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Challenging the Paradigm of Clinical Triazole Resistance in *Aspergillus fumigatus*

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Challenging the Paradigm of Clinical Triazole Resistance in *Aspergillus fumigatus*

Abstract

Invasive aspergillosis is a leading cause of morbidity and mortality among immunocompromised populations and is predicted to cause more than 200,000 life-threatening infections each year. *Aspergillus fumigatus* is the most prevalent pathogen isolated from patients with invasive aspergillosis, accounting for more than 60% of all cases. Currently, the only antifungal agents available with consistent activity against *A. fumigatus* are the mold-active triazoles and amphotericin B, of which the triazoles commonly represent both front-line and salvage therapeutic options. Unfortunately, the treatment of infections caused by *A. fumigatus* has recently been further complicated by the global emergence of triazole resistance among both clinical and environmental isolates, and a large proportion of this resistance remains unexplained. In this work, we characterize the contributions of previously identified mechanisms of triazole resistance, including mutations in the sterol-demethylase-encoding gene *cyp51A*, overexpression of sterol-demethylase genes, and overexpression of the efflux pump-encoding gene *abcC*, among a large collection of highly triazole-resistant clinical *A. fumigatus* isolates. Upon revealing that these mechanisms alone cannot substantiate the majority of triazole resistance exhibited by this collection, we then characterize the direct contribution of two additional efflux pump-encoding genes, *abcA* and *atrI*. Increased expression of *abcA* and *atrI* has previously been associated with triazole resistance in clinical isolates of *A. fumigatus*, and both of these genes exhibit a high degree of homology with the well characterized *Candida albicans* triazole efflux pump-encoding gene, *CDR1*. However, deletion of either *abcA* or *atrI* in triazole-resistant clinical isolates which overexpress these genes, did not result in a significant change in triazole susceptibility. Finally, upon demonstrating that the canonical mechanisms of triazole resistance poorly explain the high level of triazole resistance observed in this collection of clinical isolates, we subsequently describe the identification and characterization of a novel genetic determinant of triazole resistance. Mutations in the HMG-CoA reductase encoding gene, *hmg1*, were identified in a majority of triazole-resistant clinical isolates in our collection. Introduction of three different *hmg1* mutations, predicted to encode residue alterations in the conserved sterol sensing domain of Hmg1, resulted in significantly increased resistance to the triazole class of agents. Additionally, correction of an *hmg1* mutation in a pan-triazole-resistant clinical isolate of *A. fumigatus* with a novel Cas9-ribonucleoprotein (RNP) mediated system, was shown to restore clinical susceptibility to all triazole agents. Mutations in *hmg1* were also shown to lead to the accumulation of ergosterol precursors, such as eburicol, by sterol profiling, while not altering the expression of sterol-demethylase genes. Taken together, the findings described in this work serve to demonstrate that mutations in *hmg1* are a common and significant genetic determinant of triazole resistance in clinical isolates of *A. fumigatus*."

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

By
Jeffrey Michael Rybak
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DEDICATION

To my parents Michael and Stephanie, my brother Matt, and my Aunt Carol for all their love and support. To my Aunt Cindy, Uncle Mike, Grandfather Alois, and Grandfather “Hub”, you are greatly missed.

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ABSTRACT

Invasive aspergillosis is a leading cause of morbidity and mortality among immunocompromised populations and is predicted to cause more than 200,000 life-threatening infections each year. *Aspergillus fumigatus* is the most prevalent pathogen isolated from patients with invasive aspergillosis, accounting for more than 60% of all cases. Currently, the only antifungal agents available with consistent activity against *A. fumigatus* are the mold-active triazoles and amphotericin B, of which the triazoles commonly represent both front-line and salvage therapeutic options. Unfortunately, the treatment of infections caused by *A. fumigatus* has recently been further complicated by the global emergence of triazole resistance among both clinical and environmental isolates, and a large proportion of this resistance remains unexplained.

In this work, we characterize the contributions of previously identified mechanisms of triazole resistance, including mutations in the sterol-demethylase-encoding gene *cyp51A*, overexpression of sterol-demethylase genes, and overexpression of the efflux pump-encoding gene *abcC*, among a large collection of highly triazole-resistant clinical *A. fumigatus* isolates. Upon revealing that these mechanisms alone cannot substantiate the majority of triazole resistance exhibited by this collection, we then characterize the direct contribution of two additional efflux pump-encoding genes, *abcA* and *atrI*. Increased expression of *abcA* and *atrI* has previously been associated with triazole resistance in clinical isolates of *A. fumigatus*, and both of these genes exhibit a high degree of homology with the well characterized *Candida albicans* triazole efflux pump-encoding gene, *CDRI*. However, deletion of either *abcA* or *atrI* in triazole-resistant clinical isolates which overexpress these genes, did not result in a significant change in triazole susceptibility.

Finally, upon demonstrating that the canonical mechanisms of triazole resistance poorly explain the high level of triazole resistance observed in this collection of clinical isolates, we subsequently describe the identification and characterization of a novel genetic determinant of triazole resistance. Mutations in the HMG-CoA reductase encoding gene, *hmgI*, were identified in a majority of triazole-resistant clinical isolates in our collection. Introduction of three different *hmgI* mutations, predicted to encode residue alterations in the conserved sterol sensing domain of *HmgI*, resulted in significantly increased resistance to the triazole class of agents. Additionally, correction of an *hmgI* mutation in a pan-triazole-resistant clinical isolate of *A. fumigatus* with a novel Cas9-ribonucleoprotein (RNP) mediated system, was shown to restore clinical susceptibility to all triazole agents. Mutations in *hmgI* were also shown to lead to the accumulation of ergosterol precursors, such as eburicol, by sterol profiling, while not altering the expression of sterol-demethylase genes. Taken together, the findings described in this work serve to demonstrate that mutations in *hmgI* are a common and significant genetic determinant of triazole resistance in clinical isolates of *A. fumigatus*.

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CHAPTER 1. THE EMERGING THREAT OF TRIAZOLE-RESISTANT *ASPERGILLUS FUMIGATUS**

Introduction

Invasive aspergillosis is a leading cause of morbidity and mortality among immunocompromised patient populations, such as patients receiving solid organ or stem cell transplants, patients with hematologic malignancies, and those taking immunosuppressive agents.[1] *Aspergillus*, the causative agent of invasive aspergillosis, is a genus of septate, saprophytic mold containing hundreds of identified species. Of these only *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger* commonly cause disease in humans, with *A. fumigatus* representing more than 60% of all clinical isolates.[2] Worldwide, invasive aspergillosis is conservatively estimated to cause more than 200,000 life threatening infections each year.[1, 2] Mortality rates range considerably, from 30- 90%, varying by patient population as well as severity and duration of immunosuppression.[3-5] Unfortunately, the treatment of infections caused by *A. fumigatus* has recently been further complicated by the global emergence of triazole resistance among both clinical and environmental isolates.[6-9]

The Mold-Active Triazoles

Since the widespread introduction of itraconazole to clinical practice in 1992, the mold-active triazole antifungals have been utilized as both front-line and salvage therapeutic agents for the treatment of invasive aspergillosis. Voriconazole is now considered the therapy of choice, and is available in both intravenous and oral formulations.[10] Multiple studies comparing the efficacy of voriconazole to that of the previous standard of care, amphotericin B, have found voriconazole to be more efficacious and generally safer than either conventional (deoxycholate) or lipid formulations of amphotericin B.[11, 12] Isavuconazole was recently approved for the treatment of invasive aspergillosis, after demonstrating non-inferiority to the standard of care, voriconazole.[4, 10] Isavuconazole, as the prodrug isavuconazonium sulfate, is available in both intravenous and oral formulations.[13] Posaconazole, often used as prophylaxis against invasive fungal infections in high-risk patients such as those with acute myeloid leukemia, is also employed as second-line or salvage therapy for invasive aspergillosis, and is now available in both intravenous and delayed-release tablet formulations. Itraconazole is still used commonly worldwide, but its use in invasive aspergillosis has decreased due to both lower anti-aspergillus potency and the withdrawal of intravenous formulations from some markets.[10] While all these agents possess a

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triazole active moiety (from which this class of agents gained its name), they can be grouped into two distinct pharmacophores (**Figure 1-1**). The compact triazoles such as voriconazole and isavuconazole consist of a triazole ring linked to aromatic groups such as difluorobenzene via a four-carbon backbone. While the extended side-chain triazoles, including itraconazole and posaconazole, possess both a halogenated phenyl ring as well as a long lipophilic side-chain comprised of five and six membered aromatic rings terminating in a triazolone group. The structural distinctions between these agents are thought to contribute to differences in stabilizing interactions sterol-demethylase enzymes.

Mechanism of Action of the Triazoles

In fungi, ergosterol is a key membrane lipid similar to mammalian cholesterol. Under normal growth conditions, ergosterol is plentiful in the fungal cell membrane and contributes to membrane integrity and fluidity, as well the appropriate positioning of fungal proteins within the membrane. The triazoles exert antifungal activity by blocking a key enzymatic step in the biosynthesis of ergosterol. Through direct interaction between N4 of the triazole ring and an iron atom present in the catalytic heme group, the triazoles competitively inhibit sterol-demethylase enzymes (**Figure 1-2**). Inhibition of sterol-demethylase, such as Cyp51A and Cyp51B in the case of *A. fumigatus*, leads to the diversion of ergosterol precursors from the normal biosynthetic pathway and accumulation of alternative sterols including toxic 14 α -methylated sterols. It is believed that this depletion of ergosterol and buildup of 14 α -methylated sterols ultimately leads to inhibition of fungal growth.[14]

A. fumigatus possesses two genes which encode homologous but distinct sterol-demethylase enzymes, *cyp51A* and *cyp51B*. These genes share approximately 60% sequence identity, however the triazole antifungals have been observed to have significantly lower binding affinity for the Cyp51A enzyme.[15, 16] This reduced binding affinity is due in large part to the threonine amino acid present at position 301 (T301) of Cyp51A, which replaces the more common isoleucine residue present at this position in other fungal sterol-demethylase enzymes (including *A. fumigatus* Cyp51B).[17] The intrinsic fluconazole resistance of *A. fumigatus* appears to be a result of this single residue substitution. Surveillance studies of the potency of the mold-active triazoles against *A. fumigatus* clinical isolates have found isavuconazole, itraconazole, posaconazole, and voriconazole to all possess clinically relevant activity against *A. fumigatus*. [18] In general, the MIC of isavuconazole and itraconazole are higher, with MIC₅₀ and MIC₉₀ of 1 and 2mg/L, and 1 and 1mg/L, respectively (range 0.128-8mg/L: isavuconazole, 0.25-4mg/L: itraconazole). While the MIC₅₀ and MIC₉₀ for posaconazole and voriconazole are typically lower, 0.25 and 0.5mg/L for both agents (range 0.06-1mg/L: posaconazole, 0.125-2mg/L: voriconazole).[18]

The antifungal activity of the triazoles is concentration dependent. This activity has been shown to be best predicted by the free AUC to MIC ratio (fAUC:MIC).[19, 20]

Figure 1-1. Structures of the clinically relevant triazoles.

Figure 1-2. Canonical mechanisms of triazole resistance in *A. fumigatus*.

In neutropenic murine models of pulmonary aspergillosis, the *f*AUC:MIC needed to produce a 90% reduction in fungal burden (1-log reduction) was found to be roughly twice that of stasis for both posaconazole and isavuconazole. This was true for triazole-susceptible and triazole-resistant isolates.[19, 20] Additionally, the triazoles possess fungicidal activity at physiologically relevant concentrations against most triazole-susceptible *A. fumigatus*. [21] However, this fungicidal activity is also concentration dependent and varies from isolate to isolate.[22] Unlike amphotericin B, the mold-active triazoles do not possess a clinically relevant post-antifungal effect.[23]

Mechanisms of Triazole Resistance

Considerable research has led to the identification of multiple mechanisms of triazole resistance among other pathogenic fungi.[24] The most predominant mechanisms responsible for clinically encountered triazole resistance can be grouped into three distinct categories; 1) mutations in the sterol-demethylase gene, 2) overexpression of the sterol demethylase gene, and 3) overexpression of either ATP-Binding Cassette (ABC) or Major Facilitator Superfamily (MFS) type drug efflux pumps.[24] While relatively less is understood of the mechanisms of clinical triazole resistance among *A. fumigatus*, much of what is known focuses on these three paths to triazole resistance (**Figure 1-2**). [9, 25, 26]

Mutations in the *A. fumigatus* Sterol-Demethylase Gene *cyp51A*

The most commonly identified mechanism of triazole resistance among clinical isolates of *A. fumigatus* involves mutations in the sterol-demethylase gene, *cyp51A*. [8, 27] A great number of these mutations have been identified among both clinical and environmental isolates originating from across the globe, many of which are uniquely found in triazole resistant isolates (**Figure 1-3**). [8, 28, 29] A smaller subset of these mutations have been shown to directly contribute to triazole resistance through genetic manipulations in the laboratory setting (**Table 1-1**). [28, 30-36] Conversely, no mutations which are associated with clinical triazole resistance have been found in the *cyp51B* sterol-demethylase gene at this time.

Mutations in *cyp51A* associated with triazole resistance are believed to result in amino acid substitutions which either diminish the binding affinity of triazoles with the sterol-demethylase enzyme, or alter the enzyme structure in a manner which favors native substrate conversion and thus ergosterol biosynthesis. [28, 30-36] The majority of the known resistance associated mutations can be grouped into distinct regions of the sterol-demethylase enzyme. The most common and best understood of which, encode amino acid substitutions occurring along the ligand access channel (residues G54, P216, F219, and M220) or in residues which are located near the catalytic site (Y121, G138). [15] The ligand access channel associated residue substitutions have been shown to greatly decrease *A. fumigatus* susceptibility to both itraconazole and posaconazole, while having relatively less impact on voriconazole and isavuconazole susceptibilities. [30, 31, 36] It is

Tandem Repeat Associated <i>cyp51A</i> Mutations	
TR ₃₄ /L98H	
TR ₄₆ / <u>Y121F</u> /T289A	
TR ₅₃	
TR ₅₃ /L98H	
Individual <i>cyp51A</i> Mutations	
Wild Type Residue	Amino Acid Substitution
N22	D
G54	E, K, R, V, W
G138	C, R, S
P216	L
F219	C, I, L, S
M220	K, I, R, T, V, W
A284	T
<u>I367</u>	F
Y431	C, S
G432	A, C, S
G434	C
T440	A
G448	S
<u>F495</u>	L
Y491	H

Figure 1-3. *cyp51A* mutations associated with triazole resistance.
Residues shown in bold are located along the ligand access channels, residues shown underlined directly interact with voriconazole.

Table 1-1. *cyp51A* mutations by residue and relative impact on triazole MIC.

Triazole*	Ligand Access Channel Residues				Tandem Repeat Associated Residues	
	G54	P216	F219	M220	TR ₃₄ /L98H	TR ₄₆ /Y121F/T289A
Voriconazole	-	-	-	+	+	++
Posaconazole	++	+	+	+	+	+
Itraconazole	++	++	++	++	++	+

*data for isavuconazole not currently available, -; minimal to no impact on MIC, +; increase in MIC, ++; large increase in MIC

believed that this agent specific triazole resistance is a consequence of these amino acid substitutions interfering with stabilizing interactions between the long hydrophobic side chains present in itraconazole and posaconazole, but absent in the other two agents. Conversely, a mutation conferring a tyrosine to phenylalanine substitution at position 121 (Y121F) has been shown to greatly decrease voriconazole susceptibility while having minimal impact on itraconazole or posaconazole.[32, 34] In x-ray crystallography experiments voriconazole has been observed to directly interact with tyrosine 121, though it is unknown if any of the other triazoles also interact with this residue.[15]

In addition to mutations in the open reading frame of the *cyp51A* gene, sequence duplications forming a tandem repeat in the promoter region of *cyp51A* have also been observed.[37] These tandem repeats do not alter the Cyp51A enzyme itself, but significantly increase to level of *cyp51A* gene expression, and in turn the amount of Cyp51A enzyme which must be inhibited by the triazoles to block ergosterol biosynthesis.[26] The impact of these tandem repeat associated mutations on triazole resistance will be further detailed in the subsequent section.

Overexpression of *cyp51A*

The expression of *cyp51A* and *cyp51B* is regulated by a complex system of interacting transcription factors controlled by environmental conditions such as nutrient availability and stress. While differences are known to exist in the regulation of expression of the two sterol-demethylase genes, the majority of research has focused on *cyp51A*, which when overexpressed is known to contribute to clinical triazole resistance in *A. fumigatus*. [9, 26, 34, 35] It remains unknown if overexpression of *cyp51B* may also contribute to clinical resistance, but triazole-resistant clinical isolates of *A. fumigatus* have been observed to demonstrate increased expression of *cyp51B*. [38, 39]

The positive-regulation of *cyp51A* expression is controlled by the sterol regulatory element binding protein SrbA, which binds to the promoter region of *cyp51A* at two specific binding sites as a homodimer (**Figure 1-4A**). One of these two binding sites is negatively-regulated by both the multimeric CCAAT-binding complex (CBC) transcription factor (comprised of HapB, HapC, and HapE) and the iron responsive basic region leucine zipper transcription factor, HapX. It is believed that under environmental conditions that favor repression of ergosterol biosynthesis and thus repression of sterol-demethylase expression, the CBC and HapX cooperatively bind directly upstream of the 3' SrbA binding site and diminish the ability of SrbA to promote *cyp51A* expression. In the absence of either SrbA or the SrbA binding sites in the promoter region of *cyp51A*, *cyp51A* expression is essentially absent and *A. fumigatus* becomes hyper-susceptible to the triazoles.[26]

Two distinct mechanisms leading to overexpression of *cyp51A* have been identified in triazole-resistant clinical isolates of *A. fumigatus*. By far the most prevalent is the formation of a tandem repeat in the promoter region of *cyp51A*. [8, 27] Three

Figure 1-4. Regulation of *cyp51A* expression.

