New Insights into the Cyp51 Contribution to Azole Resistance in *Aspergillus* Section *Nigri*

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ABSTRACT Invasive aspergillosis (IA) is a severe condition mainly caused by *Aspergillus fumigatus*, although other species of the genus, such as section *Nigri* members, can also be involved. Voriconazole (VRC) is the recommended treatment for IA; however, the prevalence of azole-resistant *Aspergillus* isolates has alarmingly increased in recent years, and the underlying resistance mechanisms in non-*fumigatus* species remain unclear. We have determined the *in vitro* susceptibility of 36 strains from section *Nigri* to VRC, posaconazole (POS), and itraconazole (ITC), and we have explored the role of Cyp51A and Cyp51B, both targets of azoles, in azole resistance. The three drugs were highly active; POS displayed the best *in vitro* activity, while ITC and VRC showed MICs above the established epidemiological cutoff values in 9 and 16% of the strains, respectively. Furthermore, expression studies of *cyp51A* and *cyp51B* in control condition and after VRC exposure were performed in 14 strains with different VRC susceptibility. We found higher transcription of *cyp51A*, which was upregulated upon VRC exposure, but no correlation between MICs and *cyp51* transcription levels was observed. In addition, *cyp51A* sequence analyses revealed nonsynonymous mutations present in both, wild-type and non-wild-type strains of *A. niger* and *A. tubingensis*. Nevertheless, a few mutations were exclusively present in non-wild-type *A. tubingensis* strains. Altogether, our results suggest that azole resistance in section *Nigri* is not clearly explained by Cyp51A protein alteration or by *cyp51* gene upregulation, which indicates that other mechanisms might be involved.

KEYWORDS *Aspergillus* section *Nigri*, Cyp51, amino acid substitutions, azole resistance, gene expression

Aspergillus is a widely distributed genus able to cause human opportunistic infections (1), with invasive aspergillosis (IA) being the most problematic condition in terms of mortality, which ranges from 50 to 100% (2). Immunocompromised patients, such those suffering from neutropenia and cancer and patients undergoing organ or hematopoietic stem cell transplantation, are the most susceptible (3, 4). Although *A. fumigatus* is the most frequent causal agent of IA (5), species from other sections of the genus, such as *Flavi*, *Terrei*, and *Nigri*, can also be involved (6). In particular, members of the section *Nigri*, including *A. niger* and the cryptic species *A. tubingensis*, *A. brasiliensis*, *A. awamori*, *A. japonicus*, and *A. carbonarius*, among others (1), have been reported as the second leading cause of IA, along with sections *Flavi* and *Terrei* (6–11).

In the current guidelines for diagnosis and management of IA, voriconazole (VRC) constitutes the first-line therapy (12). However, in recent years there has been an alarming increase in *Aspergillus* isolates showing resistance to azoles, which is correlated with poor therapeutic outcome, entirely reducing treatment options (5, 13–15). In the particular case of *Aspergillus*, which generally displays elevated azole susceptibility (16, 17), cases of azole resistance have been constantly increasing since the first reports in the 1990s and have been associated with both azole-treated and azole-naïve patients

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(13). This acquired resistance can be explained by the fungistatic effect of azoles but also by azole exposure in the clinical and environmental settings (5, 13, 18).

The azole mode of action consists of noncompetitive binding to the Cyp51 enzyme, a 14 α -demethylase from the ergosterol biosynthetic pathway, which leads to ergosterol synthesis inhibition and, consequently, cell membrane disruption (19). In this sense, several azole resistance mechanisms in *A. fumigatus* have been postulated. The presence of amino acid substitutions in the Cyp51 target proteins and overexpression of the *cyp51* genes or efflux pumps, such as the ATP binding cassette transporter Cdr1B, constitute the most important mechanisms described thus far (7, 20).

In this context, the study of *cyp51* genes has emerged as a main topic in azole susceptibility profiling in fungal pathogens. Some filamentous fungi, including *Aspergillus*, carry multiple *cyp51* genes in their genomes. This genetic redundancy is widely believed to facilitate adaptation to fungicides exposure, leading to an increase in azole resistance (21). Specifically, *A. fumigatus*, *A. terreus*, and *A. niger* contain two *cyp51* paralogs (*cyp51A* and *cyp51B*), while *A. flavus* contains three *cyp51* genes (*cyp51A*, *cyp51B*, and *cyp51C*) (7, 22). Moreover, previous studies conducted in *A. fumigatus* have indicated functional diversification and different contribution to azole resistance of *cyp51* paralogs (22).

Since azole resistance mechanisms in non-*fumigatus* species, such as the members of section *Nigri*, remain practically unknown, characterization of *cyp51* genes might lead to increase our knowledge on azole resistance emergence.

In this study, we performed gene expression studies of *cyp51A* and *cyp51B* in a set of clinical and environmental strains belonging to *Aspergillus* section *Nigri*, as well as sequence analyses of the *cyp51A* gene, in order to potentially correlate gene expression and/or nonsynonymous mutations with VRC resistance in members of this section.

RESULTS

Antifungal susceptibility. The MICs of itraconazole (ITC), posaconazole (POS), and VRC were determined in 36 strains belonging to section *Nigri*. The results are shown in Fig. 1 and also Table S1 in the supplemental material. All the assayed drugs were active against the tested strains, with the lowest modal MIC being that of POS (0.06 μ g/ml), followed by ITC (1 μ g/ml) and VRC (2 μ g/ml). In all strains the POS MICs were below the epidemiological cutoff value (ECV) established for this drug against section *Nigri*, whereas the ITC and VRC MIC values were lower than the ECVs established in 9 and 16% of the strains, respectively. The ITC MIC was equal to the ECV in only one strain of *A. tubingensis*, while in the case of VRC, this was observed in eleven strains (species *A. niger*, *A. tubingensis*, *A. neoniger*, and *A. acidus*).

