Rapid Induction of Multiple Resistance Mechanisms in *Aspergillus fumigatus* during Azole Therapy: a Case Study and Review of the Literature

Simone M. T. Camps,^{a,b} Jan W. M. van der Linden,^{a,b} Yi Li,^{a,b} Ed J. Kuijper,^c Jaap T. van Dissel,^d Paul E. Verweij,^{a,b} and Willem J. G. Melchers^{a,b}

Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands^a; Nijmegen Institute for Infection, Inflammation and Immunity (N4i), Nijmegen, The Netherlands^b; Department of Medical Microbiology, Leiden University Medical Centre, Leiden, The Netherlands^c; and Department of Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands^d

Nine consecutive isogenic *Aspergillus fumigatus* isolates cultured from a patient with aspergilloma were investigated for azole resistance. The first cultured isolate showed a wild-type phenotype, but four azole-resistant phenotypes were observed in the subsequent eight isolates. Four mutations were found in the *cyp51A* gene of these isolates, leading to the substitutions A9T, G54E, P216L, and F219I. Only G54 substitutions were previously proved to be associated with azole resistance. Using a Cyp51A homology model and recombination experiments in which the mutations were introduced into a susceptible isolate, we show that the substitutions at codons P216 and F219 were both associated with resistance to itraconazole and posaconazole. A9T was also present in the wild-type isolate and thus considered a Cyp51A polymorphism. Isolates harboring F219I evolved further into a pan-azole-resistant phenotype, indicating an additional acquisition of a non-Cyp51A-mediated resistance mechanism. Review of the literature showed that in patients who develop azole resistance during therapy, multiple resistance mechanisms commonly emerge. Furthermore, the median time between the last cultured wild-type isolate and the first azole-resistant isolate was 4 months (range, 3 weeks to 23 months), indicating a rapid induction of resistance.

Aspergillus fumigatus is able to cause a wide range of diseases, including allergic syndromes, aspergilloma, and invasive aspergillosis. Azoles play an important role in the management of *Aspergillus* diseases, but chronic treatment may cause the development of resistance, especially in patients with cavitory lesions, such as aspergilloma (5, 14, 15). Infection with azole-resistant *A. fumigatus* is associated with a higher probability of treatment failure than infection due to isolates with a wild-type susceptibility (14, 29, 31), which is supported by experimental models of aspergillosis (6, 7, 8, 9, 17, 18). Surveillance studies indicate that the prevalence of azole resistance varies widely between countries (1, 23, 32), and there is increasing evidence that in addition to patient therapy, environmental exposure to azole compounds may be an important route of resistance development (24, 33). The most common mechanisms of resistance in *A. fumigatus* are modifications in the *cyp51A* gene (20). *cyp51A* encodes cytochrome P450 sterol 14 α -demethylase and is the target for azole drugs. Azoles bind to the heme cofactor located in the active site of the *cyp51*-encoded enzyme, thereby blocking ergosterol synthesis. Subsequent ergosterol depletion and the accumulation of unusual toxic sterols lead to inhibition of fungal growth (22, 34).

The objective of this study was to investigate the evolution of azole resistance development in a series of consecutive *A. fumigatus* isolates from a patient with aspergilloma. In addition, we reviewed the literature for other patients with development of resistant *A. fumigatus* disease.

CASE REPORT

A 48-year-old female was diagnosed with tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) in 2000. Seven years earlier, she had undergone bilobectomy of the right lung because of recurrent bacterial infections, including persistent

Mycobacterium avium infection. In 2000, she was diagnosed with aspergilloma in a cavity of the postoperative severely retracted right lung. Surgery was precluded due to the destroyed right lung, and itraconazole (ITC) treatment was initiated. Adequate serum concentrations were observed. She was treated from April 2000 to November 2007; in 2007, a decision was made to stop ITC treatment, since her computed tomography scan and chest X rays repeatedly showed a complete resolution of the aspergilloma, with no sign of recurrence over 8 months. In addition, sputum repeatedly remained negative for *Aspergillus*. Over the next year, her condition was stable, until October 2008, when *Aspergillus* was again cultured from the sputum. She was started on ITC but was switched in weeks to voriconazole (VRC). That December, posaconazole (POS) was given to replace VRC because of an insufficient clinical response. However, after several months there was radiological evidence for relapse of the aspergilloma and for *Aspergillus* localization in the left lung. A subsequently recovered *A. fumigatus* isolate cultured from the sputum exhibited a multi-azole-resistant phenotype, and treatment was switched to liposomal amphotericin B (L-AMB), which was subsequently given in combination with caspofungin (CAS). Despite 1 month of treatment with L-AMB and CAS, the patient had ongoing *Aspergillus*

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Address correspondence to Simone M. T. Camps, s.camps@mmb.umcn.nl.