A) Regulation of *cyp51A* expression with Srba homodimers under normal repression of CBC and HapX. B) Regulation of *cyp51A* expression in the presence of a tandem repeat mutation creating two additional Srba binding sites not effectively repressed by the CBC and HapX. C) Regulation of *cyp51A* expression in the presence of CBC containing the mutant HapE^{P88L}, which is unable to effectively repress the Srba mediated expression of *cyp51A*.

versions of this tandem repeat sequence have been observed (TR₃₄, TR₄₆, TR₅₃).[33, 37, 40] Each version differs in the length of the repeated sequence (34- 53 bases), but all result in the formation of two additional SrbA binding sites which are not effectively regulated by the CBC or HapX (**Figure 1-4B**). Thus, *cyp51A* expression is increased by 2-fold or more, and triazole MIC subsequently increase by approximately 4-fold.[26] Additionally, the TR₃₄ and TR₄₆ variations are, with only one known isolate being the exception, universally found in conjunction with specific mutations in the *cyp51A* gene itself, resulting in the amino acid substitutions L98H and Y121F/T289A, respectively.[32] Isolates possessing these mutations are often referred to as either having the TR₃₄/L98H or TR₄₆/Y121F/T289A genotype, and are most commonly observed to be multi- or pan-triazole-resistant. Worryingly, the prevalence of these tandem repeat mutations in both environmental and clinical isolates appears to be increasing around the world.[6, 8, 27, 37, 40]

In addition to alterations of the *cyp51A* promoter region, a single mutation in the *hapE* gene, encoding a subunit of the CBC multimer, has been shown to directly result in increased *cyp51A* expression. This mutation was originally identified in an isogenic pair of clinical isolates where resistance developed on triazole therapy, and results in a P88L amino acid substitution in HapE.[9] CBC containing the mutant HapE^{P88L} subunit bind less effectively to the promoter region of *cyp51A*. Thus, *cyp51A* expression, driven by the unimpeded SrbA, is increased (**Figure 1-4C**). Similar to what is observed with the tandem repeat *cyp51A* genotypes, this mutation in *hapE* leads to a 2-fold or greater increase in *cyp51A* expression and increases in MICs of all triazole that have been tested.[26] While screening for this *hapE* mutation is not commonly performed, to date this mechanism of triazole resistance has only been identified in the original clinical isolates of *A. fumigatus* from which it was discovered.

Overexpression of Drug Efflux Pumps

In other pathogenic fungi, efflux pump overexpression has been shown to contribute to clinical triazole resistance. The *A. fumigatus* genome is predicted to contain more than 300 efflux pumps, many of which have been observed to have their expression induced by exposure to the triazoles.[25] Furthermore, a number of these pumps have been observed to be constitutively overexpressed among triazole-resistant clinical isolates.[25, 41] However, at present relatively little is known about which of these efflux pumps contribute to clinical triazole resistance. In triazole-susceptible laboratory strains of *A. fumigatus*, the individual disruption of six drug efflux pump genes (*abcA*, *abcC*, *atrF*, *atrI*, *mdrA*, and *mdrI*) has been observed to increase triazole susceptibility.[25, 42-46] However, only the efflux pump gene, *abcA*, has been demonstrated to contribute to triazole resistance when constitutively overexpressed in a triazole-susceptible laboratory strain of *A. fumigatus*. [45] Additionally, *abcC* is the only efflux pump gene which has been demonstrated to contribute to clinical triazole resistance. This ABC efflux pump of the G- subfamily is a close homolog of well characterized drug efflux pumps from other species of pathogenic fungi and has been observed to be overexpressed in a number of triazole-resistant clinical isolates of *A. fumigatus*. Disruption of *abcC* in a triazole-

resistant clinical isolate of *A. fumigatus* decreased the itraconazole MIC by greater than 4- fold, while voriconazole and posaconazole susceptibility remained unchanged. This clinical isolate was observed to exhibit increased constitutive expression of *abcC* (>5 fold) relative to the susceptible reference strain.[25] Drug efflux pumps, have therefore been proven to be a mechanism by which triazole resistance is mediated in clinical isolates of *A. fumigatus*. However, the genetic determinants of drug efflux pump overexpression in *A. fumigatus* remains elusive. Recently, the zinc-cluster transcription factor AtrR was found to promote the expression of both *cyp51A* and *abcC*. AtrR is a close homolog of the *Saccharomyces cerevisiae* pleotropic drug response zinc-cluster transcription factor *PDR1*, and *A. fumigatus* strains with *atrR* disrupted exhibit a dramatic reduction in expression of both *abcC* and *cyp51A*. [46] However, the presence of activating mutations in *atrR* among triazole-resistant clinical isolates has yet to be identified, and its direct role in clinical triazole resistance remains unclear.

Emerging Mechanisms of Triazole Resistance

In addition to the aforementioned canonical mechanisms of triazole resistance, additional potential pathways to resistance in *A. fumigatus* are beginning to be identified. The most notable of which are directly linked to the ergosterol biosynthetic pathway. Two different studies investigating mutations acquired in laboratory strains of triazole-susceptible *A. fumigatus* which develop triazole resistance upon triazole exposure have been successful in identifying novel resistance mechanisms. In these experiments, mutations in the *hmg1* gene were found to confer resistance to voriconazole, itraconazole, and posaconazole (isavuconazole was not tested). The *hmg1* gene, encodes an *A. fumigatus* homolog of 3-hydroxy-3-methyl-glutaryl (HMG) coenzyme A reductase, a key enzyme involved in both ergosterol and siderophore biosynthesis.[47] Additionally, a mutation in the *cox10* gene, required for the normal biosynthesis of heme, was found to directly contribute to triazole resistance. Heme is utilized by a number of ergosterol biosynthetic genes, including *cyp51A* and *cyp51B*, to catalyze enzymatic conversion of sterols. The mutation in *cox10* was observed to significantly reduce itraconazole susceptibility, yet voriconazole susceptibility was not clearly impacted.[48] While the roles of these mutations in clinical triazole resistance is currently unknown, these mutations serve to demonstrate the potential impact of non-canonical mechanisms on the clinical utility of the triazole antifungals and future screening for such mutations among clinical isolates of *A. fumigatus* may be merited.

Epidemiology of Triazole Resistance

Triazole resistance among *A. fumigatus* isolates emerged as early as the 1980's in immunocompromised patients from the United States receiving itraconazole as an investigational agent. Subsequently, resistant isolates were recovered from patients in the 1990's in the Netherlands and France. However, in each of these instances, the identification and reporting of triazole resistance followed years after the original clinical cases where the isolates were cultured.

Today, analysis of the prevalence of triazole resistance among *A. fumigatus* remains limited by challenges in isolating *Aspergillus* from clinical specimens, as well as the additional resources and expertise required to perform antifungal susceptibility testing of molds.[49] However, triazole-resistant *A. fumigatus* are nonetheless being reported around the world in increasing numbers.[8, 50-53] A recent study examining the genetic diversity of *A. fumigatus* isolates observed that 6% of the 2,026 isolates obtained from 13 countries spanning four continents, were triazole-resistant.[54] This resistance appears to vary dramatically by both geographic region and patient population.

Both the increase in overall prevalence of triazole resistance and the variation in its distribution are perhaps best exemplified in epidemiology data from the Netherlands, where surveillance has been conducted since the first recovery of resistant clinical isolates. In the Netherlands, triazole resistance was originally observed to be present in 1.8% of *A. fumigatus* isolates collected from 1945 to 1998. Subsequently, examination of resistance rates across seven Dutch university medical centers from 2007- 2009 found the average triazole resistance rate to have risen to 4.6%, with medical center specific rates ranging from 0.8% to 9.5%.[7, 51] No seasonal variation in resistance was observed and there was no clear geographic influence on the distribution of resistance. Importantly, the vast majority of triazole-resistant isolates were resistant to both itraconazole and voriconazole, and the case-fatality rate among patients with triazole-resistant disease was 88%. Triazole resistance has also been observed to be more prevalent among certain high-risk patient populations. When resistance rates were tracked specifically among hematology patients at two Dutch medical centers, the prevalence of triazole resistance was found to be dramatically greater than that of the general population, ranging from 17 to 30%.[55] Similar findings were also reported in a single center study of Dutch intensive care unit patients with invasive aspergillosis, where 26% of isolates were triazole-resistant.5 Overall 90-day mortality of patients with triazole-resistant disease was 100% in this population.

In the United States, triazole resistance among *A. fumigatus* is now being reported in isolates of both clinical and environmental origin.[6, 8, 56] However, only one large, passive, surveillance study of triazole resistance among US *A. fumigatus* isolates has been performed to date. In this study 1,026 isolates were screened and 51 (5%) were found to have itraconazole MIC above the EUCAST breakpoint for susceptible (CLSI has not published triazole breakpoints for *A. fumigatus*).[57] Voriconazole susceptibility was not reported. More recently, a study characterizing triazole-resistant isolates from the US performed extensive antifungal susceptibility testing on isolates collected from 2001 to 2015. Of 26 triazole-resistant *A. fumigatus* clinical isolates tested, all were resistant to both itraconazole and posaconazole (applying EUCAST breakpoints), and only seven and eight, respectively, were susceptible to isavuconazole and voriconazole. Additionally, although the use of agricultural sterol-demethylase inhibitors is less common in the US than other parts of the world, a study conducted by collecting environmental samples from peanut fields being treated with triazole agents found 56 of 200 recovered isolates (28%) were triazole-resistant.[6]

The distribution of identifiable mechanisms contributing to triazole resistance also appears to vary dramatically by geographic region. In the Netherlands, as well as other European and Asian countries such as the United Kingdom and India, the vast majority of triazole-resistant isolates share at least one common resistance mechanism, comprised of specific combinations of mutations in the sterol-demethylase gene *cyp51A* in conjunction with the formation of tandem repeats in the *cyp51A* promoter region (TR₃₄/L98H or TR₄₆/Y121F/T289A genotype).[27] While many of these isolates likely possess additional resistance mechanisms, as suggested by the varying degrees of agent specific triazole resistance, the predominance of the TR₃₄/L98H and TR₄₆/Y121F/T289A genotypes among these resistant isolates cannot be overlooked. Conversely, in the United States only a small number of clinical isolates have been reported to possess this particular resistance mechanism. Of the 26 isolates in the aforementioned characterization of triazole-resistant *A. fumigatus* isolates, only two TR₃₄/L98H and two TR₄₆/Y121F/T289A genotype isolates were found. Yet, 16 isolates possessed other identifiable mutations in *cyp51A*. This discordance in prevalence of the TR₃₄/L98H and TR₄₆/Y121F/T289A genotypes has been linked to differences in the application of triazole related agricultural fungicides. Supporting this link, the TR₃₄/L98H and TR₄₆/Y121F/T289A genotypes are most prevalent among clinical and environmental samples from regions with higher utilization of these fungicides, and in vitro studies have demonstrated rapid acquisition of these and other *cyp51A* mutations upon exposure to agricultural triazoles. Notably, of the 56 triazole-resistant environmental samples from US peanut farms treated with triazoles, 21 (37.5%) were found to possess the TR₃₄/L98H genotype.[6] This has led some experts to call for a reduction in the reliance on agricultural sterol-demethylase inhibitors in an effort to preserve the utility of the clinical agents.[58]

While the identification and study of potential risk factors for triazole-resistant aspergillosis has been limited by the general paucity of susceptibility data available for clinical isolates, a small number of patient populations have been associated with higher prevalence of triazole-resistant aspergillosis. Multiple studies have observed rates of triazole-resistant *A. fumigatus* isolates from hematology-oncology patients in Europe, approximating and even exceeding 20%, however it is important to note that rates more similar to the general prevalence of resistance have been observed at other institutions. While prolonged use of prophylactic triazole is not uncommon in this patient population, these studies have also noted recovery of triazole-resistant isolates from patients with no prior triazole exposure, suggesting other patient factors possibly in conjunction with environmental acquisition of triazole-resistant isolates may be involved.[52, 55, 59, 60] Additionally, two epidemiologic studies examining the rates of triazole-resistant *A. fumigatus* among patients being treated in the intensive care units have also reported higher rates of resistance.[5, 59] Though both of these studies examined patients from medical centers in the Netherlands where environmental rates of triazole resistance are known to be high. It has been suggested that increased rates of triazole resistance among environmental isolates of *A. fumigatus*, may also increase the risk for triazole resistance among cases of aspergillosis.[61] This concept of environmental acquisition of resistant isolates is supported by a report of a patient admitted to the intensive care unit, with no prior history of antifungal drug exposure, suffering from proven invasive aspergillosis

caused by an *A. fumigatus* isolate harboring the TR₄₆/Y121F/T289A genotype. Environmental screening of the patient's home garden and household revealed multiple samples of a genetically indistinguishable and triazole-resistant *A. fumigatus*. [62]

Conclusions

The mold-active triazoles are essential to both prophylaxis and treatment of invasive aspergillosis, and few therapeutic alternatives exist. While the overall prevalence of triazole resistance among clinical isolates of *A. fumigatus* is currently observed to range between 1 and 10%, these estimates are limited by a relative paucity of clinical samples with accompanying susceptibility data. Additionally, the recent dramatic increase in reports of resistant isolates from both clinical and environmental sources worldwide is of clear concern. Mutations in *cyp51A*, overexpression of *cyp51A*, and efflux pump gene overexpression all are known to contribute to resistance, yet much of the triazole resistance among *A. fumigatus* still remains unexplained. Also lacking is clinical experience with therapeutic options for the treatment of triazole-resistant *A. fumigatus* infections, and mortality associated with these infections remains unacceptably high. Thus, further research is greatly needed to both better understand the emerging threat of triazole-resistant *A. fumigatus*, and to develop novel therapeutic strategies to combat these resistant infections.

CHAPTER 2. CHALLENGING THE PARADIGM OF CLINICAL TRIAZOLE RESISTANCE IN *ASPERGILLUS FUMIGATUS**

Introduction

Aspergillus fumigatus is the predominant pathogen isolated from patients with invasive aspergillosis, the most common human invasive mold infection. Invasive aspergillosis is responsible for more than 200,000 life-threatening infections each year, and afflicts up to 10% of patients with acute leukemia as well as patients receiving stem cell or solid organ transplantation.[1, 60, 63] Unfortunately, the mortality rates associated with invasive aspergillosis remain unacceptably high, often exceeding 50%, even when appropriately diagnosed and treated.[3-5, 60]

Contributing to the poor outcomes associated with the treatment of invasive aspergillosis is the relative paucity of clinically available antifungal agents with demonstrated clinical efficacy. Of the four major classes of antifungals, only the triazoles and amphotericin B are recommended as monotherapy for the treatment of aspergillosis, with the latter relegated to second-line or salvage treatment due to significant toxicities and the lack of oral formulations.[10] The triazole class of antifungals therefore have proven essential to the treatment of invasive aspergillosis, comprising both frontline treatment options such as voriconazole and isavuconazole, as well as salvage options such as posaconazole and itraconazole.[10] These agents are generally accepted to exert fungicidal activity against *Aspergillus* through the competitive inhibition of sterol-demethylase, encoded by the genes *cyp51A* (Afu4g06890) and *cyp51B* (Afu7g03740) in *A. fumigatus*, which results in abrogation of the synthesis of ergosterol, the major fungal membrane sterol.[14, 15, 64, 65] However, the treatment of invasive aspergillosis has recently been further complicated by the global emergence of triazole-resistant disease. Over the past decade, triazole resistance among clinical isolates of *A. fumigatus* has been increasingly reported on six continents, with resistance rates exceeding 20% now being reported in some studies.[6-8, 30]

Considerable research has led to the identification of three distinct molecular pathways to clinical triazole resistance in *A. fumigatus*. These three paths largely mirror the established mechanisms of resistance previously identified in other pathogenic fungi such as *Candida albicans*; 1) mutations in the sterol-demethylase gene *cyp51A*, 2) overexpression of the sterol-demethylase gene *cyp51A*, and 3) overexpression of the efflux pump-encoding gene *abcC* [9, 25, 26, 28, 31, 32, 34-36, 66]. However, it remains unknown to what extent these mechanisms explain the degree of triazole-resistance observed among clinical isolates of *A. fumigatus*, and resistant clinical isolates exhibiting

* Modified from final submission with permission. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD and Fortwendel JR. Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. MBio. 2019 Apr 2;10(2). pii: e00437-19. doi: 10.1128/mBio.00437-19.

none of these three mechanisms have been repeatedly identified.[25] Thus, the molecular mechanisms underpinning much of the clinical resistance to this essential class of anti-*Aspergillus* agents remains unexplained, limiting the discovery of therapeutic strategies to overcome triazole-resistant aspergillosis.

