing clinical isolates (104). Moreover, sequencing of the *MRR1* alleles in the *MDR1*-overexpressing isolates F5 and G5 revealed a loss of heterozygosity and retention of two copies of a mutant *MRR1* allele in both clinical isolates. Replacement of a single copy of the mutant *MRR1* allele encoding either a P683S or G997V amino acid substitution into SC5314 lacking both native copies of *MRR1* resulted in increased resistance to fluconazole and increased *MDR1* promoter activity as measured by a P_{MDR1} -*GFP* reporter construct, demonstrating the presence of gain-of-function mutations in *MRR1* that regulate *MDR1* expression in *C. albicans*.

Indeed, multiple amino acid substitutions in Mrr1 originating from both human clinical isolates and in vitro generated strains have been shown to be gain-of-function mutations driving constitutive *MDR1* expression (105). Furthermore, through DNA sequence analysis of a the F1 through F5 matched series clinical isolates, it was revealed that gain-of function mutations appear to occur in a single *MRR1* allele initially, which provides an intermediate level of resistance compared to the matched isolates collected chronologically later in the series that were homozygous for the *MRR1*^{P683S} allele. Through analysis of marker polymorphisms along chromosome 3 distal to *MRR1* and proximal to the telomere, Dunkel et al. revealed that loss-of-heterozygosity with selection

of mutated *MRR1* alleles was accomplished through mitotic recombination in clinical isolates, though chromosome loss was also observed as a cause in *MDR1* overexpressing strains developed *in vitro*.

Upc2 and increased *ERG11* expression

Upc2 is another Zn(II)-Cys(6) transcription factor involved in sterol biosynthesis and azole resistance. Initially identified as a homolog of the *S. cerevisiae* (*ScUPC2*) gene, *UPC2* in *C. albicans* was initially reported to upregulate the ergosterol biosynthesis genes *ERG2* and *ERG11* when exposed to fluconazole (106). The overexpression of *ERG11* had been shown to contribute to azole through increased production of the azole target 14 α -lanosterol demethylase (80, 99, 107, 108). However, not only was the induction of *ERG11* abolished when *UPC2* was deleted, but the deletion strain was also hypersusceptible to the azole drugs as well as the squalene epoxidase (Erg1) target drug terbinafine, the Erg2 and Erg24 target drug fenpropimorph, and the HMG-CoA reductase inhibitor lovastatin, illustrating *UPC2*'s important role in sterol biosynthesis regulation (106, 108).

Importantly, overexpression of Upc2 via expression of the ORF from the *MET3* promoter increased resistance to fluconazole and ketoconazole, and it was also demonstrated for the first time that the DNA-binding domain of Upc2 bound directly to ergosterol biosynthesis genes, binding to a putative sterol responsive element in *ERG2* (108). Furthermore, investigation of the *ERG11* promoter through deletion and linker scan mutations concluded that two 7-bp inverted repeat sequences located -231 and -251 bp upstream of the start codon form an azole-responsive element (ARE) regulated by Upc2, and that this ARE was sufficient to induce *ERG11* expression (109).

Upc2, however, was also shown to have a regulatory role in more than just sterol biosynthesis genes. Through genome-wide gene expression profiling, several ergosterol biosynthesis genes, including *ERG11*, were determined to be coordinately upregulated with *UPC2* in fluconazole resistant isolates, and ChIP-microarray location analysis identified 202 promoters bound by Upc2, including not only ergosterol biosynthesis genes (*NCPI*, *ERG11*, *ERG1*, *ERG2*, *ERG24*, *ERG4*, *ERG5*, *ERG6*, *ERG9*, *ERG10*, *ERG25*, *ERG251*, *UPC2*) but also other transcription factors and the drug transporter genes *CDR1* and *MDR1* (110-112). As with the zinc cluster transcription factors Tac1 and Mrr1, gain-of-function mutations in Upc2 were found to lead to increased gene expression of *ERG11* conferring fluconazole-resistance in clinical isolates (110, 113-115). When *UPC2* was disrupted, the $\Delta upc2$ mutants had decreased cellular ergosterol content and were more susceptible to fluconazole not only in the azole-susceptible SC5314 strain but also in a highly resistant clinical isolate 12-99 which overexpresses *CDR1*, *MDR1*, *ERG11* and possesses a mutation in *ERG11*, suggesting that *UPC2* is required for clinical azole drug resistance (115, 116).

Other zinc cluster transcription factors

Apart from the zinc cluster transcription factors Tac1, Mrr1, and Upc2, which are well-known players in *C. albicans* azole resistance, there has been significant interest in the azole-resistance role of the remaining zinc-cluster transcription factors as well. In 2013, Morschhäuser et al. demonstrated that laboratory strains exhibiting hyperactive transcription factors Aro80, Mrr2, Stb5, Cta4, Zcf25, Zcf35, and Znc1 also displayed increased azole resistance comparable to hyperactive Tac1, Mrr1, and Upc2, demonstrating that in theory other ZCFs may play a role in azole resistance in *C. albicans* (117). Additionally, in the hyperactive ZCF strains, Znc1 and Mrr2 appeared to activate the *CDR1* promoter, indicating that these ZCFs may contribute to increased efflux pump expression.

In 2015, Wang et al. described the first clinical isolate containing putative gain-of-function mutations in the ZCF Mrr2, identifying amino acid substitutions that appeared increased *CDR1*, but not *CDR2* expression (118). In contrast to this finding, a recent report investigating these mutations in *MRR2* when inserted into the native locus as well as when the gene is overexpressed failed to identify any changes in fluconazole susceptibility or *CDR1* expression compared to wild type (119). Given the contradictory findings, the clinical impact of other ZCFs outside of Tac1, Mrr1, and Upc2 in azole resistance remains in question.

Mutations in *ERG11*

Mutations in the *ERG11* gene itself is another common azole resistance mechanism in *C. albicans*, in stark contrast with the haploid organism *C. glabrata*, in which the frequency of mutations in *ERG11* appears relatively low (14, 120). Most *ERG11* mutations are thought to alter the ability of the azoles to bind and inhibit the lanosterol demethylase enzyme, either through steric hindrance in the ligand binding pocket where the azole antifungals compete with ergosterol precursor or by altering or reducing important interactions, such as the H-bond interactions with the azole ring and the heme group of the Erg11 protein.

The earliest point mutation resulting in an amino acid substitution (R467K) in the *ERG11* gene of a clinical isolate was sequenced and discovered in 1997 (121). Soon after, multiple additional amino acid substitutions in Erg11 were uncovered, and notably, the effect of these amino acid changes on triazole antifungal MICs were not uniform (122). This altered susceptibility to the azoles was noted to likely be a result of altered interaction with azole binding and overall reduced fluconazole binding affinity to the mutant 14 α -lanosterol demethylase target enzyme (123, 124). As the azole antifungals emerged as a common, first-line option in the treatment of many superficial and invasive fungal infections, documentation of new *ERG11* mutations in azole-resistant clinical isolates continued to appear, though not all documented mutations were definitively shown to be tied to azole resistance (125-138).

The isolated contribution towards azole-resistance of several predominant Erg11 amino acid substitutions found in clinical *C. albicans* isolates was investigated through homozygous replacement of the native *ERG11* alleles with a mutant *ERG11* ORF encoding either a single or double amino acid substitution in the Erg11 protein (139). Single substitutions in Erg11 showed variable responses to fluconazole and other triazoles ranging from no effect to a 16-fold increase in MIC over the fluconazole-susceptible parent, and double substitutions in Erg11 further increased the MIC beyond the individual contribution to azole resistance of any single amino acid substitution. Interestingly, while some substitutions, such as Y132F/H, K143R, G307S, and S405F, are located on the exposed active-site cavity of the Erg11 protein and presumably directly influence or interfere with azole binding, other substitutions not predicted to directly interact with azole binding, such as the G450E substitution found on the fungal CYP51-specific β 5-hairpin, may indirectly affect resistance through interaction with cytochrome P450 reductase-Erg11 interactions (140).

Recently substitutions in purified, recombinant *C. albicans* Erg11 expressed from *E. coli* revealed that Erg11 variants differ substantially in their baseline catalytic turnover and affinity for azole binding (141). Despite some substitutions showing relatively loose azole binding, their observed contribution to azole resistance in *in vitro* *C. albicans* susceptibility testing was significant; making it conceivable that Erg11 amino acid substitutions may contribute to azole resistance through more nuanced ways than simply reducing azole binding affinity.

Mutations in *ERG3* and alternative sterol biosynthesis

Alterations in sterol biosynthesis pathways in *C. albicans* remains an important, albeit rare clinical mechanism of azole antifungal resistance. Defective C5-6 desaturase, homologous to the Erg3 enzyme C-5 sterol desaturase, identified in azole-resistant isolates of *Saccharomyces cerevisiae* initially led to the currently hypothesized mode of action of the azole antifungals.

14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol, a metabolic byproduct not normally produced in significant amounts during normal ergosterol biosynthesis, was found to accumulate in *C. albicans* cells following treatment with fluconazole (72). However, isolates with defective C-5 sterol desaturase, which lacked detectable amounts of ergosterol presumably because they no longer possessed the Erg3 enzyme function required for ergosterol production, avoided accumulation of 14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol after treatment with fluconazole. The continued growth of isolates with defective Erg3 in the presence of fluconazole has led to the current belief that 14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol is toxic to the *C. albicans* cell, and its accumulation results in the inhibition of growth. Moreover, mutations and amino acid substitutions that confer defects in Erg3 also confer resistance to the azole antifungals, specifically due to the inability to produce 14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol, which requires Erg3.

While relatively few in number compared to isolates containing other more common resistance mechanisms, several mutant *ERG3* clinical isolates have been identified, and reportedly possess resistance to the azole antifungals and cross-resistance to the polyene amphotericin B (72, 130, 142-145). However, some question the clinical significance of *ERG3* mutant isolates during infection as isolates with an *ERG3* resistance phenotype also displayed hyphal growth defects and attenuated virulence (146-148). Recently, it has been shown that changes within the *ERG3* promoter that affect expression and activity may be sufficient to confer azole resistance in niche-specific instances without affecting *C. albicans* pathogenicity, which implicates changes in the *ERG3* locus as potentially more clinically relevant than previously believed (149).

Aneuploidy and loss of heterozygosity

Aneuploidy plays a role in azole resistance as well as genetic diversity in *C. albicans*. Gain or loss of chromosomes have been tied to azole resistance and attenuated virulence (150, 151). Loss of heterozygosity at gene loci of isolates serially passaged in azole-containing media as well as lab strains passaged through mice models of hematogenously disseminated disease both suggest that significant genetic rearrangements take place as a result of host and drug stress (152, 153). Studies using comparative genome hybridization in various *C. albicans* laboratory strains observed aneuploidies mainly on chromosomes 1, 2, and 5 (154), and it was later shown that some genomic changes such as isochromosome formation confer azole resistance in *C. albicans* (155).

Overrepresentation of efflux pumps, *ERG11*, or zinc cluster transcription factor genes involved in azole resistance as a result of chromosomal nondisjunctions, trisomy, or isochromosome formation is very likely to contribute to decreased drug resistance in these cells. Indeed, it had been shown for both *TAC1* and *MRR1* that loss of heterozygosity was responsible in some clinical isolates for homozygous gain-of-function mutations conferring increased efflux pump expression and azole resistance (83, 105). A recent analysis of large-scale genomic changes in 43 clinical isolates of *C. albicans* showed that while loss of heterozygosity events are persistent and often associated with resistance, aneuploidies in clinical isolates are often transient and did not correlate with drug resistance (156). The discovery of tetraploid cells of *C. albicans*, containing four homologous sets of chromosomes, and their ability to generate a diverse range of fitness and heterogeneity through increased rates of heterozygosity loss suggests that aneuploidy plays an important role in adaptive fitness to selective pressures under these circumstances (157, 158). The recent investigations into trisomy of chromosome R and chromosome 4 possibly conferring azole resistance in two different clinical isolates of *C. albicans* also underscores the importance of aneuploidy in the microevolution of drug-resistant *C. albicans* (159, 160).

Azole import

Currently there is limited evidence suggesting that reduced azole uptake has any clinical relevance to azole resistance in *C. albicans* or any other *Candida* species. However, it should be mentioned that it is plausible that reduced azole import could be a potential uncharacterized mechanism of drug resistance. In a study examining the *in vitro* accumulation of fluconazole into the *Candida* cell, it was observed that fluconazole import displayed saturation kinetics in cells de-energized with 2-deoxy-D-glucose and therefore unable to efflux drug via active transport mechanisms like the ABC transporter Cdr1 (161). Additionally, maximal accumulation of fluconazole did not progress in a temperature-dependent manner inconsistent with simple passive diffusion. The kinetics of fluconazole import, therefore, suggests that fluconazole is uptaken by means of a carrier protein via facilitated diffusion and that this mechanism is likely conserved across many fungal species (161, 162).

The carrier protein(s) responsible to importing fluconazole and presumably the other azoles, however, has/have not yet been identified despite prolific research. In theory, cells possessing defective carrier protein (via mutation, for example) or protein in low amounts would not accumulate lethal or significantly inhibitory amounts of drug, resulting in resistance.

Azole tolerance

Candida albicans is usually classified as either susceptible (and susceptible dose-dependent) or resistant when referring to clinical antifungal breakpoints, which are used in helping to predict response to therapy against fluconazole or other antifungal agents (29, 33, 163-165). Antifungal “tolerance” is an oft-used and loosely-defined term that refers generally to the residual growth of cells at or above inhibitory concentrations of drug.

Unlike resistant cells, which may be able to survive drug treatment due to gene-dependent mechanisms directly affecting the drug, drug target, or accumulation of the drug in the cell, cells displaying tolerance have been defined as those able to survive transient exposure to drug without an accompanying change in MIC (166-168). The “trailing growth” phenomenon, describing the residual population of cells that grow at supra-MIC levels observed in broth dilution assays, can be considered related to azole tolerance and has been well-documented in *C. albicans* (168-171). Recently, *RTA3*, encoding a putative lipid translocase coordinately upregulated with *CDR1* in clinical isolates with Tac1 gain-of-function mutations, has been associated with increased azole tolerance and trailing growth in *C. albicans* (172).

The presence and degree of trailing growth has been known to be affected by different parameters such as temperature and pH and can affect the MIC interpretation depending on the time of reading (24 hours versus 48 hours) (170, 173, 174). However, the clinical significance of trailing growth has been dubious at best. Past literature

suggests that the *C. albicans* isolates exhibiting trailing growth phenomena responds to azole treatment similarly to susceptible isolates in murine models and in a small sample size of HIV-infected patients with recurrent oropharyngeal candidiasis (175-177).

Importantly, a recent study delineated parameters to describe the antifungal tolerance observed with certain strains. The “fraction of growth,” or FoG, measured on antifungal disk diffusion assay and the “supra-MIC growth,” or SMG, as measured by broth microdilution assay are objective measurements assessing growth either inside the zone of inhibition or above the MIC for a given strain, respectively (178). Neither parameter correlated with MIC and instead measured a drug response distinct from MIC. Interestingly, FoG and SMG were independent of inoculum size, cell density or drug concentration, and in contrast to tolerance in bacteria, tolerant *C. albicans* cells did not have reduced growth rates. Additionally, FoG did not correlate with growth rate or colony size, indicating that high levels of FoG are not due simply to faster cell growth. Instead, *C. albicans* isolates with high levels of FoG or SMG suggests a larger subpopulation that can respond to high levels of antifungal drug stress better than isolates with low FoG or SMG.

When comparing the drug responses of clinical isolates taken from patients with persistent candidemia versus patients with candidemia that was cleared after a single treatment course of fluconazole, significantly higher FoG and SMG levels was observed in clinical isolates originating in patients with persistent candidemia (178). This was despite the fact that the tested isolates in both groups all had *in vitro* MICs considered fluconazole-susceptible, and identified FoG and SMG as potential parameters to guide treatment outcome in patients with candidemia. This tied with the identification of several adjuvant drugs that could reduce FoG but not MIC in clinical isolates suggests that drug tolerance in *C. albicans* may be an overlooked issue of significant clinical relevance.

Future Directions

As we have discussed, *C. albicans* has an arsenal of defense mechanisms that it uses, alone and in combination with each other, to overcome inhibition by the azole antifungals. Despite the numerous resistance pathways already discovered, continued investigation is required to fully characterize azole resistance in *C. albicans*. For example, the azole importer(s) in *Candida* and other fungal species has still not been identified, and its discovery would plausibly reshape our approach to overcoming azole resistance.

Ongoing research is also identifying potential novel mechanisms of azole resistance, though the clinical significance of these are still unknown. Recently, a *Nature Communications* paper published that alteration of sphingolipid synthesis mediated through *FEN1* and *FEN12* deletion results in fluconazole resistance, and these composition changes are similar to what is observed in wildtype cells treated with fluconazole (179). Furthermore, transposon-based mutagenesis and disruption of the *C.*

albicans genome in a haploid strain identified a number of potential genes involved in drug stress response through Gene Set Enrichment Analysis, providing further insight into *C. albicans* acquisition of drug resistance.

As more of the machinery driving azole resistance becomes unveiled, we can begin to isolate the cogs critical to its function. Targeting these components with novel drug development and improved use of our existing armamentarium becomes the immediate goal in addressing the rising problem of azole resistance and antifungal drug resistance in general.

Hypothesis and Objectives

Although *C. albicans* is a commensal organism that is part of normal flora in the majority of the human population (2), it is an opportunistic pathogen that has been the major infective species of *Candida* found in invasive candidiasis (17, 180, 181). For invasive fungal infections like *Candida* bloodstream infections, mortality rates remain high (182, 183). Fluconazole and other members of the azole class continue to be commonly used in the treatment of *Candida* infections, and overall have good activity against a majority of *C. albicans* isolates (15). However, azole-resistant *Candida* is of serious concern, especially in those patient with prior exposure to the azole antifungals (184-186). The ultimate, overarching goal of my research has been to improve the health outcomes of patients treated with the azoles for *C. albicans* infections. By investigating mechanisms of azole resistance in *C. albicans*, I hope to identify novel determinants in *C. albicans* that may prove useful either in the determination and detection of clinical isolates predisposed to azole non-susceptibility or as a drug target in the development of new antifungals.

In previous work performed by our research group, the major known resistance determinants, including the expression of the efflux pump encoding genes *CDR1*, *CDR2*, and *MDR1* as well as mutations in and increased expression of *ERG11*, encoding the azole target 14 α -lanosterol demethylase, were identified and characterized in a large collection of predominantly fluconazole-resistant clinical isolates (115, 116). Here it was revealed that some clinical isolates possessed higher fluconazole MICs than what could be reasonably justified based on the known mechanisms of azole resistance (**Figure 1-1**). Hence, I had reason to further investigate these clinical isolates for hidden contributors of azole resistance.

Previous literature supports the hypothesis that other mechanisms of azole resistance remain uncharacterized in clinical isolates. The critical role of the zinc cluster transcription factors Tac1, Mrr1, and Upc2 in regulating gene expression of azole resistance determinants led to identification of several other zinc cluster transcription factors in *C. albicans* that can influence azole susceptibility in laboratory strains (117). Through sequencing of the ZCF encoding gene *MRR2*, I identified several novel mutations in our clinical isolates. A recent publication suggested that this ZCF is important in the regulation of *CDR1* expression in clinical isolates (118). In some of our

Figure 1-1. Expression levels of *CDR1*, *CDR2*, *MDR1*, and *ERG11* in 63 fluconazole-resistant clinical isolates

All gene expression levels were measured in triplicate, and fold expression of genes in resistant isolates was compared to the average of the expression levels in three susceptible isolates. Results for 63 isolates with reduced susceptibility to fluconazole are represented, but only even-numbered isolates' results are labeled. Error bars show standard errors. Reprinted from final submission with permission from the American Society for Microbiology. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygas 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. doi: 10.1128/EC.00215-12.

isolates, *CDR1* expression was increased without a corresponding gain-of-function mutation in *TAC1*. Therefore, I hypothesized that gain-of-function mutations in *MRR2* was responsible for increased *CDR1* expression and increased fluconazole MICs in some of our clinical isolates. My summarized aim was to characterize these mutations with respect to their effect on fluconazole susceptibility and *CDR1* expression by placing the mutation-containing *MRR2* alleles in a fluconazole-susceptible clinical isolate.

Previously, our research group had defined the contributions of individual and combination amino acid substitutions in Erg11 (CaCYP51) to azole resistance in *C. albicans* (139). Amino acid substitutions in CaCYP51 are a well-established resistance mechanism that *C. albicans* possesses to reduce the ability of the azoles to inhibit CaCYP51 activity. The effects of these amino acid substitutions on CaCYP51 function and cell fitness, however, had not been previously studied. In a collaborative effort, our research group aimed to determine the differences in biochemical function and interaction between mutant CaCYP51 proteins, and whether this translates to reduced *in vitro* azole susceptibility in *C. albicans* cells. We hypothesized that mutant CaCYP51 protein have variable binding affinities with the azole antifungals, affecting azole susceptibility. Furthermore, we believed that amino acid substitutions not only affect CaCYP51's interaction with the azoles but also CaCYP51 native activity. My goal was to create additional mutant CaCYP51 *C. albicans* strains and evaluate the *in vitro* susceptibility data as compared to the biochemical data in this project.

Mutations in *ERG3*, a gene encoding the C-5 sterol desaturase important for ergosterol and toxic sterol production, are an oft overlooked mechanism of azole resistance due to its relative scarcity in clinical isolates. As part of the investigation into azole resistance within our clinical isolates, I also sequenced the *ERG3* gene in our collection. Interestingly, in addition to a nonsense mutation in *ERG3* in one clinical isolate with altered sterol composition, I frequently sequenced an uncharacterized A351V Erg3 amino acid substitution. Although I found no consistent correlation between ergosterol content and *ERG3* mutations, I noticed a high coincidence of *ERG3* mutations with *ERG11* mutations. I posited that amino acid substitutions in Erg3, such as the A351V mutations may play an indirect role in azole resistance by conferring either a fitness advantage to isolates with *ERG11* mutations. Preliminary data detailed in our research group's collaborative work with Erg11 mutants suggested that in addition to conferring varying changes in azole susceptibility, Erg11 amino acid substitutions also conferred changes in the Erg11 enzyme's functional efficiency and, therefore, also to the fitness of the cell. My hypothesis was that the A351V amino acid substitution in Erg3 was a permissive substitution that compensated for changes in Erg11 activity resulting from deleterious amino acid substitutions. My initial aim was to determine whether the A351V amino acid substitution conferred any advantage to cells also containing *ERG11* mutations.

Lastly, the novel investigational drugs VT-1161 and VT-1598 had been previously shown to have good activity against *Candida* and a wide variety of other fungi (187-189). However, *C. albicans* mechanisms of resistance against these two agents have not been clearly defined. Given our collection of azole-resistant clinical isolates and

laboratory strains with defined mechanisms of azole resistance, our research group endeavored to analyze the susceptibility data of our strains and isolates against both drugs. The goals here were to identify known mechanisms of azole resistance that may affect drug susceptibility to either VT-1161 or VT-1598 and to identify clinical isolates that possess increased MICs to the tetrazoles. Identifying mechanisms affecting tetrazole susceptibility would be useful in determining the role of either VT-1161 or VT-1598 as prospective therapeutic agents in invasive *Candida* infection, especially in resistant or recurrent infection. Additionally, given that our collection of clinical isolates contained isolates with unusually high azole MICs that could not be explained, we hoped that the additional tetrazole susceptibility data might provide insight on their resistance.

CHAPTER 2. THE CONTRIBUTION OF CLINICALLY-DERIVED MUTATIONS IN THE GENE ENCODING THE ZINC CLUSTER TRANSCRIPTION FACTOR MRR2 TO FLUCONAZOLE ANTIFUNGAL RESISTANCE AND *CDR1* EXPRESSION IN *CANDIDA ALBICANS*¹

Introduction

Candida spp. account for one of the most common pathogen groups implicated in nosocomial bloodstream infections in the US (12, 183). *Candida albicans* is the most common *Candida* spp. in invasive candidiasis and the primary cause of mucosal *Candida* infections such as oropharyngeal candidiasis (34, 70, 183, 190, 191). Fluconazole and other azoles are a mainstay of treatment in invasive candidiasis and oropharyngeal candidiasis, and despite the emergence of other antifungal drug classes, there has been no substantial decline in fluconazole resistance rates in *C. albicans* (29, 30, 34, 192). Moreover, the high mortality rate for *C. albicans* in invasive fungal disease provides compelling reason to further investigate how we can better utilize the available antifungal drugs in order to overcome treatment failure (190, 193, 194). Therefore, it is imperative that we achieve a comprehensive understanding of the mechanisms of azole resistance within this organism.

Current understanding of fluconazole resistance within *C. albicans* involves sterol biosynthesis gene mutations as well as zinc-cluster transcription factor-mediated changes in efflux pump activity and drug target abundance (14). Zinc-binding proteins play a crucial role in the transcriptional regulation of a vast number of genes in eukaryotes, and in particular, the zinc cluster transcription factors (ZCFs) within the fungal kingdom make up one of the largest regulatory protein families in yeast (117, 195). Regarding azole antifungal resistance in *Candida* spp., ZCFs play a major role in almost all species where azole resistance mechanisms have been elucidated, including *C. glabrata* and *C. parapsilosis* (14). Changes in the zinc-cluster transcription factors Tac1, Mrr1, and Upc2 are thought to be responsible for the bulk of fluconazole resistance in *C. albicans* (14, 78, 79, 83, 104, 113, 115, 116, 196). The presence of gain-of-function mutations in the transcription factor genes *TAC1* and *MRR1* results in increased expression of the efflux pump genes *CDR1/CDR2* and *MDR1*, respectively, while gain-of-function mutations discovered in *UPC2* lead to overexpression of the gene encoding the azole target, Erg11 (14 α -sterol demethylase) (83, 104, 113). Other mechanisms of azole resistance include changes in the azole target 14 α -sterol demethylase through mutations in its encoding gene *ERG11* and less commonly through mutations in the sterol-C5(6)-desaturase encoding gene *ERG3* (71, 121, 122, 142).

There is evidence strongly suggesting that other mechanisms of azole resistance

¹Reprinted from final submission with permission. Nishimoto AT, Zhang Q, Hazlett B, Morschhäuser, Rogers PD. (2019). The contribution of clinically-derived mutations in the gene encoding the zinc cluster transcription factor Mrr2 to fluconazole antifungal resistance and *CDR1* expression in *Candida albicans*. *Antimicrob Agents Chemother.* doi:10.1128/aac.00078-19:AAC.00078-19.

in *Candida* spp. exist. For example, it has been previously demonstrated that other ZCFs can alter susceptibility to fluconazole when transcriptionally activated (117). In particular, decreased fluconazole susceptibility was observed in a laboratory strain expressing artificially-activated Mrr2. The hyperactive ZCF Mrr2 was noted to activate the *CDR1* promoter but not the *CDR2* promoter, in contrast to activated Tac1, which is known to increase both *CDR1* and *CDR2* expression (82). Additionally, fluconazole sensitivity was restored after deletion of the *CDR1* gene in the hyperactive Mrr2 strain (117). More recently, potential activating mutations in Mrr2 which increased expression of *CDR1* but not *CDR2* have been described in fluconazole-resistant clinical isolates of *C. albicans*, indicating that not only can these mechanisms manifest *in vitro*, but also that they are clinically relevant (118). Mrr2 is a relatively understudied ZCF whose role in clinical azole resistance has not yet been completely defined, but the potential significance of Mrr2 as a contributing azole resistance determinant in *C. albicans* cannot be overlooked.

In the present study, we investigated the contributions to fluconazole resistance of mutations in *MRR2* observed among isolates within a collection of azole-resistant clinical *C. albicans* isolates. To accomplish this, we sequenced the *MRR2* open reading frame (ORF) in a collection of 57 clinical isolates with reduced susceptibility to fluconazole (MIC range: 4 to >64 µg/mL, data not shown). Screening of the strains revealed multiple polymorphisms in *MRR2*, several of which had been previously described to impact fluconazole MICs. Clinically-derived *MRR2* alleles were introduced in a fluconazole-susceptible background to generate strains containing multiple single nucleotide polymorphisms (SNPs) in the *MRR2* gene. In contrast to a previous report (118), we found no changes in fluconazole MIC or *CDR1* expression upon introduction of any of the *MRR2* SNPs observed in clinical isolates in our collection or described previously.