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TABLE 1 Isolates obtained from the patient suffering from pulmonary aspergilloma

Isolate no.	Date of isolation (day-mo-yr)	Specimen	Cyp51A substitution	MIC (mg/liter)				Microsatellite no. of repeats						Treatment
				ITC	VRC	POS	AMB	3A	3B	3C	4A	4B	4C	
v74-61	29-9-2008	Sputum	A9T	0.5	1	0.063	1	13	9	17	8	9	10	ITC
v76-03	17-11-2008	Sputum	A9T, F219I	>16	1	0.5	1	13	9	17	8	9	10	VRC
v77-41	17-12-2008	Sputum	A9T, P216L	>16	1	1	1	13	9	17	8	9	10	POS
v79-63	25-2-2009	Sputum	A9T, F219I	>16	8	>16	1	13	9	17	8	9	10	POS
v80-28	9-3-2009	Sputum	A9T, F219I	>16	8	>16	1	13	9	17	8	9	10	POS
v80-55	19-3-2009	Sputum	A9T, F219I	>16	8	>16	1	13	9	17	8	9	10	POS
v82-58	16-5-2009	Sputum	A9T, F219I	>16	4	>16	1	13	9	17	8	9	10	POS
v83-11	5-6-2009	Sputum	A9T, F219I	>16	4	>16	1	13	9	17	8	9	10	L-AMB + CAS
v83-14	7-6-2009	BAL	A9T, G54E	>16	0.5	1	1	13	9	17	8	9	10	L-AMB + CAS

disease and died because of a severe urosepsis caused by extended-spectrum- β -lactamase-positive *Escherichia coli*.

At autopsy, *Aspergillus* could not be identified in the left lung, despite extensive sampling. The right lung was severely fibrotic and showed a cavity filled with necrotic material and *Aspergillus* hyphae on microscopy, but no evidence for invasive disease was found.

MATERIALS AND METHODS

***Aspergillus fumigatus* isolates.** From our patient, nine *A. fumigatus* isolates were obtained within a 10-month period (September 2008 to June 2009). All isolates were cultured from respiratory samples, and the primary colonies were subcultured on agar plates supplemented with azole compounds (ITC, 4 mg/liter; VRC, 1 mg/liter; POS, 0.5 mg/liter) (28) as part of a national surveillance study (30). Isolates that grew on these agar plates were further analyzed. A previously cultured *A. fumigatus* isolate from this patient with a wild-type phenotype was also selected for further investigation. The isolates were identified by macroscopic and microscopic examination and ability to grow at 48°C.

Susceptibility testing. A broth microdilution test was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method (27). ITC, POS, VRC, and amphotericin B (AMB) were assayed over a 2-fold concentration range from 16 to 0.016 mg/liter. Visual readings were performed with a reading mirror, and an endpoint of 100% inhibition was used to determine the MICs. For interpretation of the azole MICs, the proposed breakpoints were used (32).

***cyp51* sequence analysis and microsatellite genotyping.** To isolate DNA, isolates were cultured on Sabouraud agar slants. Conidia were harvested and added to 200 μ l of breaking buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA, pH 8) with $\sim$ 0.1 g glass beads (diameters, 0.4 to 0.6 mm). After shaking by vortexing, conidia were incubated at 70°C for 30 min while shaking. Then, 200 μ l of phenol-chloroform-isoamyl alcohol (25:24:1) was added and samples were incubated for 5 min while they were shaken. After centrifugation for 5 min, the upper phase containing the DNA was transferred to a new tube. One microliter of DNA was used per PCR.

The *cyp51A* gene was amplified and subsequently sequenced as described previously (25). The promoter region was amplified using previously described primers P-A7 and P-A5 (19) and sequenced using the forward primer (P-A7). The *cyp51B* gene and promoter region were amplified and sequenced using primer sets 5'-CCTTTATTCCCTGCGACA-3'/5'-ACGGCAGAATACCCAGAA-3' and 5'-GGAGACTGCACAACAA CG-3'/5'-GGAACCACTGGAAGACCA-3'. To detect mutations, the sequences were compared with the *cyp51A* and *cyp51B* sequences with GenBank accession numbers AF338659 and AF338660, respectively.