In this work, we perform a comprehensive characterization of the direct contributions of previously identified mechanisms of triazole resistance in a large collection of triazole-resistant clinical isolates of *A. fumigatus*. We correct *cyp51A* mutations in 10 different triazole-resistant clinical isolates using a novel Cas9-mediated transformation system to delineate the direct impact these mutations have on clinical triazole susceptibility, and we assess the potential impact of overexpression of *cyp51A*, *cyp51B*, and *abcC* on triazole susceptibility in a collection of 21 triazole-resistant clinical isolates. We subsequently demonstrate that these mechanisms alone fail to explain the resistance observed in this collection, suggesting the presence of additional mechanisms of clinical triazole resistance yet to be identified.

Materials and Methods

Isolates, Media, and Growth Conditions Used

Twenty-one previously characterized multi-triazole-resistant clinical isolates of *A. fumigatus*, and five triazole-susceptible control clinical isolates of *A. fumigatus*, originating from the United States were obtained from the Fungus Testing Laboratory at the University of Texas in San Antonio. The laboratory strains *akuB*^{KU80} and Af293 were obtained from the Fungal Genetics Stock Center. All strains and clinical isolates used in this study were maintained on glucose minimal media (GMM) agar at 37°C. Transformants were selected for using sorbitol (1.2M) supplemented GMM agar (SMM) containing 150mg/L of hygromycin. All conidia were harvested in sterile water from three-day-old growth plates and conidia were enumerated visually using a hemocytometer.

Whole Genome Sequencing

For each isolate, genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit as previously described.[67] DNA concentrations were quantified using both the Qubit Fluorometer and Nanodrop spectrophotometer using the manufacturers' protocols. Whole genome libraries were prepared and sequenced at the University of Tennessee Health Science Center Molecular Resource Center. Bioinformatics services were provided by code4DNA (www.code4DNA.com). Sequence reads for each sample were aligned using the STAR sequence alignment tool (v2.5.0b) to the *A. fumigatus* reference sequence (version A_fumigatus_Af293_version_s03-m05-r05) which was downloaded from <http://aspgd.org/>. Duplicate alignments were marked using Picard (v1.119) and variants were called using Freebayes (v1.1.0) using the haploid population-based model.

The Freebayes output vcf file was split into individual sample vcf files using bcftools (v1.3) and variants for each sample were annotated with snpEff (v4.2) using the *A. fumigatus* reference sequence annotations.

Construction of Promoter Replacement Repair Templates and Cas9-RNP for *cyp51A*, *cyp51B*, and *abcC*

The plasmid pJMR2 was constructed by cloning the proximal 1kb of the promoter sequence from the *A. fumigatus* heat shock protein-encoding gene, *hspA*, into the plasmid pCR-HygR.[67] Then promoter replacement transformation repair templates for *cyp51A*, *cyp51B*, and *abcC* were created by PCR using primers (**Table 2-1**) which amplified both the hygromycin resistance cassette and the *hspA* promoter from the plasmid pJMR2, while also introducing approximately 40 bases of homology targeting sequences immediately upstream and downstream of the start codon for the respective gene of interest. PCR products were subsequently purified using the Gene Clean II Kit (MP Biomedicals). Cas9-RNP complexes targeting sequences immediately upstream of the open reading frame of each gene of interest were assembled as previously described.[67]

Assessment *cyp51A*, *cyp51B*, and *abcC* Expression by RTqPCR

For assessment of expression in clinical isolates, conidia from each isolate were allowed to germinate overnight in *Aspergillus* minimal media incubated at 37°C on an orbital shaker at 250 RPM. For assessment of expression in constitutive overexpression strains constructed in the *akuB*^{KU80} background, conidia from each isolate were allowed to germinate overnight in *Aspergillus* minimal media incubated at 37°C on an orbital shaker at 250 RPM, then transferred to fresh *Aspergillus* minimal media either with voriconazole 0.125mg/L (treated) or without voriconazole (untreated) for an additional 6 hours at 37°C on an orbital shaker at 250 RPM. Then RNA was extracted from mature hyphae following liquid nitrogen crush as previously described.[68] The RevertAid RT kit (Thermo Scientific) was utilized to synthesize cDNA. PCR master mix and SYBR were utilized to amplify *A. fumigatus cyp51A*, *cyp51B*, and *abcC* from cDNA by PCR per the manufacturer's instructions. **Table 2-1** lists the gene specific primers used for PCR. Conditions used for PCR were as follows: AmpliTaq Gold activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds and annealing/ extension at 60°C. The dissociation curve and threshold cycle (CT) was determined using the CFX96 Real Time PCR system (Bio-Rad). Changes in gene expression among isolates were then calculated using the 2- $\Delta\Delta$ CT method. All experiments were performed in triplicate from biological triplicates. As previously described, Δ CT values were used to calculate the standard error.[68] Statistical analysis was performed using unpaired, two-tailed, students t-test in Prism 8 for Mac OS by GraphPad Software Inc. with significance set at 0.05 and degrees of freedom equal to 10.

Table 2-1. Oligonucleotides used for *cyp51A*, *cyp51B*, and *abcC* experiments.

Oligo Type	Gene	Primer Name	Sequence (5'- 3')
PCR Primers			
	<i>cyp51A</i>	<i>cyp51A</i> KI F CRISPR	GAATACTGGGCAGCGGGCTGGAGATACT ATGGCTTTCATATGTTGCTCAGCGTCAGC ATTCTGAAACACG
	<i>cyp51A</i>	<i>cyp51A</i> KI R CRISPR	CCAGTAAGGTCTGAATAAGGGTTCAATA CAGTCATTTATTAGGCCCTCGATTTCGAGC TCCCAAATCTGTCCAG
	<i>cyp51A</i>	<i>cyp51A</i> OE <i>hspA</i> F	CATTTCCCTCATCACTGCAACTCTAATCC TCGGGCTCACCCAGCTTGCATGCCTGCA GG
	<i>cyp51A</i>	<i>cyp51A</i> OE <i>hspA</i> R	ACGGCCATGTAGGCCGTAAGCCATAGCA TCGGCACCATGCTTGGTACCTGTGAAGA AGTG
	<i>cyp51B</i>	<i>cyp51B</i> OE <i>hspA</i> F	CTACCTTTATTCCCTGCGACAGTAACTCG GTTGATCGATTTGAGCTTGCATGCCTGCA GG
	<i>cyp51C</i>	<i>cyp51B</i> OE <i>hspA</i> R	TTGCAGATGCCGTCGAGAATGAACGCGA TGAGACCCATGCTTGGTACCTGTGAAGA AGTG
	<i>abcC</i>	<i>abcC</i> OE <i>hspA</i> F	GTTTCGACTGTTTATGTGTATACCTATTGT GACGCAACCACTCAGCTTGCATGCCTGC AGG
	<i>abcC</i>	<i>abcC</i> OE <i>hspA</i> R	GGGTTGATGTTAGGATTATCGTCCCTAG AAGAGACATGCTTGGTACCTGTGAAGAA GTG
	<i>hphR</i>	<i>hphR</i> screen R	CAAATCGCCCGCAGAAGCG
crRNA Guide Sequences			
	<i>cyp51A</i>	<i>cyp51A</i> 5' crRNA	GGCTTTCATATGTTGCTCAG
	<i>cyp51A</i>	<i>cyp51A</i> 3' crRNA	TTCAAAGGATTTGGTGTGAT
	<i>cyp51A</i>	<i>cyp51B</i> 5' OE crRNA	CTCCCTGTGTCTCCTCGAAA
	<i>cyp51B</i>	<i>cyp51B</i> 5' OE crRNA	TATTCCTGCGACAGTAACT
	<i>abcC</i>	<i>abcC</i> 5' OE crRNA	GACTGAGTGTCTCACTCAAT
RTqPCR Primers			
	<i>cyp51A</i>	<i>cyp51A</i> RT F e1	CTTCTTTGCGTGCAGAGA
	<i>cyp51A</i>	<i>cyp51A</i> RT R e2	GGGGTCGTCAATGGACTA
	<i>cyp51B</i>	<i>cyp51B</i> RT F e1	CTTTTTCGACTGCCGCGC
	<i>cyp51B</i>	<i>cyp51B</i> RT R e2	AGGCGTAGTGAGTGGAGA
	<i>abcC</i>	<i>abcC</i> RT F e1	CCTGGAGAAGGTCTCAATGTCTG
	<i>abcC</i>	<i>abcC</i> RT R e2	GGCCAGACGTAGGCTCATCC

Construction of Allele Replacement Repair Templates and Cas9-RNP for the *cyp51A* Locus

The plasmid pCyp51A-HygR was created by cloning the proximal 1.5kb of promoter sequence and open reading frame of the wild-type *cyp51A* allele from the genomic reference strain Af293, upstream of the hygromycin resistance cassette in the plasmid pCR-HygR, and then cloning the proximal 1.5kb of the wild-type *cyp51A* terminator downstream of the hygromycin resistance cassette. Then, the *cyp51A*^{WT} transformation repair template was created by PCR using primers which amplified the wild-type *cyp51A* allele (including 500 bases of promoter sequence), the hygromycin resistance cassette, and 500 bases of the *cyp51A* terminator. Cas9-RNP complexes targeting sequences immediately upstream and approximately 500 bases downstream of the open reading frame of *cyp51A* were assembled as previously described.[67] Target specific guide sequences are listed in **Table 2-1**.

Aspergillus Protoplast Transformations

Transformation of *A. fumigatus* protoplasts were performed as previously described with minor modifications.[67] Approximately 2µg of each portion applicable transformation repair templates were then mixed with 200 µL of protoplasts, 26.5 µL of Cas9-RNP complexes, and 25 µL of polyethylene glycol (PEG)-CaCl₂ buffer (60% [wt/vol] PEG 3350, 50 mM CaCl₂·H₂O, 450 mM Tris-HCl, pH 7.5) as previously described. Following an incubation on ice for approximately one hour, 1.25mL PEG-CaCl₂ was added and the mixture incubated for an additional 20 minutes at room temperature. The mixture was then diluted to a total volume of 3 mL with STC buffer (1.2 M sorbitol, 7.55 mM CaCl₂·H₂O, 10 mM Tris-HCl, pH 7.5) and plated on SMM agar plates. Plates were then incubated overnight prior to being overlaid with SMM top agar (GMM supplemented with 1.2 M sorbitol and 0.7% [wt/vol] agar) supplemented with hygromycin (final concentration of 150 g/ml), and the plates were incubated at 37°C for 3 days. Transformants were isolated to separate plates containing selective agar, genomic DNA extracted, and successful CRISPR/Cas9 editing confirmed by PCR screens using gene-specific primers and Sanger sequencing.

Clinical Antifungal Susceptibility Testing

Susceptibilities for amphotericin B, voriconazole, isavuconazole, itraconazole, and posaconazole were determined for all isolates in accordance with CLSI M38-A2 methodology utilizing broth microdilution in RPMI[69]. Each antifungal was obtained from the appropriate manufacturer. All agents were suspended in dimethyl sulfoxide (DMSO) for preparation of stock solutions.

Data Availability

Whole genome sequencing data files for the 26 *Aspergillus fumigatus* isolates have been deposited to NCBI SRA under the accession number PRJNA491253.

Results

Mutations in Sterol-Demethylase Alone Poorly Explain Clinical Triazole Resistance

Mutations in the sterol-demethylase gene *cyp51A* are the most frequently identified mechanism of triazole resistance in *A. fumigatus*. [5, 8, 70] We sought to delineate the proportion of triazole resistance observed in individual clinical isolates explained by this mechanism. To accomplish this, a collection of 26 clinical isolates of *A. fumigatus* originating from the United States were obtained from the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio, including 21 multi-triazole-resistant isolates with previously reported clinical origins and characterized *cyp51A* genotypes, and 5 triazole-susceptible control isolates. [8] High quality genomic DNA was extracted from each of the 26 isolates, whole genome sequencing was performed targeting a read depth of approximately 100 reads per base aligned to the Af293 *A. fumigatus* reference genome assembly, and a total of 48,552 SNPs, insertion or deletions (indels) unique to triazole-resistant isolates were identified within 8,669 genes with annotations available. Of the 21 triazole-resistant isolates in this collection, 16 were identified as possessing peptide sequence altering *cyp51A* mutations unique to resistant isolates. Conversely, no unique *cyp51B* mutations were identified. We selected 10 of these 16 isolates with *cyp51A* mutations, which also exhibited resistance to all clinically available triazole agents, for correction of the *cyp51A* sequence to that of the wild-type consensus sequence. These isolates included examples of previously characterized *cyp51A* mutations (TR₃₄/L98H, TR₄₆/Y121F/T289A, G448S), mutations at residues where alternative mutations have previously been characterized (G138C and F219S), and mutations never before characterized to our knowledge (M263I and I367F) (Table 2-2). [28, 34, 35, 48, 71]

In order to test the direct contribution of these mutations to clinical triazole resistance in these isolates, the wild-type *cyp51A* promoter region and open reading frame from the triazole-susceptible reference strain Af293 was first cloned upstream of the hygromycin resistance cassette in plasmid pCR-HygR. [67] 1500 bases of the *cyp51A* terminator was then cloned downstream of the hygromycin resistance cassette, yielding the plasmid pCyp51A-HygR. Then, the *cyp51A*^{WT} transformation repair template was created by PCR using primers which amplified the wild-type *cyp51A* allele (including 500 bases of promoter sequence), the hygromycin resistance cassette, and 500 bases of the *cyp51A* terminator. Importantly, this repair template extends approximately 200 bases upstream of the promoter region where characterized regulatory elements and tandem repeat sequences have been identified. [26] Protoplasts of each of the 10 triazole-resistant clinical isolates, as well as the well-characterized triazole-susceptible laboratory strain

Table 2-2. Triazole MIC of clinical isolates and derivative *cyp51A*^{WT} strains.

Parent Clinical Isolate or Derivative <i>cyp51A</i> ^{WT} Strain	<i>cyp51A</i> Genotype	Triazole MIC (mg/L)		
		Voriconazole	Itraconazole	Posaconazole
DI15-117	F219S	2	≥32	2
DI15-117 <i>cyp51A</i> ^{WT}	WT	4	≥32	4
DI15-100	M263I	8	≥32	1
DI15-100 <i>cyp51A</i> ^{WT}	WT	8	≥32	0.5
DI15-116	TR ₃₄ /L98H	8	≥32	1
DI15-116 <i>cyp51A</i> ^{WT}	WT	1	0.25	0.125
DI15-102	TR ₃₄ /L98H	8	≥32	2
DI15-102 <i>cyp51A</i> ^{WT}	WT	4	≥32	0.5
DI15-108	G138C	4	≥32	1
DI15-108 <i>cyp51A</i> ^{WT}	WT	4	≥32	0.125
DI15-95	M263I	16	≥32	2
DI15-95 <i>cyp51A</i> ^{WT}	WT	16	≥32	1
DI15-98	I367F	16	16	2
DI15-98 <i>cyp51A</i> ^{WT}	WT	16	≥32	1
DI15-96	TR ₄₆ /Y121F/T289A	≥32	4	1
DI15-96 <i>cyp51A</i> ^{WT}	WT	≥32	0.5	0.25
DI15-106	TR ₄₆ /Y121F/T289A	≥32	4	1
DI15-106 <i>cyp51A</i> ^{WT}	WT	8	2	0.5
DI15-120	G448S	≥32	≥32	1
DI15-120 <i>cyp51A</i> ^{WT}	WT	≥32	≥32	1

MIC shown in bold are greater than epidemiologic cutoff values as published by the Clinical Laboratory and Standards Institute (1mg/L for voriconazole and itraconazole; 0.25mg/L for posaconazole). WT: *cyp51A* sequence matches the Af293 wild-type consensus sequence.

akuB^{KU80} (CEA17 Δ *akuB*^{KU80}) which has a wild-type *cyp51A* allele, were then transformed with Cas9-ribonucleoprotein (RNP) complexes, consisting of Cas9 protein and crRNA-trRNA duplexes targeting sequences immediately upstream and downstream of the *cyp51A* locus. The resulting transformants were selected on *Aspergillus* minimal media containing hygromycin as previously described.[67, 72] Integration of the repair template at the native *cyp51A* locus was confirmed by PCR, and acquisition of the *cyp51A*^{WT} sequence was verified by Sanger sequencing.