Results

SNPs in *MRR2* among fluconazole-resistant clinical isolates of *C. albicans*

To identify clinically-occurring amino acid substitutions in Mrr2, we sequenced the *MRR2* gene of 67 *C. albicans* clinical isolates (10 fluconazole-susceptible, 57 fluconazole-resistant) that we have previously described (115, 139). This collection of isolates had been previously characterized for known resistance mechanisms and collectively possessed every known mechanism of azole resistance (115, 139). Overall, 25 (44%) of 57 fluconazole-resistant isolates possessed SNPs resulting in amino acid changes in Mrr2 (**Appendix A**), however, many of these SNPs were also observed in fluconazole-susceptible isolates and would not be expected to influence fluconazole MICs. Seven amino acid substitutions resulting from SNPs in *MRR2* were present only among fluconazole-resistant isolates within our collection. Three of these substitutions (R45Q, A459T, V486M) had not previously been described in the literature. The remaining four substitutions (S466L, A468G, S469T, T470N) had been previously found in resistant clinical *C. albicans* isolates and reported to have an effect on fluconazole

MIC and *CDR1* expression (118). Within our collection, these four substitutions appeared in four clinical isolates and exclusively co-occurred with each other. Interestingly, constitutive *CDR1* expression levels were less than two-fold greater than that of a composite of fluconazole-susceptible isolates in five clinical isolates containing either the A459T substitution or the combination substitutions of S466L, A468G, S469T, and T470N (**Table 2-1**).

Fluconazole susceptibility in *MRR2* mutant strains

To determine if the newly discovered amino acid changes in Mrr2 affected fluconazole susceptibility, we introduced two copies of the *MRR2* allele from fluconazole-resistant isolates 23, 20, 28, and 29 containing respectively the R45Q, A459T, V486M, and combined amino acid substitutions S466L, A468G, S459T, and T470N into the *mrr2Δ/Δ* strain (SCZCF34M4A) via homologous recombination using the *SAT1* flipper technique. This subsequently yielded homozygous mutant strains *MRR2*^{R45Q, V451A} (SCMRR2R2S2), *MRR2*^{S143P, L144V, T145A, S165N, A459T, S480P} (SCMRR2R4S4), *MRR2*^{T83A, A459T, S480P, V486M} (SCMRR2R5S5), and *MRR2*^{S466L, A468G, S469T, T470N, S480P} (SCMRR2R6S6), which we hereafter will refer to as *MRR2*^{R45Q}, *MRR2*^{A459T}, *MRR2*^{A459T, V486M}, and *MRR2*^{S466L, A468G, S469T, T470N}, respectively. An additional mutant, *MRR2*^{T83A, V451A, V582L} (SCMRR2R3S3), hereafter referred to as *MRR2*^{V582L}, was similarly constructed from the *MRR2* allele of fluconazole-susceptible isolate 22 as it contained a V582L substitution not previously described in the literature. For comparison, we created a *MRR2*^{WT} (SCMRR2R1S1) strain by inserting wildtype *MRR2* amplified from fluconazole-susceptible clinical isolate SC5314 into the *mrr2Δ/Δ* strain and included the previously described hyperactive Mrr2 strain (SCZCF34GAD1), which highly expresses artificially-activated Mrr2 (117). Surprisingly, none of the *MRR2* mutant strains showed any change in fluconazole susceptibility as compared to SC5314 or *MRR2*^{WT} as measured by CLSI standard methods (Table 2). This included *MRR2*^{S466L, A468G, S469T, T470N}, whose *MRR2* alleles contained four of six mutations previously reported to have an effect on fluconazole susceptibility. As expected, hyperactive Mrr2 exhibited elevated MICs to fluconazole. Strain susceptibilities measured on RPMI-agar using E-test strips also did not show reduced fluconazole susceptibility for any polymorphism-containing *MRR2* strain compared to SC5314. However, via E-test, we observed that the *mrr2Δ/Δ* strain was slightly hypersusceptible to fluconazole compared to SC5314, as has been established previously (**Table 2-2**) (117).

MRR2 mutant strains do not overexpress *CDR1* when expressed from the *MRR2* locus

To investigate whether any of the *MRR2* mutant strains could constitutively increase expression of the efflux pump gene *CDR1*, we measured the *CDR1* mRNA abundance for each *MRR2* strain compared to SC5314 (Figure 1A) in triplicate via RT-qPCR. Expression of *CDR1* was not significantly increased compared to SC5314 in any of the created strains containing polymorphic *MRR2* alleles. *MRR2*^{R45Q}, *MRR2*^{A459T}, and

Table 2-1. Mrr2 amino acid substitutions found in azole-resistant clinical isolates expressing *CDR1*

Mrr2 amino acid substitution	Isolate ID	24H MIC_{FLU} (µg/mL)	Fold <i>CDR1</i> Expression
V486M	15	16	15.9
V486M	27	16	14.2
V486M	28	64	18.1
A459T	18	32	10.4
A459T	19	32	10.0
A459T	20	64	7.8
A459T	33	32	16.2
A459T	41	64	1.7
A459T	48	32	13.3
A459T	54	32	0.9
R45Q	23	32	16.3
S466L ^{1,2} , A468G ¹ , S469T ¹ , T470N ^{1,2}	16	8	1.6
S466L ^{1,2} , A468G ¹ , S469T ¹ , T470N ^{1,2}	26	16	0.9
S466L ^{1,2} , A468G ¹ , S469T ¹ , T470N ^{1,2}	29	>64	10
S466L ^{1,2} , A468G ¹ , S469T ¹ , T470N ^{1,2}	46	64	0.8

¹ Amino acid substitutions previously reported to increase fluconazole MICs (118).

² Amino acid substitutions previously reported to increase *CDR1* expression at least two-fold versus susceptible comparator (118).

Table 2-2. Fluconazole susceptibilities for MRR2 mutant strains

Strains	Strain description ¹	24H FLU MIC (µg/mL, CLSI)	24H FLU MIC (µg/mL, E-test)
SC5314	WT	0.25	0.125
SCZCF34M4A	<i>mrr2Δ/Δ</i>	0.25	0.064
SCZCF34GAD1A	overexpressed <i>MRR2</i> -GAD	0.5	0.75
SCMRR2R1S1	<i>MRR2</i> ^{WT}	0.25	0.094
SCMRR2R2S2	<i>MRR2</i> ^{L143P,L144V,T145A,S165N,A459T,S480P}	0.25	0.125
SCMRR2R3S3	<i>MRR2</i> ^{T83A,V451A,V582L}	0.25	0.125
SCMRR2R4S4	<i>MRR2</i> ^{R45Q,V451A}	0.25	0.125
SCMRR2R5S5	<i>MRR2</i> ^{T83A,A459T,S480P,V486M}	0.25	0.094
SCMRR2R6S6	<i>MRR2</i> ^{S466L,A468G,S469T,T470N,S480P}	0.25	0.094
SCMRR2R8S8	<i>MRR2</i> ^{C9}	0.25	0.125
SCMRR2GAD1R1S1	<i>MRR2</i> -GAD	0.5	0.19
SCΔ <i>mrr2</i> P _{ADH1} <i>MRR2</i> ^{WT} A	overexpressed <i>MRR2</i> ^{WT}	0.25	0.125
SCΔ <i>mrr2</i> P _{ADH1} <i>MRR2</i> ^{WT} B	overexpressed <i>MRR2</i> ^{WT}	0.25	0.125
SCΔ <i>mrr2</i> P _{ADH1} <i>MRR2</i> ^{C9} A	overexpressed <i>MRR2</i> ^{C9}	0.25	0.125
SCΔ <i>mrr2</i> P _{ADH1} <i>MRR2</i> ^{C9} B	overexpressed <i>MRR2</i> ^{C9}	0.25	0.125

¹GAD = 3X HA-tagged GAL4 activation domain

MRR2^{A459T, V486M}, which contained novel Mrr2 substitutions found only in fluconazole-resistant isolates, did not appear to express *CDR1* to a greater degree than *MRR2*^{WT} or SC5314. *MRR2*^{S466L, A468G, S469T, T470N}, containing four mutations thought to influence *CDR1* expression also showed no increases over *MRR2*^{WT} or SC5314, in line with our clinical isolates containing these substitutions in Mrr2 but not overexpressing *CDR1*. The strain expressing hyperactive Mrr2 showed an approximately eight-fold increase in *CDR1* over the control, consistent with its reduced susceptibility to fluconazole.

Amino acid substitutions S466L, A468G, S469T, and T470N do not influence fluconazole MICs or *CDR1* expression

The four amino acid substitutions S466L, A468G, S469T, and T470N that were introduced into *MRR2*^{S466L, A468G, S469T, T470N} failed to alter fluconazole susceptibility or change *CDR1* expression from SC5314. However, since we created this strain by inserting the entire *MRR2* allele taken from a resistant isolate, these substitutions were accompanied by other polymorphisms within the *MRR2* allele, and thus we could not rule out an interaction between these polymorphisms and the Mrr2 substitutions S466L, A468G, S469T, and T470N that could mask the increased fluconazole MICs previously associated with these substitutions. Therefore, we created the mutant *MRR2*^{S143P, L144V, T145A, H358N, E439K, V451A, S466L, A468G, S469T, T470N, S480P} strain (SCMRR2R8S8, hereafter referred to as *MRR2*^{C9}) by introducing a mutant *MRR2* allele containing 13 nucleotide changes corresponding to 11 amino acid substitutions in Mrr2 (**Table 2-3**) in order to verify the phenotype associated with these changes described in the literature (118). Surprisingly, *MRR2*^{C9} fluconazole MICs (**Table 2-2**) and *CDR1* expression (**Figure 2-1A**) were not different from SC5314, suggesting that these amino acid changes did not impact fluconazole resistance or *CDR1* expression when *MRR2* was expressed from the native promoter.

Our tested strains utilized homologous recombination to re-introduce polymorphic *MRR2* alleles back into the *mrr2Δ/Δ* strain at the native *MRR2* locus. In order to substantiate our method of strain creation, we used the *SAT1*/FLP strategy to introduce two copies of the *MRR2* allele in which the C-terminal end of the gene was fused to the *GAL4* activation domain and 3X HA-tagged, creating the mutant strain *MRR2-GAL4AD-3xHA* (SCMRR2GAD1R1S1). Fusion of the HA-tagged Gal4 activation domain to full-length ZCFs had been previously shown to constitutively activate fungal ZCFs such as Tac1, Upc2, and Mrr1 when expressed from the *ADH1* promoter (117). Here, our purpose was to create a constitutively active Mrr2 strain in which both copies of *MRR2* were natively expressed. *CDR1* expression increased ~2.5-fold from SC5314 in this strain, which resulted in slightly increased MICs to fluconazole over SC5314 as well, showing that homozygous replacement of the *MRR2* allele in the native locus using the *SAT1*/FLP method was capable of producing changes in *CDR1* expression and fluconazole MIC without foreign promoter-driven overexpression of *MRR2*. This change was smaller than the increase in *CDR1* expression and fluconazole MIC seen with the hyperactivated *MRR2* strain, in which *MRR2* is expressed from the strong *ADH1* promoter.

Table 2-3. Strains used in Chapter 2

Strains	Genotype ^{1,2}	Source or reference
SC5314	Wildtype	ATCC
SCZCF34M4 A/B	$\Delta mrr2::FRT/\Delta mrr2::FRT$	(117)
SCZCF34GAD1 A/B	<i>ADH1/adh1::P_{ADH1}-MRR2-GAL4AD-3xHA-caSAT1</i>	(117)
SCMRR2R1S1 A/B	<i>MRR2^{WT}::FRT/MRR2^{WT}::FRT</i>	This study
SCMRR2R2S2 A/B	<i>MRR2^{S143P,L144V,T145A,S165N,A459T,S480P}::FRT / MRR2^{S143P,L144V,T145A,S165N,A459T,S480P}::FRT</i>	This study
SCMRR2R3S3 A/B	<i>MRR2^{T183A,V451A,V582L}::FRT / MRR2^{T183A,V451A,V582L}::FRT</i>	This study
SCMRR2R4S4 A/B	<i>MRR2^{R45Q,V451A}::FRT / MRR2^{R45Q,V451A}::FRT</i>	This study
SCMRR2R5S5 A/B	<i>MRR2^{T183A,A459T,S480P,V486M}::FRT / MRR2^{T183A,A459T,S480P,V486M}::FRT</i>	This study
SCMRR2R6S6 A/B	<i>MRR2^{S466L,A468G,S469T,I470N,S480P}::FRT / MRR2^{S466L,A468G,S469T,I470N,S480P}::FRT</i>	This study
SCMRR2R8S8 A/B/C	<i>MRR2^{C9}::FRT / MRR2^{C9}::FRT</i>	This study
SCMRR2GADR1S1 A/B	<i>MRR2-GAL4AD::FRT / MRR2-GAL4AD-3xHA::FRT</i>	This study
SC $\Delta mrr2$ P _{ADH1} MRR2 ^{WT} A/B	$\Delta mrr2::FRT/\Delta mrr2::FRT$, <i>ADH1/adh1::P_{ADH1}-MRR2^{WT}</i>	This study
SC $\Delta mrr2$ P _{ADH1} MRR2 ^{C9} A/B	$\Delta mrr2::FRT/\Delta mrr2::FRT$, <i>ADH1/adh1::P_{ADH1}-MRR2^{C9}</i>	This study

¹Tentative activating mutations in *MRR2* described in (118) are underlined.

²Amino acid changes in *MRR2* in bold were discovered from this study. *MRR2*_{C9} refers to the *MRR2* allele of isolate C9 as described in (118) containing S143P, L144V, T145A, H358N, E439K, V451A, S466L, A468G, S469T, T470N, S480P mutations

Figure 2-1. Fold-change in expression levels of *MRR2* mutant strains compared to SC5314

(A) Fold-change in expression levels compared to SC5314 of *CDR1* for the *mrr2* Δ/Δ strain (SCZCF34M4A), the mutant *MRR2* strains containing *MRR2*^{WT} (SCMRR2R1S1), *MRR2*^{A459T} (SCMRR2R2S2), *MRR2*^{V582L} (SCMRR2R3S3), *MRR2*^{R45Q} (SCMRR2R4S4), *MRR2*^{A459T, V486M} (SCMRR2R5S5), *MRR2*^{S466L, A468G, S469T, T470N} (SCMRR2R6S6), *MRR2*^{C9} (SCMRR2R8S8) and artificially-activated Mrr2 strains expressed from either the *ADH1* promoter (SCZCF34GAD1A) or the native *MRR2* promoter (SCMRR2GAD1R1S1). (B) Fold-change in *MRR2* expression levels compared to SC5314 for the *mrr2* Δ/Δ strain (SCZCF34M4A), the mutant *MRR2*^{C9} strain (SCMRR2R8S8), the artificially-activated Mrr2 strains expressed from either the *ADH1* promoter (SCZCF34GAD1A) or the native *MRR2* promoter (SCMRR2GAD1R1S1). Expression was obtained in technical and biological triplicate for each strain tested and error bars reflect standard error of the mean.

Activated Mrr2 does not appear to regulate *MRR2* expression

To determine if Mrr2 autoregulates its expression level, we measured *MRR2* mRNA abundance of our created strains relative to SC5314 (**Figure 2-1B**). The mutant strain *MRR2*^{C9} and the artificially-activated *MRR2* strain *MRR2-GAL4AD-3xHA* did not appear to have increased expression of *MRR2* compared to SC5314. As expected, strains with hyperactive *MRR2* expressed *MRR2* ~14-fold higher compared to SC5314. Therefore, it appears only the relative overexpression of *MRR2* from the *ADH1* promoter and not activation of Mrr2 itself had any effect on increased *MRR2* expression.

Overexpression of wildtype or mutant *MRR2* in *C. albicans* strain does not affect fluconazole susceptibility or *CDR1* expression

In order to investigate whether changes in *CDR1* expression and fluconazole susceptibility required strong constitutive expression of *MRR2*, we constructed strains which lacked both native copies of *MRR2* but possessed a single copy of either the open reading frame of *MRR2*^{WT} or *MRR2*^{C9} fused to the *ADH1* promoter. In both cases, *MRR2* mRNA expression was increased >30-fold compared to SC5314 in the independently created mutant strains *mrr2Δ/Δ adh1Δ::P_{ADH1}-MRR2^{WT}/ADH1* (SCΔmrr2P_{ADH1}MRR2^{WT} A/B) and *mrr2Δ/Δ adh1Δ::P_{ADH1}-MRR2^{C9}/ADH1* (SCΔmrr2P_{ADH1}MRR2^{C9} A/B, **Figure 2B**). However, compared to SC5314, *CDR1* expression was not markedly different for any of the strains, with no increase in constitutive *CDR1* mRNA greater than 1.2-fold (**Figure 2-2A**). As expected, the strains also did not show any change in fluconazole susceptibility by either MIC or E-test (**Table 2-2**). Thus, overexpression of either *MRR2*^{WT} or *MRR2*^{C9} does not appear to influence *CDR1* expression or fluconazole susceptibility in *C. albicans*.

Discussion

Gain-of-function mutations in the genes encoding fungus-specific ZCFs Tac1, Mrr1 and Upc2 have long been known to contribute to azole resistance in *C. albicans*. However, only recently was it reported that non-synonymous mutations found in the *MRR2* gene of clinical isolates impacted fluconazole susceptibility through increased Cdr1 efflux pump expression, indicating that *MRR2* may be a clinically relevant mechanism of azole resistance. Here, we sequenced a collection of predominantly fluconazole-resistant clinical isolates in order to uncover additional mutations in the *MRR2* gene that would contribute to fluconazole resistance. In total, mutations resulting in fifteen amino acid substitutions were identified across these clinical isolates. Three (R45Q, A459T, and V486M) were found uniquely in fluconazole-resistant clinical isolates and had not been previously reported. The remaining were described in a previous report or were present in fluconazole-susceptible isolates and therefore not likely directly involved in azole resistance. Our results indicate that none of the tested SNPs in *MRR2* have an impact on susceptibility to fluconazole. Similarly, *CDR1* expression also did not appear to be affected by the presence of any of the tested SNPs.

Figure 2-2. Fold-change in *CDR1* and *MRR2* expression compared to SC5314

(A) Fold-change in expression levels compared to SC5314 of *CDR1* for strains possessing either *MRR2*^{WT} (SCΔmrr2P_{ADH1}MRR2^{WT} A and B) or mutant *MRR2*^{C9} (SCΔmrr2P_{ADH1}MRR2^{C9} A and B) expressed via the *ADH1* promoter. (B) Fold-change in *MRR2* expression levels compared to SC5314 for the same strains shown in (A). Expression was obtained in technical and biological triplicate for each strain tested and error bars reflect standard error of the mean.

In particular, four mutations identified in isolates in our collection (S466L, A468G, S469T, and T470N) had been previously shown to impact fluconazole susceptibility. The amino acid substitutions S466L and T470N had also been reported to increase *CDR1* expression by at least two-fold as compared to a control strain. In our hands the amino acid substitutions S466L, A468G, S469T, and T470N do not appear to have any effect on susceptibility to fluconazole and *CDR1* expression does not increase in the mutant strains containing these Mrr2 amino acid substitutions compared to SC5314. This finding is consistent with our clinical isolates that possess these changes in Mrr2 but do not highly express *CDR1*.

Since the publication of the original report first describing them, mutations in *MRR2* have been referenced as a clinically relevant mechanism of fluconazole resistance (160, 197, 198). While our results confirm that artificial activation of the Mrr2 ZCF indeed results in *CDR1* upregulation and a consequent increase in fluconazole resistance as previously described (117), we were unable to replicate the changes attributed to these mutations in a previous report (118). We were unsuccessful in obtaining isolate C9 from this previous report, which contained the *MRR2* mutations in question, and as such could not make a direct assessment of this clinical isolate. One possibility for the disparity seen between our results and those reported previously could be differences in methodology. We expressed *MRR2* from its native promoter, including replacement of both ORFs in the *MRR2* locus, in order to mimic natural Mrr2 expression. This is in contrast to the methods used previously whereby *MRR2* was placed in the *ADE2* locus utilizing a single copy of the ORF under control of the *ADH1* promoter. This could possibly cause altered metabolic burdens in these strains compared to ours. Furthermore, the *ADH1* promoter has been described to have variability in activity over time under different conditions (199), and therefore Mrr2 protein levels may be different depending on the time at which cells were harvested or observed for assays. Lastly, we used 50% inhibition from the fluconazole-free control well for identifying the MIC of our strains, as directed by the CLSI reference method for broth microdilution antifungal susceptibility testing of yeast (200, 201). The MICs presented in the previous report were identified by 80% inhibition of cell growth in the control well, which could explain increased MICs observed in *MRR2* mutant strains, especially if the trailing growth phenomenon was observed in these strains.

Given the lower expression of *MRR2* that we observed in our strains using the native *MRR2* promoter compared to hyperactive Mrr2, which overexpressed *MRR2* via fusion to the *ADH1* promoter, we considered that *MRR2* was not being sufficiently expressed in our strains to allow for detection of changes in *CDR1* expression or fluconazole susceptibility. However, the *MRR2-GAL4AD-3xHA* strain expressing artificially-activated Mrr2 via its native promoter demonstrates that native *MRR2* expression levels are sufficient to observe changes in fluconazole MIC and *CDR1* expression. Moreover, we believe our strains, which tested the effects of the homozygous SNPs in *MRR2* at the native locus, more closely approximates the effects these nucleotide changes would have in a clinical isolate. Lastly, we constructed our own *P_{ADH1}-MRR2* overexpressing strains to test whether there were differences in *CDR1* expression or fluconazole susceptibility between strong, constitutive expression of either

MRR2^{WT} or *MRR2*^{C9} and were unable to discern any phenotype differences between the strains. Overall, in all strains and conditions tested here we were unable to detect any change in *CDR1* expression or fluconazole susceptibility between strains containing mutant *MRR2* versus wildtype.

The ATP-binding cassette transporter Cdr1 has been shown to be regulated not only by Tac1, but also Ndt80. Furthermore, it has been shown that through artificial activation and overexpression, the zinc cluster transcription factors Mrr2, Znc1, and Stb5 also can influence Cdr1 expression levels. Thus, we speculate that there remain additional determinants of efflux pump upregulation that have clinical relevance. However, our experiments indicate that the nucleotide changes observed here, and reported previously, in *MRR2* do not impact fluconazole susceptibility or *CDR1* expression and are not clinically relevant to fluconazole resistance in *C. albicans*. Given that azole resistance cannot be fully explained by known mechanisms, further investigation of other ZCFs in azole resistance is warranted.

Materials and Methods

Strains and growth conditions

Table 2-2 lists the *C. albicans* strains used in this study. *C. albicans* isolates were obtained from a repository of clinical isolates at the University of Iowa and have been previously reported elsewhere (115). Strains were stored in 40% glycerol frozen stocks at -80° C. Routine growth of cells was performed in YPD liquid media (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. Nourseothricin-containing (200 µg/mL) YPD agar plates were used for selection of strains containing the *SAT1* marker. For plasmid propagation, DH5αTM competent *E. coli* cells (Invitrogen) were grown in Luria-Bertani (LB) broth or on LB agar plates containing either 100 µg/mL ampicillin or 50 µg/mL kanamycin.

Plasmid construction

Plasmids were derived from plasmid strain pBSS2, which contains the *SAT1* flipper disruption cassette from pSFS2 (202) placed in the pBluescript II KS+ vector. The 3' flanking region of the *MRR2* ORF was amplified from SC5314 genomic DNA using primers CaMRR2C_ (NotI) and CaMRR2D_ (SacI) and ligated into pBSS2 at the NotI and SacI restriction sites to create pMRR2CD. The 5' upstream region and *MRR2* ORF of isolates SC5314, 20, 22, 23, 28, and 29 were amplified using primers CaMRR2A_ (KpnI) and CaMRR2E_ (XhoI) and cloned into pMRR2CD to obtain plasmids pBSS2-*MRR2*^{WT}, pBSS2-*MRR2*²⁰, pBSS2-*MRR2*²², pBSS2-*MRR2*²³, pBSS2-*MRR2*²⁸, and pBSS2-*MRR2*²⁹, respectively. For pMRR2_C9.3 which replicated the *MRR2* allele of isolate C9 described by Wang et al. (118), primers CaMRR2_P1_F and CaMRR2_C9_R were used to amplify a 5' portion of the *MRR2* ORF from genomic

DNA of isolate 33. The resulting amplicon was fused to a synthesized gBlocks fragment (Integrated DNA Technologies) containing the 3' portion of the *MRR2* ORF using CaMRR2_Nested_F_KpnI and MRR2_Nested_DR_XhoI and cloned into pMRR2CD to give pMRR2_C9.2. Final changes of nucleotides were accomplished using short, overlapping extension (SOE) PCR on plasmid pMRR2_C9.2 using primer pairs pBSS2_1F.2 and CaMRR2_C9.2_R6, CaMRR2_C9.2_F6 and CaMRR2_C9.2_R7, CaMRR2_C9.2_F7 and CaMRR2_C9.2_R8, and CaMRR2_C9.2_F8 and pBSS2_MAL2_R to generate amplicon fragments of the *MRR2* ORF. Fragments were fused together via SOE PCR using CaMRR2_Nested_F_KpnI and CaMRR2_Nested_DR_XhoI and were cloned into pMRR2CD to create pMRR2_C9.3. Plasmid pMRR2GAD1 was generated using genomic DNA from SCZCF34GAD1A and primers CaMRR2GAD1A_F_KpnI and 3XHA_ACT1_R_XhoI to amplify the *MRR2* ORF including the fused *GAL4* activation domain and 3X HA-tagged C-terminal region. The resulting amplicon was cloned into pMRR2CD to yield plasmid pMRR2GAD1. Plasmids pP_{ADH1}-MRR2^{WT} and pP_{ADH1}-MRR2^{C9} were used to express the open reading frame of *MRR2* from the *ADH1* locus, utilizing the *ADH1* promoter and termination sequences. The *MRR2* open reading frame of either SC5314 or the C9 isolate was fused to the 3' *ADH1* terminator via overlap extension PCR utilizing primers CaMRR2_2F_EcoRI, CaMRR2_R_ADH1t.2, CaADH1t_F, and ADH1t_R_XhoI. The *ADH1* promoter was PCR amplified using primers CaPADH1_AF_KpnI and CaPADH1_2R_EcoI. Both the *ADH1* promoter and fused *MRR2-ADH1t* amplicons were digested with either KpnI and EcoRI or EcoRI and XhoI, respectively, and subsequently ligated into the KpnI-XhoI linearized plasmid pADH1CD, a pBSS2 derivative containing the 342 bp 3' flanking homology near the *ADH1* locus generated with primers CaADH1_C_SacII and CaADH1_D_NcoI_SacI. Successful transformants were screened on LB agar plates containing 50 µg/mL ampicillin and sequenced for the *MRR2* open reading frame corresponding to either SC5314 or isolate C9 for plasmid pP_{ADH1}-MRR2^{WT} and pP_{ADH1}-MRR2^{C9}, respectively.

***MRR2* amplification and sequencing**

Table 2-4 lists the primers used for *MRR2* amplification and sequence verification. The *MRR2* coding sequence of each isolate was PCR amplified from genomic DNA using primers CaMRR2_F_Amp and CaMRR2_R_Amp. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and product was sequenced on an ABI 3130XL genetic analyzer using *MRR2* sequencing primers. Sequencing was accomplished in duplicate in independently grown isolates.