From all isolates as well as three unrelated control isolates, six microsatellite loci (STRAf 3A, 3B, 3C, 4A, 4B, and 4C) were amplified as described before (10). The sizes of the fragments were determined, and

repeat numbers were assigned (24). Genotypes consisting of the number of repeats for each of the six microsatellites were created for all isolates.

Cyp51A sequence alignment and homology model. An alignment of 47 selected fungal Cyp51 protein sequences deposited in the NCBI protein database (<http://www.ncbi.nlm.nih.gov/protein>) was constructed using the ClustalW2 multiple-sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The Cyp51 sequences (GenBank accession numbers) included those of *Ajellomyces capsulatus* (AAU01158), *Aspergillus clavatus* Cyp51A (EAW10153), *A. clavatus* Cyp51B (EAW11788), *A. flavus* Cyp51A (EED56341), *A. flavus* Cyp51B (EED50354), *A. fumigatus* Cyp51A (EAL90099), *A. fumigatus* Cyp51B (EAL87096), *A. nidulans* (AAF79204), *Blumeria graminis* (CAE18091), *Blumeriella jaapii* (ABE01107), *Botryotinia fuckeliana* (AAF85983), *Candida albicans* (AAF00598), *C. glabrata* (AAB02329), *C. krusei* (AAO83898), *C. tropicalis* (AAA53284), *Clavospora lusitanae* (ACH87137), *Coccidioides posadasii* (AAU01157), *Cryptococcus neoformans* (AAF35366), *Cunninghamella elegans* (Q9UVC3), *Eremothecium gossypii* (Q759W0), *Erysiphe necator* (AAC49811), *Leptosphaeria maculans* (AAN28927), *Monilinia fructicola* (AAL79180), *Mycosphaerella graminicola* (AAF74756), *Nakaseomyces delphensis* (CAO98836), *Neosartorya fischeri* Cyp51A (EAW25441), *N. fischeri* Cyp51B (EAW19398), *Neurospora crassa* (EAA34813), *Oculimacula acufiformis* (AAF18468), *O. yallundae* (AAG44831), *Penicillium italicum* (CAA89824), *P. digitatum* (CAD27793), *P. digitatum* Cyp51B (ADO85403), *P. marneffeii* Cyp51B (EEA25858), *Phanerochaete chrysosporium* (ACI23621), *Pneumocystis carinii* (ABG91757), *Podosphaera fusca* (ACT56506), *Saccharomyces cerevisiae* (AAA34546), *Scheffersomyces stipitis* (ABN68111), *Schizosaccharomyces pombe* (CAA90803), *Talaromyces stipitatus* (EED21732), *Trichosporon asahii* (ADN44281), *Ustilago maydis* (CAA88176), *Venturia inaequalis* (AAF71293), *V. nashicola* (CAC85409), *Yarrowia lipolytica* (CAG82748), and *Zygosaccharomyces rouxii* (CAR28109). The positions of amino acid substitutions present in the patient isolates as well as their equivalents in other fungi were pinpointed into the alignment.

Furthermore, the locations of the observed amino acid substitutions were investigated with the help of a recently published homology model of the *A. fumigatus* Cyp51A enzyme (25).

***A. fumigatus* transformations.** Transformation experiments were performed as described before (26). Briefly, site-directed mutagenesis was used to introduce the point mutations into a cassette containing the *cyp51A* gene and promoter region together with a hygromycin selection marker gene. Mutagenic primers 5'-CTGGACAAGGGCTTTACTCTCA TCAATTTTATGCTACCG-3'/5'-CGGTAGCATAAAATTGATGAGAG TAAAGCCCTTGTCCAG-3' (for P216L) and 5'-GCTTTACTCCCATC AATATTATGCTACCGTGGGCC-3'/5'-GGCCACGGTAGCATAATA TTGATGGGAGTAAAGC-3' (for F219I) were used. Both cassettes containing either the P216L- or F219I-causing mutation were used for homologous gene replacement by electroporation in a *cyp51A* wild-type isolate. Recombinants were selected on hygromycin-containing medium and subcultured for further investigation. In addition, a cassette without