Cross-resistance between ITC and VRC was observed in four strains (9% of the total) belonging to *A. niger*, *A. tubingensis*, and *A. brasiliensis* species.

In silico identification and sequence analysis of *cyp51* genes in section *Nigri* species. We conducted an *in silico* analysis in the *A. niger*, *A. tubingensis*, and *A. brasiliensis* genomes to identify *cyp51* genes and their deduced amino acid sequences. The results of BLASTP using the amino acid sequence of *A. fumigatus* Cyp51A (Afu4g06890) as a query revealed high homology with *A. niger* An11g02230, *A. tubingensis* Asptu1_0056695, and *A. brasiliensis* Aspbr1_0190913, showing a 79.7 to 80.2% identity range. Likewise, BLASTP search using *A. fumigatus* Cyp51B (Afu7g03740) as a query revealed high homology with *A. niger* An13g0009, *A. tubingensis* Asptu1_0051806, and *A. brasiliensis* Aspbr1_0137500, showing a 84.2 to 85.1% identity range.

In addition, sequence alignment of Cyp51A and Cyp51B revealed moderate pairwise similarity among these paralogous proteins in every species, displaying identity values of 62.5% for *A. fumigatus*, and from 63 to 63.8% for the non-*fumigatus* species studied. The detailed view reveals conserved residues involving the putative azole binding site among both proteins in all species.

Unlike *A. fumigatus*, which contains a single transmembrane domain, *A. niger*, *A. tubingensis*, and *A. brasiliensis* exhibit two predicted transmembrane domains in both Cyp51A and Cyp51B, comprising amino acids 5 to 24 and 37 to 59 and amino acids 20

FIG 1 Box plot representation of *Aspergillus* section *Nigri* susceptibility pattern to the triazoles ITC (a), POS (b), and VRC (c). The box edges represent the first and third quartiles, respectively. Thick lines denote median values, while minimum and maximum values are depicted by horizontal lines.

to 42 and 54 to 73, respectively. Protein models are represented in Fig. 2. The sequence alignment, azole-binding residues, transmembrane domains, and the heme prosthetic group are represented in Fig. S1 (Cyp51A) and Fig. S2 (Cyp51B). The Cyp51A and Cyp51B sequence alignment of every species is represented in Fig. S3.

FIG 2 Protein topology of *A. fumigatus* (a) and *A. niger*, *A. tubingensis*, and *A. brasiliensis* (b) Cyp51A and Cyp51B associated with the endoplasmic reticulum membrane. The amino acid positions comprised by each protein structure are also indicated.

FIG 3 Expression analysis results of *cyp51A* (a) and *cyp51B* (b) genes in control condition and after exposure to 4 μg/ml VRC for 8 h. *actA* was used as the housekeeping gene to normalize transcription levels. MIC values expressed in μg/ml are shown below isolates labels. Statistical significance ($P \leq 0.05$) is marked by an asterisk (*).

Expression analysis of *cyp51A* and *cyp51B* in azole-inducing conditions. To analyze the expression patterns of *cyp51A* and *cyp51B* in control condition and during exposure to VRC, transcription levels of both genes were determined in 14 strains of section *Nigri* by quantitative reverse transcription-PCR. The results show higher expression of the *cyp51A* gene compared to that of the *cyp51B* in both conditions and all strains. We observed remarkable fluctuations of *cyp51A* transcription among strains, ranging from 2×10^{-1} to 5×10^2 times fold with respect to the housekeeping gene (Fig. 3). Exposure to VRC resulted in statistically significant *cyp51A* induction ($P \leq 0.008$) in six strains (43%) categorized as wild type (wt) and non-wild type (non-wt). In addition, another 43% of the tested strains showed a non-statistically significant increased transcription of this gene upon drug exposure. Exceptionally, *cyp51A* was similarly expressed in the control condition and under VRC exposure in the *A. brasiliensis* strain (Fig. 3a). Unexpectedly, the strain displaying the highest VRC MIC showed low levels of *cyp51A* transcription, which were significantly reduced in VRC presence. Therefore, we could not establish any pattern of expression of *cyp51A* along the increasing MIC scale.

Regarding *cyp51B*, expression levels ranged from 3×10^{-1} to 7 times fold respect to the housekeeping gene, and VRC exposure did not result in significant overexpression (Fig. 3b). These data suggest that *cyp51B* gene expression is not inducible by VRC, in contrast to the *cyp51A* gene.

***cyp51A* sequencing and amino acid substitutions identification.** In order to identify nonsynonymous mutations in *cyp51A* that could be correlated with VRC resistance within the section *Nigri*, Cyp51A protein sequence was analyzed in the 14 selected strains. All *A. tubingensis* strains but one and all *A. niger* strains displayed amino acid substitutions in the Cyp51A protein compared to the reference sequences.