***Candida albicans* strain construction**

The *SAT1*/FLP-containing *MRR2* replacement cassette was excised from plasmids pBSS2-MRR2^{WT}, pBSS2-MRR2²⁰, pBSS2-MRR2²², pBSS2-MRR2²³, pBSS2-MRR2²⁸, and pBSS2-MRR2²⁹, pMRR2_C9.3, and pMRR2GAD1 were digested using KpnI-HF and NcoI-HF restriction enzymes and transformed via electroporation into *C. albicans*

Table 2-4. Primers used in this study

Primer name by purpose	Sequence ¹
Amplification	
CaMRR2_F_Amp	5' – TACGAAATACTTGGAGTTATTCCCTAC – 3'
CaMRR2_R_Amp	5' – CTAGTTTTGTGTCTAGTTCTATTGTTATTG – 3'
CaMRR2GAD1A_F_KpnI	5' – CAGGGTACCAACTTGAAAAATTGCTCAACTCTTATATAGCAAAAATAAACAACCAAT AGCTTCTTCGCCAATGACCAAACGTGATCGTAC – 3'
3XHA_ACT1_R_XhoI	5' – CTACTCGAGGATTTCCAGAATTTCACTCTTA – 3'
CaPADH1_AF_KpnI	5' – GATGGTACC ACTACCACTGCAGCTGCATC – 3'
CaMRR2_2R_EcoRI	5' – CTTTTTGAGTTTTTGGGATTTGTTCTGAATTC AATTGTTTTTGTATTTGTTGTTGTTGTTG – 3'
CaMRR2_2F_EcoRI	5' – GATGAATTCATGACCAAACGTGATCGTACAAT – 3'
CaADH1t_R_XhoI	5' – CATCTCGAGTTAACC AAAATCAACGACAAATTG – 3'
CaADH1_C_SacII	5' – GATCCGCGGCATTGATTGTTTGTGTTAGTTTTTCA – 3'
CaADH1_D_NcoI_SacI	5' – GATGAGCTCCCATGGAACACCCAGTTTAATTCCATGA – 3'
Sequencing	
CaMRR2SeqA	5' – GCAGAAGCGAGGGAACCTTGAAA – 3'
CaMRR2SeqB	5' – ACTTGGAGAAGCATACATACCGAG – 3'
CaMRR2SeqC	5' – TACTCGCTCGCCTTACATCGA – 3'
CaMRR2SeqD	5' – AATCTCAACTACATCCACCTTGTC – 3'
CaMRR2SeqE	5' – CGAAACTTCTGCCATCCTCAAT – 3'
CaMRR2SeqF	5' – GTACATCGGACGACCGTTCC – 3'
CaMRR2SeqG	5' – CTATACTTTGCTCCATTGGCGG – 3'
CaMRR2SeqH	5' – GAACGATGTTAATGGGTCAGCAAAG – 3'
Short, overlapping extension	
CaMRR2_P1_F	5' – CACTGTGATCGGTTATCTTTGTTGCAC – 3'
CaMRR2_C9_3R	5' – GTTGCTTGGGGTTGTTTTCGCCAATG – 3'
CaMRR2_Nested_F_KpnI	5' – AGCGGTACCTTGGA CTTT GACTGTT CAGA – 3'
CaMRR2_Nested_DR_XhoI	5' – CAACTCGAGGGGCGATGATTGTTAGTTGTATATT – 3'
pBSS2_1F.2	
pBSS2_MAL2_R	5' – CGTGGTTTCAGTGGCTACAAC – 3'
CaMRR2_C9.2_F6	5' – GGGCCACAGATCGACGGACATTAC – 3'
CaMRR2_C9.2_R6	5' – GTAATGTCCGTCGATCTGTGGGCCC – 3'
CaMRR2_C9.2_F7	5' – CATTAAACATCGTTCTTTAGTCTCATCCC – 3'
CaMRR2_C9.2_R7	5' – GGGATGAGACTAAAGAACGATGTTAATG – 3'

Table 2-4. (Continued)

Primer name by purpose	Sequence¹
CaMRR2_C9.2_F8	5' – CAGATTTACCAGTTGCCAAAAGAC – 3'
CaMRR2_C9.2_R8	5' – GTCTTTTGGCAACTGGTAAATCTG – 3'
CaMRR2_2F_EcoRI	5' – GATGAATTCATGACCAAACGTGATCGTACAAT – 3'
CaMRR2_R_ADH1t.2	5' – GCTATTTGCTTACGAGATTTTGAGGAAATCCC – 3'
CaADH1t_F	5' – CAAAATCTCGTAAGCAAATAGCTAAATTATATACG – 3'
CaADH1t_R_XhoI	5' – CATCTCGAGTTAACCAAAATCAACGACAAATTG – 3'
Real-time qPCR	
CaMRR2_qPCR_F	5' – TCCAAGTAAGTGTGGGTGTCC – 3'
CaMRR2_qPCR_R	5' – ATGTAAGGCGAGCGAGTAGC – 3'
CaCDR1-f_qPCR	5' – ATTCTAAGATGTCGTCGCAAGATG – 3'
CaCDR1-R_qPCR	5' – AGTTCTGGCTAAATTCTGAATGTTTTC
CaACT1-FWD_qPCR	5' – ACGGTGAAGAAGTTGCTGCTTTAGTT – 3'
CaACT1-rvs_qPCR	5' – CGTCGTCACCGGCAAAA – 3'

¹Underlined nucleotide indicates introduction of a restriction site sequence.

strain SCZCF34M4A to generate strains heterozygous for their respective *MRR2* alleles of interest. **Figure 2-3** diagrams the general strain construction method for allelic replacement of *MRR2* at the native locus (**Figure 2-3A**) and inserted at the *ADHI* locus (**Figure 2-3B**). The nourseothricin marker in all strains was recycled by FLP recombinase induction after 48 hours growth in YPD liquid media. Repeat transformation of the resultant strains generated the homozygous *MRR2* allele replacements *MRR2*^{WT} (SCMRR2R1S1), *MRR2*^{S143P, L144V, T145A, S165N, A459T, S480P} (SCMRR2R2S2), *MRR2*^{T83A, V451A, V582L} (SCMRR2R3S3), *MRR2*^{R45Q, V451A} (SCMRR2R4S4), *MRR2*^{T83A, A459T, S480P, V486M} (SCMRR2R5S5), *MRR2*^{S466L, A468G, S469T, T470N, S480P} (SCMRR2R6S6), *MRR2*^{C9} (SCMRR2R8S8), and *MRR2-GAL4AD-3xHA* (SCMRR2GAD1R1S1), respectively. *MRR2* allelic replacements were confirmed by Southern blot and confirmation of polymorphisms or fused domains present in generated strains were accomplished through Sanger sequencing. For strains SCΔ*mrr2*P_{ADHI}*MRR2*^{WT} and SCΔ*mrr2*P_{ADHI}*MRR2*^{C9}, plasmids pP_{ADHI}-*MRR2*^{WT} and pP_{ADHI}-*MRR2*^{C9} were similarly digested with KpnI-HF and NcoI-HF to excise and purify the *MRR2*-containing *SAT1*/FLP cassette targeting the *ADHI* locus. Paired doubled strand breaks near the 5' and 3' ends of the *ADHI* were induced utilizing the CRISPR-Cas9 technology using previously described methods (203). Briefly, 100 pmol Alt-R® CRISPR-Cas9 tracrRNA (Integrated DNA Technologies, Inc.) was duplexed with an equal amount of crRNA targeting either the 5' or 3' region of the *ADHI* locus at 95°C for 5 minutes. The duplexed guide RNA was then complexed with 2 ug of Alt-R® S.p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies, Inc.) to form the resulting ribonucleoprotein complex. Approximately 1 ug of purified digests from either pP_{ADHI}-*MRR2*^{WT} or pP_{ADHI}-*MRR2*^{C9} were transformed via electroporation along with Cas9 ribonucleoprotein complex into SCZCF34M4A to create SCΔ*mrr2*P_{ADHI}*MRR2*^{WT} and SCΔ*mrr2*P_{ADHI}*MRR2*^{C9} expressing a single copy of the *MRR2* open reading frame from the *ADHI* locus.

Azole susceptibility testing

Fluconazole MICs were determined using the broth microdilution methods as described by the Clinical Laboratory and Standards Institute (200, 201), with slight modification by addition of 2% glucose to Roswell Park Memorial Institute 1640 (RPMI) media to reduce trailing growth in wells. Fluconazole concentration for MICs ranged from 256 to 0.06 µg/mL. Measurements were read visually at 24 and 48 hours after incubation at 35° C for a 50% reduction in growth from drug-free control wells. For fluconazole susceptibility testing using E-test strips (bioMérieux), cells were diluted to an OD₆₀₀ of 0.100 and swabbed using sterile cotton tips onto RPMI-agar plates. MICs were visually read at the border of the zone of inhibition after 24 and 48 hours of incubation at 35° C.

Figure 2-3. Representative schematic of *MRR2* mutant strain construction in *C. albicans*

The diagram shows the *MRR2/CaSAT1*/FLP cassette integration at either (A) the native *MRR2* gene locus or (B) replacing the *ADH1* open reading frame at the *ADH1* locus. The black line represents the sequence of a single allele of genomic DNA. Open reading frames are depicted as filled arrows along the genomic sequence with gene names labeled. Promoter regions, terminators, and the FLP recognition target sequences are represented by bent arrows, hairpins, and black triangles, respectively. The double slashes interrupting the genomic DNA sequence in (B) represent the CRISPR-Cas9 cutting sites. The homologous 5' and 3' flanking sequences for targeted fragment integration are represented as forward-striped and backwards-striped rectangles, respectively.

Quantitative real-time reverse transcription PCR

First strand cDNAs were synthesized from 1 µg of total RNA using the SuperScript® VILO™ for qRT-PCR (Invitrogen). Real-time PCR primers for *ACT1*, *CDR1* and *MRR2* were synthesized by Integrated DNA Technologies (**Table 2-4**). Relative mRNA abundance of *CDR1* and *MRR2* was measured in triplicate on the StepOnePlus Instrument (Applied Biosystems) against the endogenous control gene *ACT1* and the reference strain SC5314. To assess statistical significance, standard error of the mean of the average expression levels of each isolate measured in triplicate was calculated.

Nucleotide sequence accession numbers

The coding sequences of the *MRR2* alleles described in this study have been deposited in GenBank under the accession numbers [MK332630] through [MK332702].

CHAPTER 3. MUTATIONS IN *ERG11* HAVE VARYING CONTRIBUTIONS TO AZOLE RESISTANCE, *ERG11* PROTEIN FUNCTION, AND COMPETITIVE GROWTH FITNESS OF THE CELL²

Introduction

Candida albicans is a polymorphic, opportunistic pathogen responsible for causing a wide range of infections. In the United States, *C. albicans* is the most common cause of invasive candidiasis (59, 190). Morbidity and mortality rates for invasive candida infections such as candidemia remain high. Such infections have a significant impact on the health care costs. In 2014, it was estimated that *Candida* infections in the US accounted for more than 25,000 hospitalizations totaling \$1.4 billion, with invasive candidiasis contributing to 84% of the incurred costs (68). Even the less serious superficial *Candida* infections such as vulvovaginal or oropharyngeal candidiasis were responsible for over 3.5 million outpatient visits that year at an expense of nearly \$2.1 billion.

In cases of both invasive and non-invasive *Candida* infection, the azole antifungals have been and continue to be an important agent used in treatment. The azoles work through inhibition of the *ERG11*-encoded protein 14 α -lanosterol demethylase, also known as the CYP51 enzyme. While resistance in *C. albicans* to azoles like fluconazole has remained relatively constant in recent years, azole-resistant *Candida* continues to be a serious threat needing to be addressed (34, 184, 204).

Mechanisms of azole resistance often involve the regulation and overexpression of genes controlled by the zinc-cluster transcription factors (ZCFs), a fungal-specific transcription factor family. The ZCFs Tac1 and Mrr1 have been well-studied in their regulation of the expression of the ATP-binding cassette transporters Cdr1 and Cdr2 and the major facilitator superfamily transporter Mdr1, respectively (82, 83, 104, 105). The ZCF Upc2 is responsible for the regulation of most ergosterol biosynthesis genes, including *ERG11*, which encodes for the azole drug target (Erg11/14 α -lanosterol demethylase, hereafter referred to as CaCYP51), and can also influence azole susceptibility through overexpression of *ERG11* (108, 110). Mutations in *ERG11* resulting in amino acid substitutions that directly alter the drug target 14 α -lanosterol demethylase's protein sequence are another common azole resistance mechanism (121, 122, 131). It is commonly a combination of these mechanisms that confer azole resistance in clinical isolates of *C. albicans* (107).

²Portions of chapter from previously published article; final submission modified with open access permission. Warrilow AG, Nishimoto AT, Parker JE, Price CL, Flowers SA, Kelly DE, Rogers PD, Kelly SL. 2019. The Evolution of Azole Resistance in *Candida albicans* Sterol 14 α -Demethylase (CYP51) through Incremental Amino Acid Substitutions. *Antimicrob Agents Chemother*. doi:10.1128/AAC.02586-18.

To date, over 160 CaCYP51 amino acid substitutions have been reported in clinical isolates of *C. albicans* (131, 134, 136, 139). While mutation hot spots have been noted where amino acid substitutions cluster, the breadth of substitutions in CaCYP51 covers virtually the entire length of the 528 amino acid protein (**Figure 3-1**) (205). However, the effects of these substitutions are variable – while some have been shown to confer resistance to fluconazole and other azoles, others have never been shown to affect susceptibility to the azoles. A previous report by Flowers et al. showed the individual contributions of several amino acid substitutions in CaCYP51 and also found that several double substitutions conferred greater azole resistance than any single substitution, indicating that the presence of multiple CaCYP51 mutations may be beneficial for the cell in the presence of azoles (139).

More recently, the crystal structures of CaCYP51 complexed with azole antifungals have been analyzed and residues of significance in the catalytic domain or in contact with the drug ligands have been identified (140, 206). For example, while some residues like Tyr-132 lie exposed on the active site cavity of CaCYP51 and predictably would interfere with fluconazole binding, other residues such as the Ser-405 do not appear to contact the azoles, yet amino acid substitutions at this residue still confer azole resistance. Differences in the relative locations on CaCYP51 present an interesting question as to how amino acid substitutions might have variable effects on CYP51 enzyme function.

There have been significant recent findings regarding the differences in catalytic activity between amino acid substitutions in CaCYP51 activity, biochemical tolerance to the azoles, and azole binding affinity (141). In an effort to characterize functional and biochemical differences between enzymes containing substitutions in CYP51, purified recombinant CaCYP51 modified for expression in *E. coli* and 4x histidine-tagged for purification was synthesized for biochemical analysis. Twenty-four single amino acid substitution CaCYP51 mutants and five double amino acid substitution mutants were synthesized and purified, representing documented mutations found in clinical isolates. A wild type enzyme lacking any mutations representing the native CaCYP51 enzyme found in azole-susceptible clinical isolates was also synthesized for comparison. The catalytic turnover rate of the functioning enzyme was determined by quantification of sterol product form after addition of the CaCYP51 substrate lanosterol and the human cytochrome P450 reductase to the CaCYP51 enzyme. Even in the absence of azoles, CaCYP51 mutants varied widely in their catalytic turnover compared to the wild type. For example, while the V456I and Q474K substitutions seemingly increased relative velocity of the enzyme over the wild type by more than 50% at baseline, the Y118A, G307S, Y132H&K143R, and G307S&G450E substitutions all resulted in catalytic turnover of less than 10% that of the wild type enzyme's turnover (**Table 3-1**).

In the presence of either 4μM fluconazole, voriconazole, itraconazole, and posaconazole, the wild type CaCYP51 enzyme retained less than 1% of its residual activity (**Table 3-2**). Many CaCYP51 mutants behaved in a similar fashion in the presence of the azoles, suggesting that they presumably would not influence azole

1 MAIVETVIDG INY**F**LSLS**V**T **Q**QISILLGVP FV**Y****N**LVWQ**Y**L YSL**R**KDRAP**L**
 51 VFY**W**IP**W**FGS **A**ASYGQQPYE **F**FESCRQ**K**Y**G** **D**V**F**SF**M**LL**G**K IMTVYLGP**K****G**
 101 HEFV**F**NA**K**L**S** DVSA**E**DAY**K**H LTTPV**F**G**K**G**V** IYDC**P****N**S**R**L**M** EQ**K**K**F**A**K**F**A**L
 151 TT**D**S**F**K**R**Y**V**P **K**IR**E**EILNYF VTDE**S**F**K**LKE KTHGVANVMK TQPEITIFT**A**
 201 SRS**L****F**GD**E**MR RIFDRSFAQL **Y**SD**L**D**K**G**F****T**P INFV**F****P****N**LPL PHYWRRDAAQ
 251 KKIS**A**T**Y**M**K**E IKSR**R****E**R**G**DI DPN**R**D**L**I**D**S**L** LI**H**ST**Y**K**D**GV KMT**D**Q**E**I**A**N**L**
 301 LI**G**IL**M**G**G**Q**H** TSASTSAWFL LHLGEKPHLQ DVI**Y**Q**E**V**V**E**L** L**K**E**K**G**G**DLND
 351 LTYEDLQ**K**L**P** SVNNT**I**K**E**T**L** RM**H**M**P**L**H**S**I****F** **R**KVT**N****P**L**R**IP **E**TNYIV**P****K****G****H**
 401 YVL**V**S**P**GYAH TSERYFDN**P**E **D**FD**P**T**R**WD**T**A **A**A**K****A****N**S**V**S**F****N** **S****S****D**E**V****D****Y****G****F****G**
 451 **K**V**S**K**G**V**S**S**P****Y** LP**F****G****G****R****H****R**C **I****G****E****Q**FAYV**Q****L** GT**I**L**T**T**F**V**Y****N** **L**R**W****T**IDGYKV
 501 **P****D****P****D**YSS**M****V**V LP**T**EP**A**E**I****I****W** **E**KRETCMF*

Figure 3-1. Reported locations of CaCYP51 amino acid substitutions in *C. albicans*

Amino acid sequence of the 528 amino acid length of the CaCYP51 protein. Highlighted amino acids represent documented substitution(s). Yellow, green, blue, and purple highlights represent one, two, three and four possible different amino acid substitutions discovered, respectively. Bold lettering represents a difference in the SC5314 CaCYP51 reference sequence from the reported source's original amino acid residue at that location.

Table 3-1. Yield and relative velocities of CaCYP51 proteins

CaCYP51 substitution	Yield¹ (nmol L⁻¹)	Relative velocity²
Wild type	119	1.000 ±0.047
Y118A	49	0.029 ±0.001
F126V	235	0.298 ±0.008
Y132F	53	0.795 ±0.005
Y132H	28	0.135 ±0.002
K143R	143	0.599 ±0.010
F145L	35	0.476 ±0.021
P230L	71	0.250 ±0.003
Y257H	93	0.554 ±0.029
D278N	177	0.645 ±0.028
S279F	152	0.289 ±0.017
G307S	193	0.031 ±0.001
S405F	66	0.758 ±0.011
V437I	37	0.796 ±0.013
Y447H	36	0.965 ±0.019
G448E	35	0.407 ±0.007
F449Y	44	0.548 ±0.088
G450E	163	0.517 ±0.019
V456I	157	1.560 ±0.018
G464S	29	0.609 ±0.023
R467K	38	0.471 ±0.023
I471T	32	0.634 ±0.015
Q474K	35	1.601 ±0.035
V488I	152	1.167 ±0.032
Y132F&K143R	67	0.208 ±0.014
Y132H&K143R	79	0.068 ±0.001
Y132F&F145L	93	0.405 ±0.012
D278N&G464S	48	0.327 ±0.002
G307S&G450E	136	0.077 ±0.008

¹Yield of purified CaCYP51 protein isolates from one liter of *E. coli* expression culture.

²Relative velocity of 1.000 relates to a catalytic turnover number of 0.574 min⁻¹ obtained with the wild type CaCYP51 protein in the CYP51 reconstitution assay.

Table 3-2. Residual CYP51 activity in the presence of 4 μ M triazole antifungal

CaCYP51 substitution	Residual CYP51 activity (%) $\pm$ SD ¹							
	Fluconazole		Voriconazole		Itraconazole		Posaconazole	
Wild type	0.11	$\pm$ 0.09	0.11	$\pm$ 0.01	0.03	$\pm$ 0.02	0.66	$\pm$ 0.02
Y118A	1.51	$\pm$ <0.01	0.28	$\pm$ 0.04	1.30	$\pm$ 0.59	0.46	$\pm$ 0.35
F126V	0.09	$\pm$ 0.06	0.19	$\pm$ 0.06	0.22	$\pm$ 0.05	0.10	$\pm$ 0.01
Y132F	7.05	$\pm$ 0.27	1.09	$\pm$ 0.05	0.19	$\pm$ 0.04	0.93	$\pm$ <0.01
Y132H	0.86	$\pm$ 0.07	0.33	$\pm$ 0.19	0.07	$\pm$ <0.01	0.18	$\pm$ 0.06
K143R	28.14	$\pm$ 2.04	3.12	$\pm$ 0.27	6.27	$\pm$ 0.51	13.09	$\pm$ 0.02
F145L	6.71	$\pm$ 0.15	0.37	$\pm$ 0.11	1.99	$\pm$ 0.04	1.37	$\pm$ 1.21
P230L	0.05	$\pm$ 0.03	0.13	$\pm$ 0.12	0.06	$\pm$ 0.01	0.21	$\pm$ 0.05
Y257H	3.96	$\pm$ 0.18	0.63	$\pm$ 0.27	1.37	$\pm$ 0.28	6.42	$\pm$ 0.59
D278N	7.59	$\pm$ 0.58	1.34	$\pm$ 0.05	3.00	$\pm$ 0.19	14.82	$\pm$ 0.92
S279F	17.99	$\pm$ 1.67	6.15	$\pm$ 0.66	5.97	$\pm$ 0.35	20.64	$\pm$ 0.10
G307S	0.95	$\pm$ 0.17	0.41	$\pm$ 0.04	0.53	$\pm$ 0.21	0.71	$\pm$ 0.04
S405F	11.56	$\pm$ 1.41	1.94	$\pm$ 0.12	2.73	$\pm$ 0.13	9.38	$\pm$ 0.53
V437I	0.22	$\pm$ 0.06	0.02	$\pm$ <0.01	0.13	$\pm$ 0.02	0.59	$\pm$ 0.20
Y447H	4.34	$\pm$ 0.51	2.07	$\pm$ 1.04	1.45	$\pm$ 0.06	8.19	$\pm$ 3.58
G448E	10.46	$\pm$ 0.20	1.89	$\pm$ 0.61	1.75	$\pm$ 0.26	7.89	$\pm$ 0.29
F449Y	0.76	$\pm$ 0.08	0.56	$\pm$ 0.12	0.84	$\pm$ 0.57	5.42	$\pm$ 0.04
G450E	18.93	$\pm$ 0.94	3.50	$\pm$ 0.17	7.66	$\pm$ 0.54	15.57	$\pm$ 0.21
V456I	3.68	$\pm$ 0.95	0.79	$\pm$ 0.07	3.21	$\pm$ 0.07	6.66	$\pm$ 0.05
G464S	3.05	$\pm$ 0.17	0.10	$\pm$ 0.01	0.54	$\pm$ 0.11	0.54	$\pm$ 0.09
R467K	3.34	$\pm$ 0.61	0.52	$\pm$ 0.18	0.45	$\pm$ 0.17	3.62	$\pm$ 0.09
I471T	4.35	$\pm$ 0.33	0.92	$\pm$ 0.05	1.21	$\pm$ 0.01	7.71	$\pm$ 0.58
Q474K	0.71	$\pm$ 0.08	0.34	$\pm$ 0.04	0.49	$\pm$ 0.04	3.23	$\pm$ 0.18
V488I	1.06	$\pm$ 0.04	0.13	$\pm$ 0.08	0.98	$\pm$ <0.01	2.11	$\pm$ 0.14
Y132F&K143R	62.04	$\pm$ 1.21	8.86	$\pm$ 1.09	0.23	$\pm$ 0.13	0.86	$\pm$ 0.11
Y132H&K143R	68.81	$\pm$ 5.68	39.59	$\pm$ 8.63	0.70	$\pm$ 0.03	0.74	$\pm$ 0.05
Y132F&F145L	49.10	$\pm$ 1.76	13.58	$\pm$ 0.19	0.91	$\pm$ 0.05	2.64	$\pm$ 0.08
D278N&G464S	18.26	$\pm$ 0.08	4.79	$\pm$ 0.11	3.19	$\pm$ 0.46	14.97	$\pm$ 1.16
G307S&G450E	56.75	$\pm$ 2.30	0.41	$\pm$ 0.10	0.20	$\pm$ 0.04	0.26	$\pm$ 0.11

¹Residual activity is expressed as a percentage of the observed CYP51 activity in the absence of triazole antifungals. Each assay was performed in duplicate. Residual CYP51 activities greater than 3% are indicated in '**bold**'.

susceptibility. However, substitutions such as K143R, S279F, G450E, and G307S&G450E had at least 3% residual activity versus all tested azoles. Indeed, since many of these substitutions have been described in fluconazole-resistant isolates, and likely arose after exposure to the antifungal, it is unsurprising that certain substitutions conferred much higher residual activities to fluconazole versus any other tested azole. The double mutations Y132F&K143R, Y132H&K143R, Y132F&145L, and G307S&G450E, for example, had residual activities of about 50% or greater with fluconazole, but significantly less activity when exposed to voriconazole, itraconazole, or posaconazole.

In a comparison looking at the IC₅₀ values of each variant CaCYP51 for fluconazole, voriconazole, itraconazole, and posaconazole, multiple single amino acid substitutions, including K143R, D278N, S279F, S405F, G448E, and G450E conferred IC₅₀ values ≥ 2 times the IC₅₀ of the wild type (**Table 3-3**). Furthermore, the single substitutions K143R, D278N, S279F, and G450E had similarly high IC₅₀ values relative to wild type for voriconazole and posaconazole in addition to fluconazole. All double substitutions in CaCYP51 tested also showed high values against fluconazole and voriconazole, ranging from 2- to >20-fold increases in IC₅₀. Interestingly, the azole binding affinity assay did not always reflect the biochemical tolerance observed in the IC₅₀ assay. While the IC₅₀ values of some substitutions implied some degree of biochemical tolerance to fluconazole or other azoles, the K_d values of these same substitutions revealed relatively higher affinity for the azole drug, in contrast to what might be expected if these substitutions conferred azole resistance through weakening of the azole-CaCYP51 interaction.

These above findings confirm the suspicion that in addition to varied biochemical tolerance and susceptibility to the azoles, mutations in *ERG11* can also alter functional efficiency of the enzyme. Altering CaCYP51 function to retain better activity in the presence of the azoles, however, often comes at the cost of worse basal catalytic turnover and possibly at the cost of overall growth fitness to the cell. In light of these recent results, we present our findings of the *in vitro* susceptibilities of select amino substitutions in CaCYP51 in *C. albicans*.

Results

In order to determine the impact of CYP51 amino acid substitutions on *in vitro* azole susceptibility in *C. albicans*, we selected several mutations representative of those that led to the greatest biochemical effect on the CYP51 enzyme. When mutant alleles were introduced into the fluconazole-susceptible parent strain SC5314, CaCYP51 amino acid substitutions displayed variable effects on azole drug concentration (**Table 3-4**). Nine single CYP51 amino acid substitutions and three double CYP51 amino acid substitutions were tested against fluconazole, voriconazole, itraconazole and posaconazole.

Table 3-3. Triazole antifungal IC₅₀ values obtained with CaCYP51 proteins

CaCYP51 substitution	IC ₅₀ (μM) ¹			
	Fluconazole	Voriconazole	Itraconazole	Posaconazole
Wild type	0.384 ±0.019	0.197 ±0.009	0.389 ±0.013	0.195 ±0.009
Y118A	0.194 ±0.010	0.202 ±0.002	0.170 ±0.016	0.206 ±0.004
F126V	0.278 ±0.027	0.341 ±0.020	0.206 ±0.009	0.304 ±0.070
Y132F	0.606 ±0.066	0.429 ±0.012	0.309 ±0.003	0.343 ±0.020
Y132H	0.220 ±0.013	0.197 ±0.029	0.150 ±0.028	0.195 ±0.003
K143R	1.100 ±0.313	0.424 ±0.035	0.450 ±0.016	0.789 ±0.003
F145L	0.450 ±0.083	0.231 ±0.031	0.300 ±0.013	0.359 ±0.018
P230L	0.334 ±0.086	0.241 ±0.042	0.265 ±0.038	0.238 ±0.002
Y257H	0.512 ±0.047	0.350 ±0.039	0.336 ±0.014	0.611 ±0.017
D278N	0.838 ±0.027	0.555 ±0.004	0.545 ±0.044	0.908 ±0.020
S279F	1.046 ±0.325	0.487 ±0.002	0.569 ±0.121	0.912 ±0.016
G307S	0.526 ±0.211	0.439 ±0.018	0.225 ±0.007	0.288 ±0.042
S405F	0.820 ±0.042	0.362 ±0.086	0.350 ±0.019	0.637 ±0.054
V437I	0.281 ±0.004	0.304 ±0.002	0.293 ±0.022	0.319 ±0.009
Y447H	0.483 ±0.018	0.377 ±0.055	0.299 ±0.039	0.439 ±0.135
G448E	0.796 ±0.087	0.339 ±0.011	0.238 ±0.013	0.542 ±0.051
F449Y	0.245 ±0.028	0.361 ±0.010	0.222 ±0.011	0.366 ±0.037
G450E	1.078 ±0.247	0.427 ±0.031	0.458 ±0.070	0.908 ±0.132
V456I	0.483 ±0.022	0.469 ±0.023	0.603 ±0.002	0.602 ±0.017
G464S	0.455 ±0.047	0.278 ±0.038	0.282 ±0.006	0.253 ±0.024
R467K	0.476 ±0.080	0.298 ±0.049	0.235 ±0.018	0.380 ±0.011
I471T	0.600 ±0.017	0.318 ±0.015	0.218 ±0.002	0.522 ±0.064
Q474K	0.358 ±0.008	0.325 ±0.002	0.246 ±0.006	0.301 ±0.017
V488I	0.520 ±0.057	0.457 ±0.016	0.449 ±0.016	0.481 ±0.052
Y132F&K143R	5.857 ±0.524	0.580 ±0.099	0.190 ±0.002	0.246 ±0.024
Y132H&K143R	8.471 ±1.271	3.074 ±0.042	0.237 ±0.007	0.196 ±0.007
Y132F&F145L	3.889 ±0.077	1.158 ±0.231	0.364 ±0.023	0.433 ±0.102
D278N&G464S	1.243 ±0.064	0.552 ±0.045	0.403 ±0.011	0.930 ±0.009
G307S&G450E	4.980 ±0.054	0.433 ±0.033	0.318 ±0.017	0.312 ±0.015

¹IC₅₀ determinations were performed in duplicate. Mean IC₅₀ values together with standard deviations are shown. Bold values indicate IC₅₀ values that are over 2-fold greater than those obtained with the wild type protein.