	A9	G54	P216	F219
<i>C.albicans</i>	***SLSVTQ	QISIL***SAASYG	MQPYE***DKGFT	IN
<i>C.tropicalis</i>	***SLSLTQ	QITIL***SAIPFG	MAPYK***DKGFT	IN
<i>S.stipitis</i>	***SLSLTQ	QVSII***SAASYG	QQPYE***DKGFT	LN
<i>C.lusitaniae</i>	***SLGLLS	KIALL***SAVVG	MQPYE***DKGFT	IN
<i>C.krusei</i>	***TFSLLQ	QTLTV***SAIPYG	MQPYE***DKGFT	LN
<i>C.glabrata</i>	***ALPLAQ	RVSIM***SAIPYG	TKPYE***DKGFT	IN
<i>N.delphensis</i>	***ELPLAQ	RISIL***SAVPYG	TRPYE***DKGFT	IN
<i>S.cerevisiae</i>	***ALPLAQ	RISLI***SAVVG	GMKPYE***DKGFT	IN
<i>Z.rouxii</i>	***ELSLSQ	QISII***SAVPYG	TRPYE***DRGFT	IN
<i>E.gossypii</i>	***ALSWTQ	QLSIV***SALAY	GRAPYE***DKGFT	LN
<i>Y.lipolytica</i>	***GWHILG	LAIL***SSIPY	GIDPYE***DNGFT	IN
<i>A.fumigatus_Cyp51A</i>	***MLWLT	YMAVA***STISY	GIDPYK***DKGFT	IN
<i>N.fischeri_Cyp51A</i>	***MLLLT	YMAVA***STISY	GIDPYK***DKGFT	IN
<i>A.clavatus_Cyp51A</i>	***LTLFGL	YLVA***STITY	GIDPCK***DRCFT	PN
<i>A.flavus_Cyp51A</i>	***FTLVSA	YAAAG***STVTY	GMDPYA***DRGFT	IN
<i>A.nidulans</i>	***PTLFS	AYVLVG***STISY	GMNPN***EMGFT	IN
<i>P.italicum</i>	***ILGIAY	YTTGL***STIAY	GMDPYQ***DLGFS	IN
<i>P.digitatum_Cyp51A</i>	***IKCIA	YTTGL***STVAY	GMDPYQ***DLGFT	IN
<i>B.graminis</i>	***SIALAS	GIIISL***STISY	GMNPN***DMGFT	IN
<i>P.fusca</i>	***SFLAL	AGIGTF***STIPY	GIDPYK***DMGFS	IN
<i>E.necator</i>	***IFMVT	IAFSI***STISY	GIDPYK***DMGFT	IN
<i>O.acuformis</i>	***GVVIA	AGFAAF***STVTY	GIDPYK***DMGFS	IN
<i>O.yallundae</i>	***GVVIA	AGFAAF***STVTY	GIDPYK***DMGFS	IN
<i>B.jaapii</i>	***AVVIT	AGFAF***STITY	GIDPYR***DMGFS	IN
<i>M.fruticicola</i>	***GTIIA	AGVAAF***STITY	GIDPYR***DMGFS	IN
<i>B.fuckeliana</i>	***GVVVA	AGVAAF***STITY	GMDPYK***DMGFS	IN
<i>N.crassa</i>	***VSQIG	VAIASF***STITY	GMDPPR***DMGFS	IN
<i>L.maculans</i>	***FVLAIT	GFASF***STVTY	GMDPYA***DMGFS	PN
<i>A.fumigatus_Cyp51B</i>	***WVLVG	IGLLSI***STISY	GIDPYK***DMGF	IN
<i>N.fischeri_Cyp51B</i>	***WVLVG	IGLLSI***STISY	GIDPYK***DMGF	IN
<i>A.clavatus_Cyp51B</i>	***WMLGG	VGLLSI***STISY	GIDPYK***DMGF	IN
<i>A.flavus_Cyp51B</i>	***-----	-----MDPYR***DMGF	IN	IN
<i>P.digitatum_Cyp51B</i>	***YTLAS	IGALSF***STVS	GMDPYT***DKGFS	IN
<i>P.marneffeii_Cyp51B</i>	***YVLAG	LGAIV***NTISY	GIDPYK***DMGF	IN
<i>T.stipitatus</i>	***YVLAG	LGFATV***NTISY	GMDPYK***DMGF	IN
<i>C.posadasii</i>	***WTLVL	LGCIF***STISY	GIDPYK***DMGF	IN
<i>A.capsulatus</i>	***WALLL	AGSATF***STISY	GIDPYK***DMGFT	IN
<i>V.inaequalis</i>	***YTLAS	FG--F***NTVY	GIDPIK***DMGFS	IN
<i>V.nashicola</i>	***YTLAS	FG--F***NTVY	GIDPIK***DMGFS	IN
<i>M.graminicola</i>	***WKLVG	LGLAF***STITY	GIDPYK***DMGFT	IN
<i>S.pombe</i>	***---LV	SILLSI***SAVAY	GMDPYV***DQGF	PN
<i>P.jirovecii</i>	***ENFTL	SFVII***STIQY	GMDPYK***DGGFT	IN
<i>C.neoformans</i>	***SLKVA	IAVVG***SAAYY	GEDPYK***DGGFT	LN
<i>T.asahii</i>	***AQKLA	AVFIGL***SAAAY	GMDPYK***DKGFT	PN
<i>P.chrysosporium</i>	***SRLVL	ELLINI***SAAAY	GEDPIK***DGGFT	LN
<i>U.maydis</i>	***WIKTI	IVLVLL***SAIYY	GIDPYK***DSGFT	IN
<i>C.elegans</i>	***TIISM	GYSVLA***NAVQ	FGMNPVA***DQGF	IN

