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Fluconazole Susceptibility in *Cryptococcus gattii* Is Dependent on the ABC Transporter Pdr11

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***Cryptococcus gattii* isolates from the Pacific Northwest have exhibited higher fluconazole MICs than isolates from other sites. The mechanism of fluconazole resistance in *C. gattii* is unknown. We sought to determine the role of the efflux pumps Mdr1 and Pdr11 in fluconazole susceptibility. Using biolistic transformation of the parent isolate, we created a strain lacking Mdr1 (*mdr1*Δ) and another strain lacking Pdr11 (*pdr11*Δ). Phenotypic virulence factors were assessed by standard methods (capsule size, melanin production, growth at 30 and 37°C). Survival was assessed in an intranasal murine model of cryptococcosis. Antifungal MICs were determined by the M27-A3 methodology. No differences in key virulence phenotypic components were identified. Fluconazole susceptibility was unchanged in the Mdr1 knockout or reconstituted isolates. However, fluconazole MICs decreased from 32 μg/ml for the wild-type isolate to <0.03 μg/ml for the *pdr11*Δ strain and reverted to 32 μg/ml for the reconstituted strain. In murine models, no difference in virulence was observed between wild-type, knockout, or reconstituted isolates. We conclude that Pdr11 plays an essential role in fluconazole susceptibility in *C. gattii*. Genomic and expression differences between resistant and susceptible *C. gattii* clinical isolates should be assessed further in order to identify other potential mechanisms of resistance.**

Cryptococcosis is an invasive fungal infection most commonly caused by one of two species of encapsulated yeasts: *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* has a worldwide distribution, while *C. gattii* has traditionally been associated with tropical and subtropical regions (1–3). The recent identification of *C. gattii* in the temperate climate of the Pacific Northwest (4) has caused investigators to question the true distribution of this emerging pathogen, and additional cases have since been reported throughout the United States, the Mediterranean region, and northern Europe (5–8).

The Infectious Diseases Society of America (IDSA) treatment guidelines outline similar treatment principles for *C. gattii* infection and *C. neoformans* infection, although they do confirm the need for additional investigation into the role of drug resistance in infections caused by *C. gattii* (9). Elevated fluconazole MICs are most commonly observed for *C. gattii* isolates from the Pacific Northwest, particularly those of the VGII molecular type (261/449 [58%] VGII isolates exhibited fluconazole MICs of ≥8 μg/ml) (10–13), with VGIIc subtype isolates exhibiting the highest fluconazole MICs (for 41/42 VGIIc isolates, fluconazole MICs were ≥8 μg/ml) (10).

Past studies examining the molecular mechanisms behind triazole resistance in *C. neoformans* have demonstrated that resistance most commonly occurs due to a modification in the quality or quantity of the target enzyme, reduced access of the drug to the target, or some combination of these mechanisms (10). In the first instance, point mutations in the gene (*ERG11*) encoding the target enzyme, 14-α-demethylase, lead to an altered target with decreased affinity for azoles (14–16). In the second, overexpression of *ERG11* or a change in the *ERG11* copy number results in the production of high concentrations of the target enzyme, creating the need for higher intracellular azole concentrations to effectively inhibit the target enzyme present in the cell (17). The last major mechanism described for cryptococcal isolates involves active efflux of azole antifungal agents from the cell through the action of multidrug efflux transporters, and there is evidence that these mechanisms may act individually or in concert (18).

The ABC transporters are a large class of drug efflux pumps that use ATP hydrolysis to translocate compounds across cell membranes. Mdr1 is a drug efflux pump that belongs to the multidrug resistance (MDR) subclass of ABC transporters (19). Overexpression of Mdr1 promotes azole resistance in *C. neoformans*. A second subclass of ABC transporters is the pleiotropic drug resistance (PDR) family, which includes the Afr1 protein. The Afr1 drug efflux transporter has been linked to azole resistance and heteroresistance in *C. neoformans*. The *C. gattii* orthologs of *C. neoformans MDR1* (Cn*MDR1*) and Cn*AFR1* have been identified as *C. gattii MDR1* (Cg*MDR1*) and Cg*PDR11*. Whether Mdr1 or Pdr11, or both, are the mechanisms responsible for the fluconazole resistance of *C. gattii* is not known.