In the particular case of *A. tubingensis*, the amino acid change H467Q was exclusively detected in environmental non-wt strains in combination with substitutions K64E or

TABLE 1 Nonsynonymous nucleotide mutations in gDNA and their corresponding amino acid substitutions in the deduced Cyp51A protein sequence^a

Species	FMR strain	Nucleotide change	Amino acid substitution ^b	VRC MIC ($\mu\text{g/ml}$) ^c	Origin
<i>A. tubingensis</i>	14630	C475T C1293T	A140V P413S	4	Environmental
	14635			4	Environmental
	14712	G1185A C1457G	V377I H467Q	4	Environmental
	17207	A190G C1457G	K64E H467Q	4	Environmental
	11906	C475T C1293T	A140V P413S	2	Clinical
	15389	G1017A	A321T	2	Environmental
<i>A. niger</i>	11900	A169G A740G	T57A Q228R	16	Clinical
	15441	A740G	Q228R	8	Environmental
	15385	A169G A740G	T57A Q228R	2	Environmental
	11254	A169G A740G	T57A Q228R	1	Clinical
	15388	A169G A740G	T57A Q228R	1	Environmental
		T1095A	S346R		
	11894	A169G A571G (CGC)610(AGT) A740G C782T A1189G A1318C (CTG)1321(ATC)	T57A I172V A185S Q228R T242M I378V M421L V422I	0.25	Clinical
	11898	A169G A740G	T57A Q228R	0.25	Clinical
<i>A. brasiliensis</i>	15386			8	Environmental

^aNomenclature refers to the nucleotide/amino acid present in the reference sequence, followed by its position and the nucleotide/amino acid substitution in the analyzed strain.

^bAmino acid substitutions indicated in boldface have a potential role in VRC resistance.

^cMIC values indicated in boldface are greater than the established ECV for VRC (2 $\mu\text{g/ml}$).

V377I. In addition, other mutations were found in both wt and non-wt strains of this species, such as A140V and P413S, whereas the amino acid change A321T was only found in an environmental strain that displayed elevated susceptibility to VRC (Table 1). Regarding *A. niger*, none of the substitutions found in the Cyp51A protein could be linked to resistant phenotypes. Q228R was a common change in all strains, which was frequently found in combination with T57A, located in the second transmembrane domain of the *A. niger* Cyp51A. In addition to these, an environmental wt strain showed the S346R change. Multiple substitutions were observed in the case of the clinical wt strain FMR 11894 beyond Q228R and T57A: I172V, A185S, T242M, I378V, M420L, and V422I. The *A. brasiliensis* non-wt strain did not display any amino acid substitution in the Cyp51A protein.

The detected nucleotide and amino acid modifications compared to the deposited sequences in the *Aspergillus* Genome Database are summarized in Table 1. In addition, Cyp51A sequence alignments with domain identifications and the amino acid changes found in our strains are marked in Fig. S1.

DISCUSSION

Although resistance mechanisms in *A. fumigatus* have been extensively studied, there are very few studies carried out on members of *Aspergillus* section *Nigri*. Thus, we sought to test the *in vitro* susceptibility of clinical and environmental isolates of relevant species of *Aspergillus* section *Nigri*, as well as to characterize possible resistance mechanisms present in our set of strains.

The three azoles tested here (VRC, PSC, and ITC) showed good activity, with POS being the most active one, a finding in accordance with previous studies (23–25). The low modal POS MIC and its narrow MIC range are also indicative of its elevated *in vitro* activity against these fungi. Both ITC and VRC exhibited good *in vitro* activity, even though low susceptibility to VRC was more common than to ITC, which is also in line with previous results (26). In addition, ITC non-wt isolates were found more frequently among *A. tubingensis* isolates than in the other species of this section that we tested, as already described (23, 26, 27). In accordance with previous works (23, 27), cross-resistance was not common in our isolates.

Since Cyp51 proteins constitute the targets for azoles, a high level of their transcripts could confer azole resistance by maintaining the cellular ergosterol levels. In fact, it has been documented that the introduction of extra copies of the *A. nidulans cyp51A* gene (*pdmA*) in *A. fumigatus* caused a decrease in ITC susceptibility in the latter (28), suggesting that *cyp51A* overexpression might confer azole resistance. However, this mechanism does not seem to be the key factor conditioning azole resistance, since no overexpression of *cyp51A* was detected in a set of non-wt *A. fumigatus* strains in a subsequent study (29). Great variability in expression patterns was also described in studies focusing on other species of the genus, thus providing controversial results. Specifically, in *A. flavus*, *cyp51* transcript levels were not correlated to VRC-resistant phenotypes in a study (30), whereas 80% of non-wt strains displayed *cyp51A* overexpression in another one (31), although in the latter work resistance was linked to multidrug efflux pump overexpression as well.

Our results suggest, for the first time in section *Nigri* to our knowledge, that *cyp51A* actively participates in the transcriptional response to azole stress in these fungi, which is clearly upregulated after exposure to VRC. Nevertheless, transcript levels of *cyp51A* showed no correlation to the VRC MICs in this section, meaning that azole resistance is not explained by *cyp51A* overexpression. This has also been discussed in a recent study in which the basal expression of *cyp51A* in a few species of section *Nigri* did not correlate with the susceptibility patterns they displayed (26).

In contrast, *cyp51B* showed notably lower and stable expression in all the conditions we tested, suggesting a minor role in azole response. Accordingly, *cyp51A* has also been described to be the key player in *A. fumigatus* azole response, since its disruption results in reduced resistance to azoles, while azole susceptibility patterns are not altered in *cyp51B* knockout mutants (22, 32). However, this perception is not shared by everyone, since it has been suggested that *cyp51B* also contributes to azole resistance in *A. fumigatus* due to its induction upon ITC exposure, although only in one strain after ITC exposure (29). This was also observed in our study since a slight but not significant upregulation of *cyp51B* was detected upon VRC exposure. Considering that these are isolated facts, the involvement of *cyp51B* in azole resistance seems negligible.