any mutation in the *cyp51A* gene was incorporated by electroporation as a transformation control. The complete cassette was sequenced to confirm the presence of the mutation and to ensure that no other mutations were present. To confirm that only one copy of *cyp51A* was incorporated, Southern blotting was performed. The susceptibility profiles of the recombinants, the transformation control, and the transformation recipient isolate were determined by the EUCAST broth microdilution test as described above.

Literature review. We reviewed the literature for cases with acquired azole resistance development in *A. fumigatus* isolates during azole therapy. Only patients from whom at least one isogenic susceptible isolate was obtained were included, because in these cases it is very likely that resistance was acquired during treatment and not obtained from the environment. Information on *Aspergillus* disease, age, sex, underlying disease(s), treatment regimen, the number of isolates, the time needed for resistance to develop, and the resistance mechanisms observed was collected.

RESULTS

Characterization of patient isolates. Results of susceptibility testing of the nine isolates obtained from our patient are shown in Table 1, together with Cyp51A substitutions and the number of repeats for each microsatellite maker. Microsatellite typing showed identical genotypes for all isolates, indicating that the collection of isolates was isogenic. The unrelated control isolates showed aberrant genotypes (data not shown).

The first isolate showed a wild-type susceptibility phenotype, while all other isolates showed an ITC MIC of >16 mg/liter. VRC MICs varied between 0.5 and 8 mg/liter, and POS MICs were between 0.063 and >16 mg/liter. In total, four different azole susceptibility profiles were observed. *cyp51A* sequence analysis showed a conversion of alanine (A) to threonine (T) at codon 9 (codon 9 was changed from GCC to ACC) in all isolates. All eight isolates with a non-wild-type azole susceptibility profile had an additional point mutation in the *cyp51A* gene. Six isolates showed a point mutation resulting in the substitution of phenylalanine (F) for isoleucine (I) at codon 219 (change of codon 219 from TTT to ATT). One isolate contained a mutation at codon 216 (changed from CCC to CTC), resulting in the replacement of proline (P) by leucine (L). The last isolate contained a point mutation leading to the substitution of glycine (G) for glutamic acid (E) at codon 54 (GGG was changed to GAG). No mutations in *cyp51B* were observed in any of the nine isolates.

Cyp51 sequence alignments and homology model. The Cyp51A amino acid substitutions observed in the patient isolates were studied in more detail. Cyp51 sequence alignments (Fig. 1) showed that residue A9 is not well conserved. In contrast, G54, P216, and F219 are highly conserved within the fungal kingdom. Only *C. posadasii* showed a different amino acid at the position similar to *A. fumigatus* P216.

Because A9T is not well conserved and is also observed in the susceptible patient isolate and G54E is already described as being a cause of azole resistance (11), we focused on the P216L and F219I substitutions. To check for the position of these amino acids in the Cyp51A enzyme, P216L and F219I were pinpointed into a Cyp51 homology model. The model showed that both amino acids were

FIG 2 Locations of residues P216 and F219 indicated in the Cyp51A homology model. The green tubes represent the two ligand access channels leading to the heme center of the enzyme.

located close to the opening of one of the two ligand access channels of the Cyp51 protein (Fig. 2).