To elucidate the mechanisms responsible for decreased fluconazole susceptibility in *C. gattii*, we performed a series of experiments focused on the mechanisms most likely to be responsible

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for resistance and constructed mutants lacking drug efflux pumps that are likely to play a role in altered triazole susceptibility.

MATERIALS AND METHODS

Isolates, growth media, and susceptibility testing. Deletion strains were generated from isolate GRT78, a VGII clinical isolate obtained from a patient seen at the UC Davis Medical Center. This patient was a 76-year-old male with no underlying medical problems who presented with a right upper lobe infiltrate that was found on bronchoscopy to be *C. gattii*. He was treated with 200 mg voriconazole every 12 h for 6 months. He remains symptom free 3 years after the cessation of antifungal therapy.

Prior work on *C. gattii* has focused primarily on the outbreak strains R265 (VGIIa) and R272 (VGIIb); however, susceptibility testing using the M27-A3 broth microdilution reference method (20) revealed fluconazole MICs of 8 and 4 μg/ml, respectively, for these isolates (21), while GRT78 yielded a fluconazole MIC of 32 μg/ml on repeated testing and thus was our choice for use in this assessment of molecular mechanisms responsible for the elevated fluconazole MICs for some *C. gattii* VGII strains.

The media used during this study included yeast extract-peptone-dextrose (YPD, comprising 1% yeast extract, 2% peptone, and 2% glucose) liquid medium and agar (2%) plates. YPD medium containing 200 μg/ml hygromycin or 100 μg/ml nourseothricin was used to select transformants.

In vitro susceptibility to fluconazole was also determined by Etest (bioMérieux) according to the manufacturer's instructions and was used in the screening and evaluation of mutants constructed during the conduct of this study. Isolates were subcultured at least twice on Sabouraud dextrose agar (Becton Dickinson, Sparks, MD) prior to *in vitro* susceptibility testing. The Etest MICs were read as the points at which a prominent reduction in growth intersected the Etest strip, as described previously (40). All isolates used for analysis then underwent broth microdilution susceptibility testing for MIC confirmation using the standard M27-A3 methodology as mentioned above. For posaconazole, voriconazole, itraconazole, fluconazole, and flucytosine, the MIC was defined as a 50% reduction in turbidity from that for growth controls and was read at 72 h. For amphotericin B, the MIC was defined as 100% inhibition relative to growth controls. Briefly, minimum fungicidal concentrations (MFCs) were determined by subculturing 20-μl aliquots from each well that showed complete inhibition after MIC determination (optically clear well) onto Sabouraud dextrose agar. The plates were incubated at 35°C, and the MFCs, defined as the lowest drug concentrations that resulted in no growth, were read. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality controls.

Identification of *C. gattii MDR1* and *PDR11* orthologs. The *C. gattii* orthologs of the genes encoding *C. neoformans* Mdr1 and Afr1 were identified by reciprocal BLAST searches for similarities between these two cryptococcal species. The BLAST "hit" orthologs in *C. gattii* were identified as *MDR1* (locus tag CGB_A9140C; sequence identification [ID] XP_003191799.1) and *PDR11* (locus tag CGB_N1170W; sequence ID XP_003197471.1), respectively. *MDR1* in *C. gattii* shares 93% identity at the amino acid level with its ortholog in *C. neoformans*, while *PDR11* in *C. gattii* shares 96% identity at the amino acid level with *AFR1* in *C. neoformans*.