Table 3-4. CLSI MICs($\mu\text{g mL}^{-1}$) for fluconazole, voriconazole, itraconazole, and posaconazole against strains containing CaCYP51 amino acid substitutions

CaCYP51	MIC ($\mu\text{g mL}^{-1}$)			
	Fluconazole	Voriconazole	Itraconazole	Posaconazole
None ¹	1	≤ 0.03	0.06	≤ 0.03
Y132F	8	0.5	0.25	≤ 0.03
Y132H	2	0.25	0.25	≤ 0.03
K143R	16	0.5	0.5	≤ 0.03
F145L	4	0.5	0.25	≤ 0.03
D278N	1	≤ 0.03	0.125	≤ 0.03
S405F	4	0.25	0.25	≤ 0.03
G448E	8	0.25	0.25	0.06
G450E	16	0.5	0.25	≤ 0.03
G464S	8	0.25	0.25	≤ 0.03
Y132F, K143R	32	1	0.25	≤ 0.03
Y132F, F145L	32	1	0.25	≤ 0.03
D278N, G464S	8	0.25	0.25	≤ 0.03

¹Fluconazole-susceptible parent strain SC5314.

The G450E and K143R amino acid substitutions showed the greatest relative increase in the minimum inhibitory concentration (MIC) for fluconazole, each exhibiting a 16-fold increase in MIC compared to the reference strain SC5314, which lacks any substitution in CYP51. The strains containing the CYP51 single amino acid substitutions Y132F, G448E and G464S also demonstrated resistance to fluconazole, displaying an 8-fold increase in MIC over that of SC5314. The overall strongest effect on fluconazole MIC occurred with the double substitutions Y132F&K143R and Y132F&F145L, each conferring a 32-fold increase in fluconazole MIC. The D278N amino acid substitution did not appear to alter the fluconazole MIC compared to the wild type, and the Y132H substitution showed only a 2-fold increase in fluconazole MIC, showing that these individual substitutions have minor effects, if any, on fluconazole MICs.

Against voriconazole, the single substitutions Y132F, K143R, F145L and G450E had the greatest effect on MIC, and the strongest *in vitro* effect was observed in the Y132F&K143R and Y132F&F145L double substitutions. The amino acid substitution with the weakest effect on voriconazole MIC was again D278N (≤ 0.03 $\mu\text{g/mL}$). When tested against itraconazole, most of the tested CYP51 substitution strains showed a small increase in MIC, with the strongest single amino acid substitution K143R displaying an MIC ($0.5 \mu\text{g mL}^{-1}$) greater than that of any of the double amino acid substitutions. While most substitutions causes a small 4-fold increase in MIC to itraconazole, the D278N amino acid substitution only conferred a 2-fold increase in MIC over SC5314. In contrast to the other azoles, posaconazole appeared to be very effective against all CYP51 mutant strains as the MIC to the reference strain and all other strains tested were identical with the exception the strain containing the single G448E substitution, which displayed a slight two-fold increase in MIC over SC5314.

Discussion

While previous investigations have looked into the contributions of substitutions in CaCYP51 (122, 139), the isolated contributions of the D278N and Y132H amino acid substitutions when introduced and tested in *C. albicans* have not been previously described. Susceptibility testing of these *C. albicans* strains expressing these substitutions as well as testing of previously constructed CaCYP51 mutant strains is clinically relevant as all these mutations have been associated with fluconazole-resistant clinical isolates.

Furthermore, the recent biochemical and functional findings described by Warrilow et al. in conjunction with our *in vitro* susceptibility testing in *C. albicans* provides an important insight that amino acid substitutions in CaCYP51 may confer azole resistance by multiple means. For example, the D278N&G464S conferred an 8-fold increase in fluconazole MIC over wild type CaCYP51. While the IC_{50} value reported by Warrilow et al. correlated with the increased fluconazole MIC of these substitutions, the azole ligand binding study showed a relatively low K_d value (15 ± 9 nM) of the D278N&G464S CaCYP51 enzyme for fluconazole. This suggests that these two

substitutions in combination with each other do not have a great effect on fluconazole binding.

This is in stark contrast to the conventional line of thinking that resistance-associated *ERG11* mutations result in CaCYP51 substitutions that reduce the ability of the azole antifungals to bind and inhibit the enzyme. While this remains a likely mechanism of azole resistance for many CaCYP51 substitutions (Y132F&K143R and Y132H&K143R, for example, both have K_d values for fluconazole over 300-fold larger than the wild type enzyme), our susceptibility data with these mutants suggests that azole-resistance conferred by CaCYP51 mutants may be more nuanced than simply altering fluconazole binding. As previously hypothesized, CaCYP51 substitutions could alter interactions between cytochrome P450-reductase, for instance, to affect azole resistance indirectly (140).

The amino acid substitution D278N and Y132H by themselves showed modest increases in azole MIC compared to the wild type. With Y132H, a 2-, 8-, and 4-fold increase in MIC was observed with fluconazole, voriconazole, and itraconazole, respectively. D278N failed to show anything except for a two-fold increase in itraconazole MIC compared to SC5314. Strangely, while Y132H did not show elevated IC_{50} values to any tested azole compared to the wild type enzyme, D278N had increased IC_{50} values versus fluconazole, voriconazole, and posaconazole and residual activity versus fluconazole, itraconazole, and posaconazole.

The Y132H CaCYP51 amino acid substitution had previously been shown to increase MIC to fluconazole approximately 4-fold when test in *S. cerevisiae* (122). Y132H is believed to sit in the ligand binding pocket and alter the interaction with fluconazole and other azoles (140, 206). However, the isolated contribution of the D278N amino acid substitution to azole susceptibility has not been reported, and current structural analyses of CaCYP51 do not comment on any significance of the Asp-278 residue in azole binding.

In contrast to the susceptibilities in our tested strains, previous literature showed that adding the D278N amino acid substitution to G464S increased the fluconazole MIC from 4- to 16-fold (139). However, MIC readings were taken at 48 hours, rather than at 24 hours in our study. It is possible the D278N amino acid substitution influences trailing growth and azole tolerance, which might explain the increase in MICs observed at 48 hours, but not 24 hours.

Azole resistance-causing gain-of-function mutations in the transcription factors Tac1, Mrr1, and Upc2 have been shown to also confer conditional fitness defects in *C. albicans* (207-209). Having more than one of these mutations compounds the fitness defect observed, though clinical isolates of *C. albicans* may have compensatory mechanisms by which to regain their original fitness. Given the findings that CaCYP51 substitutions may alter enzyme function, it is conceivable that these amino acid substitutions might also confer a fitness defect on the cell.

In a competitive growth fitness assay, *C. albicans* strains containing either the wild type CYP51 or mutant CYP51 were grown against the control strain SCADH1R1A containing the *CaSAT1* nourseothricin resistance marker (210). At approximately 24 hours post-inoculation, relative ratios between the test and control strain were fairly constant, regardless of the test strain used, indicative that none of the tested CaCYP51 mutants conferred an observable competitive growth defect after 24 hours (**Figure 3-2**, unpublished data, personal communication from Joachim Morschhäuser on November 2, 2018). At 48 hours, only strains containing the K143R CaCYP51 amino acid substitution showed a slight drop in ratio of growth at day 0 versus day 2, indicating a trend towards less fit growth compared to the control strain. However, the difference in competitive growth fitness between either wild type strain and the K143R substitution-containing strain was non-significant ($p = 0.12$ and 0.23 between WT A and WT B strains, respectively).

These competitive growth experiments do not indicate that any of our tested substitutions have an immediate impact on cell fitness by themselves. The K143R substitution showed a non-significant decrease in competitive growth compared to the control strain, making it plausible that in longer growth competition experiments, this strain may eventually be outcompeted versus azole-susceptible strains. Because we only tested strains with at most two substitutions in CaCYP51, it remains possible that highly mutated CaCYP51 containing 3 or more amino acid substitutions may display an exacerbated fitness defect driven by poor CaCYP51 function. In a similar vein, it is unknown whether the presence of CaCYP51 mutations in combination with gain-of-function mutations in *Tac1*, *Mrr1*, or *Upc2* might further decrease fitness in the cell compared to mutations in the ZCFs alone.

CYP51 mutations will likely continue to be a thorn in the side of clinicians and hospitalists prescribing azole antifungals, even with the expected arrival of newer azole antifungal drugs. Therefore, documenting these mutations and characterizing their effects on azole susceptibility, virulence, and fitness are relevant to overcoming azole resistance and ultimately providing better treatment outcomes for invasive fungal infections. As we advance our understanding of the azole antifungal target and effects of amino acid substitutions on drug-target interactions, further investigation should continue to identify key amino acid substitutions involved in the resistance to specific azole drugs. Such work would hopefully serve to provide greater information in the selection of currently available azole antifungals and potentially be useful in the design of future antifungals.

A 24H Competitive growth fitness of Erg11 mutants

B 48H Competitive growth fitness of Erg11 mutants

Figure 3-2. Competitive growth fitness testing of Erg11 mutants

(A) 24 hour and (B) 48 hour growth fitness testing of Erg11 mutants versus wild type Erg11. Experiments were performed in triplicate. Data used with permission. Source: Joachim Morschhäuser, personal communication, November 2, 2018.

Methods

Construction of *C. albicans* CYP51 mutant strains

Strains used in this study are shown in **Table 3-5**. Strains expressing seven single mutations (Y132F, F145L, K143R, S405F, G448E, G450E, G464S) and three double mutations (Y132F&F145L, Y132F&K143R, D278N&G464S) were selected from a previous study (139). Two additional strains expressing the Y132H and D278N CYP51 gene mutations were created utilizing the *SAT1*/FLP cassette described previously (202). Briefly, *C. albicans* isolate SC5314 was transformed via electroporation with inserts of pSFS2-derived plasmid possessing the *SAT1* nourseothricin resistance marker, FLP recombinase, and mutant CaCYP51 genes of interest. The 5' end of the DNA fragment contained either by the Y132H or D278N CYP51 ORF and the 3' end possessed homology downstream of the CaCYP51 stop codon to ensure homologous recombination at the CYP51 gene locus. Successful transformants were screened on yeast-peptone-dextrose agar plates containing nourseothricin and confirmed via Southern hybridization.

Genomic DNA from nourseothricin-screened transformants were digested with HindIII restriction enzyme and the resulting DNA digests were loaded on 1% agarose gels containing ethidium bromide. Digested samples were visualized briefly under ultraviolet light after approximately 2 hours of electrophoresis (120V) to confirm appropriate band separation. Lanes containing digested DNA in the gel was transferred onto a nitrocellulose membrane via vacuum suction and DNA was crosslinked to the membrane through UV exposure. PCR amplification of the genomic DNA from parent isolate SC5314 using primers ERG11_Probe_F (5' – AGTTCAATGGTGGTTTTACCTACT – 3') and ERG11_Probe_R (5' – ATTTCTGATTGAGTCATCCTAACA – 3') generated a 254 bp product used as a probe for the *ERG11* gene locus. Probe was labelled and prepared using the Amersham™ AlkPhos Direct Labelling and Detection System (GE Life Sciences) and hybridized to the membrane through overnight incubation at 55°C. Bands containing the hybridized, alkaline phosphatase-labelled probe corresponding to either wildtype *ERG11* alleles (2678 bp) or successfully replaced alleles with the *SAT1*/FLP cassette included (4318 bp) or recycled (3119 bp) were visualized with CDP-*Star* chemiluminescent detection reagent.

To confirm sequence of the replaced *ERG11* allele, a DNA fragment containing the *ERG11* ORF and approximately 435 bp upstream of the start codon and 229 bp downstream of the *ERG11* stop codon was PCR amplified using primers CaERG11_A_(ApaI) (5' – GGGCCCGGGTTATTTGAGAACAGCC – 3') and CaERG11_E_(XhoI) (5' – CTCGAGCCAGTGGACAAAAACCATCA – 3'). The resulting DNA fragment was purified and Sanger sequenced on an ABI Model 3130XL Genetic Analyzer with sequencing primers CaERG11SeqA (5' – GCCACCACACCCTATGGCTATT – 3'), CaERG11SeqB (5' –

Table 3-5. Strains used in Chapter 3

Strains ^a	Genotype	Source or reference
SC5314	<i>ERG11-1/ERG11-2</i>	ATCC
Constructed laboratory strains		
20E1I1B1	<i>ERG11^{Y132F}::FRT / ERG11^{Y132F}::FRT</i>	Flowers et al 2015
SCERG11R1S1C1	<i>ERG11^{Y132H}::FRT / ERG11^{Y132H}::FRT</i>	This study
10B1A3A	<i>ERG11^{K143R}::FRT / ERG11^{K143R}::FRT</i>	Flowers et al 2015
2A1A18A	<i>ERG11^{F145L}::FRT / ERG11^{F145L}::FRT</i>	Flowers et al 2015
SCERG11R3S3C1	<i>ERG11^{D278N}::FRT / ERG11^{D278N}::FRT</i>	This study
21C1M1B1	<i>ERG11^{S405F}::FRT / ERG11^{S405F}::FRT</i>	Flowers et al 2015
20NA11A57A	<i>ERG11^{G448E}::FRT / ERG11^{G448E}::FRT</i>	Flowers et al 2015
15A3A108A	<i>ERG11^{G450E}::FRT / ERG11^{G450E}::FRT</i>	Flowers et al 2015
19A1A1C1	<i>ERG11^{G464S}::FRT / ERG11^{G464S}::FRT</i>	Flowers et al 2015
9A14A21A	<i>ERG11^{Y132F,K143R}::FRT / ERG11^{Y132F,K143R}::FRT</i>	Flowers et al 2015
27A5A33A	<i>ERG11^{Y132F,F145L}::FRT / ERG11^{Y132F,F145L}::FRT</i>	Flowers et al 2015
13A5A57A	<i>ERG11^{D278N,G464S}::FRT / ERG11^{D278N,G464S}::FRT</i>	Flowers et al 2015

^a All laboratory strains have SC5314 as background.

TATTTTCACTGCTTCAAGATCT – 3'), CaERG11SeqC (5' – CCAAAAGGTCATTATGTTTTAG – 3'), CaERG11SeqD (5' – CATACAAGTTTCTCTTTTTTCC – 3'), CaERG11SeqE (5' – CATTTAGGTGAAAAACCTCATT – 3'), and CaERG11SeqF (5' – TACTCCAGTTTTCGGTAAAGGG – 3').

Susceptibility testing of *C. albicans* CYP51 mutant strains

Azole minimum inhibitory concentrations (MICs) were determined using the broth microdilution methods as described by the Clinical Laboratory and Standards Institute (200, 201). Cells were incubated in 96-well microtiter plates containing Roswell Park Memorial Institute 1640 (RPMI) media and serially-diluted concentrations of either fluconazole, itraconazole, voriconazole, or posaconazole. Concentration for MICs ranged from 0.125 to 64 $\mu\text{g ml}^{-1}$ for fluconazole and 0.03 to 16 $\mu\text{g ml}^{-1}$ for itraconazole, voriconazole, and posaconazole. Measurements were read visually at 24 hours after incubation at 35°C for a 50% reduction in growth from drug-free control wells. MIC measurements were performed in duplicate and the MIC was reported as the higher of two values for all strains tested. In cases where the duplicate MIC values were not identical, the higher of the two MIC values were reported. Overall the MIC values were consistent with 92% (184/200) MICs within a single dilution of each other for a given strain and drug combination. Additionally, no MICs differed by more than two dilutions for a given strain and drug.

Competitive growth assay

Competitive growth assays performed by Morschhäuser et al. were performed in triplicate as previously described (208, 210). Briefly, each strain was compared to the control strain SCADH1R1A containing the nourseothricin resistance marker *CaSAT1*. Each test strain and SCADH1R1A were inoculated in liquid YPD media in equal ratios ($\text{OD}_{600} = 0.002$) and subsequently grown for 26h at 30°C. Cells from the culture were plate at 0 hours, 24 hours and 48 hours onto YPD-agar plates and YPD-agar with either 200 $\mu\text{g/mL}$ or 15 $\mu\text{g/mL}$ nourseothricin. Colonies on YPD with 200 $\mu\text{g/mL}$ nourseothricin represented the nourseothricin-resistant SCADH1R1A, and smaller colonies on YPD with 15 $\mu\text{g/mL}$ nourseothricin represented the nourseothricin-sensitive test strain. Colonies were counted to determine the percentage in the co-culture at each timepoint.

CHAPTER 4. THE ROLE OF *ERG3* MUTATIONS TO AZOLE RESISTANCE AND *ERG11* MUTABILITY IN *C. ALBICANS*

Introduction

Candida albicans is a commensal organism and opportunistic pathogen that can cause a wide array of infections, ranging from superficial mucosal infection to life-threatening invasive disease. In the United States, *Candida* remains a common cause of healthcare-associated infection (12, 183). In particular, mortality associated with *Candida* bloodstream infections remains high, and proper treatment of the infection increases hospitalization costs and length of stay considerably (59, 68, 182). Additionally, mucosal infections such as vaginal candidiasis and, in immunocompromised patients, oropharyngeal candidiasis are common infections in which *C. albicans* remains one of the most frequent causes of disease (211-213).

The azole antifungal drugs are commonly used in treatment and prophylaxis of candidiasis. By inhibiting 14 α -lanosterol demethylase, an enzyme critical to the production of the important yeast cellular membrane sterol ergosterol, the azoles inhibit cell growth. The primarily fungistatic action of the azole antifungals is thought to be linked the accumulation of the toxic sterol 14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol within the cell (71, 72).

C. albicans is able to avoid the inhibitory effects of the azole antifungals through several mechanisms. Commonly, *C. albicans* isolates resistant to the azole have increased expression of genes encoding for efflux pumps that reduce the intracellular concentrations of azoles inside the cell, such as the ABC transporters Cdr1 and Cdr2 and the MFS transporter Mdr1 (78, 79, 99). Changes to the azole drug target 14 α -lanosterol demethylase brought about by nucleotide changes in its encoding gene *ERG11* can also reduce the effectiveness of the azole antifungals to bind or otherwise inhibit the activity of the enzyme (107, 139, 140). Additionally, increases in the amount of azole target through upregulation of *ERG11* expression can also overcome to some degree the presence of the azole antifungals inside the cell (106, 214, 215).

The above mechanisms are among the most commonly reported determinants of azole resistance in clinical isolates, however, it has been less commonly reported that alterations in the *C. albicans* sterol biosynthesis pathway mediated by mutations in *ERG3* can also confer azole resistance. *ERG3* encodes for the 386 amino acid length C-5 sterol desaturase in *Candida albicans* a component essential to the production of ergosterol (71, 216). It had been shown that defective Erg3 confers azole-resistance in *C. albicans* by avoiding accumulation of toxic sterol inside the cell and that Erg3-defective cells had significantly different sterol profiles (72, 144, 149). This signifies that overall without the presence of a functional C-5 sterol desaturase, the *C. albicans* cells utilizes alternate sterol biosynthesis pathways in order to survive. Since the toxic 14 α -methyleergosta-8,24(28)-dien-3 β , 6 α -diol required Erg3 for its production, mutant isolates lacking functional C-5 sterol desaturase would not accumulate the toxic sterol even in the

presence of the Erg11-inhibiting azoles (**Figures 4-1 and 4-2**). Therefore, isolates lacking in or defective for Erg3 activity were rendered completely resistant to the azole antifungal class (142, 148).

Previously, we had characterized a collection of predominantly azole-resistant clinical isolates of *C. albicans* (115). Based on this data, we identified several members among this collection which could not be explained by common mechanisms of resistance in *C. albicans*. While mutations in *ERG3* can confer azole resistance to clinical isolates, their rarely reported frequency and debate over attenuated virulence in these isolates makes the clinical significance of *ERG3* mutations questionable. However, Erg3 is inexorably tied to Erg11 as both proteins are crucial to the biosynthesis of ergosterol (147). Moreover, a recent finding by Warrilow et al. demonstrated that mutations in *ERG11* can have variable effects on 14 α -lanosterol demethylase activity, and while amino acid substitutions in Erg11 conferred azole resistance, they often came at a cost to the catalytic turnover of the enzyme (141).

Since loss of demethylase activity by treatment with the azoles triggers accumulation of the toxic sterol intermediates in *C. albicans*, it is plausible that some *ERG11* mutations may result in markedly poorer demethylase activity and therefore may accumulate higher levels of toxic sterol intermediates. Furthermore, since the toxic sterol production is dependent on Erg3 activity, we believe that mutations in Erg3 that alter activity or substrate specificity of the enzyme may alleviate accumulation of toxic sterols. Therefore, it is possible that mutations in *ERG3* allows conditional *ERG11* mutations in *C. albicans* to exist. In other words, *ERG3* mutants may increase the mutability of *ERG11*, thereby increasing the opportunistic potential to develop azole-resistance through *ERG11* mutation. In this study we identified the mutations in *ERG3* across our collection of clinical isolates and posit that mutations in *ERG3* may be more common than popularly believed and may play a larger role in azole resistance than previously believed.

Results

Frequency of Erg11 and Erg3 amino acid substitutions in *C. albicans* clinical isolates

Sequencing of *ERG11* and *ERG3* within 57 fluconazole-resistant (MIC ≥ 8 μ g/mL) clinical *C. albicans* isolates revealed 51 of 57 (89%) isolates with Erg11 amino acid substitutions (**Table 4-1**). Overall, 20 of 57 (35%) clinical isolates possessed amino acid substitutions in Erg3. In particular, a C>T nucleotide change at position 1052 in *ERG3* corresponding to an A351V amino acid substitution appeared either alone or together with another Erg3 substitution in 18 (90%) of all *ERG3* mutant isolates. Among the 51 isolates with Erg11 amino acid substitutions, concomitant Erg3 amino acid substitutions occurred in 19 (37%) isolates (**Table 4-1**). Interestingly, all Erg3 mutants containing either a homozygous or heterozygous A351V amino acid substitution possessed at least one substitution in Erg11, and 15 of 18 (83%) of those isolates

Figure 4-1. Normal ergosterol biosynthesis pathway inhibition by the azole antifungals in *C. albicans*

Simplified representation of the later stages of ergosterol biosynthesis converting lanosterol to ergosterol is interrupted with fluconazole treatment. Fluconazole is represented by the light red rectangle. The azole target enzyme is shown in green. Grayed areas represent blocked biosynthesis pathways. T-shaped representation of pathway inhibition is shown at inhibition sites. A skull and crossbones represents the accumulation of toxic sterol.

Figure 4-2. Ergosterol biosynthesis pathway inhibition by the azole antifungals in *C. albicans* with defective C-5 sterol desaturase

Simplified representation of the later stages of ergosterol biosynthesis converting lanosterol to ergosterol is interrupted with fluconazole treatment, but the toxic azole cannot be produced due to defective Erg3. Fluconazole is represented by the light red rectangle. The azole target enzyme is shown in green. Grayed areas represent blocked biosynthesis pathways. T-shaped representation of pathway inhibition is shown at inhibition sites. Erg3 is represented in yellow.

Table 4-1. Fluconazole MIC, ergosterol content, and mutations in *ERG11* and *ERG3* in clinical isolates of *C. albicans*

Isolate	MIC _{FLU} (µg/mL)	% Ergosterol	ERG11 mutation ^a	ERG3 amino acid substitution ^a
11	8	75.5	E266D, V488I	A351V
12	32	73.5	K143R	None
13	32	54.1	K143R	None
14	16	72.3	K143R	None
15	16	64.2	E266D, V488I	A351V (h), A353T (h)
16	8	27.7	Q21R (h)	A351V
18	32	49.1	S405F	None
19	32	47.8	S405F	None
20	64	52.3	S405F	None
21	32	45.0	A114V, E266D, H283R	A351V (h), A353T (h)
23	32	53.2	I483V, G450E	A351V (h)
24	16	41.2	G450E	None
25	16	53.6	D446E	None
26	16	47.5	G307S, G448R	A351V (h)
27	16	53.9	E266D, V488I	A351V (h), A353T (h)
28	64	50.7	E266D, V488I	A351V (h), A353T (h)
29	>64	58.1	Y132F, F145L	A351V (h), A353T (h)
30	32	51.1	Y132F, H283Y	None
31	>64	66.9	G448E	None
32	8	71.0	G464S	None
33	32	60.5	F145L, E266D	None
34	64	40.4	G450E	None
35	16	75.5	D446E	None
36	64	76.0	None	None
37	64	68.1	None	None
38	>64	74.0	G464S	None
39	>64	70.0	G464S	None
40	>64	70.0	F449V	None
41	64	55.7	S405F	None
42	64	58.8	G464S	None
43	8	74.0	D278N, G464S	None
44	>64	60.9	D446E	None
45	64	76.0	E266D, G464S	A351V
46	64	45.7	G307S, L403F, G448R	A351V (h)
47	>64	73.9	G450E	A351V (h)
48	32	70.5	Y132F	L195V (h), A351V (h)
49	>64	77.7	V437I, Y447S	H28Y (h)
50	>64	72.0	Y132F, K143R	None
51	>64	57.5	D278N, G464S	None
54	32	61.5	G307S, G450E	A351V (h)

Table 4-1. (Continued)

Isolate	MIC_{FLU} (µg/mL)	% Ergosterol	ERG11 mutation¹	ERG3 amino acid substitution¹
55	>64	66.0	None	None
56	>64	74.2	None	None
58	>64	80.9	None	None
59	>64	71.7	Y132F, T229A, F449I	A351V (h)
60	>64	0.8	None	W131*
61	>64	46.6	Y132F, K143R	None
62	32	69.8	D278N, G464S	None
63	32	70.3	G450E	None
64	32	52.6	G450E	None
65	64	74.5	G448E	None
66	64	78.3	G448E	None
67	>64	82.4	G448E	None
68	>64	60.2	Y132F, E266D, I471M, V488I	None
69	64	76.0	G448E	None
70	32	71.5	M258L, G464S (h)	A351V
71	>64	71.0	Y132F, V437I, F449L	A351V (h)
72	16	69.5	A114V, D153E, E266D, G450E	A351V (h)

¹(h) indicated a heterozygous amino acid substitution. Pink highlighted rows indicate *ERG3*^{A351V} mutant clinical isolates.

contained more than one amino acid substitution in Erg11. By comparison, among isolates lacking Erg3 amino acid substitutions, 32 of 38 (84%) had at least one Erg11 substitution and only 8 (21%) isolates possessed multiple Erg11 amino acid substitutions. The frequency with which the A351V Erg3 amino acid substitution coincided with more than one Erg11 amino acid substitution in clinical isolates and the relatively lower frequency of multiple Erg11 amino acid substitutions in isolates lacking the A351V substitution suggested that this substitution may possibly be associated with hypermutability of *ERG11*.

Incidentally, we also discovered four novel Erg3 substitutions in select isolates (**Table 4-1**). Isolate 49 contained a heterozygous H28Y amino acid substitution, and the Erg3 of isolate 60 was truncated due to mutation encoding a premature stop codon after only 130 amino acids. The L195V and A353T substitutions only appeared heterozygously and only in combination with the heterozygous A351V substitution in isolates 48 and isolates 15, 21, 27, 28, and 29, respectively.

Sterol content in clinical isolates

In an effort to determine whether substitutions in Erg3 altered sterol content, whole cell membrane sterol contents from the clinical isolates (**Table 4-2**). Ergosterol, normally the major sterol component of the fungal cell membrane, made up on average approximately 62% (median: 67.5%, range: 0.8% - 82.4%) of whole cell membrane sterol content in our fluconazole-resistance clinical isolates. In isolates lacking Erg3 substitutions the average ergosterol content was approximately 64% (median: 68.5%, range: 40.4% - 82.4%) and in the Erg3 mutants containing the A351V substitution, the cell membrane ergosterol content was approximately 60.4% (median: 62.9%, range: 27.7% - 76%). Thus, the presence or absence of the A351V substitution in Erg3 did not seem to affect ergosterol content in the cell under normal growth conditions.