***A. fumigatus* recombinants.** *A. fumigatus* recombinants were constructed with either the P216L or the F219I substitution. None of the recombinants had additional mutations in *cyp51A*, the promoter region, the 3' region, or the hygromycin selection marker gene. Southern blotting confirmed that integration occurred at a single chromosomal locus in all recombinants (data not shown). As shown in Tables 1 and 2, the P216L recombinant exhibited a susceptibility phenotype similar to that of patient isolate v77-41, with resistance to ITC and an elevated MIC of POS. The phenotype of the F219L recombinant was similar to that of patient isolate v76-03 but different from the phenotypes of five other patient isolates with the F219I substitution (v79-63, v80-28, v80-55, v82-58, and v83-11) that were cultured later in the course of the disease.

Previously described cases of acquired azole resistance. Including the present case, eight cases with acquired azole resistance caused by *A. fumigatus* have been described (Fig. 3). From each patient, between two and nine isogenic or nearly isogenic isolates of *A. fumigatus* were obtained, and the first isolate(s) showed a wild-type azole susceptibility phenotype. Except for patient 7, the *cyp51A* gene was sequenced and mutations at codons G54 (8 isolates from 5 patients), M220 (2 isolates from 2 patients), G448 (2 isolates from 2 patients), P216 (1 isolate), and F219 (6 isolates) were found. In five out of these seven cases, multiple resistance mechanisms emerged (patients 1, 3, 5, 6, and 8). All patients had azole exposure before the identification of the first resistant iso-

FIG 1 Alignment of fungal Cyp51 proteins. In case Cyp51 was specified as either Cyp51A or Cyp51B, this is indicated after the name of the fungus. The positions of *A. fumigatus* A9, G54, P216, and F219 are indicated in black; aligned positions in the other fungal species are indicated in gray. For each of the amino acids we are interested in, only 10 amino acids of surrounding sequence is added to the alignment. Other surrounding sequence is indicated by ***. The *A. flavus* Cyp51B sequence was relatively short compared to the other sequences. The alignment of *A. flavus* Cyp51B started at the position of residue 55 in *A. fumigatus* Cyp51A, so there is no comparable amino acid for *A. fumigatus* A9 and G54 in *A. flavus* Cyp51B.

TABLE 2 MICs of recombinants with either the P216L or F219I substitution compared to those of the recipient isolate and the transformation control isolate

Isolate	Base change in <i>cyp51A</i> ^a		MIC (μg/ml)		
	Codon 216	Codon 219	ITC	VRC	POS
Transformation recipient isolate	CCC	TTT	0.5	0.5	0.063
Transformation control	CCC	TTT	0.5	0.5	0.063
Recombinant P216L	CTC	TTT	>16	0.5	1
Recombinant F219I	CCC	ATT	>16	0.5	1

^a Bold indicates the base change.

late. The antifungals used for therapy included ITC in two patients (patients 1 and 6), and in all other patients, multiple antifungal treatments had been used (Fig. 3), mainly because of the development of resistance. The patient that we present here also underwent multiple therapy changes, as described above. The time between the last culture of an isolate with a wild-type susceptibility phenotype and the first with an azole-resistant phenotype varied between 3 weeks and 23 months (median, 4 months).

DISCUSSION

Azole resistance has been reported to emerge in patients with chronic pulmonary aspergillosis and pulmonary aspergilloma during azole therapy. Here, we describe a patient with pulmonary aspergilloma from whom isogenic azole-resistant *A. fumigatus* isolates with various resistance phenotypes were cultured during azole therapy. The resistant isolates showed different mutations in

the *cyp51A* gene resulting in G54E, P216L, and F219I substitutions. In addition, all isolates contained a mutation leading to the A9T substitution. It is unlikely that this mutation will have an impact on azole susceptibility because the sensitive isolate also contained the A9T substitution and A9 is not well conserved among fungal species. Mutations at codon G54 are already known to be associated with azole resistance in *A. fumigatus* (5, 11, 12, 16, 21) and are even mentioned to be hot-spot mutations (13) because mutations at this site are commonly found in azole-resistant isolates. Clinical and laboratory-induced mutant *A. fumigatus* isolates with the G54E substitution in Cyp51A usually show the following susceptibility profile: ITC MIC, >8 mg/liter; VRC MIC, 0.25 to 0.5 mg/liter; and POS MIC, 1 mg/liter (11, 12, 16). This profile is consistent with that of the isolate obtained from our patient. The Cyp51A P216L substitution was described before in a patient with chronic cavitary pulmonary aspergillosis with aspergilloma treated with ITC (14). However, this substitution is not yet confirmed to be a cause of resistance by the construction of recombinants. To our knowledge, the F219I substitution has not been described before.