Disruption of the *MDR1* and *PDR11* genes. (i) *MDR1* disruption. The *mdr1*Δ mutant was generated by overlap PCR as described previously (22). A 5,600-bp fragment of the wild-type *MDR1* gene was amplified by PCR in a 50-μl master mix containing 0.25 μl of TaKaRa HS DNA polymerase, 5 μl of 10× HS *Ex Taq* buffer, 4 μl of a 2.5 mM deoxynucleoside triphosphate (dNTP) mixture, 1 μl of each primer (gMDR1_F57 and gMDR1_R5637), 1 μl of 50-ng/μl genomic DNA, and 38.75 μl of nuclease-free water (see Table S1 in the supplemental material). Immediately following PCR, 5 μl of the reaction mixture was aliquoted and was confirmed by agarose electrophoresis, while 0.2 μl of Qiagen *Taq* polymerase (nonproofreading) and 0.5 μl of a dNTP mixture were added to the remaining reaction mixture, which was then incubated at 72°C for 10 min. The fragment was cloned into plasmid pCR2.1 by following the manufacturer's instructions for the TOPO TA cloning kit (Life Technologies), generating the pCR2.1::*MDR1* construct. More than 50 white colonies were screened via PCR using the same primers as those used to amplify the genes and M13 primers. Plasmids were extracted from the transformants via the QIAprep Spin Miniprep kit and were submitted to the UC Davis core sequencing facility with M13 primers. The sequences were analyzed using Geneious software (version 7.7.1), and the identity of the cloned fragment was confirmed.

The *MDR1* disruption construct (pCR2.1::*mdr1*Δ::*HYG*) was generated by replacing the middle region of the *MDR1* gene in the pCR2.1::*MDR1* construct with the selectable marker *HYG*. To ligate the two fragments, two unique restriction sites (AvrII and PacI) were introduced into the primers. The fragment from the pCR2.1::*MDR1* construct was amplified with primers MDR_topo PacI F5098 and MDR_topo AvrII R1323, and the *HYG* fragment was amplified with primers pJAF15&1 AvrII 001F and pJAF15&1 PacI 001R, as described above (see Table S1 in the supplemental material). The two PCR fragments were purified using a QIAquick purification kit (Qiagen), digested with the DpnI, AvrII, and PacI restriction enzymes, and ligated via T4 DNA ligase according to the manufacturer's instructions (New England BioLabs). This generated the pCR2.1::*mdr1*Δ::*HYG* construct, which was then transformed into chemically competent *Escherichia coli* TOP10 cells (from the TOPO TA cloning kit). The transformants were confirmed by PCR and restriction digestion. The A14 transformant was selected as the *MDR1* disruption construct (pCR2.1::*mdr1*Δ::*HYG*), propagated, and biolistically transformed into 3- to 4-day-old cells of the *C. gattii* parental strain GRT78. The mutants were confirmed by PCR and reverse transcriptase PCR (RT-PCR).

(ii) *PDR11* disruption. The *PDR11* disruption construct pCR2.1::*pdr11*Δ::*HYG* was generated by following the same protocol as that for the *mdr1*Δ strain discussed above. A 5,622-bp fragment of the wild-type *PDR11* gene was amplified from the parental strain GRT78 with primers cgPDR11_F2192 and cgPDR11_R7814 (see Table S1 in the supplemental material) and was cloned into pCR2.1. To replace the middle region of the *PDR11* gene in the pCR2.1::*PDR11* construct, a 5,116-bp fragment was amplified with primers CgPDR11_PacI F5166 and CgPDR11_AvrII R2584, digested with DpnI, AvrII, and PacI, and ligated with the same *HYG* fragment that was used for *mdr1*Δ mutants. This generated the *PDR11* disruption construct (pCR2.1::*pdr11*Δ::*HYG*), which was then transformed into chemically competent *E. coli* TOP10 cells. Once the construct was confirmed, 2.5 μg to 5 μg of the purified construct was biolistically transformed into the parental strain GRT78. *pdr11*Δ mutants were confirmed by PCR and RT-PCR.

Reconstitution of *MDR1* and *PDR11* genes. The nourseothricin selectable marker (*NAT*) from plasmid pCH233 (23) was used to reconstitute the mutant strains with the wild-type *MDR1* and *PDR11* genes. The entire wild-type *MDR1* gene (7,207-bp fragment), including the promoter and terminator, was amplified from the parental strain GRT78 with primers gMDR_F912 and gMDR_R8119 by following the same PCR protocol as that described above (see Table S1 in the supplemental material), confirmed by agarose electrophoresis, and purified. A 2.5-μg portion of the purified fragment and 2.5 μg of plasmid pCH233 (*NAT* selectable marker) were cobiolistically transformed into the knockout strains. Transformants were selected on YPD with 100 μg/ml of *NAT*.