Clinical susceptibility testing was performed for each of the 10 triazole-resistant clinical isolates and the corresponding *cyp51A*^{WT} derivative strains for voriconazole, itraconazole, and posaconazole according to the Clinical Laboratory Standards Institute M38-A2 standards (Table 1).[69] All of the resistant clinical isolates exhibited triazole minimum inhibitory concentrations (MIC) consistent with those previously reported for these isolates under clinical susceptibility testing conditions, and all MIC for each agent tested were above epidemiologic cut-off values previously reported by CLSI (1mg/L for voriconazole and itraconazole, 0.25mg/L for posaconazole).[8, 73] Among the *cyp51A*^{WT} strains, only one strain (DI15-116 *cyp51A*^{WT}) was observed to have full restoration of wild-type triazole susceptibility, and two strains (DI15-96 *cyp51A*^{WT} and DI15-108 *cyp51A*^{WT}) were observed to have restoration of wild-type susceptibility to at least one triazole. Additionally, two strains (DI15-102 *cyp51A*^{WT} and DI15-106 *cyp51A*^{WT}) were observed to have at least one triazole MIC decrease by more than one dilution. Five of the 10 *cyp51A*^{WT} derivative strains exhibited no change in susceptibility to any triazole agent tested. Importantly, replacement of the native wildtype *cyp51A* allele with the *cyp51A*^{WT} cassette in the *akuB*^{KU80} background had no effect on triazole MIC. Taken together, these data demonstrate that while mutations in *cyp51A* are common among triazole-resistant clinical isolates of *A. fumigatus*, and a number of these mutations have been demonstrated to increase triazole MIC when introduced into a triazole susceptible background, mutations in *cyp51A* alone poorly explain the high level of triazole resistance observed in this collection of clinical isolates.

Analysis of the Expression of the Sterol-Demethylases Genes *cyp51A* and *cyp51B* Among Triazole-Resistant Clinical Isolates.

Triazole-resistant clinical isolates of *A. fumigatus* exhibiting overexpression of *cyp51A* and *cyp51B*, relative to susceptible comparator isolates, have previously been reported.[25, 39] Additionally, both the formation of tandem repeats (TR₃₄, TR₄₆, and TR₅₃) in the promoter region of *cyp51A* and a mutation (encoding the amino acid substitution P88L) in *hapE*, a CCAAT-binding complex protein-encoding gene which participates in the negative regulation *cyp51A* expression, have been shown to increase both *cyp51A* expression and triazole MICs.[9, 26, 34] Following the observation that mutations in *cyp51A* alone do not explain the degree of triazole resistance observed in this collection of clinical isolates, we sought to evaluate the potential contributions of sterol-demethylase gene overexpression to clinical triazole resistance. To accomplish this, each of the 26 clinical isolates in our collection were grown overnight at 37 °C in *Aspergillus* minimal media in biological triplicate, and RNA was extracted from mature

hyphae following liquid nitrogen crush as previously described.[68] Reverse transcription quantitative-PCR (RTqPCR) was then utilized to quantify the relative expression of both *cyp51A* and *cyp51B* in each of the 21 triazole-resistant clinical isolates as compared to the expression level observed among the 5 triazole-susceptible control isolates.

The expression of *cyp51A* and *cyp51B* in all clinical isolates was compared to the triazole-susceptible isolate DI16-6 (voriconazole MIC 0.5 mg/L). Among triazole-susceptible control isolates, the expression of *cyp51A* ranged from 0.5 to 1.6-fold that of isolate DI16-6 (**Figure 2-1A**), with isolates DI16-5, DI16-7, and DI16-9 exhibiting statistically significant lower expression of *cyp51A*. While the expression of *cyp51A* among triazole-resistant clinical isolates ranged from 0.7 to 3.5 –fold that of the susceptible comparator isolate. Twelve of the 21 triazole-resistant isolates were observed to have *cyp51A* expression that exceeded the highest expression level observed among triazole-susceptible isolates. Of these 12 isolates, 8 were found to have *cyp51A* expression levels statistically greater than the susceptible comparator isolate. This included 2 isolates (DI15-102 and DI15-116) which possess the TR₃₄/L98H *cyp51A* mutation, 2 isolates (DI15-96 and DI15-106) which possess the TR₄₆/Y121F/T289A *cyp51A* mutation, and one isolate (DI15-105) which possesses the previously characterized P88L-encoding mutation in *hapE*. However, no previously characterized mutations known to contribute to *cyp51A* overexpression, were identified in the remaining 3 isolates. The expression of *cyp51B* among the triazole-susceptible control isolates ranged from 0.7 to 1.3-fold that of isolate DI16-6 (**Figure 2-1B**), with isolate DI16-8 exhibiting statistically greater expression of *cyp51B* (1.3 fold). The expression of *cyp51B* among triazole-resistant clinical isolates ranged from 0.7 to 2.1-fold that of the susceptible comparator isolate, with 4 isolates (DI15-95, DI15-97, DI15-102 and DI15-108) exhibiting expression of *cyp51B* exceeding that of the highest expression level observed among triazole-susceptible isolates, 3 of which (DI15-95, DI15-102 and DI15-108) were statistically significant.

Constitutive Overexpression of *cyp51B* Decreases Triazole Susceptibility

Overexpression of *cyp51A* has previously been shown to contribute to triazole resistance, but it remains unknown if overexpression of the paralogous gene, *cyp51B*, has a similar effect on triazole susceptibility. We sought to employ a novel Cas9-RNP mediated promoter replacement system to delineate the direct impact of constitutive overexpression of *cyp51B* on triazole MIC. To accomplish this, the previously characterized, strong, constitutive promoter from the heat shock protein encoding gene, *hspA* (Afu1g07440), was cloned upstream of the hygromycin resistance cassette in the plasmid pCR-HygR.[74] A transformation repair template for the *cyp51B* promoter replacement was then constructed by amplifying the *hspA* promoter and hygromycin resistance cassette from the resulting plasmid (pJMR2) with primers that introduced approximately 50 bases of homology for the 3' end of the native promoter region and the 5' end of the *cyp51B* open reading frame. Protoplasts of the well-characterized triazole-

Figure 2-1. Relative expression of *cyp51A* and *cyp51B* among triazole-resistant clinical isolates.

A) Expression of *cyp51A* in clinical isolates relative to triazole-susceptible isolate DI16-6, identified mutations known to contribute to *cyp51A* overexpression are shown on the corresponding bar **B)** expression of *cyp51B* in clinical isolates relative to triazole-susceptible isolate DI16-6; Triazole-susceptible isolates shown as open bars, triazole-resistant isolates shown as filled bars. Triazole-resistant isolates shown in order of escalating voriconazole MIC from left to right. Horizontal dotted lines denote highest and lowest expression level observed among the 5 triazole-susceptible isolates. Comparisons with the susceptible control isolate DI16-6 with statistically significant ($p < 0.05$) results are noted with an Asterix.

susceptible laboratory strain *akuB*^{KU80} were then transformed with Cas9-RNP complexes, consisting of Cas9 protein and crRNA-trRNA duplexes targeting sequences immediately upstream of the *cyp51B* start codon, yielding the strain *akuB*^{KU80}*_P_{hspA}cyp51B* (**Figure 2-2**).[75] As a comparator, the same methodology was employed to create the constitutive *cyp51A* overexpressing strain, *akuB*^{KU80}*_P_{hspA}cyp51A*.

The relative expression of both *cyp51A* and *cyp51B* in the strains *akuB*^{KU80}*_P_{hspA}cyp51A* and *akuB*^{KU80}*_P_{hspA}cyp51B* was then compared to the parental *akuB*^{KU80} strain under both normal growth conditions and following voriconazole treatment. Conidia from each strain were grown in *Aspergillus* minimal media at 37 °C until germination, and then were transferred to fresh *Aspergillus* minimal media containing either no voriconazole, or 0.125mg/L of voriconazole (half of the MIC of the parental *akuB*^{KU80} strain) for an additional 6 hours at 37 °C. RNA was extracted from each sample as previously described and RTqPCR was performed. The expression of *cyp51B* in *akuB*^{KU80} was 2-fold higher after treatment with voriconazole than in untreated conditions (**Figure 2-3A**). Expression of *cyp51B* in the *akuB*^{KU80}*_P_{hspA}cyp51B* strain was 35.9-fold higher under untreated conditions, and 36.9-fold higher following voriconazole exposure, as compared to the expression measured in the parental *akuB*^{KU80} under untreated conditions. This increased expression was statistically greater than in the parental strain under both treated and untreated conditions. The expression of *cyp51B* in the *akuB*^{KU80}*_P_{hspA}cyp51A* strain approximated that of *akuB*^{KU80} under both untreated and voriconazole-treated conditions. The expression of *cyp51A* in *akuB*^{KU80} was 4.1-fold higher following treatment with voriconazole as compared to untreated conditions. Expression of *cyp51A* in the *akuB*^{KU80}*_P_{hspA}cyp51A* strain was 14.1-fold higher under untreated conditions, and 18.5-fold higher following voriconazole exposure, as compared to the expression measured in the parental *akuB*^{KU80} under untreated conditions. This increased expression was statistically greater than in the parental strain under both treated and untreated conditions. The expression of *cyp51A* in the *akuB*^{KU80}*_P_{hspA}cyp51B* strain was similar to that of *akuB*^{KU80} under both untreated and voriconazole-treated conditions.

Upon confirmation of the constitutive overexpression of *cyp51A* and *cyp51B* in the *akuB*^{KU80}*_P_{hspA}cyp51A* and *akuB*^{KU80}*_P_{hspA}cyp51B* strains, respectively, voriconazole MIC were performed in triplicate according to CLSI M38-A2 standards.[69] The parental *akuB*^{KU80} strain exhibited a voriconazole MIC of 0.25mg/L. The *akuB*^{KU80}*_P_{hspA}cyp51A* strain was observed to have a 2-fold increase in voriconazole MIC (0.5mg/L), which is similar to the decrease in susceptibility previously reported when expression of this gene is increased as a result of either the formation of tandem repeats in the *cyp51A* promoter or a mutation in *hapE* (**Figure 2-3B**)[9, 26, 34, 35]. Interestingly, the voriconazole MIC of the *akuB*^{KU80}*_P_{hspA}cyp51B* strain was 1mg/L, 4-fold higher than the parental isolate. Taken together, these results demonstrate that overexpression of either sterol-demethylase gene can decrease triazole susceptibility. However, even when the expression of either of these genes increase by as much as 36.9-fold, MIC did not exceed the epidemiological cutoff-value for voriconazole reported by CLSI (1mg/L).[73]

Figure 2-2. Schematic of Cas9-RNP mediated promoter replacement system. Constitutive overexpression repair templates were amplified from pJMR2 using primers incorporating microhomology targeting genes of interest (GOI), then mixed with Cas9-RNP targeting immediately upstream of the start codon of the GOI, and protoplasts created from the strain *akuB*^{KU80}.

Figure 2-3. Relative expression of *cyp51A* and *cyp51B*, and voriconazole MIC in promoter replacement strains *akuB*^{KU80}_P_{hspA}*cyp51A* and *akuB*^{KU80}_P_{hspA}*cyp51B*. The expression of A) *cyp51A* and *cyp51B* in *akuB*^{KU80}, *akuB*^{KU80}_P_{hspA}*cyp51A*, and *akuB*^{KU80}_P_{hspA}*cyp51B* was determined by RTqPCR following growth in *Aspergillus* minimal media at 37 °C both with voriconazole (0.125mg/L) treatment for 6 hours, and without voriconazole treatment. The expression level for each sample is shown relative to that of the respective gene in *akuB*^{KU80} without voriconazole treatment. B) Voriconazole MIC for *akuB*^{KU80}, *akuB*^{KU80}_P_{hspA}*cyp51A*, and *akuB*^{KU80}_P_{hspA}*cyp51B*. Comparisons with statistically significant ($p < 0.05$) results are noted with an Asterisk, while those that are not are noted with “ns”.

Analysis of the Expression of the Efflux Pump-Encoding Gene *abcC* Among Triazole-Resistant Clinical Isolates

In addition to sterol-demethylase-mediated mechanisms of triazole resistance, increased constitutive expression of the ATP-binding cassette efflux pump encoding gene, *abcC* (Afu1g14330; also known as *abcB*, *cdr1B*, and *abcG1*), has been associated with triazole resistance in *A. fumigatus*. [25, 48, 66] Furthermore, deletion of *abcC* in both triazole-susceptible laboratory strains of *A. fumigatus*, as well as a triazole-resistant clinical isolate, has been shown to increase triazole susceptibility. [25, 48, 66] As *cyp51A* and *cyp51B*-mediated mechanisms alone do not adequately explain the high level of triazole-resistance in this collection of clinical isolates, we sought to characterize the expression of *abcC* and its potential impact on triazole susceptibility. To accomplish this, each of the 26 clinical isolates in our collection were grown overnight at 37 °C in *Aspergillus* minimal media in biological triplicate, and RNA was extracted as previously described. RTqPCR was then utilized to quantify the relative expression of *abcC* in each of the 21 triazole-resistant clinical isolates as compared to the expression level observed among the 5 triazole-susceptible control isolates.

The expression of *abcC* in all clinical isolates was compared to the triazole-susceptible isolate DI16-6. Among triazole-susceptible control isolates, the expression of *abcC* ranged from 0.7 to 2-fold that of isolate DI16-6 (**Figure 2-4A**), with both isolates DI16-5 and DI16-8 exhibiting statically greater *abcC* expression (1.3 and 2.0-fold respectively) than DI16-6. The expression of *abcC* among triazole-resistant clinical isolates ranged from 1.2 to 33.2-fold that of the susceptible comparator isolate. Nineteen of the 21 triazole-resistant isolates were observed to have *abcC* expression that was statistically greater than the expression observed in the susceptible control isolate DI16-6, 14 of which exhibited *abcC* expression which also exceeded the highest expression level observed among triazole-susceptible isolates (2-fold). While the majority of these 14 isolates exhibited modestly elevated *abcC* expression (2.4 to 4.1-fold that of the comparator susceptible isolate) isolates DI15-110, DI15-120, and DI15-106 exhibited markedly higher levels of *abcC* expression (6.5, 10.4, and 33.2-fold, respectively). Notably, the pan-triazole-resistant isolate DI15-110, which exhibited the highest level of *abcC* expression, possesses no mutations in *cyp51A* and did not exhibit increased constitutive expression of either sterol-demethylase gene.

As previous studies associating increased triazole resistance to *abcC* have entirely relied upon deletion of the *abcC* gene among either clinical or laboratory isolates of *A. fumigatus*, we next sought to utilize our Cas9-RNP mediated promoter replacement system to delineate the direct impact of constitutive overexpression of *abcC* on triazole MIC. To accomplish this, protoplasts of the triazole-susceptible laboratory strain *akuB*^{KU80} were transformed with Cas9-ribonucleoprotein (RNP) complexes, consisting of Cas9 protein and crRNA-trRNA duplexes targeting sequences immediately upstream of the *abcC* start codon, as well as the *P_{hspA}abcC* transformation repair template which contains both the *hspA* promoter and hygromycin resistance cassette flanked by 50-bases of micro-homology targeting the *abcC* locus, yielding the strain *akuB*^{KU80}*-P_{hspA}abcC*. Subsequently, the relative expression of *abcC* in both *akuB*^{KU80}*P_{hspA}abcC* and the

Figure 2-4. Relative expression of *abcC* in triazole-resistant clinical isolates and the constitutive overexpression strain *akuB*^{KU80}_P_{hspA}*abcC*.

Triazole-susceptible isolates shown as open bars, triazole-resistant isolates shown as filled bars. Triazole resistant isolates shown in order of escalating voriconazole MIC from left to right. Horizontal dotted lines denote highest and lowest expression level observed among the 5 triazole-susceptible isolates. a) expression of *abcC* relative to triazole-susceptible isolate DI16-6. b) The expression of *abcC* in strains *akuB*^{KU80} and *akuB*^{KU80}_P_{hspA}*abcC* was determined by RTqPCR following growth in *Aspergillus* minimal media at 37 °C both with voriconazole (0.125mg/L) treatment for 6 hours, and without voriconazole treatment. The expression level for each sample is shown relative to that of the respective gene in *akuB*^{KU80} without voriconazole treatment. Comparisons with statistically significant ($p < 0.05$) results are noted with an Asterix, while those that are not are noted with “ns”.

parental strain *akuB*^{KU80} was assessed by RTqPCR following growth in *Aspergillus* minimal media at 37 °C both with voriconazole treatment (0.125mg/L) for 6 hours and without.

In the parental *akuB*^{KU80} strain, *abcC* expression was significantly (9.7-fold) higher following voriconazole treatment than was observed without voriconazole treatment (**Figure 2-4B**). By comparison, the *akuB*^{KU80} *P_{hspA}abcC* strain exhibited 7.8-fold and 10.4-fold *abcC* expression, under untreated and voriconazole-treated conditions, respectively, as compared to the expression of *abcC* measured in the parental *akuB*^{KU80} under untreated conditions. *abcC* expression was not found to be significantly different between *akuB*^{KU80} *P_{hspA}abcC* and the parental strain when both had been treated with voriconazole for 6 hours. Surprisingly, even with an approximate 10-fold increase in the constitutive expression of *abcC*, the *akuB*^{KU80} *P_{hspA}abcC* strain did not demonstrate any change in voriconazole susceptibility relative to the parental *akuB*^{KU80} strain (MIC 0.25mg/L).