The average ergosterol content when looking at all isolates containing Erg3 substitutions of any sort was considerably skewed lower (58.3%) due to the only 0.8% ergosterol content in isolate 60, containing a nonsense mutation (W131*) in *ERG3*. This premature termination of the Erg3 protein very likely would result in a dysfunctional or nonfunctional enzyme unable to produce significant amount of ergosterol, possibly explaining the isolate's low ergosterol content (**Figures 4-3** and **4-4**).

Allelic replacement of *ERG3*

The A351V amino acid substitution in Erg3 does not directly affect *in vitro* fluconazole susceptibility

To determine whether the A351V mutation had a direct effect on azole resistance, we created an *erg3Δ/Δ* strain as well as an *ERG3*^{A351V} mutant in the azole-susceptible

Table 4-2. Percent total cell membrane sterol content composition in *C. albicans* clinical isolates

Strain (<i>C. albicans</i>)	Lanosterol	Eburicol	Fecosterol	Episterol	Ergosterol ¹	Ergosta- 7,22-dienol	14-methyl fecosterol	14 α -methyl ergosta-8,24(28)- dien-3 β ,6 α -diol
1	1.3		0.5	1.0	91.4			
2	4.2	0.5	2.5	6.2	65.8	1.1		
3	3.4	0.1	1.1	2.1	76.7			
4	0.7		1.6	2.3	69.1			
5	6.3	0.7	5.0	6.5	55.0			
6	8.3	10.4	1.1	0.5	58.9	0.2		0.4
7	2.1	0.1	1.8	5.4	74.1			
8	2.0		3.5	8.6	55.7	6.3		
9	7.2	0.5	4.9	9.0	46.4	1.6		
10	11.1	3.6	1.8		62.5			
11	4.2	0.3	1.1	2.5	75.5	0.4		
12	6.1	3.2	0.8	1.7	73.5			
13	11.8	9.6	2.5	3.1	54.1			
14	5.0	2.4	2.3	4.5	72.3			0.0
15	3.6	0.8	2.7	6.3	64.2	0.4		
16	18.0	5.2	5.1	12.8	27.7	0.4		0.7
17	3.1	0.2	1.7	3.0	75.2			
18	14.5	19.0	0.7	1.2	49.1		2.8	1.9
19	17.2	19.6	0.6	0.7	47.8		3.1	2.0
20	22.1	6.7	1.7	2.5	52.3		1.1	0.5
21				1.9	45.0			4.3
22	8.1	2.8	2.0	4.1	60.5		1.0	
23	25.6		0.4	1.8	53.2		3.6	

Table 4-2. (Continued)

Strain (<i>C. albicans</i>)	Lanosterol	Eburicol	Fecosterol	Episterol	Ergosterol	Ergosta- 7,22-dienol	14-methyl fecosterol	14 α -methyl ergosta-8,24(28)- dien-3 β ,6 α -diol
24	29.5	19.2	0.2	1.5	41.2	1.3	1.2	
25	3.0		3.2	6.0	53.6			
26	20.8	14.4		0.7	47.5		3.4	6.4
27	13.7	1.6	3.9	7.5	53.9			
28	10.2	1.0	4.3	10.3	50.7			
29	12.5	2.2	4.7	5.0	58.1			
30	22.4	2.9	1.3	2.6	51.1			
31	9.0	12.1	0.4	1.4	66.9		1.2	1.3
32	5.7	0.2	1.9	3.8	71.0			
33	13.2	1.1	1.5	4.2	60.5			
34	15.7	8.0	1.5	4.6	40.4	0.4	1.5	
35	1.1		2.0	4.7	75.5			
36	1.0		1.2	3.0	76.0			
37	1.4		3.4	5.0	68.1			
38	2.4		1.6	3.2	74.0			
39	3.1		1.9	4.4	70.0			
40	5.8	4.6	0.3	1.4	70.0			
41	6.8	0.3	2.2	4.2	55.7			
42	7.6	1.9	1.2	4.6	58.8	0.7		
43	5.2	0.3	0.5	1.2	74.0			
44	4.3	0.7	2.9	7.9	60.9	0.7		
45	2.9	0.5	0.6	2.2	76.0			
46	24.9	11.0	1.0	1.0	45.7		0.9	
47	5.6	3.7	0.4	0.9	73.9			

Table 4-2. (Continued)

Strain (<i>C. albicans</i>)	Lanosterol	Eburicol	Fecosterol	Episterol	Ergosterol	Ergosta- 7,22-dienol	14-methyl fecosterol	14 α -methyl ergosta-8,24(28)- dien-3 β ,6 α -diol
48	7.4	3.2	1.2	2.6	70.5	0.9	1.3	
49	6.0	1.6	0.8	1.4	77.7			
50	13.0	3.3	0.6	0.2	72.0			
51	13.1	1.3	0.8	2.1	57.5			
52	3.5	0.4	2.4	5.1	67.5			
53	2.6	0.6	1.0	2.1	73.9			
54	7.6	20.3	0.5		61.5			
55	1.7	0.1	3.7	4.1	66.0			
56	1.3	0.2	3.6	5.1	74.2			
57	1.4	3.7	1.7	3.2	68.8			
58	1.2	1.0	1.3	2.4	80.9			
59	2.0	0.4	1.9	3.9	71.7			
60	0.9		2.0	4.8	0.8	62.1		
61	15.9	11.0	0.7	1.5	46.6			
62	8.5	2.1	0.5	1.5	69.8			
63	5.5	3.8			70.3			
64	11.6	11.7	1.0	2.6	52.6			
65	4.0	1.4	1.2	2.9	74.5			
66	5.2	3.3		1.4	78.3			
67	3.0	1.0		1.3	82.4			
68	10.0	6.2	1.3	3.2	60.2			
69	3.9	1.6	0.8	2.0	76.0			

Table 4-2. (Continued)

Strain (<i>C. albicans</i>)	Lanosterol	Eburicol	Fecosterol	Episterol	Ergosterol	Ergosta- 7,22-dienol	14-methyl fecosterol	14 α -methyl ergosta-8,24(28)- dien-3 β ,6 α -diol
70	3.2	0.6	1.0	2.3	71.5			
71	6.8	4.7		0.9	71.0			
72	9.2	5.7		0.8	69.5			

¹Percent ergosterol content is reflected in the center of the table and colored to indicate increased (greener) or decreased (redder) total ergosterol content. Isolate 60, whose sterol content closely resembles a typical *erg3* Δ sterol profile that lacks ergosterol, is highlighted in yellow. Blank entries under each sterol heading indicates there was none of that particular sterol detected for each isolate.

Figure 4-3. Normal ergosterol biosynthesis pathway in *C. albicans*

Simplified representation of the later stages of ergosterol biosynthesis converting lanosterol to ergosterol. The azole target enzyme Erg11 is shown in green. Red arrows represent alternate biosynthesis pathways. T-shaped representation of pathway inhibition is shown at inhibition sites.

Figure 4-4. Alternative sterol biosynthesis pathway in *C. albicans* cells with defective C-5 sterol desaturase

Simplified representation of the later stages of ergosterol biosynthesis converting lanosterol to ergosterol shows that ergosterol biosynthesis cannot be completed without Erg3 activity. The azole target enzyme Erg11 is shown in green. Erg3 is shown in yellow. Red arrows represent alternate biosynthesis pathways. T-shaped representation of pathway inhibition is shown at inhibition sites. Grayed areas represent blocked biosynthesis pathways.

isolate SC5314. Deletion of the *ERG3* gene from both native loci resulted in complete resistance to fluconazole (**Table 4-3**), consistent with the traditional *ERG3* resistance phenotype. However, when the *ERG3* allele containing the nucleotide mutation corresponding to the A351V amino acid substitution was replaced again in both native loci, the strains appeared to have no direct effect on fluconazole MICs compared to the parent isolate. Thus, in our hands, the A351V amino acid substitution does not appear to directly affect fluconazole resistance as measure by CLSI broth microdilution assay. However, given the prevalence of *ERG3* mutations in resistant clinical isolates and their co-occurrence with *ERG11* mutations, the possibility remained that mutations in *ERG3* were conferring a compensatory fitness benefit to the cell in the presence of *ERG11* mutations that impair native enzyme function.

A homozygous *ERG11*^{Y132H,K143R} mutant is conditionally constructed in combination with *ERG3*^{A351V}

To this end, we aimed to create double mutant strains containing two copies of either the *ERG3*^{WT} allele or the *ERG3*^{A351V} allele in combination with mutant *ERG11* alleles. These mutant *ERG11* alleles encoded for select amino acid substitutions previously shown to affect basal catalytic turnover rate of the Erg11 enzyme. The original intent in creating these mutants was to determine whether the strains containing the mutant *ERG3*^{A351V} allele conferred any additional benefit to fitness over *ERG3*^{WT} when either is combined with activity-altering *ERG11* mutations. In the case of the double amino acid substitution Y132H&K143R in Erg11, we were able to successfully insert both copies of *ERG11* in an *ERG3*^{A351V} background, but not in an *ERG3*^{WT} background. Repeated attempts to insert two copies of the *ERG11*^{Y132H,K143R} allele in SC5314 yielded a single copy of the mutant allele, and interestingly, the Y132H unaccompanied by K143R on the second replaced allele, as revealed by Sanger sequencing. Susceptibility testing of the two independently created strains SCERG11R2S2L1 and SCERG11R2S2L2 (*ERG11*^{Y132H,K143R}::FRT / *ERG11*^{Y132H}::FRT) had a fluconazole MIC of 1 µg/mL, a 4-fold increase in MIC compared to SC5314 (0.25 µg/mL). Comparatively, the strains SCERG3R1S1ERG11R2S2A and SCERG3R1S1ERG11R2S2B, which contained two copies of both *ERG3*^{A351V} and *ERG11*^{Y132H,K143R}, displayed an 8-fold increase (MIC_{FLU}: 2 µg/mL) over SC5314, likely because of the additional K143R substitution present in the second allele.

A homozygous *ERG11*^{G307S} mutant cannot be constructed

Surprisingly, we were unable to retain both copies of the *ERG11*^{G307S} allele in our strains. The single amino acid substitution G307S was unable to be produced in either *ERG3* background strain. Repeated attempts were made to construct the above strains, and Southern blotting in conjunction with screening using a nourseothricin drug selection marker seemed to confirm successful integration of allelic replacement cassettes. Despite this, subsequent nucleotide sequencing of tentatively successful strains repeatedly

Table 4-3. 24-hour fluconazole MICs for *C. albicans* strains in Chapter 4

Strain	MIC _{FLU} ($\mu\text{g mL}^{-1}$)	<i>ERG3</i> genotype
SC5314	0.25	WT
SCERG3R1S1A	0.25	<i>ERG3</i> ^{A351V} :: <i>FRT</i> / <i>ERG3</i> ^{A351V} :: <i>FRT</i>
SCERG3R1S1E	0.25	<i>ERG3</i> ^{A351V} :: <i>FRT</i> / <i>ERG3</i> ^{A351V} :: <i>FRT</i>
SCERG11R2S2L1	1	<i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i> / <i>ERG11</i> ^{Y132H} :: <i>FRT</i>
SCERG11R2S2L2	1	<i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i> / <i>ERG11</i> ^{Y132H} :: <i>FRT</i>
SCERG3R1S1ER11GR2S2A	2	<i>ERG3</i> ^{A351V} :: <i>FRT</i> / <i>ERG3</i> ^{A351V} :: <i>FRT</i> <i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i> / <i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i>
SCERG3R1S1ERG11R2S2B	2	<i>ERG3</i> ^{A351V} :: <i>FRT</i> / <i>ERG3</i> ^{A351V} :: <i>FRT</i> <i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i> / <i>ERG11</i> ^{Y132H,K143R} :: <i>FRT</i>
SCERG3M4A	>64	<i>erg3</i> Δ :: <i>FRT</i> / <i>erg3</i> Δ :: <i>FRT</i> Δ
SCERG3M4B	>64	<i>erg3</i> Δ :: <i>FRT</i> / <i>erg3</i> Δ :: <i>FRT</i> Δ

revealed retention of only a single copy of either the *ERG11*^{G307S} mutant allele. The inability to retain both copies of the mutant *ERG11* alleles suggests that there may be a selective disadvantage for *C. albicans* to have the G307S amino acid substitution in Erg11 in the absence of drug or other cell stressors.

Discussion

We have known for some time that mutations in the *ERG11* gene can have variable effects on azole resistance. However, the differences observed in Erg11 enzyme function has only recently been discovered. Warrilow et al. measured the relative velocities of purified recombinant *C. albicans* Erg11 protein expressed from *E. coli* and found that compared to the wild type enzyme, enzymes containing even a single amino acid substitution altered the protein's catalytic turnover significantly (141). From these findings, it was revealed that changes in the amino acid sequence could lower the relative velocity of the Erg11 enzyme to as low as 3% of the wild type enzyme, and double amino acid substitutions generally resulted in even poorer relative velocities compared to single amino acid substitutions. Importantly, many of these amino acid changes also resulted in higher residual activity in the presence of fluconazole compared to the wild type Erg11.

While the benefit of having such substitutions during azole stress is clear, it raises questions about the potential deleterious effects of Erg11 amino acid changes and fitness costs to the cell in the absence of drug. Amino acid changes, such as the Y132H&K143R double substitution and the G307S substitution, both of which retain less than 7% of the catalytic turnover of the wild type Erg11, would not be predicted to be advantageous to a cell in the absence of azole drug stress (141). The low enzyme rate even raises questions whether such an isolate, without compensatory changes elsewhere in the genome, would be viable. Poor Erg11 function as measured by the low catalytic turnover in these cases could have a so-called azole-mimetic effect on the cell, whereby the bottleneck of the ergosterol biosynthesis pathway is exacerbated by innately poor Erg11 function (**Figure 4-5**). Ergosterol precursors in this case could possibly be shunted to alternative metabolic pathways, producing unwanted byproducts and accumulation of the 14 α -methylergosta-8,24(28)-dien-3 β , 6 α -diol toxic sterol.

As production of the toxic sterol is dependent on C-5 sterol desaturase activity, it is plausible that changes affecting either the activity of Erg3 or substrate specificity of Erg3 may ameliorate toxic sterol accumulation. Moreover, changes within the genome that might normally incur higher 14 α -methylergosta-8,24(28)-dien-3 β , 6 α -diol levels may be more permissible when there are accompanying changes in either facet of Erg3 activity. Thus, such Erg3 changes may be the gateway for increased genetic diversity in the ergosterol biosynthetic genes that might otherwise not be possible.

Here, we attempted to determine whether amino acid changes in Erg3, namely the A351V amino acid substitution, acted as permissive substitutions that allowed for Erg11 variants with slow enzymatic function to persist in *C. albicans* isolates. Substitutions in

Figure 4-5. Proposed sterol biosynthesis pathway in *C. albicans* cells with *ERG11* mutations resulting in slow Erg11 enzyme function

Simplified representation of proposed consequences to the sterol biosynthesis pathway when Erg11 activity is slowed or lowered as a result of *ERG11* mutations. The azole target enzyme Erg11 is shown in green and the mutant Erg11 protein with poor function is labeled with an asterisk. Erg3 is shown in yellow. Red arrows represent alternate biosynthesis pathways. Dashed arrows represent the biosynthetic pathway affected by low Erg11 catalytic turnover.

Erg3 that might reduce toxic sterol production through alterations of enzymatic activity would be expected to also reduce susceptibility to the azole antifungals directly. However, no detectable changes in susceptibility were observed in *ERG3*^{A351V} strains. It is possible that changes in susceptibility were too subtle to be detected through broth microdilution assay or that perhaps lower concentration ranges for fluconazole should be used going forward.

By obtaining whole cell membrane sterol profiles, we hoped to readily identify differences in sterol content between *ERG11* mutants with and without *ERG3* mutations. Isolates with multiple Erg11 mutations or mutations known to affect enzyme activity might be expected to possess lower percentages of ergosterol and potentially higher levels of 14 α -methylergosta-8,24(28)-dien-3 β , 6 α -diol. However, we were unable to identify significant differences between the ergosterol content of mutant and non-mutant *ERG3* isolates. One possible explanation for this may be that alterations to total sterol content may be relatively minor. It is also possible that the alterations in sterol content and accumulation of toxic sterols may occur more slowly in these isolates than expected, and that sterol profiles changes would be more apparent after longer periods of growth than what was performed here.

Lastly, we were unable to generate the *ERG11*^{Y132H,K143R} and *ERG11*^{G307S} mutants in a background with *ERG3*^{WT}. Previous data suggests that both Erg11 variants retain extremely poor function relative to the wild type enzyme (141). By comparing these variant Erg11 proteins with and without the Erg3 A351V amino acid substitution, we hoped to observe differences in fitness based on our selection of Erg11 variants with particularly slow turnover. To our knowledge, the Y132H&K143R substitutions and the G307S substitution in Erg11 have not been reported in clinical isolates solely by themselves (i.e. without additional, potentially compensatory Erg11 substitutions) and without an accompanying substitution in Erg3. It is possible that mutations resulting in these substitutions are a “double-edged sword” for the cell, trading normal Erg11 enzyme function for azole resistance. Such mutations would only persist in the presence of azole stress and possibly also only with accompanying compensatory mutations.

Our ability to generate the *ERG11*^{Y132H&K143R} mutant in the *ERG3*^{A351V} strain but not the *ERG3*^{WT} strain raises interesting questions regarding the role of *ERG3* mutations in azole resistance. C-5 sterol desaturase-mediated azole resistance has so far only been described to occur as a result of amino acid substitutions in Erg3 that cripple or abolish desaturase activity. Abolition of Erg3 function results in an inability to metabolize the toxic sterol precursor, 14 α -methylfecosterol to 14 α -methylergosta-8,24(28)-dien-3 β , 6 α -diol in the presence of azoles, conferring azole resistance. This is most likely the same mechanism of azole resistance occurring in isolate 60, which had a nonsense mutation in *ERG3*, preventing transcription and subsequent translation of the full length C-5 sterol desaturase.

However, our findings leave open the possibility that substitutions in Erg3 may also play a peripheral role in azole resistance, enabling *C. albicans* to increase mutability of *ERG11* and possibly other ergosterol biosynthesis genes with mutations that would

otherwise be unfavorable. If true, mutations in *ERG3* could prove to be a valuable predictor of patient response to azole treatment, as *ERG3* mutations may indicate the cells proclivity to develop additional mutations in the azole-resistance gene *ERG11*. While this remains a possibility, further investigation must be conducted to determine the role of the *ERG3* mutations observed in clinical isolates of *C. albicans*.

Materials and Methods

Strains and growth conditions

The *C. albicans* strains used in this study are listed in **Table 4-3**. Cells were streaked on the YPD agar plates from frozen 40% glycerol stocks and grown overnight at 30° C. Colonies from YPD agar plates were grown overnight in YPD liquid media (1% yeast extract, 2% peptone, 2% dextrose) at 30° C for all routine genomic DNA extraction. Luria-Bertani (LB) liquid media and agar plates, both containing 100 µg/mL ampicillin or 50 µg/mL kanamycin, were used to grow *E. coli* cells used in plasmid construction and propagation.

Plasmid construction

Table 4-4 shows the primers used in this study. To construct plasmids containing our transformation cassettes for gene *ERG3* and *ERG11* gene knockout or replacement, we utilized the pSFS2-derived plasmid pBSS2 containing the *SAT1* nourseothricin resistance marker and FLP recombinase, described previously (202). To generate the *ERG3* deletion cassette, flanking sequences of the 5' and 3' ends of the *ERG3* open-reading frame (ORF) was amplified from SC5314 genomic DNA using primers CaERG3_AF_ApaI and CaERG3_BR_XhoI or CaERG3_CF_SacII and CaERG3_DR_SacI, respectively. Fragments were digested and ligated to plasmid pBSS2 to create plasmid pBSS2-*ERG3*ABCD. The replacement cassette used in putting two copies of the *ERG3*^{A351V} allele back into *C. albicans* was constructed by amplifying the *ERG3* ORF using primers CaERG3_AF_ApaI and CaERG3_ER_XhoI. The resulting fragment and plasmid pBSS2-*ERG3*ABCD were digested with restriction enzymes ApaI and XhoI and ligated together to form pBSS2-*ERG3*AECD.

To construct the mutant *ERG11*^{Y132H,K143R} allele, primer sets CaERG11-1F & CaERG11SOE-3R Y132H and CaERG11SOE-2F Y132H & CaERG11-4R were used to amplify fragments of the *ERG11* gene from isolate 13 containing the *ERG11*^{K143R} allele previously characterized (139). For the *ERG11*^{G307S} allele, primer sets CaERG11-1F & CaERG11SOE-6R and CaERG11SOE-5F & CaERG11-4R amplified *ERG11* gene fragments from isolate 26 genomic DNA. Each respective set fragments was combined and amplified using short-overlapping extension PCR utilizing primers CaERG11-AF (ApaI) and CaERG11-BR (XhoI) to make a full length *ERG11* allele containing either the Y132H&K143R or G307S amino acid substitutions. Fused PCR products were digested

Table 4-4. Primers used in Chapter 4

Primer	Oligonucleotide sequence ^a
Amplification	
CaERG3_F_HindIII	5' – TTGTTAAGCTTTTTTTTCTTCCACCTATTTTG – 3'
CaERG3_R_BAMHI	5' – CACACGGATCCATATAACCCCTTGATTCC – 3'
CaERG11-AF (ApaI)	5' – GGGCCCGGGTTATTTGAGAACAGCC – 3'
CaERG11-BR (XhoI)	5' – CTCGAGCCAGTGGACAAAAACCATCA – 3'
Short, overlapping extension	
CaERG11-1F	5' – CAATCTATACGACGACATTTAACTTTTT – 3'
CaERG11SOE-3R Y132H	5' – GACAATCATGAATAACCCCTTACCGAAAA – 3'
CaERG11SOE-2F Y132H	5' – GGGGTTATTATGATTGTCCAAATTCCAGA – 3'
CaERG11SOE-6R	5' – ACCTTTTTCTTTCAATAATTCAACAAC – 3'
CaERG11SOE-5F	5' – ATTATTGAAAGAAAAAGGTGGTGATTG – 3'
CaERG11-4R	5' – GCTGTTGTTGTTGTTGAAAGAAA – 3'
Sequencing	
CaERG3SeqA	5' – GACCTAAGATTCCTACAATC – 3'
CaERG3SeqB	5' – CAGCTACTGATTTCAATTAATAC – 3'
CaERG3SeqC	5' – GGTTCCTCCAATCTTTACC – 3'
CaERG3SeqD	5' – GGAAAAATAGTCAATGGTCC – 3'
CaERG3SeqE	5' – GTAAACAGATGGCCAGTG – 3'
CaERG3SeqF	5' – TCTGAAAATGTTTGATCTGGC – 3'
CaERG11SeqA	5' – GCCACCACACCCTATGGCTATT – 3'
CaERG11SeqB	5' – TATTTTCACTGCTTCAAGATCT – 3'
CaERG11SeqC	5' – CCAAAAGGTCATTATGTTTTAG – 3'
CaERG11SeqD	5' – CATAACAAGTTTCTCTTTTTTCC – 3'
CaERG11SeqE	5' – CATTTAGGTGAAAAACCTCATT – 3'
CaERG11SeqF	5' – TACTCCAGTTTTTCGGTAAAGGG – 3'
Deletion and Replacement	
CaERG3_AF_ApaI	5' – ATCTGATTTATATATGGGCCCCAAGTGTTTG – 3'
CaERG3_BR_XhoI	5' – AAAAGATAATAGTCTCGAGTTTCTAGTACG – 3'
CaERG3_CF_SacII	5' – ATAGCCGCGGTAACTCTTACAGAAGACC – 3'
CaERG3_DR_SacI	5' – TGTGATGTGAGCTCGTTAGTATTATTTTCA – 3'
CaERG3_ER_XhoI	5' – TGTGATGTACTCGAGTTAGTATTATTTTCA – 3'

^aUnderlined bases indicate a restriction enzyme cutting sequence, red bases indicate a base change from reference sequence for base specific mutagenesis

with restriction enzymes *ApaI* and *XhoI* and ligated into pBSS2, a derivative of pSFS2 containing the *CaSAT1/FLP* cassette in a pBluescript backbone (217). The resulting ligation yielded plasmid pERG11R2 and pERG11R4, containing the *ERG11*^{Y132H,K143R} and *ERG11*^{G307S} allele, respectively.

***ERG3* and *ERG11* amplification and sequencing**

For sequencing of *ERG3* in our *C. albicans* clinical isolate collection, the ORF for *ERG3* and *ERG11* was amplified using primers CaERG3_F_HindIII & CaERG3_R_BAMHI and CaERG11-AF (*ApaI*) & CaERG11-BR (*XhoI*), respectively. The QIAquick® PCR Purification Kit (Qiagen) was used to purify PCR products. Approximately 100 – 150 ng of purified PCR product containing the *ERG3* ORF was used in sample preparation with the *ERG3* sequencing primers CaERG3SeqA, CaERG3SeqB, CaERG3SeqC, CaERG3SeqD, CaERG3SeqE, and CaERG3SeqF for Sanger sequencing by capillary electrophoresis. For *ERG11*, the sequencing primers used were CaERG11SeqA, CaERG11SeqB, CaERG11SeqC, CaERG11SeqD, CaERG11SeqE, and CaERG11SeqF. An ACI 3130XL genetic analyzer was used in processing sequence reads and sequencing was accomplished in duplicate in independently grown isolates.

***C. albicans* strain construction**

A DNA fragment containing the *SAT1/FLP* cassette flanked by sequences at the 5' and 3' end of the *ERG3* gene was excised by *ApaI* and *XhoI* restriction enzymes. The resulting fragment was transformed via electroporation into *C. albicans* isolate SC5314, disrupting a single copy of *ERG3* in successfully transformed cells. Cells were screened on YPD-agar plates containing (200 µg/mL) nourseothricin and successful cells were grown in YPD for 24 hours to recycle the *SAT1/FLP* marker. A repeat transformation and screening was performed to generate the *erg3Δ/Δ* strain SCERG3M4A. For strains containing the *ERG3*^{A351V} mutant allele, the replacement cassette was excised out of plasmid pBSS2-ERG3AECD with *ApaI* and *XhoI* and transformed via electroporation into SCERG3M4A. As above, recycling of the nourseothricin marker, repeat transformation and screening on YPD-agar with nourseothricin ensured both alleles contained the replacement cassette, generating strains SCERG3R1S1A1, SCERG3R1S1A2, SCERG3R1S1E1, and SCERG3R1S1E2. To generate strains possessing both mutant *ERG11* and *ERG3* alleles, we transformed the *ERG11* replacement cassettes from pERG11R2 and pERG11R4 into SCERG3R1S1A or SC5314. Two sequential integrations of the replacement cassette of pERG11R2 generated homozygous replacement of the native *ERG11* alleles with two copies of *ERG11*^{Y132H&K143R}. Southern blotting visually confirmed homozygous allelic deletion and/or replacement of all strains using methods previously described (141). Confirmation of allelic sequences were confirmed by Sanger sequencing of either the *ERG3* or *ERG11* ORF.

Azole susceptibility testing

MICs for fluconazole were determined by broth microdilution assay in accordance with the Clinical Laboratory and Standards Institute (200, 201). To reduced trailing growth observed in isolates, a slight modification through addition of 2% glucose to RPMI media was made. Assay was prepared in 96-well round bottomed plates and incubated for 24 hours at 35° C. Measurements were visually read after 24 hours. MIC was defined as the concentration at which approximately 50% growth inhibition was seen compared to isolate or strain growth in drug-free control wells.

CHAPTER 5. *IN VITRO* ACTIVITIES OF THE NOVEL INVESTIGATIONAL TETRAZOLE VT-1161 AND VT-1598 COMPARED TO THE TRIAZOLE ANTIFUNGALS AGAINST AZOLE-RESISTANCE STRAINS AND CLINICAL ISOLATES OF *CANDIDA ALBICANS*³

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 (16, 69, 185, 211, 218). 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 (12). Moreover, resistance to currently available antifungal agents continues to be a problem, particularly given the relatively limited armamentarium against fungal infections (32, 219-222). In particular, azole antifungal resistance in *Candida* spp. threatens to diminish the efficacy of arguably the most widely used antifungal drug class (223). 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 (83, 84, 104, 196). Second, increased production of the azole target 14 α -lanosterol demethylase (CYP51) can attenuate the inhibitory effects of the azoles drug class (106, 108, 115). 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 (122-124, 139). 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 (72, 142, 145, 224).

VT-1161 and VT-1598 are novel tetrazole antifungal agents with high specificity for fungal CYP51 compared to human CYP enzymes (188, 189, 225), 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

³Reprinted from final submission with permission. Nishimoto AT, Wiederhod NP, Flowers SA, Zhang Q, Kelly SL, Morschhäuser J, Yates CM, Hoekstra WJ, Schotzinger RJ, Garvey EP, Rogers PD. (2019) *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*. *AntiMicrob Agents Chemother.* doi:10.1128/aac.00341-19:AAC.00341-19.