In order to create the *PDR11* reconstituted strain, the *PDR11* gene (7,097-bp fragment), including the promoter and terminator, was PCR amplified from the parental strain GRT78 with primers cgPDR11_F1011 and cgPDR11_R8108. Reconstituted strains were confirmed by PCR and Etest.

RNA extraction and RT PCR. RNA was extracted using the MasterPure yeast RNA purification kit (Epicentre). The quality of the RNA was confirmed via denatured agarose formaldehyde electrophoresis. cDNA was synthesized from RNA using the SuperScript III First-Strand Synthesis system (Invitrogen), followed by PCR using the same primers used to clone the genes into pCR2.1.

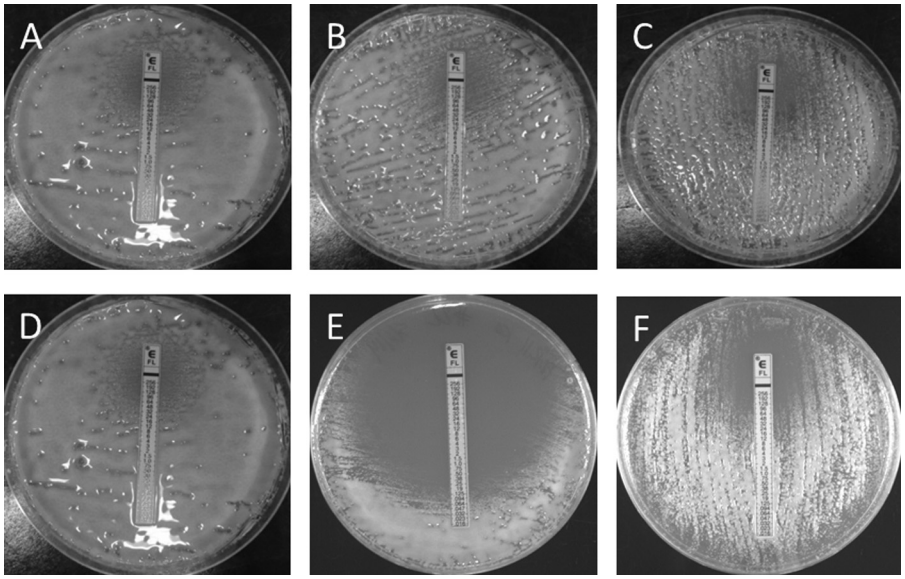

FIG 1 MICs of fluconazole, obtained by Etest, for GRT78 strains. (A and D) Wild-type GRT78 strains with fluconazole MICs of 32 μg/ml. (B) *mdr1*Δ mutant strain (MIC, 32 μg/ml). (C) *MDR1* reconstituted strain (*mdr1*::*MDR1*) (MIC, 32 μg/ml). (E) *pdr11*Δ mutant strain, for which the fluconazole MIC was significantly reduced, to 0.06 μg/ml. (F) *PDR11* reconstituted strain (*pdr11*::*PDR11*), with reversion to wild-type fluconazole susceptibility (MIC, 32 μg/ml).

Phenotypic testing. Capsule formation, melanin production, growth at 30°C and 37°C, and resistance to H_2O_2 were evaluated using previously described protocols (21, 24, 25). The development of heteroresistance was assessed by taking subclones from each strain and using stepwise exposure to YPD plates containing various concentrations of fluconazole (0.5 to 512 μg/ml). Growth was read after 72 h of incubation at 30°C. In order to obtain highly resistant subclones, the clones that grew on different concentrations of fluconazole were isolated and plated on YPD agar with stepwise (2-fold) increases in the fluconazole concentration (1 to 512 μg/ml). The culture plates of each passage were incubated at 30°C for 72 to 96 h as described previously (26).