Conclusions

In this work, we performed a comprehensive characterization of the direct contributions of previously identified mechanisms of triazole resistance in a collection of triazole-resistant clinical isolates of *A. fumigatus*. We observed that correction of *cyp51A* mutations in 10 pan-triazole-resistant clinical isolates only restored wild-type susceptibility, to even a single triazole agent, in 3 isolates. We then demonstrated that while constitutive overexpression of *cyp51A*, and to a lesser extent *cyp51B*, was common among isolates in this collection, increasing expression of either sterol-demethylase gene by as much as 18 to 36-fold in the triazole-susceptible *akuB*^{KU80} background, had a limited effect on triazole MIC. Analogously, we then observed that while the expression of the ABC-type efflux pump-encoding gene *abcC* was higher among 14 of the triazole-resistant isolates in this collection, constitutive overexpression of this gene in the *akuB*^{KU80} background did not have any effect on voriconazole MIC.

Taken together, these data serve to demonstrate that while the canonical mechanisms of triazole resistance, even when considered collectively, do not adequately explain the triazole resistance observed in this collection of *A. fumigatus* clinical isolates, and additional mechanisms yet to be identified are likely present in this collection.

CHAPTER 3. THE *ASPERGILLUS FUMIGATUS* EFFLUX PUMP-ENCODING GENES *ABCA* AND *ATRI* ARE NOT SIGNIFICANT CONTRIBUTORS TO CLINICAL TRIAZOLE RESISTANCE*

Introduction

Aspergillus fumigatus is the principal etiological agent of invasive aspergillosis, a devastating disease state responsible for more than 200,000 life-threatening infections each year, and predominantly afflicting the expanding population of immunocompromised patients.[1, 2] Unfortunately, even among patients appropriately diagnosed and receiving the best available treatment, clinical outcomes associated with invasive aspergillosis remain poor with mortality rates ranging from 30-90%.[3-5, 60]

Potentially contributing to these poor outcomes, is the paucity of therapeutic options for the treatment of invasive aspergillosis. Of the relatively limited armamentarium of clinically available antifungals, only the triazoles, the echinocandins, and the polyene amphotericin B, are commonly utilized for the treatment of invasive aspergillosis. However, amphotericin B is most often recommended as salvage or second-line therapy, because of both limitations in available formulations and a less favorable safety profile, and the echinocandins are most frequently used as adjunctive therapy due to a lack of fungicidal activity against *Aspergillus*. [10] Thus, the anti-*Aspergillus* triazoles, including voriconazole, isavuconazole, itraconazole, and posaconazole, have proven essential to the treatment of invasive aspergillosis, and are commonly relied upon for both front-line and salvage therapies. Yet, the emergence isolates of *Aspergillus fumigatus* from both clinical and environmental samples, represents a critical threat to the future utility of this crucial class of anti-*Aspergillus* agents.[5, 6, 8, 37, 52]

Over the last several years, the identification of triazole-resistant *A. fumigatus* has been increasingly reported worldwide. In certain regions such as northern Europe, where the rate of triazole-resistant *A. fumigatus* had previously approximated the global average of 5%, resistance rates exceeding 15% are now being reported.[7, 51, 55] While a growing body of evidence supports the agricultural use of antifungals as a significant contributor to this emergence of resistance, the underlying molecular mechanisms of triazole resistance are less well understood.[6, 76-78] The majority of the molecular mechanisms of triazole resistance which have been characterized to date involve the genes encoding sterol-demethylase, the enzymes which the triazoles competitively inhibit.[79] A number of mutations encoding amino acid substitutions in one of the *A. fumigatus* sterol-demethylase enzymes, Cyp51A, are known to decrease triazole susceptibility when introduced into susceptible laboratory strains of *A. fumigatus*. [79]

* Modified from final submission with permission. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD and Fortwendel JR. Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. MBio. 2019 Apr 2;10(2). pii: e00437-19. doi: 10.1128/mBio.00437-19

Additionally, mutations altering both cis- and trans-regulatory elements controlling the expression of *cyp51A* have also been shown to contribute to triazole resistance. However, in our previous studies we have demonstrated that mutations in *cyp51A* and overexpression of sterol demethylase genes cannot explain the high level of triazole resistance observed among many clinical isolates, suggesting the contribution of additional mechanisms not yet identified.

Another common mechanism of triazole antifungal resistance in pathogenic fungi is the up-regulation of efflux pump-encoding gene expression. The *A. fumigatus* genome is predicted to contain more than 300 efflux pump-encoding genes, many of which have been observed to have their expression induced by exposure to the triazoles.[25, 42-44, 66] Furthermore, a number of these transporters have been observed to be constitutively overexpressed among triazole resistant clinical isolates.[25] In triazole-susceptible laboratory strains of *A. fumigatus*, the individual disruption of six efflux pump genes (*abcA*, *abcC*, *atrF*, *atrI*, *mdr1*, and *mdrA*) has been observed to increase triazole susceptibility. [25, 42-44, 66] However, only the small molecule transporter gene, *abcA*, has been demonstrated to contribute to triazole resistance when constitutively overexpressed in a triazole-susceptible laboratory strain of *A. fumigatus*. Additionally, *abcC* (also called *cdr1B*, *abcB*, and *abcG1*) is the only transporter gene which has been demonstrated to contribute to clinical triazole resistance. Disruption of *abcC* in a triazole-resistant clinical isolate of *A. fumigatus* decreased the itraconazole minimum inhibitory concentration (MIC) by greater than 4-fold, while voriconazole and posaconazole susceptibility remained unchanged. This clinical isolate was observed to exhibit increased constitutive expression of *abcC* (>5 fold) relative to the susceptible reference strain. Efflux pumps have therefore been proven to be a mechanism by which triazole resistance is mediated in clinical isolates of *A. fumigatus*. However, the extent of the contribution of overexpression of these transporters to clinical triazole resistance is not known.

In this work, we identify the *A. fumigatus* efflux pumps with the highest degree of homology to *C. albicans* Cdr1, a well characterized efflux pump known to significantly contribute to clinical triazole resistance.[80, 81] We then perform transcriptional profiling on a collection of twenty-one highly triazole-resistant clinical isolates of *A. fumigatus* as well as five triazole-susceptible control isolates to identify which of the genes encoding the identified efflux pumps are differentially expressed among triazole-resistant isolates. Of these genes, *abcA* and *atrI* were identified as the candidates of greatest interest, and selected for deletion in clinical isolates observed to constitutively overexpress these genes using a Cas9-mediated gene transformation system. We subsequently demonstrate that the deletion of either *abcA* or *atrI* in highly triazole resistant clinical isolates of *A. fumigatus* known to overexpress these efflux pump-encoding genes, is insufficient to significantly restore triazole susceptibility.

Materials and Methods

Isolates, Media, and Growth Conditions Used

Twenty-one previously characterized multi-triazole-resistant clinical isolates of *A. fumigatus*, and five triazole-susceptible control clinical isolates of *A. fumigatus*, originating from the United States were obtained from the Fungus Testing Laboratory at the University of Texas in San Antonio.[8] All clinical isolates were maintained on glucose minimal media (GMM) agar at 37°C. Transformants were selected for using sorbitol (1.2M) supplemented GMM agar (SMM) containing 150mg/L of hygromycin. All conidia were harvested in sterile water from three-day-old growth plates and conidia were enumerated visually using a hemocytometer.

Transcriptional Profiling

For assessment of the expression of efflux pump-encoding genes of interest in clinical isolates, conidia from each isolate were allowed to germinate overnight in *Aspergillus* minimal media incubated at 37°C on an orbital shaker at 250 RPM in biological triplicate. Then RNA was isolated by TriZol based extraction from mature hyphae following liquid nitrogen crush as previously described.[68] RNA samples were purified using the Qiagen® RNeasy kit. Barcoded libraries were prepared and sequenced on the Illumina MiSeq platform. Bowtie2 was utilized to create the *A. fumigatus* Af293 reference genome index, followed by alignment of RNAseq reads to the reference genome by TopHat2. Transcript assemblies will then be generated from the aligned RNAseq reads by Cufflinks, and Cuffmerge was utilized to create a single annotated transcriptome. Transcript counts from each sample, normalized by reads per kilobase of exon length per million mapped reads, were then compared to the triazole susceptible control clinical isolate DI-16-6 (voriconazole MIC 0.25mg/L) to obtain fold change values. Statistical analysis was performed using unpaired, two-tailed, students t-test in Prism 8 for Mac OS by GraphPad Software Inc. with significance set at 0.05 and degrees of freedom equal to 10.

Construction of Efflux Pump-Encoding Gene Deletion Repair Templates

Repair templates consisting of the resistance marker (*hphR*) and 50 bases of microhomology targeting the genes of interest were prepared by PCR as previously described.[67] Briefly, the hygromycin B resistance cassette, including the *gdpA* promoter and *hphR* gene was amplified by PCR from the pUCGH plasmid using primers that introduced approximately 50 bases of homology with the regions immediately upstream and downstream of either the *abcA* or *atrI* open reading frame. Subsequently, the Gene Clean II Kit (MP Biomedicals) was used to purify PCR products following confirmation of product size by gel electrophoresis. Cas9-RNP complexes targeting sequences immediately upstream and downstream of the open reading frame of *abcA* and

atrI were assembled as previously described.[67] Target specific primers and guide sequences are listed in **Table 3-1**.

***Aspergillus* Protoplast Transformations**

Transformation of *A. fumigatus* protoplasts were performed as previously described with minor modifications.[67] Approximately 2µg of each portion applicable transformation repair templates were then mixed with 200 µL of protoplasts, 26.5 µL of Cas9-RNP complexes, and 25 µL of polyethylene glycol (PEG)-CaCl₂ buffer (60% [wt/vol] PEG 3350, 50 mM CaCl₂·H₂O, 450 mM Tris-HCl, pH 7.5) as previously described. Following an incubation on ice for approximately one hour, 1.25mL PEG-CaCl₂ was added and the mixture incubated for an additional 20 minutes at room temperature. The mixture was then diluted to a total volume of 3 mL with STC buffer (1.2 M sorbitol, 7.55 mM CaCl₂·H₂O, 10 mM Tris-HCl, pH 7.5) and plated on SMM agar plates. Plates were then incubated overnight prior to being overlaid with SMM top agar (GMM supplemented with 1.2 M sorbitol and 0.7% [wt/vol] agar) supplemented with hygromycin (final concentration of 150 g/ml), and the plates were incubated at 37°C for 3 days. Transformants were isolated to separate plates containing selective agar, genomic DNA extracted, and successful CRISPR/Cas9 editing confirmed by PCR screens using gene-specific primers.

Clinical Antifungal Susceptibility Testing

Susceptibilities for voriconazole, isavuconazole, itraconazole, and posaconazole were determined for all isolates in accordance with Clinical Laboratory Standards Institute (CLSI) M38-A2 methodology utilizing broth microdilution in RPMI in technical triplicate.[69] Each antifungal was obtained from the appropriate manufacturer. All agents were suspended in dimethyl sulfoxide (DMSO) for preparation of stock solutions.

Results

Analysis of the Expression of the Efflux Pump-Encoding Genes *abcA* and *atrI* Among Triazole-Resistant Clinical Isolates

To identify candidate efflux-pumps encoding genes which may contribute to triazole resistance in clinical isolates of *A. fumigatus*, the genes in the reference Af293 *A. fumigatus* genome with a predicted peptide sequences possessing the highest homology to *C. albicans* Cdr1 were identified by BLASTP. Twelve genes (**Table 3-2**) were identified to have markedly higher homology than any other gene (BLAST P scores greater than 800, E values of 0.0, and greater than 35% matching residue identities). Of these, four have been previously associated with triazole resistance in *A. fumigatus*

Table 3-1. Oligonucleotides used for *abcA* and *atrI* experiments.

Oligo Type	Gene	Primer Name	Sequence (5'- 3')
PCR Primers			
	<i>abcA</i>	<i>abcA</i> KO F CRISPR	GTATTTCTTCCTGCCATGACCAGGCAGTCA TCCCAGAGGTTGAGCTTGCATGCCTGCAGG
	<i>abcA</i>	<i>abcA</i> KO R CRISPR	CTATCAGTGCTGCAAGTCATGCAGGGTACA TATACATCACAGCGCCCTTCCGAGCTCCC
	<i>atrI</i>	<i>atrI</i> KO F CRISPR	CCACTGTGGATTCTCGGCATCGTCATTCAT TTTTCTTCCGAGCTTGCATGCCTGCAGG
	<i>atrI</i>	<i>atrI</i> KO R CRISPR	GGCGGCAGGAAAGATGTGGATTTTCTATGT ATAAGAATAGCGCCCTTCCGAGCTCCC
	<i>abcA</i>	<i>abcA</i> screen F	GTTCGACTGTTTATGTGTATACCTATTGTG ACGCAACCACTCAGCTTGCATGCCTGCAGG
	<i>atrI</i>	<i>atrI</i> screen F	GGGTTGATGTTAGGATTTATCGTCCCTAGA AGAGACATGCTTGGTACCTGTGAAGAAGT G
	<i>hphR</i>	<i>hphR</i> screen R	CAAATCGCCCGCAGAAGCG
crRNA Guide Sequences			
	<i>abcA</i>	<i>abcA</i> 5' crRNA	TGGTGTGAGTCGTGCAGCGA
	<i>abcA</i>	<i>abcA</i> 3' crRNA	CTTATTGCTGTTCGTCAGCT
	<i>atrI</i>	<i>atrI</i> 5' crRNA	ATTATGGGTGTATAGATGAA
	<i>atrI</i>	<i>atrI</i> 5' crRNA	CATGTACACACCCACTATCA

Table 3-2. Relative expression of efflux pump-coding genes of interest among triazole-resistant clinical isolates.

Gene (Name)	Susceptible Isolates					Resistant Isolates																				
	DI16-6	DI16-5	DI16-9	DI16-8	DI16-7	DI15-111	DI15-99	DI15-107	DI15-109	DI15-115	DI15-103	DI15-117	DI15-118	DI15-97	DI15-114	DI15-100	DI15-110	DI15-116	DI15-105	DI15-102	DI15-108	DI15-95	DI15-98	DI15-96	DI15-106	DI15-120
Afu2g15130 (abcA)	0.9	1.4	0.6	<u>1.5</u>	1.0	2.1	2.4	0.6	2.1	0.7	4.2	1.7	2.5	1.8	7.4	19.0	238.6	1.4	15.1	1.3	1.1	2.3	3.3	11.6	6.6	1.8
Afu1g14330 (abcC)	0.9	<u>3.1</u>	1.2	1.2	1.5	1.2	0.8	0.8	1.9	0.8	1.4	1.4	1.1	3.1	3.2	3.7	83.4	3.3	2.4	1.9	0.5	1.7	2.4	6.8	4.0	6.3
Afu1g17440	<u>1.1</u>	0.7	0.7	0.3	0.7	1.3	0.8	0.9	2.2	2.5	1.6	1.1	0.4	1.3	2.1	2.3	3.2	1.7	1.6	2.6	8.7	1.7	1.0	1.1	2.6	1.6
Afu5g00790	0.8	0.5	0.4	<u>1.4</u>	0.6	0.3	0.3	0.4	0.4	0.2	0.3	0.1	0.2	0.4	0.8	1.5	0.6	0.2	0.2	0.2	1.8	1.1	1.2	0.6	0.5	0.2
Afu3g07300 (atrI)	1.1	0.6	0.8	<u>1.3</u>	0.9	2.0	1.4	1.3	1.2	1.7	0.6	1.5	1.9	1.6	2.1	2.2	8.3	1.8	2.0	3.5	1.7	2.0	2.3	4.6	8.1	3.3
Afu5g02260	<u>0.9</u>	0.6	0.8	0.7	0.5	1.5	0.5	0.5	0.8	0.3	0.7	0.6	0.7	1.5	0.9	0.9	1.1	1.0	0.9	1.1	1.6	1.3	1.6	1.3	1.4	0.6
Afu6g08020	1.1	1.5	1.3	1.3	<u>1.6</u>	1.8	1.0	1.0	3.2	1.5	5.6	0.9	2.6	1.3	1.1	1.9	3.6	1.3	22.4	7.9	1.1	3.3	3.4	2.4	6.1	1.4
Afu3g01400	<u>1.1</u>	0.5	0.6	0.5	0.6	0.7	0.5	0.4	0.6	0.6	0.5	0.6	0.6	0.7	0.4	1.3	2.3	0.7	0.5	1.4	1.0	0.7	0.8	1.8	1.7	1.0
Afu6g04360 (atrF)	0.9	0.4	2.3	<u>6.9</u>	1.3	2.3	1.4	2.2	4.1	1.6	1.8	1.7	2.3	2.6	2.7	1.2	1.0	3.8	5.3	8.4	4.6	2.0	6.9	4.6	1.6	2.2
Afu4g01050	<u>1.1</u>	0.3	0.4	0.7	0.6	0.3	0.5	0.8	0.8	0.6	0.6	0.3	0.9	0.3	0.5	0.2	0.8	0.2	1.5	0.5	0.8	0.4	1.4	0.3	0.4	0.5
Afu5g09460	0.9	0.9	0.9	<u>4.7</u>	1.3	1.1	0.7	0.8	0.9	0.6	0.5	0.2	0.6	1.7	0.3	1.3	0.8	0.4	0.8	0.9	0.8	1.9	0.5	1.4	0.6	0.7
Afu8g02650	1.1	1.2	1.2	1.2	<u>1.6</u>	0.6	0.8	1.3	1.0	0.9	0.6	0.9	1.2	1.8	1.2	1.4	1.1	1.6	1.6	1.7	1.3	2.2	1.4	1.5	1.7	1.0

A. fumigatus clinical isolates are ordered by escalating voriconazole MIC from left to right. *A. fumigatus* genes are listed by degree of homology with *C. albicans* Cdr1 from top to bottom. Genes shown in bold have previously been associated with triazole resistance in *A. fumigatus*. Fold expression values are shown as the average of three independent biological samples relative to the triazole-susceptible control isolate DI16-6. Fold expression values shown underlined are the highest for that gene among susceptible isolates. Fold expression values among resistant isolates shown in bold are at least two times higher than the highest measured value for the same gene among the triazole susceptible control isolates (underlined value).