Interestingly, an opposite pattern was detected in a transcriptome analysis performed on a strain of *A. fumigatus* with elevated azole susceptibility in which both *cyp51* genes decreased their expression after VRC exposure (33). Surprisingly, we have observed this inhibition in the strain showing the highest VRC MIC (FMR 11900), which displayed a drastic reduction of *cyp51A* transcription under VRC. These controversial data reinforce the hypothesis that other mechanisms and not *cyp51* expression could be contributing to the appearance of resistant phenotypes in *Aspergillus*.

In another line, differences in the Cyp51A protein sequence and structure are also important parameters to be considered, as changes in the conformation of the protein can vary its stability and flexibility, reducing azole binding affinity (34, 35). It should be noted that the detailed study of the Cyp51 proteins revealed some structural and sequence differences among the studied species of section *Nigri* and *A. fumigatus*, such as the presence of two transmembrane domains in section *Nigri* in contrast to only one of *A. fumigatus*. Although the transmembrane domain present in *A. fumigatus* has been described already, there are slight differences between the previously reported amino acids that comprise the transmembrane region in this species and our predicted data (36).

In *A. fumigatus*, many cases of azole resistance have been linked to *cyp51A* single point mutations that lead to amino acid alterations in the Cyp51A protein, as well as tandem repeats in the promoter region of the *cyp51A* gene that enhance its expression (37–41). Interestingly, no amino acid substitutions in the Cyp51B protein have been correlated to azole resistance so far, which is also indicative of its smaller role in azole response.

However, there has been some controversy, since some of the Cyp51A mutations previously described have also been found in highly azole-susceptible *A. fumigatus* and other *Aspergillus* spp. (23, 27, 42, 43). In fact, some of these amino acid differences believed to confer azole resistance have been related to different regional origins rather than resistance events (42).

Regarding our results in the species of section *Nigri*, sequence analysis of Cyp51A revealed no general correlation between amino acid changes and azole resistance, since most of the mutations were present in both wt and non-wt strains. This is the case for A140V and P413S substitutions in *A. tubingensis* or T57A and Q228R in *A. niger*, which were previously reported by others as well (23, 26). Moreover, amino acid A321 in *A. tubingensis* Cyp51A has been described as a residue that could confer azole resistance (26). Our results though, seem to contradict this, since five of our strains with different VRC MICs presented Ala in this position and a non-wt strain presented Thr in the same position. This straightforwardly suggests that amino acid changes in this position are not related to azole resistance. In addition, we found that two non-wt strains belonging to *A. tubingensis* (FMR 14635) and *A. brasiliensis* (FMR 15386) did not present any amino acid substitution, meaning that elevated VRC MICs found for these strains could be due to other mechanisms. Nevertheless, other non-wt strains (FMR 14712 and FMR 17207) presented in an exclusive manner a few Cyp51A substitutions. To our knowledge, the mutations H467Q, V377I, or K64E found in these isolates have not been reported to date; however, it remains unclear whether they are the cause of azole resistance in these strains, and further studies are needed to clarify their exact role. In particular, the substitutions K64E, V377I, and H467Q do not correspond to any characterized azole binding site residues, and therefore azole union should not be altered. Nonetheless, it remains unknown whether these mutations have any effect on the conformation of the protein or its binding pocket, preventing optimal binding.

Although *cyp51* expression levels and protein affinity to VRC may be crucial to dealing with azole toxicity inside the cell, our data show that neither *cyp51A* transcription nor amino acid changes are straightforwardly linked to resistant phenotypes in *Aspergillus* section *Nigri*. This contrasts with what occurs in *A. fumigatus* (26, 27). Together, these findings suggest that resistance is a complex phenomenon in which other molecular mechanisms are involved, especially in non-*fumigatus* species. In this sense, more studies with larger sets of strains are needed, and potential new resistance mechanisms should be further explored.

MATERIALS AND METHODS

Strains, media, and growth conditions. A total of 36 environmental and clinical strains belonging to *Aspergillus* section *Nigri* (*A. niger*, *A. tubingensis*, *A. brasiliensis*, *A. awamori*, *A. japonicus*, *A. carbonarius*, *A. neoniger*, and *A. acidus*), previously identified by ITS, *benA*, and *cam* gene marker sequencing, were included in the *in vitro* susceptibility study (see Table S1 in the supplemental material).

Fourteen of these strains, belonging to *A. niger*, *A. tubingensis*, and *A. brasiliensis*, were selected for further studies on the basis of their different *in vitro* VRC susceptibility to characterize potential resistance mechanisms (Table S1). Five of the strains were clinical and had been previously isolated from sputum or skin lesions in Texas, Brazil, and Spain. The rest had been isolated from environmental sites in different countries (Spain, Venezuela, Mexico, Vietnam, and Brazil).

Strains were stored as mineral oil cultures or lyophilized cultures and before used they were grown on potato dextrose agar (PDA) media (Conda-Pronadisa) for 3 to 5 days at 35°C twice. For conidiation, strains were grown for 3 to 5 days on PDA at 35°C, and conidia were collected by flooding the PDA culture plates with 5 ml of 0.1% Tween 20 solution and gentle agitation. For genomic DNA (gDNA) extraction, cultures were incubated in potato dextrose broth (PDB) as previously described with some modifications (44). Briefly, conidial suspensions were incubated in PDB for 24 h at 37°C and 180 rpm, and mycelia were collected by monodur filtration, washed with distilled sterile water, and stored at –80°C until use. For gene expression analyses, 1×10^7 conidia/ml were germinated in YG medium (0.5% yeast extract, 2% glucose) for 12 h at 37°C and 180 rpm. Mycelia were harvested by filtration and aseptically