Inhalational model. Virulence was assessed using female A/JCr mice (15 to 20 g; Jackson Laboratory). Strains were cultured in YPD broth for 18 to 20 h at 30°C, harvested, washed three times with sterile phosphate-buffered saline (PBS), and counted using a hemocytometer to determine cell concentrations. Inocula for all experiments were confirmed by plating on YPD and counting CFU. Six mice per strain were infected by intranasal inhalation with 5×10^4 CFU in 50 μl PBS as described previously (27). During the conduct of all studies, animals that appeared moribund or in pain were euthanized. All experiments were in compliance with the ethical regulations established by the UC Davis Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. Mortality and time to mortality were evaluated for statistical significance by Kaplan-Meier survival analysis and the Mann-Whitney U test. *P* values were obtained from a log rank test. Tissue fungal burdens were assessed using analysis of variance with Tukey's posttest. A *P* value of <0.05 was considered statistically significant.

RESULTS

Generation of *PDR11*- and *MDR1*-deficient and reconstituted strains of *C. gattii*. We generated a set of isogenic *C. gattii* strains from isolate GRT78, a VGII strain with a fluconazole MIC of 32 μg/ml. The *PDR11* gene was disrupted as described above, and the resulting disruption allele, *pdr11*::*HYG*, was introduced into the wild-type strain GRT78. Of the 60 transformants produced, 4 with presumptive evidence of *PDR11* disruption were evaluated by RT-PCR. These analyses confirmed the absence of *PDR11* transcripts in one of these transformants, and this *pdr11*Δ mutant was selected for subsequent experiments. Similarly, the *MDR1* gene was disrupted, and the resulting disruption allele, *mdr1*::*HYG*, was introduced into the initial GRT78 strain. Three mutants with presumptive evidence of *MDR1* disruption were evaluated by RT-PCR, and one was chosen for inclusion in this study.

Deletion of *PDR11* but not *MDR1* has a significant effect on susceptibility to fluconazole. Deletion mutations of both *PDR11* and *MDR1* were created. The susceptibilities of the corresponding knockout mutants to fluconazole, screened by Etest (Fig. 1), revealed a significant reduction in fluconazole MICs for the *pdr11*Δ mutants (<0.06 μg/ml) but no corresponding reduction in fluconazole MICs for the *mdr1*Δ isolates (≥ 32 μg/ml). Fluconazole MICs returned to 32 μg/ml, the same as that for the wild-type isolate, for the *pdr11*::*PDR11* and *mdr1*::*MDR1* reconstituted strains.

Additional susceptibility testing of GRT78 revealed the following MICs: for itraconazole and posaconazole, 2 μg/ml; for voriconazole, 1 μg/ml; and for amphotericin B and flucytosine, 0.125 μg/ml. For the *pdr11*Δ mutant, the itraconazole, posaconazole, and voriconazole MICs decreased to 0.5, 0.5, and 0.25 μg/ml, respectively, while the amphotericin B and flucytosine MICs were not significantly changed from those for the parental strain. Susceptibility testing of the *mdr1*Δ mutant and the reconstituted isolates yielded results identical to those for GRT78 (Table 1).

Minimum fungicidal concentrations (MFCs) were also determined for each isolate in order to ascertain whether gene disruption and/or reconstitution played any role. Except for the *pdr11*Δ mutant (fluconazole MFC, 4 μg/ml), all mutants and reconstituted isolates exhibited fluconazole MFCs of ≥ 128. *MDR1* thus had no effect on fluconazole MICs or MFCs (Table 1).

***In vitro* phenotypic analysis of *PDR11* and *MDR1* deletion and reconstituted strains.** There were no statistically significant differences in capsule formation, melanin production, growth at 30°C or 37°C, or resistance to H_2O_2 between any of the strains (21). All isolates examined in this study also underwent serial

TABLE 1 MICs and MFCs[a] for clones used during the conduct of this study

Isolate[b]	MIC (MFC) (μg/ml) of:					
	Fluconazole	Itraconazole	Posaconazole	Voriconazole	Amphotericin B	Flucytosine
GRT78	32 (>128)	2	2	1	0.125	0.125
*pdr11*Δ strain	0.06 (4)	0.5	0.5	0.25	0.125	0.125
PDR1 strain	32 (>128)	2	2	1	0.125	0.125
*mdr1*Δ strain	32 (>128)	2	2	1	0.125	0.125
MDR1 strain	32 (>128)	2	2	1	0.125	0.25