(*abcA*, *abcC*, *atrF*, and *atrI*). Additionally, disruption of *abcC* has previously been demonstrated to increase triazole susceptibility in both susceptible laboratory strains and a triazole-resistant clinical isolate. [25, 42-44, 66]

To assess the whether the constitutive expression of each the identified efflux pump-encoding genes of interest may be greater among triazole-resistant clinical isolates of *A. fumigatus*, we obtained twenty-six clinical isolates from the Fungus Testing Laboratory in San Antonio, Texas. This collection of isolates includes five triazole-susceptible clinical isolates, and twenty-one triazole-resistant clinical isolates that have MIC for at least two triazole agents above published CLSI epidemiological cut-off values (ECV; voriconazole, isavuconazole, and itraconazole 1mg/L, posaconazole 0.25mg/L).[73, 82] RNA was then isolated from each clinical isolate in biological triplicate following overnight growth in *Aspergillus* minimal media at 37°C on an orbital shaker at 250 RPM, and transcriptional profiling was performed. Normalized read counts were for each sample were compared to the read counts measured in the triazole-susceptible control isolate DI16-6 (voriconazole MIC 0.25mg/L) to generate fold change values for each of the efflux pump-encoding genes of interest (**Table 3-2**).

Of the twelve efflux pump-encoding genes of interest, six were observed to be expressed at least 2-fold higher in one or more triazole-resistant isolate, as compared to the triazole-susceptible isolate with the highest measured level of expression for each gene of interest (*abcA*, *abcC*, Afu1g17440, *atrI*, Afu6g08020, and Afu3g01400). Ten resistant isolates were observed to overexpress more than one gene of interest, and this was more frequently observed among more highly triazole resistant clinical isolates. *abcA*, which has been shown to increase triazole resistance when overexpressed in a triazole-susceptible laboratory strain and which has the highest degree of homology with *C. albicans* Cdr1, was observed to be overexpressed in eight triazole-resistant clinical isolates (3.3 to 238.6-fold that of DI16-6). *abcC*, which has previously been shown to contribute to clinical triazole resistance in *A. fumigatus*, was overexpressed in three triazole-resistant clinical isolates (6.3 to 83.4-fold that of DI16-6). Afu1g17440, which has not previously been implicated in triazole resistance, was overexpressed in seven triazole-resistant clinical isolates (2.2 to 8.7-fold). *atrI*, which when deleted in a triazole-susceptible laboratory strain of *A. fumigatus* leads to hyper-susceptibility, was overexpressed in five triazole-resistant isolates (3.3 to 8.3-fold that of DI16-6). Afu6g08020 and Afu3g014000 have not previously been associated with triazole resistance and were overexpressed in eight (3.2 to 22.4-fold that of DI16-6) and one (2.3 fold that of DI16-6) triazole-resistant isolates, respectively. As *abcA* and *atrI* both have previously been implicated in triazole resistance, were both found to be overexpressed in multiple triazole-resistant isolates in this collection, and have not yet been demonstrated to contribute to triazole resistance among clinical isolates of *A. fumigatus*, these efflux pump-encoding genes were selected for further interrogation.

Deletion of the Efflux Pump-Encoding Genes *abcA* and *atrI* Has Minimal Impact on Clinical Triazole Resistance

Following the identification of *abcA* and *atrI* as the efflux pump-encoding genes of greatest interest, we sought to characterize the direct impact of these two genes on triazole resistance in clinical isolates of *A. fumigatus*. To accomplish this, we utilized a Cas9-mediated transformation system to delete *abcA* and *atrI* in triazole-resistant clinical isolates observed to exhibit constitutive overexpression of each of these genes.[67] As eight resistant clinical isolates were observed to exhibit overexpression of *abcA* (at least 2-fold above the highest expression among triazole-susceptible isolates), and seven of these were found to be statistically significant (as compared to DI16-6; **Figure 3-1A**), we selected both DI15-100 (19-fold the *abcA* expression of DI16-6) and DI15-106 (6.6-fold the *abcA* expression of DI16-6) for deletion of *abcA*. Importantly, DI15-110 (238.6-fold the *abcA* expression of DI16-6) was not selected as this isolate exhibits a slow growth rate, altered growth morphology, and diminished conidiation, all of which make genetic manipulations technically challenging. Of the five triazole-resistant isolates observed to overexpress *atrI* (at least 2-fold above the highest expression among triazole-susceptible isolates), all of which were statistically significant as compared to DI16-6 (**Figure 3-1B**), DI15-106 (8.1-fold the *atrI* expression of DI16-6) was selected for deletion of *atrI*. Subsequently, two independent, positive transformants were constructed for each gene of interest as previously described.[67] Triazole MIC for all clinically available agents were then determined following CLSI methods for the parental triazole-resistant clinical isolates and all efflux pump-deletion strains.[69]

The voriconazole, isavuconazole, itraconazole, and posaconazole MIC for DI15-100, DI-106, and all derivative efflux pump deletion strains are shown in **Table 3-3**. For both triazole-resistant clinical isolates, the MIC for voriconazole, isavuconazole, and itraconazole were greater than previously published ECV. Notably, the voriconazole and isavuconazole MIC for DI15-106 were above the CLSI recommended testing range (0.03 to 16mg/L) and at least 32-fold above the ECV (1mg/L) for these agents.[69] Deletion of *abcA* in either the DI5-100 or DI15-106 clinical isolate backgrounds had no impact on isavuconazole or posaconazole MIC. In the *abcA* deletion strains constructed in the DI15-100 background (DI15-100_*abcA*_A and DI15-100_*abcA*_B), voriconazole MIC were reduced by one dilution (8 to 4mg/L). In the *abcA* deletion strains constructed in the DI15-106 background (DI15-106_*abcA*_A and DI15-106_*abcA*_B), itraconazole MIC were reduced by one dilution (2 to 1mg/L). While these changes in triazole MIC were reproducible, they are also within the accepted assay error range.[69] Similarly, MIC for voriconazole, isavuconazole, and itraconazole were unchanged upon deletion of *atrI* in the DI15-106 background. Posaconazole MIC were observed to increase by one dilution (from 0.125 to 0.25mg/L).

Figure 3-1. Relative expression of *abcA* and *atrI* among triazole-resistant clinical isolates.

A) Expression of *abcA* in clinical isolates relative to triazole-susceptible isolate DI16-6

B) expression of *atrI* in clinical isolates relative to triazole-susceptible isolate DI16-6;

Triazole-susceptible isolates shown as open bars, triazole-resistant isolates shown as filled bars. Triazole-resistant isolates shown in order of escalating voriconazole MIC from left to right. Horizontal dotted lines denote highest and lowest expression level observed among the five triazole-susceptible isolates. Comparisons with the susceptible control isolate DI16-6 with statistically significant ($p < 0.05$) results are noted with an Asterix.

Table 3-3. Triazole MIC of clinical isolates and derivative efflux pump-encoding gene deletion strains.

Parent Clinical Isolate or Derivative Strain	Triazole MIC (mg/L)			
	Voriconazole	Isavuconazole	Itraconazole	Posaconazole
DI15-100	8	4	4	0.125
DI15-100_abcA_A	4	4	4	0.125
DI15-100_abcA_B	4	4	4	0.125
DI15-106	≥32	≥32	2	0.125
DI15-106_abcA_A	≥32	≥32	1	0.125
DI15-106_abcA_B	≥32	≥32	1	0.125
DI15-106_atrI_A	≥32	≥32	2	0.25
DI15-106_atrI_B	≥32	≥32	2	0.25

MIC shown in bold are greater than epidemiologic cutoff values as published by the Clinical Laboratory and Standards Institute (1mg/L for voriconazole and itraconazole; 0.25mg/L for posaconazole).

Conclusions

In this work we attempted to identify efflux pump-encoding genes which may contribute to triazole resistance in clinical isolates of *A. fumigatus*. We identified twelve efflux pump encoding genes in the Af293 reference *A. fumigatus* genome with predicted peptides sequences that have the highest degree of homology with the well characterized *C. albicans* triazole efflux pump Cdr1. Importantly, four of the identified genes of interest have previously been associated with triazole resistance, and one of these, *abcC*, has been demonstrated to directly contribute to clinical triazole resistance. [25, 42-44, 66] The remaining eight genes have not previously been well characterized.

We then performed transcriptional profiling on a collection of twenty-one multi-triazole-resistant clinical isolates of *A. fumigatus*, as well as five triazole-susceptible control clinical *A. fumigatus* isolates, to identify which of these efflux pump-encoding genes are constitutively overexpressed among triazole-resistant clinical isolates. Transcriptional profiling revealed that six of the twelve efflux pump-encoding genes of interest were more highly expressed among triazole resistant isolates. These six genes included *abcA*, *abcC*, and *atrI*, which have previously been implicated in triazole resistance, as well as Afu1g17440, Afu6g08020, and Afu3g01400 which have not. Interestingly, ten triazole-resistant isolates were observed to overexpress two or more genes of interest, and this was observed more frequently among isolates which are more highly triazole-resistant. Additionally, *atrF*, which has also previously been implicated in triazole resistance, was not observed to be more highly expressed among the triazole-resistant isolates in this collection.

We subsequently identified both *abcA* and *atrI* as the genes of greatest interest for further investigation, due to both the observed degree of overexpression among triazole-resistant isolates in our collection, and the findings of previous studies implicating these genes in clinical triazole resistance. Surprisingly, upon deletion of *abcA* in both the DI15-100 and DI15-106 triazole-resistant clinical isolate backgrounds, no pronounced change in triazole susceptibility was observed. Similarly, when *atrI* was deleted in the DI15-106 background, no decrease in triazole resistance occurred. Yet, it remains possible that these highly triazole-resistant clinical isolates harbor multiple triazole resistance mechanisms, such as mutations in the sterol-demethylase gene *cyp51A*, which may mask smaller changes in triazole susceptibility upon deletion of the efflux pump encoding genes of interest. In fact, in previous studies we have shown that DI15-100 and DI15-106 both harbor mutations in *cyp51A* (encoding M263I and TR₄₆/Y121F/T289A, respectively). However, we have also demonstrated that correction of these *cyp51A* mutations in DI15-100 and DI15-106 also failed to restore the MIC of any triazole agent to wild-type susceptible ranges.

Taken together, the findings of this study serve to demonstrate that *A. fumigatus* efflux pump-encoding genes with homology to known triazole resistance genes are constitutively overexpressed among triazole-resistant clinical isolates. However, deletion of either *abcA* or *atrI* in resistant clinical isolates which constitutively overexpress them was insufficient to significantly restore triazole susceptibility. Future studies are needed

to further characterize the potential role of these and other *A. fumigatus* efflux pump-encoding genes in clinical triazole resistance, the genetic determinants which lead to increased efflux pump gene expression among triazole-resistant clinical isolates of *A. fumigatus*.

CHAPTER 4. MUTATIONS IN THE *ASPERGILLUS FUMIGATUS* HMG-COA REDUCTASE GENE, *HMG1*, A NOVEL GENETIC DETERMINANT OF CLINICAL TRIAZOLE RESISTANCE*

Introduction

The most common invasive mold infection is invasive aspergillosis, with *Aspergillus fumigatus* being identified as the etiological agent for more than 72% of all cases.[2] Worldwide invasive aspergillosis is implicated in more than 200,000 life-threatening infections each year, and is estimated to affect more than 10% of all patients with acute leukemia or having received bone marrow or other transplants.[1] While substantial healthcare resources are allocated to the treatment of invasive aspergillosis, rates of clinical failure remain unacceptably high, approximating 40%.[4, 83, 84]

The triazole class of antifungals are essential to the treatment of invasive aspergillosis. Routinely relied upon as both front-line (voriconazole and isavuconazole) and salvage (itraconazole and posaconazole) therapeutic options, these agents are available in a range of oral and intravenous formulations and generally are well tolerated.[10, 13, 79] The triazoles exert fungicidal activity against *Aspergillus* through the inhibition of the biosynthesis of ergosterol, and essential constituent of fungal cell membranes.[21] The triazoles impede ergosterol biosynthesis through competitive inhibition of the sterol-demethylase proteins, Cyp51A and Cyp51B. Inhibition of these two enzymes ultimately leads to a depletion of cellular ergosterol and the accumulation of methylated ergosterol precursors.[14] Unfortunately, the treatment of invasive aspergillosis has been further complicated by the emergence of *A. fumigatus* isolates resistant to the triazoles. While the rate of triazole resistance in the United States is currently estimated to be 5%, globally reports of triazole-resistant *A. fumigatus* clinical isolates are increasing. In countries such as the United Kingdom and the Netherlands, where triazole resistance rates were previously comparable to those in the United States, resistance rates now exceed 15%.[6-8, 30]

A limited number of triazole resistance mechanisms have been identified in *A. fumigatus*, largely mirroring mechanisms of triazole resistance previously identified in other pathogenic fungi such as *Candida albicans*. Single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions in one of the two sterol-demethylase enzymes, Cyp51A, overexpression of the gene encoding this sterol-demethylase enzyme, *cyp51A*, and overexpression of the efflux pump-encoding gene, *abcC*, have all been shown to contribute to triazole resistance among clinical isolates of *A. fumigatus*. [25, 28, 34, 35, 42-44, 48, 66, 71, 79] However, we have previously demonstrated that these

* Modified from final submission with permission. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD and Fortwendel JR. Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. MBio. 2019 Apr 2;10(2). pii: e00437-19. doi: 10.1128/mBio.00437-19

mechanisms alone poorly explain the high level of triazole resistance observed among many clinical isolates of *A. fumigatus*. Thus, the molecular mechanisms underpinning much of the clinical resistance to this essential class of anti-Aspergillus agents remains unexplained, limiting the discovery of therapeutic strategies to overcome triazole-resistant aspergillosis.

We describe here for the first time a novel mechanism of triazole resistance, not mediated by mutations in sterol-demethylase and never before observed among pathogenic fungi, which was found to be present in a majority of triazole-resistant clinical isolates of *A. fumigatus* in our collection. Mutations in the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase encoding gene, *hmg1* (Afu2g03700), were found to result in dramatically increased resistance to all clinically available triazole agents. Additionally, the restoration of *hmg1* to the wild-type sequence in a clinical *A. fumigatus* isolate exhibiting high-level pan-triazole resistance was found to restore clinical susceptibility to the triazole class. We demonstrate that mutations in *hmg1* result in maintained or even increased ergosterol production as well as accumulation of multiple ergosterol precursors, including eburicol, the substrate of the sterol-demethylase enzymes which the triazole antifungals competitively inhibit.

Materials and Methods

Isolates, Media, and Growth Conditions Used

Twenty-one previously characterized multi-triazole-resistant clinical isolates of *A. fumigatus*, and five triazole-susceptible control clinical isolates of *A. fumigatus*, originating from the United States were obtained from the Fungus Testing Laboratory at the University of Texas in San Antonio.[8] The laboratory strains *akuB*^{KU80} and Af293 were obtained from the Fungal Genetics Stock Center. All strains and clinical isolates used in this study were maintained on glucose minimal media (GMM) agar at 37°C. Transformants were selected for using sorbitol (1.2M) supplemented GMM agar (SMM) containing 150mg/L of hygromycin. All conidia were harvested in sterile water from three-day-old growth plates and conidia were enumerated visually using a hemocytometer.

Construction of Allele Replacement Repair Templates and Cas9-RNP for the *hmg1* Locus

Two component repair templates consisting of a split hygromycin resistance marker (*hphR*) and *hmg1* alleles of interest were prepared by PCR. Briefly, *hmg1* alleles of interest including the open reading frame and approximately 500 downstream bases were amplified by PCR from DI15-98 (S305P), DI15-100 (I412S), DI15-105 (F262del), and *akuB*KU80 (wild-type control) using a 3' primer which introduced the terminal 80 bases with homology to the 3' end of the *hphR* hygromycin B resistance gene open

reading frame. A partial hygromycin B resistance cassette, including the *gdpA* promoter and a truncated *hphR* gene lacking the terminal 40 bases, was then amplified by PCR from the pUCGH plasmid using primers that introduced approximately 70 bases of homology with the downstream region of *hmg1*. PCR products were subsequently purified using the Gene Clean II Kit (MP Biomedicals). Cas9-RNP complexes targeting sequences immediately upstream and approximately 500 bases downstream of the open reading frame of *hmg1* were assembled as previously described.[67] Target specific guide sequences and primers are listed in **Table 4-1**.