TABLE 2 Primers used in this study designed with Oligo7 software^a

Primer	Sequence (5'–3')	Species	Use
cyp51A-F1	GAACCCAGACGAGGAGAAG	All ^b	RT-qPCR/Seq
cyp51A-R1	TCGCAGCATGATCCAAGAAC	<i>A. niger</i> - <i>A. brasiliensis</i>	RT-qPCR
cyp51A-R2	CGCAACATAATCCAAGAGCTA	<i>A. tubingensis</i>	RT-qPCR
cyp51B-F1	GTTTCCATTATAGGTAGCAC	All	RT-qPCR
cyp51B-R1	ACTTCTTCAGCACAGACATCA	All	RT-qPCR
act-F2	ACCCTCAGATACCCATTGA	All	RT-qPCR
act-R2	CTGGGTCATCTTCTCACGG	All	RT-qPCR
An-F2	CGATAGTCTTAAATGTCACGC	<i>A. niger</i>	Seq
An-R3	GCTCCCAACCCACTATAGC	<i>A. niger</i>	Seq
Anb-R2	TGAGGCGAGTAGAACATGGT	<i>A. niger</i> - <i>A. brasiliensis</i>	Seq
Ax-R3	ACCTCCTCGCCCTGCAAC	All	Seq
Asptu-F1	ATGGCATATCTTGCTGTTGCA	<i>A. tubingensis</i>	Seq
Asptu-R1	TTAGTTCAAGGACCCCTTGGA	<i>A. tubingensis</i>	Seq
Asptu-R2	CTGGTGAGGCGAGTAGAAC	<i>A. tubingensis</i>	Seq
Asptu-R3	CTTGGAGTTGTCTTGATGC	<i>A. tubingensis</i>	Seq
Aspbt-F2	TGCTCGTTGCGATAGTCTTG	<i>A. tubingensis</i> - <i>A. brasiliensis</i>	Seq
Aspbr-F1	ATGGCATTCTTGCTATCGC	<i>A. brasiliensis</i>	Seq
Aspbr-R1	TCAGTTCGATGACCCCTTGG	<i>A. brasiliensis</i>	Seq
Aspbr-R3	CGGAGAAGAGAGTCGAGTAA	<i>A. brasiliensis</i>	Seq

^aAbbreviations: F, forward; R, reverse; RT-qPCR, quantitative real-time PCR; Seq, sequencing.

^bAll = *A. niger*, *A. tubingensis*, and *A. brasiliensis*.

transferred to fresh YG medium supplemented with VRC (Pfizer, Inc.) at a final concentration of 4 µg/ml when required. Cultures were incubated at 37°C and 180 rpm for 8 h and mycelia were collected by monodur filtration, washed with distilled water, and stored at –80°C until use.

Antifungal susceptibility testing. *In vitro* susceptibility testing was carried out according to Clinical and Laboratory Standards Institute (CLSI) protocol M38 for broth microdilution (45). Briefly, stock solutions of ITC (Janssen Pharmaceutica), POS (Schering-Plough Research Institute), and VRC (Pfizer, Inc.) were prepared in sterile water or dimethyl sulfoxide. All drugs were diluted in RPMI 1640 medium and dispensed in 96-well microdilution trays, which were inoculated with a conidial suspension of every strain previously adjusted by using a hemocytometer count. MICs of triazoles were determined after 48 h of incubation at 35°C without light nor agitation by direct visualization with an inverted mirror. MICs corresponded to the lowest drug concentration that completely inhibited fungal growth. *A. flavus* strain ATCC 204304 was used as the quality control strain.

Strains were classified as wt and non-wt according to the currently established ECVs for ITC (4 µg/ml), POS (2 µg/ml), and VRC (2 µg/ml) and section *Nigri*, since no clinical breakpoints are available yet for *Aspergillus* (46). The strains displaying ITC, POS, or VRC MICs above the ECVs were categorized as non-wt, while those displaying MICs below the ECVs were categorized as wt.

DNA and RNA isolation. For DNA and RNA extraction, mycelia were ground in liquid nitrogen with mortar and pestle. Total gDNA was extracted from ground mycelia according to a previously reported protocol (47). Total RNA was extracted from ground mycelia with TRIzol reagent (Thermo Fisher) as previously described (48). RNA was purified with a NucleoSpin RNA kit (Macherey-Nagel, Germany), including on-column DNase digestion. The resulting DNA and RNA pellets were resuspended in DNase- and RNase-free purified water, and the quantity and quality were checked by running aliquots in RedSafe-stained agarose gels and spectrophotometric analysis in NanoDrop 2000 spectrophotometer (Thermo Fisher).

Gene and protein identification and analysis in section *Nigri*. *cyp51A*, *cyp51B*, and actin (*actA*) orthologs were identified in *A. niger*, *A. tubingensis*, and *A. brasiliensis* by BLASTP searches (Basic Local Alignment Search Tool) in the *Aspergillus* Genome Database, using *A. fumigatus* Af293 protein sequences as templates. Alignments of the deduced amino acid sequence were performed with ClustalW algorithm of MegaLign software (DNASTAR). Sequence analysis and identification of the putative azole-binding residues and heme prosthetic group in *A. niger*, *A. tubingensis*, and *A. brasiliensis* was performed by comparison with *A. fumigatus* protein structure deposited in the SWISS-MODEL database (IDs: 4uy1.2, 4uym.1, and 5fbr.1) and data published by others (49–51). Prediction of transmembrane helices and topology of proteins was performed with the TMHMM v2.0 server and the InterPro platform.