[a] MFCs, minimum fungicidal concentrations.
[b] The *MDR1* and *PDR11* strains are reconstituted strains (*mdr1*::*MDR1* and *pdr11*::*PDR11*, respectively).

passage in a fluconazole-containing medium for assessment of heteroresistance. Reduced growth was not observed for GRT78 subclones until a fluconazole concentration of 256 μg/ml was reached, and growth was completely inhibited at 512 μg/ml. The *pdr11*Δ mutants had visibly fewer colonies at a fluconazole concentration of 2 μg/ml and were unable to grow at 4 μg/ml. The *mdr1*Δ mutant and both reconstituted isolates exhibited a level of fluconazole tolerance unchanged from that of the wild-type isolate (visibly fewer colonies at 256 μg/ml and complete inhibition of growth at 512 μg/ml).

Inhalational model (virulence and *in vivo* treatment effects). The possible involvement of *PDR11* and *MDR1* in the virulence of *C. gattii* was assessed in an inhalational model of infection. As shown in Fig. 2, there were no significant differences in survival time between any of the strains through 60 days postinfection. Necropsies revealed disseminated infection to the brain, spine, kidneys, spleen, and liver and showed no significant differences between strains (P, >0.05 for all groups).

DISCUSSION

The emergence of *C. gattii* as an important pathogen has resulted in numerous studies to define its epidemiology and clinical features, as well as the virulence components responsible for the lower treatment response rates than those observed with *C. neoformans* (21, 28–32). In some regions, *C. gattii* has been observed to exhibit reduced *in vitro* susceptibility to fluconazole and other triazole agents (33). Susceptibility differs between *C. gattii* molecular types, and several studies have shown that VGII isolates frequently have higher fluconazole MICs than other genotypes

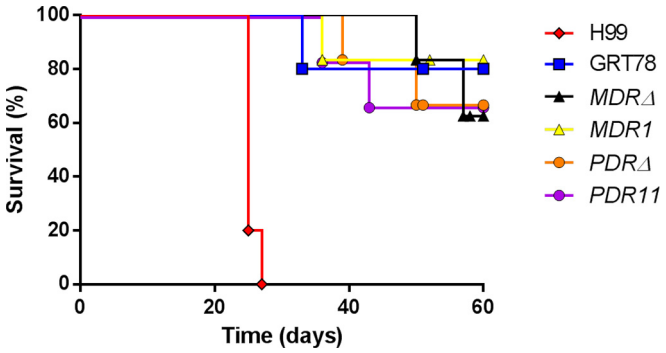

FIG 2 The wild-type strain (GRT78), the *mdr1*Δ and *pdr11*Δ mutant strains, and the *mdr1*::*MDR1* and *pdr11*::*PDR11* reconstituted strains do not differ significantly in a murine intranasal inhalational model of virulence. All *C. gattii* strains evaluated showed lower levels of virulence than *C. neoformans* strain H99 ($P < 0.01$).

(11–13, 34, 35). Despite the potential clinical importance of reduced susceptibility, the molecular mechanism behind elevated MICs in selected VGII strains has not been established previously.

We have demonstrated that elevated fluconazole MICs for a clinical *C. gattii* VGII isolate are dependent on *PDR11* expression. Deletion of this ABC transporter resulted in a fluconazole MIC markedly reduced (from 32 to 0.06 μg/ml) from that for the parent isolate, and the elevated fluconazole MIC was completely restored with the reconstitution of the *PDR11* gene. In contrast, *MDR1* appeared to play no role in *in vitro* or *in vivo* resistance to fluconazole in this isolate.