***Aspergillus* Protoplast Transformations**

Transformation of *A. fumigatus* protoplasts were performed as previously described with minor modifications.[67] Approximately 2µg of each portion applicable transformation repair templates were then mixed with 200 µL of protoplasts, 26.5 µL of Cas9-RNP complexes, and 25 µL of polyethylene glycol (PEG)-CaCl₂ buffer (60% [wt/vol] PEG 3350, 50 mM CaCl₂·H₂O, 450 mM Tris-HCl, pH 7.5) as previously described. Following an incubation on ice for approximately one hour, 1.25mL PEG-CaCl₂ was added and the mixture incubated for an additional 20 minutes at room temperature. The mixture was then diluted to a total volume of 3 mL with STC buffer (1.2 M sorbitol, 7.55 mM CaCl₂·H₂O, 10 mM Tris-HCl, pH 7.5) and plated on SMM agar plates. Plates were then incubated overnight prior to being overlaid with SMM top agar (GMM supplemented with 1.2 M sorbitol and 0.7% [wt/vol] agar) supplemented with hygromycin (final concentration of 150 g/ml), and the plates were incubated at 37°C for 3 days. Transformants were isolated to separate plates containing selective agar, genomic DNA extracted, and successful CRISPR/Cas9 editing confirmed by PCR screens using gene-specific primers and Sanger sequencing.

Clinical Antifungal Susceptibility Testing

Susceptibilities for amphotericin B, voriconazole, isavuconazole, itraconazole, and posaconazole were determined for all isolates in accordance with CLSI M38-A2 methodology utilizing broth microdilution in RPMI.[69] Each antifungal was obtained from the appropriate manufacturer. All agents were suspended in dimethyl sulfoxide (DMSO) for preparation of stock solutions.

Comprehensive Sterol Profiling.

Fresh conidial suspensions of each strain to be studied were prepared in saline with tween 80 from AMM agar plates. Conidia were grown in 25mL RPMI supplemented with 0.2% glucose buffered to pH 7.0 with MOPS for 24 hours in an orbital shaker at 180 RPM and 35°C. Cells were then flash frozen using liquid nitrogen, dry weights obtained, and alcoholic KOH was utilized to extract nonsaponifiable lipids. A vacuum centrifuge (Heto) was then used to dry samples, prior to being derivatized by the addition of 200µL

Table 4-1. Oligonucleotides used for *hmg1*, *cyp51A*, and *cyp51B* experiments.

Oligo Type	Gene	Primer Name	Sequence (5'- 3')
PCR Primers			
	<i>hmg1</i>	<i>hmg1</i> F	GACAGGCGGCTTACACCGCCTCTCCTGCT CGCCATTTTGTCTATGATCACTGATGGCT ACCTCTCTGATT
	<i>hmg1</i>	<i>hmg1</i> split <i>hphR</i> R	TCTGGACCGATGGCTGTGTAGAAGTACT CGCCGATAGTGGAACCGACGCCCCAGC ACTCGTCCGAGGGCAAAGGAATAGGCTG CGTTACTCGGTCTTGG
	<i>hphR</i>	<i>hphR</i> split F	CCACTATCGGCGAGTACTTCTACAC
	<i>hphR</i>	<i>hphR</i> 3'UTR <i>hmg1</i> R	CCCACCTGGGATGAAGCAAAAGCGGGTA CATAGATAGGTTTATGGGGACTTTGGGA AGTGAAATATGGGCGAGCTCCCAAATCT GTCCAG
	<i>hmg1</i>	<i>hmg1</i> screen F	GGTGTCCGTGGTTCCCACC
	<i>hphR</i>	<i>hphR</i> screen R	CAAATCGCCCGCAGAAGCG
crRNA Guide Sequences			
	<i>hmg1</i>	<i>hmg1</i> 5' crRNA	ATTTTGTCTATGATAGACAA
	<i>hmg1</i>	<i>hmg1</i> 3' crRNA	CCAACGATTGCCAAAGGTCA
RTqPCR Primers			
	<i>cyp51A</i>	<i>cyp51A</i> RT F e1	CTTCTTTGCGTGCAAGAGA
	<i>cyp51A</i>	<i>cyp51A</i> RT R e2	GGGGTCGTCAATGGACTA
	<i>cyp51B</i>	<i>cyp51B</i> RT F e1	CTTTTTCGACTGCCGCGC
	<i>cyp51B</i>	<i>cyp51B</i> RT R e2	AGGCGTAGTGAGTGGAGA

of anhydrous pyridine (Sigma), 100 μ L of N,O- bis(trimethylsilyl)trifluoroacetamide (BSTFA)- 10% trimethylsilyl (TMS) (Sigma), and two hours of heating at 80°C. Gas chromatography-mass spectrometry (GC-MS) (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer; Thermo Scientific) was then used to analyze and identify TMS-derivatized sterols. Known standards were referenced for fragmentation spectra and retention times. Sterol profiles for each isolate were then created using Xcalibur software (Thermo Scientific) to analyze GC- MS data.[16] Statistical analysis of both sterol profiles and total ergosterol per dry weight were performed using GraphPad Prism 7. In all cases, 6 independent biological replicates were measured and included in analysis, two-tailed unpaired t-test were performed in Prism 8 for Mac OS by GraphPad Software Inc. with significance set at 0.05 and degrees of freedom equal to 10.

Assessment *cyp51A* and *cyp51B* Expression by RTqPCR

Conidia from each strain were allowed to germinate overnight on an orbital shaker at 250 RPM incubated at 37°C in *Aspergillus* minimal media. RNA was then extracted from mature hyphae following liquid nitrogen crush as previously described.[68] cDNA was synthesized using the RevertAid RT kit (Thermo Scientific). *A. fumigatus cyp51A* and *cyp51B* were amplified from cDNA by PCR using PCR master mix and SYBR per the manufacturer's instructions. **Table 4-1** lists the gene specific primers used for PCR. Conditions used for PCR were as follows: AmpliTaq Gold activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds and annealing/ extension at 60°C. The dissociation curve and threshold cycle (CT) was determined using the CFX96 Real Time PCR system (Bio-Rad). Changes in gene expression among isolates were then calculated using the $2^{-\Delta\Delta CT}$ method. All experiments were performed in triplicate from biological triplicates. As previously described, ΔCT values were used to calculate the standard error.[68] Statistically analysis was performed using unpaired, two-tailed, students t-test in Prism 8 for Mac OS by GraphPad Software Inc. with significance set at 0.05 and degrees of freedom equal to 10.

Results

Mutations in *hmg1* Are Common Among Triazole-Resistant Clinical Isolates of *A. fumigatus*

Following our prior findings that the previously characterized mechanisms of triazole-resistance did not explain the high level of triazole resistance observed in this collection of clinical *A. fumigatus* isolates, we sought to interrogate the previously generated whole genome sequencing data to identify novel mechanisms of resistance. As the triazoles exert antifungal activity through the inhibition of biosynthesis of the predominant fungal membrane sterol, ergosterol, initial whole genome sequencing analysis targeted genes encoding proteins known to participate in ergosterol biosynthesis. In addition to the previously identified *cyp51A* mutations, mutations unique to triazole-resistant isolates were also observed in *erg3B* (Afu2g00320), *erg3C* (Afu8g01070),

erg4B (Afu1g07140), *erg5* (Afu1g03950), and *erg6* (Afu4g03630). However, mutations occurred in these genes among only a small number of the clinical isolates (**Table 4-2**). Intriguingly, 11 of the 21 triazole-resistant clinical isolates (52%) were found to possess mutations in the *hmg1* gene resulting in peptide sequence changes not observed among the triazole-susceptible control isolates (**Figure 4-1A**). The *A. fumigatus* *hmg1* gene encodes a HMG-CoA reductase enzyme previously reported to be essential. HMG-CoA reductase catalyzes the first committed step in ergosterol biosynthesis and has previously been shown in other eukaryotic organisms to participate in the regulation of sterol biosynthesis through direct interactions between sterols and a conserved sterol sensing-domain.[85-88] Of particular interest, eight of the eleven isolates with *hmg1* mutations were found to have mutations affecting residues predicted to reside within the conserved sterol-sensing domain of Hmg1 (**Figure 4-1B**). We additionally accessed publicly available whole genome sequencing data for 11 triazole-resistant clinical isolates (<https://www.ebi.ac.uk/arrayexpress/experiments/E-SYBR-1/samples/>) and found 5 to have mutations in *hmg1*, 2 of which alter residues in the predicted sterol sensing domain (P309L and I412T). As mutations in *hmg1* were observed in a large proportion of clinical triazole-resistant isolates, and *hmg1* mutations have previously been observed among laboratory isolates of *A. fumigatus* grown in the presence of triazole antifungals and the closely related agricultural sterol-demethylase inhibitors, the observed mutations in *hmg1* were selected for further interrogation.[47, 89]

Mutations in *hmg1* Confer Resistance to the Triazole Class of Antifungals

To test the hypothesis that mutations in *hmg1* directly contribute to clinical triazole resistance in *A. fumigatus*, three mutations encoding amino acid substitutions or in-frame codon deletions in different transmembrane regions of the predicted *hmg1* sterol-sensing domain (F262del, S305P, and I412S) were selected for characterization (**Figure 4-1B**). Each of these mutations and a wild-type control allele were directly introduced to the native *hmg1* locus of the triazole-susceptible laboratory strain *akuB*^{KU80} using a novel Cas9-RNP editing technique incorporating a split hygromycin B resistance marker (**Figure 4-2**). Briefly, *hmg1* alleles including the open reading frame and approximately 500 downstream bases were amplified by PCR from DI15-98 (S305P), DI15-100 (I412S), DI15-105 (F262del), and *akuB*^{KU80} (wild-type control) using a 3' primer which introduced the terminal 80 bases with homology to the 3' end of the *hphR* hygromycin B resistance gene open reading frame. A partial hygromycin B resistance cassette, including the *gdpA* promoter and a truncated *hphR* gene lacking the terminal 40 bases, was then amplified by PCR from the pUCGH plasmid using primers that introduced approximately 70 bases of homology with the downstream region of *hmg1*. [90] Protoplasts of *akuB*^{KU80} were then transformed with RNP complexes, consisting of Cas9 protein and crRNA-trRNA duplexes targeting sequences immediately upstream and approximately 500 bases downstream of the open reading frame of *hmg1*, and 2µg of each portion of the split marker repair template (a single *hmg1* allele and the truncated hygromycin B resistance cassette), to produce *hmg1* mutant strains

Table 4-2. Mutations in genes involved in ergosterol biosynthesis which are unique to triazole resistant isolates.

Isolate	D115-95	D115-96	D115-97	D115-98	D115-99	D115-100	D115-102	D115-103	D115-105	D115-106	D115-107	D115-108	D115-109	D115-110	D115-111	D115-114	D115-115	D115-116	D115-117	D115-118	D115-120
<i>erg3A</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>erg3B</i>	H40R	-	-	-	-	H40R	-	-	-	-	-	-	-	L88P	-	-	-	-	-	-	L88P
<i>erg3C</i>	-	P197fs, R203S, I205V, F252I, Q310H	-	-	-	-	P317S	-	-	P197fs, R203S, I205V, F252I, Q310H	-	-	-	-	-	-	-	P317S	-	-	-
<i>erg4A</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>erg4B</i>	D42H	-	T90I, H93N, H97N	-	H97N	D42H	-	-	H97N, A505S	-	-	D42H	-	-	D42H	-	D42H	-	-	-	-
<i>erg5</i>	-	K363R	F68L	-	-	-	-	-	-	K363R	-	-	-	-	-	-	-	K363R	-	-	-
<i>erg6</i>	-	-	-	-	-	-	-	-	F148C	-	-	-	-	-	F311del	M187L	-	A291T	L234I	-	-
<i>erg25A</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>erg25B</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>hmg1</i>	I412S	G466V, S541G	S305P, V995I	S305P	-	I412S	E105K	-	F262del	-	-	Y250H	V995I	L413P	-	G307D	-	-	-	-	-

Figure 4-1. Mutations in *hmg1* observed in this collection of triazole-resistant clinical *A. fumigatus* isolates.

A) *hmg1* mutations observed in each clinical isolate relative to the Af293 reference genome, with mutations affecting residues predicted to reside within the conserved sterol-sensing domain shown in bold. **B)** Schematic depicting the predicted transmembrane domains of *A. fumigatus* Hmg1. Residues in light blue comprise the predicted sterol-sensing domain, residues in dark blue represent those affected by *hmg1* mutations observed in this collection of clinical isolates, residues shown in green represent those affected by *hmg1* mutations observed in this collection of clinical isolates and further characterized in this work.

Figure 4-2. Schematic of Cas9 ribonucleoprotein (RNP) editing technique incorporating a split hygromycin B resistance marker for creation of *hmg1* mutant strains.

akuB^{KU80}*_hmg1*^{F262del}, *akuB*^{KU80}*_hmg1*^{S305P}, *akuB*^{KU80}*_hmg1*^{I412S}, and *akuB*^{KU80}*_hmg1*^{WT}.

Antifungal susceptibility testing was subsequently performed for *akuB*^{KU80} and each of the derivative strains for voriconazole, isavuconazole, itraconazole, posaconazole and amphotericin B according to the Clinical Laboratory Standards Institute M38-A2 standards (**Figure 4-3A**).[69] The parental *akuB*^{KU80} strain was susceptible to all agents with MIC of 0.25, 0.5, 0.125, 0.06, and 0.5 mg/L for voriconazole, isavuconazole, itraconazole, posaconazole, and amphotericin B, respectively. The *akuB*^{KU80}*_hmg1*^{WT} control strain exhibited MIC exactly matching those of the parental strain. Conversely, all strains harboring a clinically derived mutation in *hmg1* were found to exhibit a 4 to 8-fold increase in MIC for all tested triazoles, exceeding the CLSI reported epidemiologic cut-off values for at least one agent in each mutant strain.[73, 82] Notably amphotericin B MIC were observed to decrease by two-fold in both the *akuB*^{KU80}*_hmg1*^{S305P} and *akuB*^{KU80}*_hmg1*^{F262del} strains (0.5 versus 0.25mg/L), but not in *akuB*^{KU80}*_hmg1*^{I412S}.

In an effort to delineate the extent of *hmg1*-mutation-mediated triazole resistance within a clinical isolate from our collection, the mutant *hmg1* allele in the pan-triazole-resistant clinical isolate DI15-105 was replaced with the wild-type *hmg1* allele from *akuB*^{KU80} using the same Cas9-RNP-mediated gene editing described above. The resultant DI15-105 *_hmg1*^{WT} strain exhibited complete restoration of triazole susceptibility with an eight-fold or greater decrease in MIC for each triazole agent (Figure 6b). Intriguingly, the amphotericin B MIC was observed to increase by two-fold in DI15-105 *_hmg1*^{WT} relative to DI15-105 (0.125 versus 0.25mg/L).

Mutations in *hmg1* Lead to Accumulation of Ergosterol Precursors

As the sterol-sensing domain of HMG-CoA reductase has previously been shown to interact with ergosterol precursors and participate in the negative regulation of HMG-CoA reductase activity in both human cells and the model fission yeast *Schizosaccharomyces pombe*, we hypothesized that the mutations identified in the sterol sensing domain of *A. fumigatus hmg1* may be leading to dysregulation of the ergosterol biosynthesis pathway resulting in the observed triazole resistance (**Figure 4-4A**).[85-88] To test this hypothesis, comprehensive sterol profiling including assessment of the relative distribution of cell sterols and the measurement of total ergosterol content per dry weight was performed on *akuB*^{KU80}, *akuB*^{KU80}*_hmg1*^{F262del}, *akuB*^{KU80}*_hmg1*^{S305P}, *akuB*^{KU80}*_hmg1*^{I412S}, *akuB*^{KU80}*_hmg1*^{WT}.

To assess the relative distribution of sterols among *A. fumigatus* harboring *hmg1* mutations, freshly harvested conidia stocks of each strain were grown in six biological replicates in RPMI media supplemented with 0.2% glucose and buffered with MOPS (pH 7.0) for 24 hours before cells were flash frozen with liquid nitrogen, dry weights were obtained, and nonsaponifiable lipids were extracted, derivatized, and analyzed by gas-chromatography mass spectrometry as described previously.[16, 91] In agreement with the hypothesis that mutations in *hmg1* precipitate dysregulation of the ergosterol

Figure 4-3. Impact of *hmg1* mutations on antifungal susceptibility.

A) Triazole MIC for *akuB*^{KU80} and derivative mutant *hmg1* strains **B)** Triazole MIC for DI15-105 and derivative *hmg1*^{WT} strain.

Figure 4-4. Relative distribution of sterols and total ergosterol content of *hmg1* mutant strains.