Retrotranscription and quantitative real-time PCR. For gene expression studies, total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad) and following the manufacturer's indications. Reverse transcription-PCRs were performed in a StepOne Plus real-time PCR system (Applied Biosystems) using 7.5 µl of FastStart Universal SYBR green Master mix (Roche Diagnostics), 6.9 µl of cDNA template, and 300 nM of each gene-specific primer (Table 2) in a final reaction volume of 15 µl. All primer pairs amplified products of 162 to 207 bp. The thermal cycling conditions were as follows: 94°C (5 min); 45 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s); and finally 80°C (20 s). A melting curve was obtained immediately after PCR completion to check amplification specificity under the following conditions: 95°C (15 s), 60°C (15 s), and 95°C (15 s). Expression levels were calculated by normalizing *cyp51A* and *cyp51B* cycle thresholds (C_T) with those of the housekeeping gene *actA* (52) using the efficiency (E) corrected ΔC_T method (53). With this method, the relative expression

ratio is calculated from the quantitative real-time PCR (RT-qPCR) E value assessed for the three genes in the study (Fig. S4), which were within an acceptable E value range (54), and the C_T in each case.

cyp51A sequencing and protein analysis. Amplification of *cyp51A* was carried out by standard PCR using the following thermal cycling protocol: 98°C (1 min); followed by 35 cycles of 98°C (10 s), 62°C (30 s), and 72°C (1.5 min); and finally 72°C (10 min).

Amplicons were sequenced with the primers listed in Table 2 at Macrogen Europe (Macrogen, Inc., Madrid, Spain), and sequences were analyzed using SeqMan (DNASTAR) and MEGA version 7 software. Alignments of the deduced amino acid sequences were performed using the ClustalW algorithm of MegaAlign software (DNASTAR).

Statistical analysis. A Mann-Whitney test was used to compare relative gene expression values. All statistical analyses were performed with GraphPad Prism 6.0 for Windows. *P* values of ≤ 0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00543-19>.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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REFERENCES

- Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud Mycol* 78:141–173. <https://doi.org/10.1016/j.simyco.2014.07.004>.
- Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. *Sci Transl Med* 4:165rv13.
- Taccone F, Van den Abele A-M, Bulpa P, Misset B, Meersseman W, Cardoso T, Paiva J-A, Blasco-Navalpotro M, De Laere E, Dimopoulos G, Rello J, Vogelaers D, Blot SI. 2015. Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes. *Crit Care* 19:7. <https://doi.org/10.1186/s13054-014-0722-7>.
- García-Rubio R, Cuenca-Estrella M, Mellado E. 2017. Triazole resistance in *Aspergillus* species: an emerging problem. *Drugs* 77:599–613. <https://doi.org/10.1007/s40265-017-0714-4>.
- Chowdhary A, Sharma C, Meis JF. 2017. Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis* 216: S436–S444. <https://doi.org/10.1093/infdis/jix210>.
- Lass-Flörl C, Cuenca-Estrella M. 2017. Changes in the epidemiological landscape of invasive mould infections and disease. *J Antimicrob Chemother* 72:i5–i11. <https://doi.org/10.1093/jac/dkx028>.
- Hagiwara D, Watanabe A, Kamei K, Goldman GH. 2016. Epidemiological and genomic landscape of azole resistance mechanisms in *Aspergillus* fungi. *Front Microbiol* 7:1382. <https://doi.org/10.3389/fmicb.2016.01382>.
- Vermeulen E, Maertens J, Meersseman P, Saegeman V, Dupont L, Lagrou K. 2014. Invasive *Aspergillus niger* complex infections in a Belgian tertiary care hospital. *Clin Microbiol Infect* 20:333–335.
- Ergene U, Akcali Z, Ozbalki D, Nese N, Senol S. 2013. Disseminated aspergillosis due to *Aspergillus niger* in immunocompetent patient: a case report. *Case Rep Infect Dis* 2013:385190. <https://doi.org/10.1155/2013/385190>.
- Atchade E, Jean-Baptiste S, Houzé S, Chabut C, Massias L, Castier Y, Brugière O, Mal H, Montravers P. 2017. Fatal invasive aspergillosis caused by *Aspergillus niger* after bilateral lung transplantation. *Med Mycol Case Rep* 17:4–7. <https://doi.org/10.1016/j.mmc.2017.05.002>.
- Person AK, Chudgar SM, Norton BL, Tong BC, Stout JE. 2010. *Aspergillus niger*: an unusual cause of invasive pulmonary aspergillosis. *J Med Microbiol* 59:834–838. <https://doi.org/10.1099/jmm.0.018309-0>.
- Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JAH, Bennett JE. 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis* 63:e1–e60. <https://doi.org/10.1093/cid/ciw326>.
- Wiederhold N, Patterson T. 2015. Emergence of azole resistance in *Aspergillus*. *Semin Respir Crit Care Med* 36:673–680. <https://doi.org/10.1055/s-0035-1562894>.
- Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. 2017. The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect Dis* 17:e383–e392. [https://doi.org/10.1016/S1473-3099\(17\)30316-X](https://doi.org/10.1016/S1473-3099(17)30316-X).
- Wiederhold N. 2017. Antifungal resistance: current trends and future strategies to combat. *Infect Drug Resist* 10:249–259. <https://doi.org/10.2147/IDR.S124918>.
- Delarue E, Sanglard D. 2015. Defining the frontiers between antifungal resistance, tolerance, and the concept of persistence. *Drug Resist Updat* 23:12–19. <https://doi.org/10.1016/j.drug.2015.10.001>.
- Nett JE, Andes DR. 2016. Antifungal agents: spectrum of activity, pharmacology, and clinical indications. *Infect Dis Clin North Am* 30:51–83. <https://doi.org/10.1016/j.idc.2015.10.012>.
- Chowdhary A, Kathuria S, Xu J, Meis JF. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog* 9:e1003633. <https://doi.org/10.1371/journal.ppat.1003633>.
- Parker JE, Warrilow AGS, Price CL, Mullins JGL, Kelly DE, Kelly SL. 2014. Resistance to antifungals that target CYP51. *J Chem Biol* 7:143–161. <https://doi.org/10.1007/s12154-014-0121-1>.
- Fraczek MG, Bromley M, Buied A, Moore CB, Rajendran R, Rautemaa R, Ramage G, Denning DW, Bowyer P. 2013. The *cdr1B* efflux transporter is associated with non-*cyp51A*-mediated itraconazole resistance in *Aspergillus fumigatus*. *J Antimicrob Chemother* 68:1486–1496. <https://doi.org/10.1093/jac/dkt075>.
- Hawkins NJ, Cools HJ, Sierotzki H, Shaw MW, Knogge W, Kelly SL, Kelly DE, Fraaije BA. 2014. Paralog re-emergence: a novel, historically contingent mechanism in the evolution of antimicrobial resistance. *Mol Biol Evol* 31:1793–1802. <https://doi.org/10.1093/molbev/msu134>.
- Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodríguez-Tudela JL. 2001. Identification of two different 14- α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol* 39:2431–2438. <https://doi.org/10.1128/JCM.39.7.2431-2438.2001>.
- Iatta R, Nuccio F, Immediato D, Mosca A, De Carlo C, Miragliotta G, Parisi A, Crescenzo G, Otranto D, Cafarchia C. 2016. Species distribution and *in vitro* azole susceptibility of *Aspergillus* section *Nigri* from clinical and environmental settings. *J Clin Microbiol* 54:e01075-16.
- Asano M, Kano R, Kamata H, Makimura K, Hasegawa A. 2011. Molecular typing and *in-vitro* activity of azoles against clinical isolates of *Aspergillus fumigatus* and *A. niger* in Japan. *J Infect Chemother* 17:483–486. <https://doi.org/10.1007/s10156-010-0202-1>.
- Badali H, Fakhim H, Zarei F, Nabili M, Vaezi A, Poorzad N, Dolatabadi S,