These findings suggest that *PDR11* alone may be responsible for the triazole-resistant *C. gattii* isolates from the Pacific Northwest. Prior work has revealed that neither overexpression nor mutations within *ERG11*, the triazole drug target, could explain the marked elevation in fluconazole MICs associated with Pacific Northwest *C. gattii* isolates (36). Subsequent work by the same group used [^{3}H]fluconazole to examine the role of drug efflux pumps in *C. gattii* and found an association between a decline in intracellular radiolabeled fluconazole and fluconazole MICs (37). Expression of *C. gattii* and *C. neoformans* drug efflux pumps in a *Saccharomyces cerevisiae* strain allowed for additional scrutiny of these pumps and confirmed that in a model system, they may increase triazole MICs. Our study adds to this work by confirming the essential role of *PDR11* in a clinical *C. gattii* isolate with elevated triazole MICs. In contrast to the work by Basso et al. (37), we did not confirm a role for *MDR1* in antifungal drug resistance.

The kinetics of *C. gattii* ABC transporters are also noteworthy. The K_m values of these enzymes are similar for *C. neoformans* and *C. gatiii*; however, the V_{max} values of both species exceed those of Cdr1p and Cdr2p in *Candida albicans*, and the V_{max} values of *C. gattii* efflux pumps are substantially higher than those observed for *C. neoformans* (37). However, it remains unclear whether these species-specific differences are related to the quantity of protein expressed and the stability of the enzyme, or whether subtle sequence differences in these regions confer added affinity and thereby heightened efflux of antifungal agents. These efflux pumps also likely exhibit variable affinity and efficiency in the transport of different triazoles—differences that account for the divergent triazole MICs in our mutant and reconstituted strains.

The MICs of other triazoles (itraconazole, posaconazole, and voriconazole) were also elevated for our initial isolate. In results similar to those observed with fluconazole, deletion of *PDR11*, but not *MDR1*, played a significant role in altering fungal susceptibility to these mold-active triazoles as well to all agents experiencing at least a 2-dilution reduction in their MICs for the *pdr11*Δ mutant. The importance of elevated MICs of other extended-spec-

trum triazoles for *C. gattii* isolates should not be overlooked. The emergence of elevated fluconazole MICs for VGII strains has become well known, and some patients may be treated empirically with alternative triazoles based on epidemiologic studies demonstrating lower MICs of itraconazole, posaconazole, and voriconazole than of the more commonly used agent fluconazole (12). In view of this, we recommend susceptibility testing for all *C. gattii* isolates from areas known to harbor VGII isolates prior to, or concurrently with, the initiation of antifungal therapy.

Drug efflux transporters not only are important for resistance to antifungal agents and xenobiotics but also have been implicated in fungal pathogenesis and virulence (38). The PDR family is one of the largest families of ABC transporters in pathogenic fungi, and most ABC transporters have been demonstrated to have multiple substrates, including phospholipids, steroids, host-specific toxins, and host-derived compounds that would inhibit fungal growth in addition to providing resistance to antifungal agents. However, in our strains, no differences were found between the parental isolates, disruption mutants, and reconstituted strains in virulence studies. This is in contrast to the results of a previous study examining the role of *AFR1* (*PDR11* ortholog) in a murine model of *C. neoformans* infection (39). However, the study by Sanguinetti et al. used a fluconazole-susceptible *C. neoformans* isolate and a fluconazole-resistant isolate created by serial passage in a fluconazole-containing medium (39). This study occurred prior to the recognition of chromosomal aneuploidy as a major mechanism behind the stress response in cryptococcal species, and although the authors carefully attempted to assess the quantity of known virulence factors in different isolates by RT-PCR, aneuploidy likely played a large role in the virulence of the strains used in their study, as has been noted by others (26).

In conclusion, we present evidence that the efflux pump gene *PDR11* plays a critical role in the antifungal resistance of a VGII clinical strain. These results demonstrate, for the first time, that antifungal resistance in VGII isolates may be entirely dependent on *PDR11* alone. Future work should focus on a quantitative assessment of efflux pump expression in *C. gattii* isolates of different molecular types obtained from different locations and with different triazole susceptibility patterns. With the declining costs of whole-genome and transcriptome-sequencing (RNA-seq) analysis, we are hopeful that potential differences in the gene sequence, expression, and drug affinity of efflux pumps from different *C. gattii* molecular types will soon be determined.

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