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 $\mu\text{g/mL}$) and possessed multiple known azole resistance mechanisms (**Appendix B, Table A-1 to B-3**) (115). Both VT-1161 and VT-1598 had lower MIC₅₀ values (0.06 and 0.125 $\mu\text{g/mL}$, respectively), and VT-1598 had a lower MIC₉₀ value (0.25 $\mu\text{g/mL}$) when compared to the other tested azole antifungals (**Table 5-1**). VT-1161 and VT-1598 MICs were ≤ 0.015 $\mu\text{g/mL}$ against the 11 fluconazole-susceptible isolates within the collection, and the VT-1598 MICs were 0.03 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$, range 0.06 to >8 $\mu\text{g/mL}$) 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 $\mu\text{g/mL}$, range 0.06 to >8 $\mu\text{g/mL}$) against 49 fluconazole-resistant isolates. Five clinical isolates displayed highly elevated VT-1598 and VT-1161 MICs (range 4 to >8 $\mu\text{g/mL}$) 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 (115). 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

Table 5-1. Geometric mean MICs, MIC₅₀, MIC₉₀, and ranges (µg/mL) for tested compounds against 68 clinical isolates of *C. albicans*

Drug	GM MIC	MIC₅₀	MIC₉₀	Range
VT-1161	0.15	0.125	1	≤0.015 - >8
VT-1598	0.05	0.06	0.25	≤0.015 - >8
Fluconazole	20.2	32	>64	≤0.125 - >64
Voriconazole	0.32	0.5	2	≤0.03 - >16
Itraconazole ¹	0.31	0.25	1	≤0.03 - >16
Posaconazole ¹	0.2	0.5	2	≤0.03 - >16

¹ n = 66 clinical isolates for itraconazole and posaconazole

between the log₂-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 log₂-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 (115). 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 5-1**). 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 5-2**). 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

Figure 5-1. Comparison of the MICs of VT-1161 and VT-1598 against the MICs of triazoles

VT-1161 and VT-1598 MICs versus 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.

aMICs 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 5-2. 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 $\Delta cdr1$ derivatives of SCTAC1GAD1A (SC $\Delta cdr1$ TAC1GAD1A and -B), $\Delta mdr1$ derivatives of SCMRR1GAD1A and -B (SC $\Delta mdr1$ 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 (μ g/mL)

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

B

MICs (μ g/mL)

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

C

MICs (μ g/mL)

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

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 (94).

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 (113, 115). 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) (115). 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 B-2, Figure 5-3**). The Y132F single substitution caused a substantial (16-fold) increase in VT-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

Figure 5-3. 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.

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 (226), and has shown improved survival and reduced fungal burden in murine models of CNS coccidioidomycosis (227) and cryptococcosis (228). 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 (187). 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 (229). 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 MIC₅₀ and MIC₉₀ 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 (139). 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 (139, 140). 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 (140). 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 MIC₅₀ and MIC₉₀ 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 (230). 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 $\Delta 5,6$ -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 (142, 145, 147). The inhibition of the azole target 14 α -lanosterol demethylase causes accumulation of the toxic sterol intermediate, 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol, which is thought to be the source of the fungistatic effect seen with azole antifungal class (71, 72, 224). 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 5-2 lists the constructed strains used in this study. Ten single *ERG11* mutations and four double mutations were selected from previous studies (139). Two additional strains expressing the Y132H and D278N *ERG11* gene mutations were created in a previous study utilizing the *SAT* flipper cassette (141, 202). 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 B-3**). 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 fragments were generated by primers pairs Ca*ERG11*_1F with CaERG11SOE-6R using template genomic DNA from clinical

Table 5-2. Constructed strains used in Chapter 5

Strains ¹	Genotype	Source or reference
SC5314	<i>ERG11-1/ERG11-2</i>	ATCC
Constructed laboratory strains ^a		
20E1I1B1	<i>ERG11^{Y132F}::FRT / ERG11^{Y132F}::FRT</i>	Flowers et al., 2015
SCERG11R1S1C1	<i>ERG11^{Y132H}::FRT / ERG11^{Y132H}::FRT</i>	Warrilow et al., 2019
10B1A3A	<i>ERG11^{K143R}::FRT / ERG11^{K143R}::FRT</i>	Flowers et al., 2015
2B1A51A	<i>ERG11^{F145L}::FRT / ERG11^{F145L}::FRT</i>	Flowers et al., 2015
29NA29A23A	<i>ERG11^{E266D}::FRT / ERG11^{E266D}::FRT</i>	Flowers et al., 2015
SCERG11R3S3C1	<i>ERG11^{D278N}::FRT / ERG11^{D278N}::FRT</i>	Warrilow et al., 2019
21C1M1B1	<i>ERG11^{S405F}::FRT / ERG11^{S405F}::FRT</i>	Flowers et al., 2015
20NA11A57A	<i>ERG11^{G448E}::FRT / ERG11^{G448E}::FRT</i>	Flowers et al., 2015
7A5A5A	<i>ERG11^{F449V}::FRT / ERG11^{F449V}::FRT</i>	Flowers et al., 2015
15A3A108A	<i>ERG11^{G450E}::FRT / ERG11^{G450E}::FRT</i>	Flowers et al., 2015
19A1A1C1	<i>ERG11^{G464S}::FRT / ERG11^{G464S}::FRT</i>	Flowers et al., 2015
22B12A58A	<i>ERG11^{D466E}::FRT / ERG11^{D466E}::FRT</i>	Flowers et al., 2015
9A14A21A	<i>ERG11^{Y132F,K143R}::FRT / ERG11^{Y132F,K143R}::FRT</i>	Flowers et al., 2015
27A5A33A	<i>ERG11^{Y132F,F145L}::FRT / ERG11^{Y132F,F145L}::FRT</i>	Flowers et al., 2015
13A5A57A	<i>ERG11^{D278N,G464S}::FRT / ERG11^{D278N,G464S}::FRT</i>	Flowers et al., 2015
8A4A1A	<i>ERG11^{G450E,I483V}::FRT / ERG11^{G450E,I483V}::FRT</i>	Flowers et al., 2015
SCTAC1GAD1A and -B	<i>ADH1/adh1::P_{ADH1}-TAC1-GAL4AD-3xHA-caSATI</i>	Schillig et al., 2013
SCΔ <i>cdr1</i> /TAC1GAD1A and -B	<i>ADH1/adh1::P_{ADH1}-TAC1-GAL4AD-3xHA-caSATI</i>	Schillig et al., 2013
SCMRR1GAD1B and -B	<i>cdr1Δ::FRT/cdr1Δ::FRT; ADH1/adh1::P_{ADH1}-MRR1-GAL4AD-3xHA-caSATI</i>	Schillig et al., 2013
SCΔ <i>mdr1</i> /MRR1GAD1A and -B	<i>mdr1Δ::FRT/mdr1Δ::FRT; ADH1/adh1::P_{ADH1}-MRR1-GAL4AD-3xHA-caSATI</i>	This study
SCUPC2GAD1A and -B	<i>ADH1/adh1::P_{ADH1}-UPC2-GAL4AD-3xHA-caSATI</i>	Schillig et al., 2013
SCUPC2R14A and -B	<i>UPC2^{G648D}::FRT / UPC2^{G648D}::FRT</i>	Heilmann et al., 2010

¹All laboratory strains have SC5314 as background.

isolate 43 and CaERG11SOE-5F with CaERG11_4R using SC5314 genomic DNA. The resulting fragments were again fused into a single fragment containing either the D278N-containing mutant *ERG11* open reading frame (ORF) using nested primers CaERG11-AF_ (ApaI) and CaERG11-BR_ (XhoI). In constructing the plasmids used in the transformation of both strains, the *ERG11* 3' flanking sequence was amplified from SC5314 genomic DNA and primers CaERG11_C-F' and CaERG11_D-R'. The inserts 3' of the *ERG11* ORF were digested with restriction enzymes NotI and SacII, and cloned into the pSFS2-derived plasmid pBSS2 previously described by Vasicek et al. containing the *SAT1* flipper cassette from Reuß et al. (202, 217) to create plasmid pERG11CD. The *ERG11* ORF-containing fragments with either the Y132H or D278N mutations were digested using restriction enzymes ApaI and XhoI, gel excised, and cloned into the plasmid pERG11CD to generate plasmids pERG11A1 and pERG11A3. Plasmids were digested with restriction enzymes ApaI and SacII and used to transform strain SC5314 by electroporation to generate heterozygous *ERG11* mutants. Recycling of the nourseothricin selection marker through 24 hours of growth in YPD and repeat transformation of the resultant strains generated the homozygous *ERG11* allele replacements SCERG11R1S1C1 and SCERG11R3S3C1, confirmed via Southern hybridization and Sanger sequencing. The artificially-activated Tac1, Mrr1, and Upc2 mutants used in this study as well as SC Δ *cdr1*TAC1GAD1A and -B, containing the artificially activated *TAC1* allele in a *cdr1* Δ background, were described in a previous study (117). Strains SC Δ *mdr1*MRR1GAD1A and -B were constructed by introducing the artificially activated *MRR1* allele from plasmid pMRR1-GAD1 (32) into the *mdr1* Δ mutants SCMDR1M4A and -B (112), respectively.

***ERG11* amplification and sequencing**

Table B-3 lists the primers used for *ERG11* amplification and sequence verification. The *ERG11* ORF of each isolate was PCR amplified from genomic DNA using primers Ca*ERG11*_F_Amp and Ca*ERG11*_R_Amp. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and product was sequenced on an ABI 3130XL genetic analyzer using sequencing primers. Sequencing was accomplished in duplicate in independently grown isolates.

Relative gene expression by real-time PCR

Expression levels of the genes *CDR1*, *MDR1*, and *ERG11* in clinical *C. albicans* isolates were measured in a previous study, and *CDR1*, *MDR1*, and *ERG11* expression of laboratory strains were measured similarly using previously described methods (115). Briefly, first-strand cDNA was generated from 1 µg of extracted RNA for each strain using the SuperScript® VILO™ Master Mix (Invitrogen) reaction kit. Quantitative PCRs were performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in technical and biological triplicate for each sample. SYBR green PCR master mix (Applied Biosystems) was used for amplification detection of candidate genes against the *CaACT1* normalizing gene. Calculation of the relative quantitation values of

CDR1, *ERG11*, and *MDR1* gene expression was accomplished using the StepOne Software v2.3 (Applied Biosystems). Primers used in the amplification of genes measured via qPCR are listed in **Table B-3**.

Susceptibility testing

Minimum inhibitory concentrations (MIC) of VT-1161, VT-1598, fluconazole, voriconazole, posaconazole, and itraconazole were measured using broth microdilution methods in accordance with the Clinical Laboratory and Standards Institute (200, 201). 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 B-1** and **B-2**), though 98% (592/606) of MIC duplicate readings were identical or within a single dilution of each other. The geometric mean MIC (GM MIC), MIC₅₀, and MIC₉₀ was reported for clinical *C. albicans* isolates for each triazole and tetrazole agent used in this study. The MIC₅₀ and MIC₉₀ 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.

Transparency Declaration

NPW has received research support to the UT Health San Antonio from Astellas, bioMerieux, Cidara, F2G, Merck, and Viamet, and has served on advisory boards for Merck, Astellas, Toyama, and Viamet. CMY, RJS, and EPG are employees of Viamet Pharmaceuticals, Inc. All other authors have no conflicts of interest.

CHAPTER 6. CONCLUSIONS

Introduction

Candida albicans is the leading etiologic cause of *Candida*-related infection. While azole-resistance rates in *C. albicans* remains relatively low, treatment failures still occur resulting from the emergence of highly azole-resistant *C. albicans* isolates. For the foreseeable future, fluconazole and other azole antifungals will continue to be among the most commonly used and prescribed antifungal medications. However, the increased use of this drug class in combination with imperfect clinical practice with antifungal drug dosing will increase risk of azole-resistance development. As has been discussed, *C. albicans* has several known mechanisms enabling it to survive in the presence of azoles, and commonly it is the combination of these resistance mechanisms that arise in clinical isolates that permit them to thrive, even with appropriate treatment. The main objective of my dissertation has been to investigate additional possible mechanisms of azole resistance and resistance-adaptation in *C. albicans* from mutations derived from a collection of predominantly azole-resistant clinical isolates. Amino acids substitutions in the zinc cluster transcription factor Mrr2, and the sterol biosynthetic proteins Erg11 and Erg3 were the primary focus of this investigation. Furthermore, the novel tetrazole compounds VT-1161 and VT-1598 proved to be valuable agents to screen for unknown azole resistance mechanisms present within this collection. Here, I discuss and interpret the results of each aim of my investigation.

Summary and Conclusions of Chapter 2

In Chapter 2, I sequenced the *MRR2* gene in 57 *C. albicans* clinical isolates with elevated fluconazole MICs, and identified 15 amino acid substitutions in resistant isolates. Four of these substitutions (S466L, A468G, S469T, T470N) had been previously reported to reduce fluconazole susceptibility through increased expression of the efflux pump encoding gene *CDR1* (118). Interestingly, these four amino acid substitutions were present in clinical isolates that did not possess significantly increased expression of *CDR1* relative to a composite of susceptible isolates. This initially brought into question the validity of the claim that these amino acid substitutions impacted *CDR1* expression and required further investigation.

I tested the effects of these combined substitutions in Mrr2 as well as the novel substitutions R45Q, A459T, V486M, and V582L by placing the alleles of clinical isolates containing the *MRR2* mutations into the native *MRR2* locus of the fluconazole-susceptible parent SC5314. Broth microdilution assay and qPCR did not reveal any increases in either fluconazole resistance or *CDR1* expression among any of my created strains. Previous work looking at either artificially hyperactive Mrr2 and clinically-derived mutant Mrr2 increased *CDR1* expression and fluconazole resistance (117, 118). However, in both cases, the created strains artificially overexpressed the *MRR2* gene to observe these effects. I therefore constructed *C. albicans* strains overexpressing either

wild type *MRR2* or a replica of the *MRR2* gene containing mutations previously reported to show the strongest increase in fluconazole MIC. Even with strong expression of *MRR2*, no change in *CDR1* expression or fluconazole MIC compared to SC5314 was observed.

Lastly, by creating an artificially activated *MRR2* strain with native promoter expression. I showed two things. Firstly, artificial activation of *MRR2* does not result in auto-upregulation of the *MRR2* gene. The ZCF genes *UPC2* and *TAC1* both have been shown to be target genes of their respective proteins, and their expression is upregulated through gain-of-function activity (93, 231). Our data suggests that activated Mrr2 does not do this, though further investigation would be required. Secondly, native expression levels of artificially activated *MRR2* is sufficient to confer increased *CDR1* expression and fluconazole MIC. This suggests that any activating effects from *MRR2* mutations should be observable in our created strains expressing mutant *MRR2* from the native promoter. The lack of any change in MIC in these strains coupled with the unchanged expression of *CDR1* suggests that these mutations are not tentative gain-of-function mutations in *MRR2*, and more importantly, that they are not clinically relevant for fluconazole resistance.

Though this is ultimately a negative finding, I strongly emphasize the importance of these results. A far-reaching goal of my research is to impact and improve patient outcomes in those with fungal infections treated with the azoles. To this end, discovery and improvement in the established knowledge of fungal pathogens and resistance within these organisms is critical. Publishing contrasting findings to previously established results is important in shaping and correcting the constantly evolving body of scientific knowledge, from which new discoveries and advancements in treatments and therapy is derived. Currently, there are published reported referencing mutations in *MRR2* as a relevant mechanism of azole resistance (160, 197, 198). My findings run strongly to the contrary of these published findings and are therefore important to disseminate in the interest of my research goals.

Summary and Conclusions of Chapter 3

My own research in Chapter 3 was limited to the creation of the additional Erg11 mutant strains containing the D278N and Y132H amino acid substitutions, and the analysis of the susceptibility testing data of these new additions to our collection of Erg11 mutant strains. Since *ERG11* mutations play a large role in azole resistance, the continued investigation characterizing additional mutations and their effects and interactions in the *C. albicans* cell remains critically important. My work was contributory to a collaborative research aim to investigate these effects.

The results of the investigation showed that Erg11 mutants vary considerably in their baseline activities as well as their activity in the presence of the azoles (141). Furthermore, the increased ability to retain activity around the azoles did not, however, always correlate with decreased binding to the azole. Interestingly, these biochemical

effects did not always translate into what was seen in *C. albicans in vitro* susceptibility testing of the *ERG11* mutations. This suggests that some *ERG11* mutations may work to alter interaction with other components, such as NADPH-cytochrome P450 reductase, that work with CaCYP51 in sterol biosynthesis. Overall, my work in combination with the biochemical data on these Erg11 mutants shed additional light on the mode of action by which Erg11 amino acid substitutions interfere with growth inhibition by the azoles. It also identifies the possibility that these substitutions may impact normal cell sterol biosynthesis production due to the altered catalytic rates exhibited by the Erg11 mutant proteins. This notion was important in the justification of my investigation of another sterol biosynthesis gene, *ERG3*, which is detailed next.

Summary and Conclusions of Chapter 4

In chapter 4, I sequenced the *ERG3* gene of our research group's collection of clinical *C. albicans* isolates in an initial effort to define additional azole resistance mechanisms present in the collection. We had previously reported the majority of the major resistance mechanisms present among these isolates (115). However, sterol profiles on this collection identified isolates with lower ergosterol content. *ERG3* mutants resulting in defective C-5 sterol desaturase have been reported as having little to no ergosterol content and increased amounts of ergosta-7,22-dienol instead (224). One such clinical isolates matched this "textbook" *ERG3* mutant phenotype, and subsequent sequencing of the open-reading frame confirmed a premature stop codon at the normal Trp-131 residue. Interestingly, my sequence work identified multiple *ERG3* mutations present in these clinical isolates. Investigating whether these mutations may have any role in azole resistance, I noticed the high co-occurrence of the A351V *ERG3* mutation with *ERG11* mutations.

Given our suspicion that *ERG11* mutations may come at a fitness cost and may require possible compensatory mutations in order to adapt, I proceeded to make *ERG3* and *ERG11* combination mutants to test whether *ERG3* had an indirect role in azole resistance. Unfortunately, growth competition and fitness testing between *ERG11* mutants with and without mutant *ERG3* could not be completed since I was unsuccessful in creating the G307S and Y132H, K143R Erg11 mutants. Based on data from our collaborators, these two sets of amino acid substitutions conferred the poorest catalytic turnover rates for the Erg11 enzyme, and would be expected to show the greatest fitness defects. I was only successful in creating the *ERG11*^{Y132H, K143R} mutant in the presence of the *ERG3*^{A351V} mutation. While not conclusive, it is preliminary evidence that perhaps the *ERG3*^{A351V} mutation may confer a compensatory fitness benefit on *C. albicans* isolates that accrue multiple *ERG11* mutations.

While further work needs to be done to investigate any possible beneficial effects for the cell, the possibility of mutations in *ERG3* conferring a compensatory fitness benefit raises interesting questions about azole resistance in clinical isolates. If certain *ERG3* mutations are acquired early on by the cell, then the mutagenic potential of the *ERG11* gene, and therefore of azole resistance development, could increase as well. The

identification of such an evolution of azole resistance could make *ERG3* mutations an intriguing diagnostic marker in the prediction of azole treatment failures through acquired resistance.

Summary and Conclusions of Chapter 5

Lastly, my work in Chapter 5 involved the analysis of the susceptibilities of our *C. albicans* clinical isolates and resistant laboratory strains to the established azole antifungals used in invasive fungal infection alongside the new tetrazole antifungal agents VT-1161 and VT-1598. Both newer agents showed potent activity against predominantly azole-resistant strains and isolates. In particular, VT-1598 displayed activity against the vast majority of clinical isolates as well as strains overexpressing *CDR1*, *MDR1*, or *ERG11* through gain-of-function ZCF mutations. In comparison, the currently available antifungal agent posaconazole displayed similar activity to VT-1598, while voriconazole and itraconazole were more comparable VT-1161's activity against clinical isolates. Both tetrazoles also appeared to be unaffected by several, but not all, *ERG11* mutations, similar to the triazole posaconazole. Overall, the tetrazole compounds appear to be promising agents that could be utilized in the future as alternative therapies in invasive candidiasis.

An important finding in the analysis of this susceptibility data is the identification of isolates 55, 56, 58, 60, and 65 that all showed greatly increased MICs to the tetrazoles and all other azoles. Apart from isolate 60, which possesses an early stop codon in *ERG3*, the other isolates do not possess any unique traits with respect to known azole resistance mechanisms that sets them apart from other clinical isolates. This indicates that these isolates possess as yet undiscovered mechanisms of azole resistance and provides an interesting future project to investigate these isolates further.

A Final Word

The findings of my research projects leave several questions that remain to be answered in *C. albicans*. Whether mutations in additional ZCFs may play a role in azole resistance is currently an ongoing investigation. The role of compensatory mutations in *ERG3* and other sterol biosynthesis genes is a plausible but as yet unproven mechanism that could influence azole resistance development. Do mutations in *ERG11* also alter CaCYP51 interaction with peripheral components outside of the azole antifungals that may influence susceptibility? And what are the means by which some clinical isolates possess such strongly reduced susceptibility to the azole antifungals, if not by any of the currently known mechanisms? One can speculate that some of these questions may ultimately prove to be important in changing the clinical approach to dealing with azole-resistant *Candida*. For the immediate future, though, we must remember to put one foot in front of the other – assessing the need for further investigation into these ever-shifting questions based on the research we gather: one gel, one blot, one cell at a time.

LIST OF REFERENCES

1. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, Lewis JD, Bushman FD. 2013. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PLoS One* 8:e66019.
2. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, Gillevet PM. 2010. Characterization of the Oral Fungal Microbiome (Mycobiome) in Healthy Individuals. *Plos Pathogens* 6.
3. Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA, Schoenfeld D, Nomicos E, Park M, Program NIHISCCS, Kong HH, Segre JA. 2013. Topographic diversity of fungal and bacterial communities in human skin. *Nature* 498:367-70.
4. Merenstein D, Hu H, Wang C, Hamilton P, Blackmon M, Chen H, Calderone R, Li D. 2013. Colonization by *Candida* species of the oral and vaginal mucosa in HIV-infected and noninfected women. *AIDS Res Hum Retroviruses* 29:30-4.
5. Arendrup MC, Patterson TF. 2017. Multidrug-Resistant *Candida*: Epidemiology, Molecular Mechanisms, and Treatment. *J Infect Dis* 216:S445-S451.
6. Muskett H, Shahin J, Eyres G, Harvey S, Rowan K, Harrison D. 2011. Risk factors for invasive fungal disease in critically ill adult patients: a systematic review. *Crit Care* 15:R287.
7. Diekema D, Arbefeville S, Boyken L, Kroeger J, Pfaller M. 2012. The changing epidemiology of healthcare-associated candidemia over three decades. *Diagn Microbiol Infect Dis* 73:45-8.
8. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. 1999. Nosocomial bloodstream infections in United States hospitals: A three-year analysis. *Clinical Infectious Diseases* 29:239-244.
9. Kullberg BJ, Oude Lashof AM. 2002. Epidemiology of opportunistic invasive mycoses. *Eur J Med Res* 7:183-91.
10. Bongomin F, Gago S, Oladele RO, Denning DW. 2017. Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. *J Fungi (Basel)* 3.
11. Lamoth F, Lockhart SR, Berkow EL, Calandra T. 2018. Changes in the epidemiological landscape of invasive candidiasis. *J Antimicrob Chemother* 73:i4-i13.

12. 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.
13. Kett DH, Azoulay E, Echeverria PM, Vincent JL, Extended Prevalence of Infection in ICUSGoI. 2011. Candida bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. *Crit Care Med* 39:665-70.
14. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. 2016. Azole Antifungal Resistance in *Candida albicans* and Emerging Non-*albicans* *Candida* Species. *Front Microbiol* 7:2173.
15. Castanheira M, Messer SA, Rhomberg PR, Pfaller MA. 2016. Antifungal susceptibility patterns of a global collection of fungal isolates: results of the SENTRY Antifungal Surveillance Program (2013). *Diagn Microbiol Infect Dis* 85:200-4.
16. 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.
17. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. 2018. Invasive candidiasis. *Nat Rev Dis Primers* 4:18026.
18. Tan BH, Chakrabarti A, Li RY, Patel AK, Watcharananan SP, Liu Z, Chindamporn A, Tan AL, Sun PL, Wu UI, Chen YC, Asia Fungal Working G. 2015. Incidence and species distribution of candidaemia in Asia: a laboratory-based surveillance study. *Clin Microbiol Infect* 21:946-53.
19. Cheng YR, Lin LC, Young TG, Liu CE, Chen CH, Tsay RW. 2006. Risk factors for candidemia-related mortality at a medical center in central Taiwan. *J Microbiol Immunol Infect* 39:155-61.
20. Colombo AL, Nucci M, Park BJ, Nouer SA, Arthington-Skaggs B, da Matta DA, Warnock D, Morgan J, Brazilian Network Candidemia S. 2006. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J Clin Microbiol* 44:2816-23.
21. Doi AM, Pignatari AC, Edmond MB, Marra AR, Camargo LF, Siqueira RA, da Mota VP, Colombo AL. 2016. Epidemiology and Microbiologic Characterization of Nosocomial Candidemia from a Brazilian National Surveillance Program. *PLoS One* 11:e0146909.

22. Chowdhary A, Becker K, Fegeler W, Gugnani HC, Kapoor L, Randhawa VS, Mehta G. 2003. An outbreak of candidemia due to *Candida tropicalis* in a neonatal intensive care unit. *Mycoses* 46:287-92.
23. Farooqi JQ, Jabeen K, Saeed N, Iqbal N, Malik B, Lockhart SR, Zafar A, Brandt ME, Hasan R. 2013. Invasive candidiasis in Pakistan: clinical characteristics, species distribution and antifungal susceptibility. *J Med Microbiol* 62:259-68.
24. Hachem R, Hanna H, Kontoyiannis D, Jiang Y, Raad I. 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. *Cancer* 112:2493-9.
25. Sipsas NV, Lewis RE, Tarrand J, Hachem R, Rolston KV, Raad, II, Kontoyiannis DP. 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001-2007): stable incidence but changing epidemiology of a still frequently lethal infection. *Cancer* 115:4745-52.
26. Chowdhary A, Sharma C, Meis JF. 2017. *Candida auris*: A rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PLoS Pathog* 13:e1006290.
27. Nobile CJ, Johnson AD. 2015. *Candida albicans* Biofilms and Human Disease. *Annu Rev Microbiol* 69:71-92.
28. Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 20:133-63.
29. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis* 62:e1-50.
30. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, Rodloff A, Fu W, Ling TA, Global Antifungal Surveillance G. 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J Clin Microbiol* 48:1366-77.
31. Lyon GM, Karatela S, Sunay S, Adiri Y, Candida Surveillance Study I. 2010. Antifungal susceptibility testing of *Candida* isolates from the *Candida* surveillance study. *J Clin Microbiol* 48:1270-5.
32. 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.
33. Pfaller MA, Espinel-Ingroff A, Canton E, Castanheira M, Cuenca-Estrella M, Diekema DJ, Fothergill A, Fuller J, Ghannoum M, Jones RN, Lockhart SR, Martin-Mazuelos E, Melhem MS, Ostrosky-Zeichner L, Pappas P, Pelaez T, Peman J, Rex J, Szeszs MW. 2012. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B, flucytosine, and itraconazole and *Candida* spp. as determined by CLSI broth microdilution. *J Clin Microbiol* 50:2040-6.
 34. Lockhart SR, Iqbal N, Cleveland AA, Farley MM, Harrison LH, Bolden CB, Baughman W, Stein B, Hollick R, Park BJ, Chiller T. 2012. Species identification and antifungal susceptibility testing of *Candida* bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. *J Clin Microbiol* 50:3435-42.
 35. Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Wernecke J, Hofs S, Gratacap RL, Robbins J, Runglall M, Murciano C, Blagojevic M, Thavaraj S, Forster TM, Hebecker B, Kasper L, Vizcay G, Iancu SI, Kichik N, Hader A, Kurzai O, Luo T, Kruger T, Kniemeyer O, Cota E, Bader O, Wheeler RT, Gutschmann T, Hube B, Naglik JR. 2016. Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature* 532:64-8.
 36. Kasper L, Konig A, Koenig PA, Gresnigt MS, Westman J, Drummond RA, Lionakis MS, Gross O, Ruland J, Naglik JR, Hube B. 2018. The fungal peptide toxin Candidalysin activates the NLRP3 inflammasome and causes cytolysis in mononuclear phagocytes. *Nat Commun* 9:4260.
 37. Moyes DL, Richardson JP, Naglik JR. 2015. *Candida albicans*-epithelial interactions and pathogenicity mechanisms: scratching the surface. *Virulence* 6:338-46.
 38. Taschdjian CL, Burchall JJ, Kozinn PJ. 1960. Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. *AMA J Dis Child* 99:212-5.
 39. Simonetti N, Strippoli V, Cassone A. 1974. Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in *Candida albicans*. *Nature* 250:344-6.
 40. Buffo J, Herman MA, Soll DR. 1984. A characterization of pH-regulated dimorphism in *Candida albicans*. *Mycopathologia* 85:21-30.
 41. Berman J, Sudbery PE. 2002. *Candida Albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genet* 3:918-30.

42. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* 183:5385-94.
43. Fox EP, Nobile CJ. 2012. A sticky situation: untangling the transcriptional network controlling biofilm development in *Candida albicans*. *Transcription* 3:315-22.
44. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. 2009. Our current understanding of fungal biofilms. *Crit Rev Microbiol* 35:340-55.
45. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. 2005. *Candida* biofilms: an update. *Eukaryot Cell* 4:633-8.
46. Lan CY, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, Agabian N. 2002. Metabolic specialization associated with phenotypic switching in *Candida albicans*. *Proc Natl Acad Sci U S A* 99:14907-12.
47. Moreno-Ruiz E, Galan-Diez M, Zhu W, Fernandez-Ruiz E, d'Enfert C, Filler SG, Cossart P, Veiga E. 2009. *Candida albicans* internalization by host cells is mediated by a clathrin-dependent mechanism. *Cell Microbiol* 11:1179-89.
48. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. 2011. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol* 10:112-22.
49. Phan QT, Myers CL, Fu Y, Sheppard DC, Yeaman MR, Welch WH, Ibrahim AS, Edwards JE, Jr., Filler SG. 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* 5:e64.
50. Zhu W, Phan QT, Boontheung P, Solis NV, Loo JA, Filler SG. 2012. EGFR and HER2 receptor kinase signaling mediate epithelial cell invasion by *Candida albicans* during oropharyngeal infection. *Proc Natl Acad Sci U S A* 109:14194-9.
51. Holt RJ, Azmi A. 1978. Miconazole-resistant *Candida*. *Lancet* 1:50-1.
52. Holt RJ. 1979. Clinical isolates of *Candida albicans*. *Infection* 7:154-5.
53. Horsburgh CR, Jr., Kirkpatrick CH. 1983. Long-term therapy of chronic mucocutaneous candidiasis with ketoconazole: experience with twenty-one patients. *Am J Med* 74:23-9.
54. Warnock DW, Johnson EM, Richardson MD, Vickers CF. 1983. Modified response to ketoconazole of *Candida albicans* from a treatment failure. *Lancet* 1:642-3.

55. Smith KJ, Warnock DW, Kennedy CT, Johnson EM, Hopwood V, Van Cutsem J, Vanden Bossche H. 1986. Azole resistance in *Candida albicans*. *J Med Vet Mycol* 24:133-44.
56. Lucatorto FM, Franker C, Hardy WD, Chafey S. 1991. Treatment of refractory oral candidiasis with fluconazole. A case report. *Oral Surg Oral Med Oral Pathol* 71:42-4.
57. White A, Goetz MB. 1994. Azole-resistant *Candida albicans*: report of two cases of resistance to fluconazole and review. *Clin Infect Dis* 19:687-92.
58. Evans TG, Mayer J, Cohen S, Classen D, Carroll K. 1991. Fluconazole failure in the treatment of invasive mycoses. *J Infect Dis* 164:1232-5.
59. Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, Lockhart SR, Magill SS, Derado G, Park BJ, Chiller TM. 2012. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008-2011. *Clin Infect Dis* 55:1352-61.
60. Arendrup MC, Fuursted K, Gahrn-Hansen B, Jensen IM, Knudsen JD, Lundgren B, Schonheyder HC, Tvede M. 2005. Seminal surveillance of fungemia in Denmark: notably high rates of fungemia and numbers of isolates with reduced azole susceptibility. *J Clin Microbiol* 43:4434-40.
61. Arendrup MC, Bruun B, Christensen JJ, Fuursted K, Johansen HK, Kjaeldgaard P, Knudsen JD, Kristensen L, Moller J, Nielsen L, Rosenvinge FS, Roder B, Schonheyder HC, Thomsen MK, Truberg K. 2011. National surveillance of fungemia in Denmark (2004 to 2009). *J Clin Microbiol* 49:325-34.
62. Puig-Asensio M, Padilla B, Garnacho-Montero J, Zaragoza O, Aguado JM, Zaragoza R, Montejo M, Munoz P, Ruiz-Camps I, Cuenca-Estrella M, Almirante B, Project C, Geih G, Reipi. 2014. Epidemiology and predictive factors for early and late mortality in *Candida* bloodstream infections: a population-based surveillance in Spain. *Clin Microbiol Infect* 20:O245-54.
63. Trouve C, Blot S, Hayette MP, Jonckheere S, Patteet S, Rodriguez-Villalobos H, Symoens F, Van Wijngaerden E, Lagrou K. 2017. Epidemiology and reporting of candidaemia in Belgium: a multi-centre study. *Eur J Clin Microbiol Infect Dis* 36:649-655.
64. Hesstvedt L, Gaustad P, Andersen CT, Haarr E, Hannula R, Haukland HH, Hermansen NO, Larssen KW, Mylvaganam H, Ranheim TE, Sandven P, Nordoy I, Norwegian Yeast Study G, Kanestrom A, Grub C, Onken A, Thielsen C, Skaare D, Tofteland S, Sonsteby LJ, Hjetland R, Hide R, Vik E, Kummel A, Asheim S.

2015. Twenty-two years of candidaemia surveillance: results from a Norwegian national study. *Clin Microbiol Infect* 21:938-45.
65. Chapman B, Slavin M, Marriott D, Halliday C, Kidd S, Arthur I, Bak N, Heath CH, Kennedy K, Morrissey CO, Sorrell TC, van Hal S, Keighley C, Goeman E, Underwood N, Hajkiewicz K, Hofmeyr A, Leung M, Macesic N, Botes J, Blyth C, Cooley L, George CR, Kalukottege P, Kesson A, McMullan B, Baird R, Robson J, Korman TM, Pendle S, Weeks K, Liu E, Cheong E, Chen S, Australian, New Zealand Mycoses Interest G. 2017. Changing epidemiology of candidaemia in Australia. *J Antimicrob Chemother* 72:1103-1108.
 66. Govender NP, Patel J, Magobo RE, Naicker S, Wadula J, Whitelaw A, Coovadia Y, Kularatne R, Govind C, Lockhart SR, Zietsman IL, group TR-SA. 2016. Emergence of azole-resistant *Candida parapsilosis* causing bloodstream infection: results from laboratory-based sentinel surveillance in South Africa. *J Antimicrob Chemother* 71:1994-2004.
 67. Chakrabarti A, Sood P, Rudramurthy SM, Chen S, Kaur H, Capoor M, Chhina D, Rao R, Eshwara VK, Xess I, Kindo AJ, Umabala P, Savio J, Patel A, Ray U, Mohan S, Iyer R, Chander J, Arora A, Sardana R, Roy I, Appalaraju B, Sharma A, Shetty A, Khanna N, Marak R, Biswas S, Das S, Harish BN, Joshi S, Mendiratta D. 2015. Incidence, characteristics and outcome of ICU-acquired candidemia in India. *Intensive Care Med* 41:285-95.
 68. Benedict K, Jackson BR, Chiller T, Beer KD. 2018. Estimation of direct healthcare costs of fungal diseases in the United States. *Clin Infect Dis* doi:10.1093/cid/ciy776.
 69. Askinyte D, Matulionyte R, Rimkevicius A. 2015. Oral manifestations of HIV disease: A review. *Stomatologija* 17:21-8.
 70. Redding SW, Zellars RC, Kirkpatrick WR, McAtee RK, Caceres MA, Fothergill AW, Lopez-Ribot JL, Bailey CW, Rinaldi MG, Patterson TF. 1999. Epidemiology of oropharyngeal *Candida* colonization and infection in patients receiving radiation for head and neck cancer. *J Clin Microbiol* 37:3896-900.
 71. 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.
 72. 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.

73. Rybak JM, Marx KR, Nishimoto AT, Rogers PD. 2015. Isavuconazole: Pharmacology, Pharmacodynamics, and Current Clinical Experience with a New Triazole Antifungal Agent. *Pharmacotherapy* 35:1037-51.
74. Shirley M, Scott LJ. 2016. Isavuconazole: A Review in Invasive Aspergillosis and Mucormycosis. *Drugs* 76:1647-1657.
75. Wiederhold NP, Xu X, Wang A, Najvar LK, Garvey EP, Ottinger EA, Alimardanov A, Cradock J, Behnke M, Hoekstra WJ, Brand SR, Schotzinger RJ, Jaramillo R, Olivo M, Kirkpatrick WR, Patterson TF. 2018. In Vivo Efficacy of VT-1129 against Experimental Cryptococcal Meningitis with the Use of a Loading Dose-Maintenance Dose Administration Strategy. *Antimicrob Agents Chemother* 62.
76. Nishimoto AT, Wiederhold NP, Flowers SA, Zhang Q, Kelly SL, Morschhäuser J, Yates CM, Hoekstra WJ, Schotzinger RJ, Garvey EP, Rogers PD. 2019. *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*. *Antimicrobial Agents and Chemotherapy* doi:10.1128/aac.00341-19:AAC.00341-19.
77. Prasad R, De Wergifosse P, Goffeau A, Balzi E. 1995. Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. *Curr Genet* 27:320-9.
78. 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 (Pt 2):405-16.
79. Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J. 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* 39:2378-86.
80. 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* 41:1482-7.
81. Niimi M, Niimi K, Takano Y, Holmes AR, Fischer FJ, Uehara Y, Cannon RD. 2004. Regulated overexpression of CDR1 in *Candida albicans* confers multidrug resistance. *J Antimicrob Chemother* 54:999-1006.
82. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. 2004. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters CDR1 and CDR2. *Eukaryot Cell* 3:1639-52.

83. 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.
84. 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.
85. Manoharlal R, Gorantala J, Sharma M, Sanglard D, Prasad R. 2010. PAP1 [poly(A) polymerase 1] homozygosity and hyperadenylation are major determinants of increased mRNA stability of CDR1 in azole-resistant clinical isolates of *Candida albicans*. *Microbiology* 156:313-26.
86. Siikala E, Rautemaa R, Richardson M, Saxen H, Bowyer P, Sanglard D. 2010. Persistent *Candida albicans* colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. *J Antimicrob Chemother* 65:2505-13.
87. Sasse C, Schillig R, Dierolf F, Weyler M, Schneider S, Mogavero S, Rogers PD, Morschhauser J. 2011. The transcription factor Ndt80 does not contribute to Mrr1-, Tac1-, and Upc2-mediated fluconazole resistance in *Candida albicans*. *PLoS One* 6:e25623.
88. Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, d'Enfert C, Berman J, Sanglard D. 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot Cell* 6:1889-904.
89. de Micheli M, Bille J, Schueller C, Sanglard D. 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. *Mol Microbiol* 43:1197-214.
90. Hernaez ML, Gil C, Pla J, Nombela C. 1998. Induced expression of the *Candida albicans* multidrug resistance gene CDR1 in response to fluconazole and other antifungals. *Yeast* 14:517-26.
91. Liu Z, Myers LC. 2017. Mediator Tail Module Is Required for Tac1-Activated CDR1 Expression and Azole Resistance in *Candida albicans*. *Antimicrob Agents Chemother* 61.
92. Prasad R, Banerjee A, Khandelwal NK, Dhamgaye S. 2015. The ABCs of *Candida albicans* Multidrug Transporter Cdr1. *Eukaryot Cell* 14:1154-64.

93. Liu TT, Znaidi S, Barker KS, Xu L, Homayouni R, Saidane S, Morschhauser J, Nantel A, Raymond M, Rogers PD. 2007. Genome-wide expression and location analyses of the *Candida albicans* Tac1p regulon. *Eukaryot Cell* 6:2122-38.
94. 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.
95. Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM. 2004. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14 α -sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob Agents Chemother* 48:568-74.
96. Cheng S, Clancy CJ, Nguyen KT, Clapp W, Nguyen MH. 2007. A *Candida albicans* petite mutant strain with uncoupled oxidative phosphorylation overexpresses MDR1 and has diminished susceptibility to fluconazole and voriconazole. *Antimicrob Agents Chemother* 51:1855-8.
97. Fling ME, Kopf J, Tamarkin A, Gorman JA, Smith HA, Koltin Y. 1991. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol Gen Genet* 227:318-29.
98. Ben-Yaacov R, Knoller S, Caldwell GA, Becker JM, Koltin Y. 1994. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob Agents Chemother* 38:648-52.
99. Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhauser J. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob Agents Chemother* 42:3065-72.
100. Lopez-Ribot JL, McAtee RK, Lee LN, Kirkpatrick WR, White TC, Sanglard D, Patterson TF. 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob Agents Chemother* 42:2932-7.
101. Wirsching S, Michel S, Morschhauser J. 2000. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical *Candida albicans* isolates. *Mol Microbiol* 36:856-65.
102. Wirsching S, Michel S, Kohler G, Morschhauser J. 2000. Activation of the multiple drug resistance gene MDR1 in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a trans-regulatory factor. *J Bacteriol* 182:400-4.

103. Harry JB, Oliver BG, Song JL, Silver PM, Little JT, Choiniere J, White TC. 2005. Drug-induced regulation of the MDR1 promoter in *Candida albicans*. *Antimicrob Agents Chemother* 49:2785-92.
104. 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.
105. Dunkel N, Blass J, Rogers PD, Morschhauser J. 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. *Mol Microbiol* 69:827-40.
106. 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.
107. 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* 45:2676-84.
108. 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.
109. Oliver BG, Song JL, Choiniere JH, White TC. 2007. cis-Acting elements within the *Candida albicans* ERG11 promoter mediate the azole response through transcription factor Upc2p. *Eukaryot Cell* 6:2231-9.
110. 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* 7:1180-90.
111. Znaidi S, Weber S, Al-Abdin OZ, Bomme P, Saidane S, Drouin S, Lemieux S, De Deken X, Robert F, Raymond M. 2008. Genomewide location analysis of *Candida albicans* Upc2p, a regulator of sterol metabolism and azole drug resistance. *Eukaryot Cell* 7:836-47.
112. 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.

113. 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.
114. Hoot SJ, Smith AR, Brown RP, White TC. 2011. An A643V amino acid substitution in Upc2p contributes to azole resistance in well-characterized clinical isolates of *Candida albicans*. *Antimicrob Agents Chemother* 55:940-2.
115. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gyax 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.
116. Vasicek EM, Berkow EL, Flowers SA, Barker KS, Rogers PD. 2014. UPC2 is universally essential for azole antifungal resistance in *Candida albicans*. *Eukaryot Cell* 13:933-46.
117. 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.
118. Wang Y, Liu JY, Shi C, Li WJ, Zhao Y, Yan L, Xiang MJ. 2015. Mutations in transcription factor Mrr2p contribute to fluconazole resistance in clinical isolates of *Candida albicans*. *Int J Antimicrob Agents* 46:552-9.
119. Nishimoto AT, Zhang Q, Hazlett B, Morschhäuser J, Rogers PD. 2019. The contribution of clinically-derived mutations in the gene encoding the zinc cluster transcription factor Mrr2 to fluconazole antifungal resistance and *CDR1* expression in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* doi:10.1128/aac.00078-19:AAC.00078-19.
120. Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G. 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob Agents Chemother* 49:668-79.
121. White TC. 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in *Candida albicans*. *Antimicrob Agents Chemother* 41:1488-94.
122. 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.

123. Kelly SL, Lamb DC, Kelly DE. 1999. Y132H substitution in *Candida albicans* sterol 14 α -demethylase confers fluconazole resistance by preventing binding to haem. *FEMS Microbiol Lett* 180:171-5.
124. Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. 1999. The G464S amino acid substitution in *Candida albicans* sterol 14 α -demethylase causes fluconazole resistance in the clinic through reduced affinity. *Biochem Biophys Res Commun* 262:174-9.
125. Wang YB, Wang H, Guo HY, Zhao YZ, Luo SQ. 2005. [Analysis of ERG11 gene mutation in *Candida albicans*]. *Di Yi Jun Yi Da Xue Xue Bao* 25:1390-3.
126. Xu Y, Chen L, Li C. 2008. Susceptibility of clinical isolates of *Candida* species to fluconazole and detection of *Candida albicans* ERG11 mutations. *J Antimicrob Chemother* 61:798-804.
127. Wang H, Kong F, Sorrell TC, Wang B, McNicholas P, Pantarat N, Ellis D, Xiao M, Widmer F, Chen SC. 2009. Rapid detection of ERG11 gene mutations in clinical *Candida albicans* isolates with reduced susceptibility to fluconazole by rolling circle amplification and DNA sequencing. *BMC Microbiol* 9:167.
128. Feng LJ, Wan Z, Wang XH, Li RY, Liu W. 2010. Relationship between antifungal resistance of fluconazole resistant *Candida albicans* and mutations in ERG11 gene. *Chin Med J (Engl)* 123:544-8.
129. Kim TH, Lee MK. 2010. Evaluation of the V404I and V509M amino acid substitutions of ERG11 gene in *Candida albicans* isolates by pyrosequencing. *Folia Microbiol (Praha)* 55:301-4.
130. Martel CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL. 2010. A clinical isolate of *Candida albicans* with mutations in ERG11 (encoding sterol 14 α -demethylase) and ERG5 (encoding C22 desaturase) is cross resistant to azoles and amphotericin B. *Antimicrob Agents Chemother* 54:3578-83.
131. Morio F, Loge C, Besse B, Hennequin C, Le Pape P. 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn Microbiol Infect Dis* 66:373-84.
132. Oliveira Carvalho V, Okay TS, Melhem MS, Walderez Szeszs M, del Negro GM. 2013. The new mutation L321F in *Candida albicans* ERG11 gene may be associated with fluconazole resistance. *Rev Iberoam Micol* 30:209-12.

133. Strzelczyk JK, Slempl-Migiel A, Rother M, Golabek K, Wiczkowski A. 2013. Nucleotide substitutions in the *Candida albicans* ERG11 gene of azole-susceptible and azole-resistant clinical isolates. *Acta Biochim Pol* 60:547-52.
134. Xiang MJ, Liu JY, Ni PH, Wang S, Shi C, Wei B, Ni YX, Ge HL. 2013. Erg11 mutations associated with azole resistance in clinical isolates of *Candida albicans*. *FEMS Yeast Res* 13:386-93.
135. Ying Y, Zhao Y, Hu X, Cai Z, Liu X, Jin G, Zhang J, Zhang J, Liu J, Huang X. 2013. In vitro fluconazole susceptibility of 1,903 clinical isolates of *Candida albicans* and the identification of ERG11 mutations. *Microb Drug Resist* 19:266-73.
136. Zhang L, Yang HF, Liu YY, Xu XH, Ye Y, Li JB. 2013. Reduced susceptibility of *Candida albicans* clinical isolates to azoles and detection of mutations in the ERG11 gene. *Diagn Microbiol Infect Dis* 77:327-9.
137. Zhao J, Xu Y, Li C. 2013. Association of T916C (Y257H) mutation in *Candida albicans* ERG11 with fluconazole resistance. *Mycoses* 56:315-20.
138. Oliveira-Carvalho V, Del Negro GM. 2014. Is the S405F mutation in *Candida albicans* ERG11 gene sufficient to confer resistance to fluconazole? *J Mycol Med* 24:241-2.
139. 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.
140. 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 14 α -demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. *J Biol Chem* 292:6728-6743.
141. Warrilow AG, Nishimoto AT, Parker JE, Price CL, Flowers SA, Kelly DE, Rogers PD, Kelly SL. 2019. The Evolution of Azole Resistance in *Candida albicans* Sterol 14 α -Demethylase (CYP51) through Incremental Amino Acid Substitutions. *Antimicrob Agents Chemother* doi:10.1128/AAC.02586-18.
142. 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.

143. Miyazaki Y, Geber A, Miyazaki H, Falconer D, Parkinson T, Hitchcock C, Grimberg B, Nyswaner K, Bennett JE. 1999. Cloning, sequencing, expression and allelic sequence diversity of ERG3 (C-5 sterol desaturase gene) in *Candida albicans*. *Gene* 236:43-51.
144. Howell SA, Mallet AI, Noble WC. 1990. A comparison of the sterol content of multiple isolates of the *Candida albicans* Darlington strain with other clinically azole-sensitive and -resistant strains. *J Appl Bacteriol* 69:692-6.
145. 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.
146. Miyazaki T, Miyazaki Y, Izumikawa K, Kakeya H, Miyakoshi S, Bennett JE, Kohno S. 2006. Fluconazole treatment is effective against a *Candida albicans* erg3/erg3 mutant in vivo despite in vitro resistance. *Antimicrob Agents Chemother* 50:580-6.
147. 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.
148. Chau AS, Gurnani M, Hawkinson R, Laverdiere M, Cacciapuoti A, McNicholas PM. 2005. Inactivation of sterol Delta5,6-desaturase attenuates virulence in *Candida albicans*. *Antimicrob Agents Chemother* 49:3646-51.
149. Luna-Tapia A, Willems HME, Parker JE, Tournu H, Barker KS, Nishimoto AT, Rogers PD, Kelly SL, Peters BM, Palmer GE. 2018. Loss of Upc2p-Inducible ERG3 Transcription Is Sufficient To Confer Niche-Specific Azole Resistance without Compromising *Candida albicans* Pathogenicity. *MBio* 9.
150. Perepnikhatka V, Fischer FJ, Niimi M, Baker RA, Cannon RD, Wang YK, Sherman F, Rustchenko E. 1999. Specific chromosome alterations in fluconazole-resistant mutants of *Candida albicans*. *J Bacteriol* 181:4041-9.
151. Chen X, Magee BB, Dawson D, Magee PT, Kumamoto CA. 2004. Chromosome 1 trisomy compromises the virulence of *Candida albicans*. *Mol Microbiol* 51:551-65.
152. Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB, Kohn LM. 2000. Evolution of drug resistance in experimental populations of *Candida albicans*. *J Bacteriol* 182:1515-22.

153. Forche A, May G, Magee PT. 2005. Demonstration of loss of heterozygosity by single-nucleotide polymorphism microarray analysis and alterations in strain morphology in *Candida albicans* strains during infection. *Eukaryot Cell* 4:156-65.
154. Selmecki A, Bergmann S, Berman J. 2005. Comparative genome hybridization reveals widespread aneuploidy in *Candida albicans* laboratory strains. *Mol Microbiol* 55:1553-65.
155. Selmecki A, Forche A, Berman J. 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* 313:367-70.
156. Ford CB, Funt JM, Abbey D, Issi L, Guiducci C, Martinez DA, Delorey T, Li BY, White TC, Cuomo C, Rao RP, Berman J, Thompson DA, Regev A. 2015. The evolution of drug resistance in clinical isolates of *Candida albicans*. *Elife* 4:e00662.
157. Legrand M, Lephart P, Forche A, Mueller FM, Walsh T, Magee PT, Magee BB. 2004. Homozygosity at the MTL locus in clinical strains of *Candida albicans*: karyotypic rearrangements and tetraploid formation. *Mol Microbiol* 52:1451-62.
158. Hickman MA, Paulson C, Dudley A, Berman J. 2015. Parasexual Ploidy Reduction Drives Population Heterogeneity Through Random and Transient Aneuploidy in *Candida albicans*. *Genetics* 200:781-94.
159. Li X, Yang F, Li D, Zhou M, Wang X, Xu Q, Zhang Y, Yan L, Jiang Y. 2015. Trisomy of chromosome R confers resistance to triazoles in *Candida albicans*. *Med Mycol* 53:302-9.
160. Anderson MZ, Saha A, Haseeb A, Bennett RJ. 2017. A chromosome 4 trisomy contributes to increased fluconazole resistance in a clinical isolate of *Candida albicans*. *Microbiology* 163:856-865.
161. Mansfield BE, Oltean HN, Oliver BG, Hoot SJ, Leyde SE, Hedstrom L, White TC. 2010. Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. *PLoS Pathog* 6:e1001126.
162. Esquivel BD, Smith AR, Zavrel M, White TC. 2015. Azole drug import into the pathogenic fungus *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 59:3390-8.
163. Pfaller MA, Andes D, Arendrup MC, Diekema DJ, Espinel-Ingroff A, Alexander BD, Brown SD, Chaturvedi V, Fowler CL, Ghannoum MA, Johnson EM, Knapp CC, Motyl MR, Ostrosky-Zeichner L, Walsh TJ. 2011. Clinical breakpoints for voriconazole and *Candida* spp. revisited: review of microbiologic, molecular, pharmacodynamic, and clinical data as they pertain to the development of species-specific interpretive criteria. *Diagn Microbiol Infect Dis* 70:330-43.

164. Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Jones RN. 2011. Triazole and echinocandin MIC distributions with epidemiological cutoff values for differentiation of wild-type strains from non-wild-type strains of six uncommon species of *Candida*. *J Clin Microbiol* 49:3800-4.
165. European Committee on Antimicrobial Susceptibility Testing. 2018. Antifungal Agents: Breakpoint tables for interpretation of MICs.
166. Fridman O, Goldberg A, Ronin I, Shores N, Balaban NQ. 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* 513:418-21.
167. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol* 14:320-30.
168. Delarze E, Sanglard D. 2015. Defining the frontiers between antifungal resistance, tolerance and the concept of persistence. *Drug Resist Updat* 23:12-19.
169. Marchetti O, Moreillon P, Glauser MP, Bille J, Sanglard D. 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob Agents Chemother* 44:2373-81.
170. Arthington-Skaggs BA, Lee-Yang W, Ciblak MA, Frade JP, Brandt ME, Hajjeh RA, Harrison LH, Sofair AN, Warnock DW, Candidemia Active Surveillance G. 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* 46:2477-81.
171. Lee MK, Williams LE, Warnock DW, Arthington-Skaggs BA. 2004. Drug resistance genes and trailing growth in *Candida albicans* isolates. *J Antimicrob Chemother* 53:217-24.
172. Whaley SG, Tsao S, Weber S, Zhang Q, Barker KS, Raymond M, Rogers PD. 2016. The RTA3 Gene, Encoding a Putative Lipid Translocase, Influences the Susceptibility of *Candida albicans* to Fluconazole. *Antimicrob Agents Chemother* 60:6060-6.
173. Luna-Tapia A, Butts A, Palmer GE. 2019. Loss of C-5 Sterol Desaturase Activity in *Candida albicans*: Azole Resistance or Merely Trailing Growth? *Antimicrob Agents Chemother* 63.

174. Marr KA, Rustad TR, Rex JH, White TC. 1999. The trailing end point phenotype in antifungal susceptibility testing is pH dependent. *Antimicrob Agents Chemother* 43:1383-6.
175. 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* 36:153-6.
176. 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* 42:129-34.
177. 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 after fluconazole treatment in a murine model of invasive candidiasis. *Antimicrob Agents Chemother* 44:2081-5.
178. Rosenberg A, Ene IV, Bibi M, Zakin S, Segal ES, Ziv N, Dahan AM, Colombo AL, Bennett RJ, Berman J. 2018. Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia. *Nat Commun* 9:2470.
179. Gao J, Wang H, Li Z, Wong AH, Wang YZ, Guo Y, Lin X, Zeng G, Liu H, Wang Y, Wang J. 2018. *Candida albicans* gains azole resistance by altering sphingolipid composition. *Nat Commun* 9:4495.
180. McCarty TP, Pappas PG. 2016. Invasive Candidiasis. *Infect Dis Clin North Am* 30:103-24.
181. Kullberg BJ, Arendrup MC. 2015. Invasive Candidiasis. *N Engl J Med* 373:1445-56.
182. Morgan J, Meltzer MI, Plikaytis BD, Sofair AN, Huie-White S, Wilcox S, Harrison LH, Seaberg EC, Hajjeh RA, Teutsch SM. 2005. Excess mortality, hospital stay, and cost due to candidemia: a case-control study using data from population-based candidemia surveillance. *Infect Control Hosp Epidemiol* 26:540-7.
183. Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, Lynfield R, Maloney M, McAllister-Hollod L, Nadle J, Ray SM, Thompson DL, Wilson LE, Fridkin SK, Emerging Infections Program Healthcare-Associated I, Antimicrobial Use Prevalence Survey T. 2014. Multistate point-prevalence survey of health care-associated infections. *N Engl J Med* 370:1198-208.