A) *A. fumigatus* ergosterol biosynthetic pathway **B)** Relative fold change of cellular sterols lanosterol, eburicol, 4,4-dimethyl ergosta 8,24(28) dienol, fecosterol, episterol, and ergosterol in *hmg1* mutant strains as compared to the parental *akuB*^{KU80} strain **C)** Total cellular ergosterol per dry weight for *hmg1* mutant strains and the parental *akuB*^{KU80} strain. Comparisons with the susceptible parental isolate *akuB*^{KU80} with statistically significant ($p < 0.05$) results are noted with an Asterix.

biosynthetic pathway, the distribution of sterols was dramatically altered in all *hmg1* mutant strains relative to *akuB*^{KU80} (**Figure 4-4B**). Most notably, a significant decrease in the relative proportion of ergosterol, along with corresponding increases in the proportions of several ergosterol precursors downstream of Hmg1, including eburicol, 4,4-dimethyl ergosta 8,24(28) dienol, and fecosterol, were observed in all mutant *hmg1* strains. Additionally, the relative proportion of episterol was observed to be significantly elevated in *akuB*^{KU80}*_hmg1*^{F262del} and *akuB*^{KU80}*_hmg1*^{S305P}. Importantly, no significant differences in the relative distribution of sterols was observed between *akuB*^{KU80} and *akuB*^{KU80}*_hmg1*^{WT} (not shown).

While the distribution of cellular sterols among *A. fumigatus* harboring *hmg1* mutations demonstrated a significant decrease in the relative proportion of cellular ergosterol, total ergosterol content per dry weight was not lower among any of the mutant *hmg1* strains (**Figure 4-4C**). In fact, relative to the parental *akuB*^{KU80}, *akuB*^{KU80}*_hmg1*^{F262del} was observed to exhibit a significant increase in total ergosterol (4.56 v. 7.06 µg ergosterol/ mg dry weight; p<0.01). Taken together, these findings demonstrate *hmg1* mutations precipitate a relative increase in ergosterol precursors downstream of Hmg1, while maintaining or in some cases even increasing cellular ergosterol, leading to significantly altered cellular sterol profiles among mutant *hmg1* strains.

Mutations in *hmg1* Do Not Lead to Increased Expression of Sterol-Demethylase Genes

As our sterol profiling studies revealed significant accumulations of ergosterol precursors both upstream and downstream of sterol-demethylase, and we had previously observed that increased expression of either sterol-demethylase gene contributes to triazole resistance, we sought to investigate if the triazole resistance associated with mutations in *hmg1* were at least in part mediated by altered expression of sterol-demethylase genes. To accomplish this, conidia suspensions of *akuB*^{KU80}, *akuB*^{KU80}*_hmg1*^{F262del}, *akuB*^{KU80}*_hmg1*^{S305P}, *akuB*^{KU80}*_hmg1*^{I412S}, and *akuB*^{KU80}*_hmg1*^{WT} were grown in biological triplicate under the same conditions used for sterol profile analysis, and RNA was extracted from liquid nitrogen-pulverized samples, as previously described.[68] RTqPCR was then performed to assess expression of the sterol-demethylase genes *cyp51A* and *cyp51B* among mutant *hmg1* strains relative to the parental *akuB*^{KU80}. No significant differences in expression of either sterol-demethylase gene was observed for any of the mutant *hmg1* strains or the *akuB*^{KU80}*_hmg1*^{WT} control strain (**Figure 4-5**). Demonstrating that while mutant *hmg1* strains are observed to have relatively greater proportions of ergosterol precursors, expression levels of both *cyp51A* and *cyp51B* remain unchanged. Thus, resistance in these strains is not due to increased expression of these genes.

Figure 4-5. Expression of sterol-demethylase genes is not elevated among mutant *hmg1* strains.

Fold expression of *cyp51A* and *cyp51B* in mutant *hmg1* strains relative to the parental *akuB*^{KU80} strain as determined by reverse transcription quantitative PCR.

Conclusions

Here we describe for the first time, a novel and non-canonical mechanism of triazole antifungal resistance. Identified through whole genome sequencing, mutations in the HMG-CoA reductase encoding gene, *hmg1*, were found among the majority of triazole-resistant *A. fumigatus* clinical isolates in our collection. Of the observed mutations, most occurred in the conserved sterol sensing domain of *hmg1*, which has been previously demonstrated to participate in the regulation of sterol biosynthesis in other eukaryotic organisms including *S. pombe*. [85-88] When three of these clinically-derived *hmg1* mutations were independently introduced into a well characterized triazole-susceptible strain of *A. fumigatus* using an optimized Cas9-mediated system to replace the native *hmg1* locus, resistance to all clinically available anti-*Aspergillus* triazole antifungals greatly increased. To confirm that mutations in *hmg1* are a novel mechanism of clinical triazole resistance, we employed the same system to correct a *hmg1* mutation identified in a pan-triazole resistant clinical isolate of *A. fumigatus* and observed restoration of susceptibility to all triazoles.

As the sterol-sensing domain of HMG-CoA reductase has been shown to be essential to the regulation of sterol biosynthesis, we hypothesized that amino acid substitutions in this domain of Hmg1 may lead to impaired negative regulation of Hmg1 activity. [85-88] In an effort to test this hypothesis, we performed comprehensive sterol profiling on the mutant *hmg1* strains. This analysis identified significant accumulations of ergosterol precursors, including eburicol, 4,4-dimethyl ergosta 8,24(28) dienol, fecosterol, episterol, while total cellular ergosterol content was seen to be maintained, or in the case of the *hmg1*^{F262del} mutation, significantly increased. However, this increase in ergosterol precursors did not result in a subsequent increase in sterol-demethylase expression levels in any of the mutant *hmg1* strains. Thus, *hmg1* mutation-mediated triazole resistance is not the result of increased sterol-demethylase gene expression.

Taken together, the results of this study identify mutations in the *A. fumigatus* HMG-CoA reductase gene, *hmg1*, as a novel and non-canonical genetic determinant of clinical triazole resistance, present among a large proportion of resistant clinical isolates. While the exact mechanism by which mutations in the sterol-sensing domain of *hmg1* impart clinical triazole resistance remains unknown at this time, it is tempting to speculate that the negative regulation of Hmg1 activity, which has been shown to be dependent on the sterol-sensing domain in other eukaryotic organisms, may be altered by residue substitutions in the sterol-sensing domain of *A. fumigatus* Hmg1.

CHAPTER 5. DISCUSSION

Invasive aspergillosis is a leading cause of morbidity and mortality among immunocompromised populations and is predicted to cause more than 200,000 life-threatening infections each year.[1, 4, 83, 84] *Aspergillus fumigatus* is the predominant pathogen isolated from patients with invasive aspergillosis, and accounts for more than 60% of all cases.[2] Only three classes antifungal agents are currently available for the treatment of invasive infections caused by *Aspergillus fumigatus*. Of these, the triazoles represent both therapeutic agents of choice, such as voriconazole and isavuconazole, as well as agents commonly used for salvage therapies such as itraconazole and posaconazole. As other antifungal agents such as the polyene amphotericin B and the echinocandins possess limitations in formulation, safety profile, or lack consistent fungicidal activity against *A. fumigatus*, the triazoles have proven to be essential to the treatment of invasive aspergillosis. Thus, the recent emergence of triazole-resistant *A. fumigatus* represents a critical threat to the current treatment of invasive aspergillosis.[10]

While considerable research has been performed to interrogate the mechanisms by which *A. fumigatus* becomes resistant to triazole antifungals, the overwhelming majority of resistance has been ascribed to three canonical paths that were originally described among pathogenic fungi decades ago; 1) mutations in sterol-demethylase genes, 2) overexpression of sterol-demethylase genes, or 3) overexpression of efflux pump-encoding genes. [25, 28, 34, 35, 42-44, 48, 66, 71, 79] Of these three paths, mutations in the *A. fumigatus* sterol demethylase gene *cyp51A* are the most commonly reported in the literature. However, the proportion of clinical triazole resistance explained by these canonical pathways remains poorly characterized. Additionally, resistant clinical isolates exhibiting none of these previously identified mechanisms have been reported.[25] This gap in the current understanding of the molecular mechanisms underpinning clinical triazole resistance in *A. fumigatus* is a significant barrier to the preservation of the utility of the triazoles and the design of novel therapeutic strategies to overcome triazole-resistant aspergillosis. The overall objective of this work was to overcome this barrier by performing a comprehensive characterization of the direct contributions of previously identified mechanisms of triazole resistance in a large collection of highly triazole-resistant clinical *A. fumigatus* isolates, and to identify novel mechanisms where unexplained clinical resistance was found.

In Chapter 2 we sought to test the hypothesis that the previously identified mechanisms of triazole resistance do not fully account for the degree of triazole resistance observed among clinical isolates of *A. fumigatus*. To accomplish this, we utilized a large collection of triazole-resistant clinical *A. fumigatus* isolates and whole genome sequencing, to identify all mutations in the sterol-demethylase genes *cyp51A* and *cyp51B*, and well the previously identified sterol-demethylase cis- and trans-regulatory elements. While no mutations unique to resistant isolates were identified in *cyp51B*, unique mutations in *cyp51A* were found in the vast majority of resistant isolates. However, correction of ten of these *cyp51A* mutations to the wild-type sequence only partially or fully restored clinical triazole susceptibility in two and one clinical isolate

background, respectively. Additionally, RTqPCR revealed significant overexpression of *cyp51A* was observed in eight of the twenty-one triazole resistant isolates, five of which had mutations in either the promoter of *cyp51A* or a known transcriptional regulator of *cyp51A* (*hapE*). However, we subsequently demonstrated that the constitutive overexpression of either sterol-demethylase gene by as much as thirty-five-fold, had a limited impact on triazole susceptibility in a triazole-susceptible laboratory isolate. Next, we examined the expression of the previously characterized *A. fumigatus* triazole efflux pump *abcC* in our collection of isolates by RTqPCR. Fourteen of the twenty-one triazole-resistant clinical isolates were observed to have significantly increased expression of *abcC*. However, the majority of these isolates only exhibited a small increase in *abcC* (2.4 to 4.1-fold) while only three were found to have more marked increases in expression (6.5 to 33.2-fold), and constitutive overexpression of *abcC* (7.8-fold) in a triazole-susceptible laboratory strain yielded no change in triazole susceptibility. These findings serve to highlight the fact that even when considered collectively, the previously characterized mechanisms of resistance do not adequately explain the triazole resistance observed in this collection of *A. fumigatus* clinical isolates, and additional mechanisms yet to be identified are likely present in this collection.

Subsequently, in Chapter 3 we tested the hypothesis that the constitutive overexpression of efflux pump-encoding genes, other than *abcC*, directly contributes to clinical triazole resistance in *A. fumigatus*. To accomplish this, we utilized the collection of clinical *A. fumigatus* isolates we had previously characterized and transcriptional profiling to identify a subset of *A. fumigatus* efflux pump-encoding genes with a high degree of homology to the well characterized *C. albicans* triazole efflux pump *Cdr1*, which were uniquely, constitutively overexpressed among triazole-resistant clinical isolates. We then identified *abcA* and *atrI* as the genes of greatest interest, and utilized Cas9-mediated genetic manipulations to delineate the direct contributions of these genes to triazole resistance. Unexpectedly, we found that deletion of these genes in triazole-resistant clinical isolates known to overexpress them, did not result in meaningful changes in triazole susceptibility. However, it is worth noting that these results may have been limited by the presence of additional resistance mechanisms in these isolates, such as *cyp51A* mutations. While these findings could not definitively demonstrate a role for either *abcA* or *atrI* in clinical triazole resistance, they do serve to demonstrate the potential for additional efflux pump-encoding genes to participate in clinical triazole resistance.

Finally, in Chapter 4 we utilized our previously generated whole genome sequencing data to test the hypothesis that mutations in genes involved in the biosynthesis of ergosterol, other than sterol-demethylase, significantly contribute to clinical triazole resistance. Following careful examination of our whole genome sequencing data, we identified numerous genes of the ergosterol biosynthesis pathway (*erg3B*, *erg3C*, *erg4B*, *erg5*, *erg6*, and *hmg1*) where mutations unique to triazole-resistant isolates were present. Of these, we found mutations in the HMG-CoA-reductase gene *hmg1* to be the most intriguing, as the majority of resistant clinical isolates in our collection were observed to have mutations in this gene, and previous studies in other eukaryotic organisms such as *Schizosaccharomyces pombe* have found *hmg1* homologs

to contain conserved sterol sensing domains involved in the regulation of the ergosterol biosynthesis pathway. Additionally, the majority of the identified *hmg1* mutations were found to reside in the predicted sterol-sensing domain. As these sterol-sensing domain mutations each fell within one of three predicted transmembrane domains, we selected one representative mutation from each transmembrane domain and utilized a novel Cas9-mediated split marker transformation system to introduce these mutant *hmg1* alleles into a triazole-susceptible laboratory strain of *A. fumigatus*. Surprisingly, introduction of each of the three mutant *hmg1* alleles to the susceptible strain were found to dramatically increase MICs for all clinically available triazole agents. Moreover, the complementary experiment correcting the F262del *hmg1* mutation in the *hmg1* allele of the pan-triazole-resistant isolate DI15-105 resulted in the restoration of clinical susceptibility to all triazoles. Thus, proving that mutations in *hmg1* are a novel genetic determinant of clinical triazole resistance in *A. fumigatus*.

As the sterol-sensing domain of HMG-CoA-reductase enzymes has been shown to participate in the autoregulation of HMG-CoA-reductase activity, and subsequently the regulation of ergosterol biosynthesis, we then hypothesized that mutations in the sterol-sensing domain of *hmg1* lead to dysregulation of ergosterol biosynthesis. To test this hypothesis, we performed compressive sterol profiling on the cellular extracts from each of our mutant *hmg1* strains and the parental strain with a wild-type *hmg1* allele. In agreement with our hypothesis, sterol profiling revealed that the mutant *hmg1* strains indeed exhibited accumulation of ergosterol precursors downstream HMG-CoA-reductase activity, while also maintaining, or in some cases increasing, the total cellular ergosterol content. This finding was particularly intriguing as it is suggestive of a potential association between increased *hmg1* activity and triazole resistance. Additionally, HMG-CoA is a validated target for pharmacological inhibition, and numerous drugs which inhibit the human form of this enzyme are currently clinically available. Future studies are needed to further characterize the role of *hmg1* mutations in clinical triazole resistance, and to identify any potential therapeutic strategies for overcoming triazole resistance associated with these mutations.

While the findings of the studies described herein identify mutations in *hmg1* as a novel genetic determinant of clinical triazole resistance, we have yet to definitively identify the molecular mechanism(s) by which these mutations contribute to the observed resistance. It is however tempting to speculate, based upon these data and previously published findings, that triazole resistance may be the result of two molecular mechanisms related to altered cell sterol composition. First, the accumulation of ergosterol precursors such as eburicol, episterol, and fecosterol, as observed in our sterol profiling experiments, may alter baseline cell membrane characteristics sufficiently to diminish the impact of sterol-demethylase inhibition. It has been postulated that a portion of the antifungal effect of triazoles is a consequence of acute changes in membrane characteristics such as fluidity and integrity upon inhibition of sterol-demethylase activity. [92] Accumulation of ergosterol precursors, particularly those downstream of the sterol-demethylase enzymes such as episterol and fecosterol, may augment the impact of the triazoles on membrane characteristics by either temporarily facilitating continued ergosterol biosynthesis or through the modification of baseline membrane characteristics.

Additionally, it is possible that the accumulation of ergosterol precursors indirectly mediates triazole resistance. Previous studies have shown that the accumulation of sterols such as eburicol can precipitate activation of stress response pathways similar to those triggered by exposure to triazole antifungals. [93] Thus, the accumulation of ergosterol precursors in isolates with mutations in *hmg1* may lead to increases in the constitutive expression of drug resistance effectors, such as efflux pump-encoding genes, and prime cells to rapidly respond to triazole exposure. However, future studies will be needed to explore the potential contribution of these molecular mechanisms to *hmg1* mutation-mediated clinical triazole resistance.

In conclusion, the findings of the work described herein serve to demonstrate that clinical triazole resistance in *A. fumigatus* is most likely the result of a complex amalgamation of resistance mechanisms, including both mechanisms currently known and yet to be identified. Furthermore, the paradigm of the molecular mechanisms of triazole resistance established in fungal pathogens such as *Candida albicans*, offers only an incomplete explanation for the triazole resistance observed in clinical isolates of *A. fumigatus*. The addition of *hmg1* mutations to the known genetic determinants of triazole resistance advances our understanding of the molecular mechanisms underpinning clinical triazole resistance, yet a substantial proportion of resistance remains to be explained. Future directions for this work include the characterization of compounded triazole resistance mechanisms, such as the combination of *cpy51A* and *hmg1* mutations, as well as the further characterization of efflux pump-encoding genes which are differentially expressed among triazole-resistant clinical isolates. Additionally, *A. fumigatus* Hmg1 must be investigated as a potential target of pharmacological inhibition and an opportunity for the development of a new therapeutic strategy to overcome clinical triazole resistance in *A. fumigatus*.

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