Sequence alignments revealed that residues P216 and F219 are conserved within the fungal kingdom, and the Cyp51A homology model showed that they are both located close to the opening of the ligand access channel, suggesting that these residues are important. As the channels are thought to be used by azole compounds to enter the active site of the protein, mutations at codons P216 and F219 might affect the docking of azole molecules. Previously, docking of azoles in a homology model of *A. fumigatus* Cyp51A showed an interaction of P216 and several other closely situated residues with POS but not with VRC (34), indicating that mutations in this region might play a role in POS resistance. We

FIG 3 Reported cases of acquired azole resistance in *A. fumigatus* (2, 3, 5, 6, 14). The treatment schedules of all eight patients are indicated with bars, and the *A. fumigatus* isolates obtained from the patients are indicated with triangles. The corresponding resistance mechanisms are also indicated. wt, azole-susceptible wild-type isolate; ?, resistant isolate without any *cyp51A* mutations; ND, *cyp51A* sequence not determined; ITC, itraconazole; VRC, voriconazole; CAS, caspofungin; POS, posaconazole; AMB, amphotericin B (in various formulations). In the isolates marked with an asterisk, the F219I resistance mechanism was found in *cyp51A*. However, this isolate continued to evolve further azole resistance by an additional and yet unknown non-*cyp51A*-related resistance mechanism. Information regarding the treatment of patients 1, 2, and 3 was kindly provided by the author (S. Howard, personal communication).

confirmed the Cyp51A P216L and F219I substitutions to be the cause of azole resistance by the generation of recombinants in which the wild-type *cyp51A* gene was replaced by a *cyp51A* cassette containing either one of the two mutations. This resulted in resistance to ITC and POS, while recombinants remained susceptible to VRC. For P216L, this was in agreement with the phenotype of the clinical isolate, but for F219I, only one of six clinical isolates harboring this substitution showed a similar phenotype. For the remaining F219I isolates, the VRC MIC had increased from 1 mg/liter to 8 mg/liter and the POS MIC had increased from 0.5 mg/liter to >16 mg/liter. As no additional *cyp51A* mutations were found in these five isolates, we assume that a second resistance mechanism not related to the *cyp51A* gene had evolved during POS treatment. This is reasonable because resistance mechanisms other than *cyp51A* mutations have been reported before in *A. fumigatus* (2, 4, 14).

Review of the literature revealed seven other cases of aspergillosis caused by *A. fumigatus* in which azole resistance emerged during azole therapy. Because we selected only for cases in which at least one isogenic isolate with a susceptible phenotype was obtained, it is very likely that resistance was induced in the patient. The patients were probably initially colonized or infected with a susceptible isolate, and over time, through azole exposure, resistance developed in this initial isolate. In one case, the resistance mechanisms were not determined, but in five of the remaining seven cases (71%), at least two resistance mechanisms emerged, indicating that different evolutionary processes within one patient might result in independent adaptation of the fungus to azole exposure. Furthermore, in the case described here, resistance might also have accumulated sequentially as one of the resistant isolates further evolved to become multiazole resistant.

All patients were diagnosed with aspergilloma and chronic pulmonary aspergillosis. It was previously suggested that in the case of aspergilloma or cavitary aspergillus disease, the fungus is able to undergo multiple generations in the patient by the asexual way of reproduction. Sporulation (in the lung) as opposed to hyphal growth may be important to facilitate the expression of the azole-resistant phenotype; hyphal growth is typically found in acute invasive aspergillosis (33).

Our literature review showed that the median time between the last cultured wild-type isolate and the first azole-resistant strain was only 4 months, indicating that resistance can be induced soon after initiating treatment. We have to address that two of the patients (patients 4 and 8; Fig. 3) received azole treatment for several years before the last cultured wild-type isolate was obtained. It is therefore possible that under azole pressure a preceding event unrelated to *cyp51A* occurred in the wild-type isolate and that the event subsequently resulted in the rapid acquisition of the resistance mutations. From every clinical sample, only one colony is usually subcultured, stored, and subjected to susceptibility testing even when more colonies grow. Therefore, it cannot be excluded that the resistance mechanisms found persisted for a longer time. Moreover, additional resistance mechanisms may have been found when multiple colonies had been tested. Furthermore, patients are not usually regularly sampled for the presence of fungi, so isolates cultured could have already been present in the patient for a long time. Although it is now shown that azole resistance can be induced in the patient within a relatively short period of time, the questions of which proportion of azole-treated patients with aspergilloma or other cavitary lung lesions develop azole resis-

tance and whether specific risk factors for resistance development can be identified remain.