184. Centers for Disease Control and Prevention. 2013. Antibiotic Resistance Threats in the United States. <https://www.cdc.gov/drugresistance/threat-report-2013/pdf/>. <https://www.cdc.gov/drugresistance/threat-report-2013/pdf/>.
185. 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.
186. Shah DN, Yau R, Lasco TM, Weston J, Salazar M, Palmer HR, Garey KW. 2012. Impact of prior inappropriate fluconazole dosing on isolation of fluconazole-nonsusceptible *Candida* species in hospitalized patients with candidemia. *Antimicrob Agents Chemother* 56:3239-43.
187. 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.
188. 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.
189. 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.
190. Pfaller M, Neofytos D, Diekema D, Azie N, Meier-Kriesche HU, Quan SP, Horn D. 2012. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004-2008. *Diagn Microbiol Infect Dis* 74:323-31.
191. Goldman M, Cloud GA, Wade KD, Reboli AC, Fichtenbaum CJ, Hafner R, Sobel JD, Powderly WG, Patterson TF, Wheat LJ, Stein DK, Dismukes WE, Filler SG, Team ACTGS, Mycoses Study Group Study T. 2005. A randomized study of the use of fluconazole in continuous versus episodic therapy in patients with advanced HIV infection and a history of oropharyngeal candidiasis: AIDS Clinical Trials Group Study 323/Mycoses Study Group Study 40. *Clin Infect Dis* 41:1473-80.
192. Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, Phelan M, Morgan J, Lee-Yang W, Ciblak MA, Benjamin LE, Sanza LT, Huie S, Yeo SF, Brandt ME, Warnock DW. 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected

- from 1998 to 2000 in a population-based active surveillance program. *J Clin Microbiol* 42:1519-27.
193. Klevay MJ, Ernst EJ, Hollanbaugh JL, Miller JG, Pfaller MA, Diekema DJ. 2008. Therapy and outcome of *Candida glabrata* versus *Candida albicans* bloodstream infection. *Diagn Microbiol Infect Dis* 60:273-7.
 194. Moriyama B, Gordon LA, McCarthy M, Henning SA, Walsh TJ, Penzak SR. 2014. Emerging drugs and vaccines for candidemia. *Mycoses* 57:718-33.
 195. MacPherson S, Larochelle M, Turcotte B. 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiol Mol Biol Rev* 70:583-604.
 196. Tsao S, Rahkhoodae 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.
 197. Hampe IAI, Friedman J, Edgerton M, Morschhauser J. 2017. An acquired mechanism of antifungal drug resistance simultaneously enables *Candida albicans* to escape from intrinsic host defenses. *PLoS Pathog* 13:e1006655.
 198. Vargas-Blanco D, Lynn A, Rosch J, Noreldin R, Salerni A, Lambert C, Rao RP. 2017. A pre-therapeutic coating for medical devices that prevents the attachment of *Candida albicans*. *Ann Clin Microbiol Antimicrob* 16:41.
 199. Partow S, Siewers V, Bjorn S, Nielsen J, Maury J. 2010. Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast* 27:955-64.
 200. 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.
 201. 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.
 202. 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.
 203. Grahl N, Demers EG, Crocker AW, Hogan DA. 2017. Use of RNA-Protein Complexes for Genome Editing in Non-*albicans* *Candida* Species. *mSphere* 2.
 204. Kao AS, Brandt ME, Pruitt WR, Conn LA, Perkins BA, Stephens DS, Baughman WS, Reingold AL, Rothrock GA, Pfaller MA, Pinner RW, Hajjeh RA. 1999. The

- epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin Infect Dis* 29:1164-70.
205. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FCS, Odds FC, Vanden Bossche H. 1999. Contribution of mutations in the cytochrome P450 14 α -demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* 145 (Pt 10):2701-2713.
 206. Keniya MV, Sabherwal M, Wilson RK, Woods MA, Sagatova AA, Tyndall JDA, Monk BC. 2018. Crystal Structures of Full-Length Lanosterol 14 α -Demethylases of Prominent Fungal Pathogens *Candida albicans* and *Candida glabrata* Provide Tools for Antifungal Discovery. *Antimicrob Agents Chemother* 62.
 207. Popp C, Hampe IAI, Hertlein T, Ohlsen K, Rogers PD, Morschhauser J. 2017. Competitive Fitness of Fluconazole-Resistant Clinical *Candida albicans* Strains. *Antimicrob Agents Chemother* 61.
 208. Sasse C, Dunkel N, Schafer T, Schneider S, Dierolf F, Ohlsen K, Morschhauser J. 2012. The stepwise acquisition of fluconazole resistance mutations causes a gradual loss of fitness in *Candida albicans*. *Mol Microbiol* 86:539-56.
 209. Lohberger A, Coste AT, Sanglard D. 2014. Distinct roles of *Candida albicans* drug resistance transcription factors TAC1, MRR1, and UPC2 in virulence. *Eukaryot Cell* 13:127-42.
 210. Morschhäuser J. 2019. Competition experiments.
 211. Sobel JD. 2007. Vulvovaginal candidosis. *Lancet* 369:1961-71.
 212. Buchacz K, Lau B, Jing Y, Bosch R, Abraham AG, Gill MJ, Silverberg MJ, Goedert JJ, Sterling TR, Althoff KN, Martin JN, Burkholder G, Gandhi N, Samji H, Patel P, Rachlis A, Thorne JE, Napravnik S, Henry K, Mayor A, Gebo K, Gange SJ, Moore RD, Brooks JT, North American ACCoR, Design of Ie DEA. 2016. Incidence of AIDS-Defining Opportunistic Infections in a Multicohort Analysis of HIV-infected Persons in the United States and Canada, 2000-2010. *J Infect Dis* 214:862-72.
 213. Coronado-Castellote L, Jimenez-Soriano Y. 2013. Clinical and microbiological diagnosis of oral candidiasis. *J Clin Exp Dent* 5:e279-86.
 214. Song JL, Harry JB, Eastman RT, Oliver BG, White TC. 2004. The *Candida albicans* lanosterol 14- α -demethylase (ERG11) gene promoter is maximally induced after prolonged growth with antifungal drugs. *Antimicrob Agents Chemother* 48:1136-44.

215. Ribeiro MA, Paula CR. 2007. Up-regulation of ERG11 gene among fluconazole-resistant *Candida albicans* generated in vitro: is there any clinical implication? *Diagn Microbiol Infect Dis* 57:71-5.
216. Arthington-Skaggs BA, Crowell DN, Yang H, Sturley SL, Bard M. 1996. Positive and negative regulation of a sterol biosynthetic gene (ERG3) in the post-squalene portion of the yeast ergosterol pathway. *FEBS Lett* 392:161-5.
217. 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.
218. 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.
219. Solomon SL, Oliver KB. 2014. Antibiotic resistance threats in the United States: stepping back from the brink. *Am Fam Physician* 89:938-41.
220. Rex JH, Rinaldi MG, Pfaller MA. 1995. Resistance of *Candida* species to fluconazole. *Antimicrob Agents Chemother* 39:1-8.
221. 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.
222. 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.
223. Kontoyiannis DP. 2017. Antifungal Resistance: An Emerging Reality and A Global Challenge. *J Infect Dis* 216:S431-S435.
224. 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.
225. 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.

226. 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.
227. 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.
228. 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.
229. 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 14 α -Demethylase Provides Insights into Its Broad-Spectrum Antifungal Activity. *Antimicrob Agents Chemother* 61.
230. 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.
231. Hoot SJ, Brown RP, Oliver BG, White TC. 2010. The UPC2 promoter in *Candida albicans* contains two cis-acting elements that bind directly to Upc2p, resulting in transcriptional autoregulation. *Eukaryot Cell* 9:1354-62.

APPENDIX A. AMINO ACID SUBSTITUTIONS IN MRR2 CLINICAL *C. ALBICANS* ISOLATES

Table A-1. Amino acid substitutions in Mrr2 clinical *C. albicans* isolates

Isolate	Amino Acid Substitutions ^{a,b}
FLU-susceptible	
1	V451A (h)
2	-
3	S143P, L144V, T145A, S165N, V451A, S480P
5	-
7	-
9	V451A
10	V451A
17	-
22	T83A, V451A, V582L
53	-
FLU-resistant	
11	V451A, S480P
12	V451A
13	-
14	-
15	T83A, A459T, S480P, V485M
16	S466L, A468G, S469T, T470N, S480P
18	S143P, L144V, T145A, S165N, A459T, S480P
19	S143P, L144V, T145A, S165N, A459T, S480P
20	S143P, L144V, T145A, S165N, A459T, S480P
21	V451A
23	R45Q, V451A
24	-
25	-
26	S143P (h), L144V (h), T145A (h), V451A (h), S466L, A468G, S469T, T470N, S480P
27	T83A, A459T, S480P, V486M
28	T83A, A459T, S480P, V486M
29	S466L, A468G, S469T, T470N, S480P
30	-
31	-
32	-
33	S143P, L144V, T145A, S165N, A459T, S480P
34	-
35	-
36	-

Table A-1. (Continued)

Isolate	Amino Acid Substitutions ^{a,b}
FLU-resistant	
37	-
38	-
39	-
40	V451A
41	S143P, L144V, T145A, S165N, A459T, S480P
42	-
43	-
44	-
45	T83A, T145A, V451A, S480P
46	S143P (h), L144 (h), T145A (h), V451A (h), S466L, A468G, S469T, T470N, S480P
47	T83A, V451A, S480P
48	S143P, L144V, T145A, S165N, A459T, S480P
49	-
50	-
51	-
54	S143P, L144V, T145A, S165N, A459T, S480P
55	-
56	-
58	-
59	T83A, L143P, L144V, T145A, S165N, V451A
60	-
61	-
62	-
63	-
64	-
65	-
66	-
67	-
68	S143P (h), L144V (h), T145A (h), S165N (h), S480P (h)
69	-
70	T83A (h), S143P (h), L144V (h), T145A (h), S165N (h), V451A, S480P (h)
71	T83A, V451A, S480P
72	-

^a (h) indicates a heterozygous amino acid substitution

^b “-” Denotes no changes in the amino acid sequence of the clinical isolate from the *MRR2* allele of SC5314.

APPENDIX B. SUPPLEMENTARY DATA TABLES TO CHAPTER 5

Table B-1. Minimum inhibitory concentration values for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, posaconazole, and known azole resistance mechanisms in clinical *C. albicans* isolates

Isolate ID	VT-1161		VT-1598		Fluconazole		Voriconazole		Itraconazole		Posaconazole		ERG11 mutations ^a	Other Resistance Mechanisms ^b
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2		
1	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	0.25	0.5	≤ 0.03	≤ 0.03	0.06	≤ 0.03	≤ 0.03	0.06	K128T ^(h) , D116E ^(h)	---
2	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.125	0.25	≤ 0.03	≤ 0.03	≤ 0.03	0.06	≤ 0.03	0.06	F198fs ^(h) , K128T, D116E	---
3	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	0.25	0.25	≤ 0.03	≤ 0.03	≤ 0.03	0.06	≤ 0.03	0.06	G129A, D116E	---
5	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	0.5	1	≤ 0.03	≤ 0.03	NA	NA	NA	NA	NA	NA
6	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	1	1	≤ 0.03	≤ 0.03	NA	NA	NA	NA	NA	NA
7	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.125	0.25	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	0.06	K128T ^(h) , D116E ^(h)	---
9	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.125	≤ 0.125	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	S457F ^(h) , V437I ^(h)	---
10	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.125	≤ 0.125	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	V437I ^(h)	---
11	0.125	0.125	0.03	≤ 0.015	8	4	0.25	0.25	0.5	0.5	0.25	0.5	E266D, V488I	↑ <i>ERG11</i> , <i>CDR1</i>
12	0.125	0.125	≤ 0.015	≤ 0.015	32	32	0.06	0.125	0.25	0.125	0.125	0.25	K143R	↑ <i>ERG11</i> , <i>CDR1</i>
13	0.06	0.06	0.03	0.06	32	32	0.125	0.25	0.25	0.5	0.25	0.5	K143R	↑ <i>CDR1</i>
14	0.06	0.03	≤ 0.015	≤ 0.015	16	16	0.06	0.06	0.25	0.25	0.125	0.25	K143R	↑ <i>CDR1</i>
15	0.125	0.06	0.06	0.03	16	16	0.25	0.25	0.5	0.5	0.25	0.5	E266D, V488I	↑ <i>ERG11</i> , <i>CDR1</i>

Table B-1. (Continued)

Isolate ID	VT-1161		VT-1598		Fluconazole		Voriconazole		Itraconazole		Posaconazole		ERG11 mutations ^a	Other Resistance Mechanisms ^b
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2		
16	≤ 0.015	0.03	≤ 0.015	0.03	4	8	≤ 0.03	0.06	0.25	0.25	0.06	0.06	Q21R ^(h)	↑ <i>MDR1</i>
17	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	2	0.06	0.5	0.125	0.25	≤ 0.03	0.06	A114S, Y257H	↑ <i>CDR1</i>
18	0.5	0.5	0.06	0.03	16	32	0.5	0.5	0.25	0.5	0.125	0.25	S405F	↑ <i>ERG11</i> , <i>CDR1</i>
19	0.5	0.5	≤ 0.015	≤ 0.015	32	32	0.5	0.5	0.5	0.5	0.125	0.25	S405F	↑ <i>ERG11</i> , <i>CDR1</i>
20	1	0.5	0.06	0.06	64	32	0.5	0.5	0.5	0.25	0.25	0.5	S405F	↑ <i>ERG11</i> , <i>CDR1</i>
21	1	0.5	0.125	0.06	32	32	0.5	0.5	0.5	0.5	0.25	0.5	A114V, E266D, H283R	↑ <i>ERG11</i> , <i>CDR1</i>
22	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	0.5	1	≤ 0.03	≤ 0.03	≤ 0.03	0.06	≤ 0.03	0.06	F145L	---
23	0.125	0.125	0.03	≤ 0.015	32	32	0.5	0.25	0.125	0.25	0.06	0.125	I483V, G450E	↑ <i>CDR1</i>
24	0.03	0.03	0.03	0.03	16	16	0.25	0.125	0.125	0.25	0.06	0.125	G450E	↑ <i>CDR1</i> , <i>MDR1</i>
25	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	16	16	0.06	0.125	0.25	0.125	≤ 0.03	0.125	D446E	↑ <i>ERG11</i> , <i>MDR1</i>
26	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	16	16	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	G307S, G448R	---
27	0.06	0.06	≤ 0.015	0.03	8	16	0.25	0.125	0.25	0.5	0.125	0.25	E266D, V488I	↑ <i>ERG11</i> , <i>CDR1</i>
28	0.5	0.5	≤ 0.015	0.06	64	64	0.5	1	0.5	0.5	0.25	1	E266D, V488I	↑ <i>ERG11</i> , <i>CDR1</i>
29	0.25	0.5	0.06	0.06	>64	>64	2	2	0.5	0.5	0.25	1	Y132F, F145L	↑ <i>CDR1</i>
30	0.25	0.5	0.06	0.06	16	32	0.5	1	0.06	0.125	≤ 0.03	≤ 0.03	F126L, Y132F, H283R	↑ <i>ERG11</i>

Table B-1. (Continued)

Isolate ID	VT-1161		VT-1598		Fluconazole		Voriconazole		Itraconazole		Posaconazole		ERG11 mutations ^a	Other Resistance Mechanisms ^b
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2		
31	0.5	0.5	0.125	0.125	>64	64	0.5	0.5	0.5	1	0.25	0.25	G448E	↑ <i>CDR1</i>
32	≤ 0.015	0.03	≤ 0.015	≤ 0.015	8	8	0.06	0.06	0.125	0.25	0.06	0.125	G464S	↑ <i>CDR1</i> , <i>MDR1</i>
33	0.125	0.125	≤ 0.015	≤ 0.015	32	32	0.5	0.5	0.25	0.5	0.25	0.25	F145L E266D	↑ <i>CDR1</i>
34	0.125	0.125	0.06	0.06	64	16	0.5	0.25	0.5	0.5	0.25	0.5	G450E	↑ <i>CDR1</i>
35	0.015	0.03	0.03	0.03	16	16	0.06	0.125	0.25	0.25	0.125	0.25	D446E	↑ <i>ERG11</i> , <i>MDR1</i>
36	0.5	0.25	0.06	0.06	64	64	0.5	1	0.25	0.25	0.125	0.25	None	↑ <i>ERG11</i> , <i>CDR1</i>
37	0.125	0.125	0.06	0.06	32	64	0.5	0.5	0.25	0.25	0.125	0.25	None	↑ <i>ERG11</i> , <i>CDR1</i>
38	1	0.5	0.125	0.06	>64	>64	2	1	0.25	0.5	0.25	0.5	G464S	↑ <i>ERG11</i> , <i>CDR1</i>
39	1	1	0.125	0.125	>64	>64	2	2	1	1	0.5	1	G464S	↑ <i>ERG11</i> , <i>CDR1</i>
40	0.5	0.25	0.125	0.125	>64	>64	1	1	0.5	0.5	0.25	0.5	F449V	↑ <i>ERG11</i> , <i>CDR1</i>
41	0.06	0.06	0.06	0.03	64	64	0.25	0.25	0.25	0.25	0.125	0.25	S405F	↑ <i>ERG11</i> , <i>MDR1</i>
42	0.25	0.125	0.06	0.06	64	32	1	0.5	0.25	0.25	0.125	0.25	G464S	↑ <i>CDR1</i>
43	0.03	0.06	≤ 0.015	0.06	8	8	0.25	0.5	0.25	0.25	0.06	0.125	D278N G464S	↑ <i>CDR1</i>
44	0.5	0.25	0.03	0.03	>64	64	1	1	0.25	0.5	0.25	0.5	D446E	↑ <i>CDR1</i>
45	0.5	0.25	0.06	0.06	64	32	1	1	0.25	0.5	0.125	0.25	E266D G464S	↑ <i>CDR1</i>

Table B-1. (Continued)

Isolate ID	VT-1161		VT-1598		Fluconazole		Voriconazole		Itraconazole		Posaconazole		ERG11 mutations ^a	Other Resistance Mechanisms ^b
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2		
46	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	32	64	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	G307S, L403F, G448R	---
47	0.125	0.125	0.03	≤ 0.015	>64	64	0.5	0.5	0.25	0.25	0.125	0.25	G450E	↑ <i>ERG11</i> , <i>CDR1</i>
48	2	2	0.06	0.03	32	32	0.5	0.5	0.25	0.25	0.06	0.125	Y132F	↑ <i>CDR1</i>
49	0.125	0.125	0.06	0.125	>64	>64	0.5	0.5	0.5	0.5	0.25	0.5	V437I, Y447S	↑ <i>ERG11</i> , <i>CDR1</i>
50	1	0.5	0.06	0.06	>64	>64	2	1	0.25	0.5	0.06	0.25	Y132F K143R	↑ <i>CDR1</i>
51	0.5	0.5	0.06	0.06	>64	64	2	1	0.125	0.25	0.125	0.25	D278N G464S	↑ <i>CDR1</i>
53	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	1	1	≤ 0.03	≤ 0.03	0.125	0.25	0.25	0.25	None	↑ <i>ERG11</i>
54	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	32	32	≤ 0.03	0.06	0.06	0.125	≤ 0.03	0.06	G307S G450E	↑ <i>ERG11</i> , <i>MDR1</i>
55	4	8	>8	>8	>64	>64	>16	>16	>16	>16	>16	>16	None	---
56	4	8	>8	>8	>64	64	>16	>16	>16	>16	>16	>16	None	↑ <i>CDR1</i>
58	4	4	>8	>8	>64	>64	>16	0.5	>16	>16	>16	>16	None	↑ <i>ERG11</i> , <i>CDR1</i>
59	0.5	0.5	0.125	0.06	>64	>64	2	2	0.25	0.25	0.06	0.125	Y132F, T229A, F449I	↑ <i>ERG11</i> , <i>CDR1</i>
60	4	>8	>8	>8	>64	>64	>16	>16	>16	>16	>16	>16	None	<i>ERG3</i> ^{W131*} , ↑ <i>ERG11</i>
61	0.5	0.5	0.03	0.06	>64	>64	1	1	0.25	0.5	0.125	0.25	Y132F K143R	↑ <i>CDR1</i>
62	0.125	0.25	≤ 0.015	≤ 0.015	32	32	0.5	0.5	0.25	0.5	0.125	0.25	D278N G464S	↑ <i>CDR1</i>

Table B-1. (Continued)

Isolate ID	VT-1161		VT-1598		Fluconazole		Voriconazole		Itraconazole		Posaconazole		ERG11 mutations ^a	Other Resistance Mechanisms ^b
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2		
63	0.06	0.125	0.125	0.06	32	32	0.25	0.25	0.5	0.5	0.25	0.25	G450E	↑ <i>CDR1</i> , <i>MDR1</i>
64	0.25	0.125	0.125	0.06	32	32	0.5	0.25	0.5	0.5	0.25	0.25	G450E	↑ <i>ERG11</i> , <i>MDR1</i>
65	4	2	>8	0.5	64	64	1	0.5	1	2	0.5	0.5	G448E	↑ <i>ERG11</i> , <i>CDR1</i>
66	0.5	0.5	0.25	0.125	64	64	0.5	0.5	0.5	0.5	0.25	0.5	G448E	↑ <i>CDR1</i>
67	0.5	0.5	>8	0.25	>64	64	1	1	1	1	0.5	0.5	G448E	↑ <i>ERG11</i> , <i>CDR1</i>
68	0.5	0.5	≤ 0.015	≤ 0.015	>64	>64	0.5	0.5	0.25	0.5	≤ 0.03	0.06	Y132F, E266D, I471M, V488I	↑ <i>ERG11</i> , <i>MDR1</i>
69	0.5	1	0.25	0.25	64	64	1	1	1	1	0.5	0.5	G448E	↑ <i>ERG11</i> , <i>CDR1</i>
70	0.06	0.03	≤ 0.015	≤ 0.015	32	32	0.5	0.5	0.25	0.5	0.125	0.125	M258L, G464S	---
71	0.25	0.125	0.06	0.06	>64	64	0.5	0.5	0.5	0.5	0.125	0.125	Y132F, V437I, F449L A114V,	↑ <i>ERG11</i> , <i>CDR1</i>
72	0.25	0.125	0.06	0.125	16	16	0.25	0.25	0.5	0.5	0.25	0.25	D153E, E266D, G450E	↑ <i>CDR1</i>

^a(h) indicates heterozygous mutation

^b↑ denotes increased expression of the gene in question

Notes: Low antifungal MICs (within a two-fold increase of that of the fluconazole-susceptible isolates) against fluconazole-resistant isolates are highlighted in yellow.

Table B-2. MICs for VT-1161, VT-1598, fluconazole, voriconazole, itraconazole, and posaconazole against *ERG11* mutant strains

Erg11 substitution	VT-1598		VT-1161		Fluconazole		Itraconazole		Posaconazole		Voriconazole	
	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2	MIC #1	MIC #2
Wildtype ^a	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	0.5	0.5	0.125	0.125	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03
Y132F	≤ 0.015	≤ 0.015	0.25	0.125	4	8	0.125	0.125	≤ 0.03	≤ 0.03	0.25	0.125
Y132H	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	1	0.125	0.125	≤ 0.03	≤ 0.03	0.125	0.06
K143R	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	4	0.06	0.125	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03
F145L	0.03	≤ 0.015	≤ 0.015	≤ 0.015	4	4	0.25	0.25	≤ 0.03	≤ 0.03	0.125	0.125
E266D	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	1	1	0.125	0.125	≤ 0.03	≤ 0.03	0.06	0.06
D278N	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	1	0.125	0.125	≤ 0.03	≤ 0.03	0.125	0.06
S405F	0.03	≤ 0.015	≤ 0.015	≤ 0.015	4	4	0.25	0.25	≤ 0.03	≤ 0.03	0.125	0.125
G448E	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	1	2	0.125	0.25	≤ 0.03	≤ 0.03	≤ 0.03	0.06
F449V	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	4	0.125	0.125	≤ 0.03	≤ 0.03	0.06	≤ 0.03
G450E	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	2	0.125	0.25	≤ 0.03	≤ 0.03	0.06	0.06
G464S	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	2	0.125	0.125	≤ 0.03	≤ 0.03	0.06	0.06
D466E	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	1	1	0.125	0.25	≤ 0.03	≤ 0.03	0.125	0.06
Y132F, K143R	≤ 0.015	≤ 0.015	0.06	0.125	32	64	0.125	0.125	≤ 0.03	≤ 0.03	0.25	0.25
Y132F, F145L	0.03	0.03	≤ 0.015	0.06	16	32	0.25	0.25	≤ 0.03	≤ 0.03	0.5	1
D278N, G464S	0.03	≤ 0.015	≤ 0.015	≤ 0.015	4	8	0.25	0.25	≤ 0.03	≤ 0.03	0.25	0.25
G450E, I483V	≤ 0.015	≤ 0.015	≤ 0.015	≤ 0.015	2	2	0.125	0.125	≤ 0.03	≤ 0.03	0.125	0.125

^aFrom azole-susceptible parent strain SC5314

Table B-3. Primers used in Chapter 5

Primer name by purpose	Sequence ^a
Amplification	
CaERG11_AF_(ApaI)	5' – <u>GGGCCC</u> GGGTATTGAGAACAGCC – 3'
CaERG11_BR_(XhoI)	5' – <u>CTCGAG</u> CCAGTGGACAAAAACCATCA – 3'
CaERG11_C-F'	5' – AAT <u>GCGGCCG</u> CATTTTAACTACTTTTGTTTA – 3'
CaERG11_D-R'	5' – CTT <u>CCGCGG</u> TAATCAATTCACCATTTTTAAC – 3'
Sequencing	
CaERG11SeqA	5' – GCCACCACACCCTATGGCTATT – 3'
CaERG11SeqB	5' – TATTTTCACTGCTTCAAGATCT – 3'
CaERG11SeqC	5' – CCAAAAGGTCATTATGTTTTAG – 3'
CaERG11SeqD	5' – CATAACAAGTTTCTCTTTTTTCC – 3'
CaERG11SeqE	5' – CATTTAGGTGAAAAACCTCATT – 3'
CaERG11SeqF	5' – TACTCCAGTTTTCGGTAAAGGG – 3'
Short, overlapping extension	
CaERG11_1F	5' – CAATCTATACGACGACATTTAACTTTTT – 3'
CaERG11SOE-3R_Y132H	5' – GACAATCATGAATAACCCCTTTACCGAAAA – 3'
CaERG11SOE-2F_Y132H	5' – GGGGTTATTCATGATTGTCCAAATTCCAGA – 3'
CaERG11_4R	5' – GCTGTTGTTGTTGTTGAAAGAAA – 3'
CaERG11SOE-5F	5' – ATTATTGAAAGAAAAAGGTGGTGATTG – 3'
CaERG11SOE-6R	5' – ACCTTTTTCTTTCAATAATTCAACAACT – 3'
Real-time qPCR	
CaCDR1-F_qPCR	5' – ATTCTAAGATGTCGTCGCAAGATG – 3'
CaCDR1-R_qPCR	5' – AGTTCTGGCTAAATTCTGAATGTTTTC – 3'
CaERG11-F_qPCR	5' – CCCCTATTAATTTTGTTTTCCCTAATTTAC – 3'
CaERG11-R_qPCR	5' – CACGTTCTCTTCTCAGTTTAATTTCTTTC – 3'
CaBMR1-F_qPCR	5' – ACATAAATACTTTGCCCATCCAGAA – 3'
CaBMR1-R_qPCR	5' – AAGAGTTGGTTTGTAATCGGCTAAA – 3'
CaACT1-FWD_qPCR	5' – ACGGTGAAGAAGTTGCTGCTTTAGTT – 3'
CaACT1-rvs_qPCR	5' – CGTCGTCACCGGCAAAA – 3'

^aUnderlined nucleotide indicates introduction of a restriction site sequence

VITA

Andrew (Andy) Tadashi Nishimoto was born in Long Beach, California in 1984 to S. Ken and Miyako Nishimoto. He enthusiastically attended Farmington Elementary, Houston Middle/High School in Germantown, Tennessee and graduated as valedictorian in 2003. He graduated with a Bachelor of the Arts in Molecular Biology from Pomona College in Claremont, California in 2007 and enrolled in the PharmD/PhD dual degree program at the University of Tennessee Health Science Center in 2009. He earned his Doctor of Pharmacy degree with Honors in 2013 and completed a one year pharmacy practice residency at Methodist University Hospital in Memphis, TN, earning his BCPS certification in 2015. Andy anticipates graduation from the Pharmaceutical Sciences program in the University of Tennessee College of Graduate Health Sciences with a Doctor of Philosophy degree in 2019.