- Mirhendi H. 2016. *In vitro* activities of five antifungal drugs against opportunistic agents of *Aspergillus Nigri* complex. *Mycopathologia* 181: 235–240. <https://doi.org/10.1007/s11046-015-9968-0>.
26. Hashimoto A, Hagiwara D, Watanabe A, Yahiro M. 2017. Drug sensitivity and resistance mechanism in *Aspergillus* section *Nigri* strains from Japan. *Antimicrob Agents Chemother* 61:1–10.
 27. Howard SJ, Harrison E, Bowyer P, Varga J, Denning DW. 2011. Cryptic species and azole resistance in the *Aspergillus niger* complex. *Antimicrob Agents Chemother* 55:4802–4809. <https://doi.org/10.1128/AAC.00304-11>.
 28. Oshervov N, Kontoyiannis DP, Romans A, May GS. 2001. Resistance to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the *A. nidulans* P-450 14 α -demethylase gene, *pdmA*. *J Antimicrob Chemother* 48:75–81. <https://doi.org/10.1093/jac/48.1.75>.
 29. Buié A, Moore CB, Denning DW, Bowyer P. 2013. High-level expression of *cyp51B* in azole-resistant clinical *Aspergillus fumigatus* isolates. *J Antimicrob Chemother* 68:512–514. <https://doi.org/10.1093/jac/dks451>.
 30. Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, Li R. 2012. The T788G mutation in the *cyp51C* gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. *Antimicrob Agents Chemother* 56: 2598–2603. <https://doi.org/10.1128/AAC.05477-11>.
 31. Fattahi A, Zaini F, Kordbacheh P, Rezaie S, Safara M, Fateh R, Farahyar S, Kanani A, Heidari M. 2015. Evaluation of mRNA expression levels of *cyp51A* and *mdr1*, candidate genes for voriconazole resistance in *Aspergillus flavus*. *Jundishapur J Microbiol* 8:e26990. <https://doi.org/10.5812/jjm.26990>.
 32. Garcia-Effron G, Mellado E, Gomez-Lopez A, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2005. Differences in interactions between azole drugs related to modifications in the 14- α sterol demethylase gene (*cyp51A*) of *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 49:2119–2121. <https://doi.org/10.1128/AAC.49.5.2119-2121.2005>.
 33. da Silva Ferreira ME, Malavazi I, Savoldi M, Brakhage AA, Goldman MHS, Kim HS, Nierman WC, Goldman GH. 2006. Transcriptome analysis of *Aspergillus fumigatus* exposed to voriconazole. *Curr Genet* 50:32–44. <https://doi.org/10.1007/s00294-006-0073-2>.
 34. Warrilow AGS, Parker JE, Price CL, Nes WD, Kelly SL, Kelly DE. 2015. *In vitro* biochemical study of CYP51-mediated azole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 59:7771–7778. <https://doi.org/10.1128/AAC.01806-15>.
 35. Liu M, Zheng N, Li D, Zheng H, Zhang L, Ge H, Liu W. 2016. *cyp51A*-based mechanism of azole resistance in *Aspergillus fumigatus*: illustration by a new 3D structural model of *Aspergillus fumigatus* CYP51A protein. *Med Mycol* 54:400–408. <https://doi.org/10.1093/mmy/myv102>.
 36. Warrilow AGS, Melo N, Martel CM, Parker JE, Nes WD, Kelly SL, Kelly DE. 2010. Expression, purification, and characterization of *Aspergillus fumigatus* sterol 14- α demethylase (CYP51) isoenzymes A and B. *Antimicrob Agents Chemother* 54:4225–4234. <https://doi.org/10.1128/AAC.00316-10>.
 37. Spiess B, Postina P, Reinwald M, Cornely OA, Hamprecht A, Hoenigl M, Lass-Flörl C, Rath P-M, Steinmann J, Miethke T, Lauten M, Will S, Merker N, Hofmann W-K, Buchheidt D. 2014. Incidence of Cyp51A key mutations in *Aspergillus fumigatus*: a study on primary clinical samples of immunocompromised patients in the period of 1995–2013. *PLoS One* 9:e103113. <https://doi.org/10.1371/journal.pone.0103113>.
 38. Rivero-Menendez O, Alastruay-Izquierdo A, Mellado E, Cuenca-Estrella M. 2016. Triazole resistance in *Aspergillus* spp.: a worldwide problem? *J Fungi* 2:21. <https://doi.org/10.3390/jof2030021>.