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REFERENCES

1. Alanio A, et al. 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J. Antimicrob. Chemother.* 66:371–374.
2. Arendrup MC, et al. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One* 5:e10080.
3. Bellete B, et al. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med. Mycol.* 48:197–200.
4. Bueid A, et al. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J. Antimicrob. Chemother.* 65:2116–2118.
5. Chen J, Li H, Li R, Bu D, Wan Z. 2005. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J. Antimicrob. Chemother.* 55:31–37.
6. Dannaoui E, et al. 2001. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* 47:333–340.
7. Dannaoui E, Borel E, Persat F, Monier MF, Piens MA. 1999. In-vivo itraconazole resistance of *Aspergillus fumigatus* in systemic murine aspergillosis. EBGA Network. European Research Group on Biotypes and Genotypes of *Aspergillus fumigatus*. *J. Med. Microbiol.* 48:1087–1093.
8. Denning DW, et al. 1997. Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of *Aspergillus fumigatus* infection. *J. Antimicrob. Chemother.* 40:401–414.
9. Denning DW, et al. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 41:1364–1368.
10. de Valk HA, et al. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J. Clin. Microbiol.* 43:4112–4120.
11. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. 2003. A point mutation in the 14 α -sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 47:1120–1124.
12. Garcia-Effron G, et al. 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.
13. Howard SJ, Arendrup MC. 2011. Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Med. Mycol.* 49(Suppl 1):S90–S95.
14. Howard SJ, et al. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* 15:1068–1076.
15. Howard SJ, et al. 2006. Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* 28:450–453.
16. Mann PA, et al. 2003. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14 α -demethylase. *Antimicrob. Agents Chemother.* 47:577–581.
17. Mavridou E, Bruggemann RJ, Melchers WJ, Mouton JW, Verweij PE. 2010. Efficacy of posaconazole against three clinical *Aspergillus fumigatus* isolates with mutations in the *cyp51A* gene. *Antimicrob. Agents Chemother.* 54:860–865.
18. Mavridou E, Bruggemann RJ, Melchers WJ, Verweij PE, Mouton JW. 2010. Impact of *cyp51A* mutations on the pharmacokinetic and pharmacodynamic properties of voriconazole in a murine model of disseminated aspergillosis. *Antimicrob. Agents Chemother.* 54:4758–4764.
19. Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-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.
20. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the

- 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob. Agents Chemother.* **48**:2747–2750.
21. Nascimento AM, et al. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* **47**:1719–1726.
 22. Odds FC, Brown AJ, Gow NA. 2003. Antifungal agents: mechanisms of action. *Trends Microbiol.* **11**:272–279.
 23. Pfaller MA, et al. 2008. In vitro survey of triazole cross-resistance among more than 700 clinical isolates of *Aspergillus* species. *J. Clin. Microbiol.* **46**:2568–2572.
 24. Snelders E, et al. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl. Environ. Microbiol.* **75**: 4053–4057.
 25. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob. Agents Chemother.* **54**:2425–2430.
 26. Snelders E, et al. 2011. The structure-function relationship of the *Aspergillus fumigatus* cyp51A L98H conversion by site-directed mutagenesis: the mechanism of L98H azole resistance. *Fungal Genet. Biol.* **48**: 1062–1070.
 27. Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). 2008. EUCAST technical note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. *Clin. Microbiol. Infect.* **14**: 982–984.
 28. van der Linden JW, Arendrup MC, van der Lee HA. 2009. Azole containing agar plates as a screening tool for azole resistance of *Aspergillus fumigatus*. *Abstr. Trends Med. Mycol.* **4**:abstr O1.1.
 29. van der Linden JW, et al. 2009. Azole-resistant central nervous system aspergillosis. *Clin. Infect. Dis.* **48**:1111–1113.
 30. van der Linden JW, et al. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009. *Emerg. Infect. Dis.* **17**: 1846–1854.
 31. van Leer-Buter C, Takes RP, Hebeda KM, Melchers WJ, Verweij PE. 2007. Aspergillosis—and a misleading sensitivity result. *Lancet* **370**:102.
 32. Verweij PE, Howard SJ, Melchers WJ, Denning DW. 2009. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resist. Updat.* **12**:141–147.
 33. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect. Dis.* **9**:789–795.
 34. Xiao L, et al. 2004. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob. Agents Chemother.* **48**:568–574.