 39. Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, Mazier D, Datry A, Fekkar A. 2014. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. *J Antimicrob Chemother* 69:3244–3247. <https://doi.org/10.1093/jac/dku316>.
 40. Krishnan Natesan S, Wu W, Cutright JL, Chandrasekar PH. 2012. *In vitro-in vivo* correlation of voriconazole resistance due to G448S mutation (*cyp51A* gene) in *Aspergillus fumigatus*. *Diagn Microbiol Infect Dis* 74: 272–277. <https://doi.org/10.1016/j.diagmicrobio.2012.06.030>.
 41. Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. 2011. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *cyp51A* gene. *Antimicrob Agents Chemother* 55:4465–4468. <https://doi.org/10.1128/AAC.00185-11>.
 42. Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. 2015. A novel Y319H substitution in CYP51C associated with azole resistance in *Aspergillus flavus*. *Antimicrob Agents Chemother* 59:6615–6619. <https://doi.org/10.1128/AAC.00637-15>.
 43. Escibano P, Recio S, Peláez T, Bouza E, Guinea J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrob Agents Chemother* 55:2460–2462. <https://doi.org/10.1128/AAC.01358-10>.
 44. Jin J, Lee Y-K, Wickes BL. 2004. Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol* 42:4293–4296. <https://doi.org/10.1128/JCM.42.9.4293-4296.2004>.
 45. CLSI. 2017. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, 3rd ed. CLSI standard M38. Clinical and Laboratory Standards Institute, Wayne, PA.
 46. Espinel-Ingroff A, Turnidge J. 2016. The role of epidemiological cutoff values (ECVs/ECOFFs) in antifungal susceptibility testing and interpretation for uncommon yeasts and moulds. *Rev Iberoam Micol* 33:63–75. <https://doi.org/10.1016/j.riam.2016.04.001>.
 47. Torres AM, Weeden NF, Martín A. 1993. Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. *Theor Appl Genet* 85:937–945. <https://doi.org/10.1007/BF00215032>.
 48. Islas-Flores I, Peraza-Echeverría L, Canto-Canché B, Rodríguez-García CM. 2006. Extraction of high-quality, melanin-free RNA from *Mycosphaerella fijiensis* for cDNA preparation. *Mol Biotechnol* 34:45–50. <https://doi.org/10.1385/MB:34:1:45>.
 49. Hargrove TY, Wawrzak Z, Lamb DC, Guengerich FP, Lepesheva GI. 2015. Structure-functional characterization of cytochrome P450 sterol 14 α -demethylase (CYP51B) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *J Biol Chem* 290:23916–23934. <https://doi.org/10.1074/jbc.M115.677310>.
 50. Alcazar-Fuoli L, Cuesta I, Rodriguez-Tudela JL, Cuenca-Estrella M, Sanglard D, Mellado E. 2011. Three-dimensional models of 14 α -sterol demethylase (Cyp51A) from *Aspergillus lentulus* and *Aspergillus fumigatus*: an insight into differences in voriconazole interaction. *Int J Antimicrob Agents* 38:426–434. <https://doi.org/10.1016/j.ijantimicag.2011.06.005>.
 51. Podust LM, Stojan J, Poulos TL, Waterman MR. 2001. Substrate recognition sites in 14 α -sterol demethylase from comparative analysis of amino acid sequences and X-ray structure of *Mycobacterium tuberculosis* CYP51. *J Inorg Biochem* 87:227–235. [https://doi.org/10.1016/S0162-0134\(01\)00388-9](https://doi.org/10.1016/S0162-0134(01)00388-9).
 52. Bohle K, Jungebloud A, Göcke Y, Dalpiaz A, Cordes C, Horn H, Hempel DC. 2007. Selection of reference genes for normalization of specific gene quantification data of *Aspergillus niger*. *J Biotechnol* 132:353–358. <https://doi.org/10.1016/j.jbiotec.2007.08.005>.
 53. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45. <https://doi.org/10.1093/nar/29.9.e45>.
 54. Rocha AJ, de Sousa Miranda R, Sousa AJS, da Silva ALC. 2016. Guidelines for successful quantitative gene expression in real-time qPCR assays. In Samadkikuchaksaraei A (ed), *Polymerase chain reaction for biomedical applications*. IntechOpen, Ltd, London, United Kingdom. <https://doi.org/10.5772/65850>.