Resistance to Voriconazole Due to a G448S Substitution in *Aspergillus fumigatus* in a Patient with Cerebral Aspergillosis

Teresa Pelaez,^{a,b} Paloma Gijón,^a Eleonora Bunsow,^a Emilio Bouza,^{a,b} Waldo Sánchez-Yebra,^c Maricela Valerio,^a Beatriz Gama,^a Manuel Cuenca-Estrella,^d and Emilia Mellado^d

Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario Gregorio Marañón and Department of Medicine, Faculty of Medicine, Universidad Complutense, Madrid, Spain^a; CIBER de Enfermedades Respiratorias (CIBERES CD06/06/0058), Palma de Mallorca, Spain^b; UGC Biotecnología, Unidad de Microbiología y Parasitología, Complejo Hospitalario Torrecárdenas, Almería, Spain^c; and Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain^d

A voriconazole-resistant isolate of *Aspergillus fumigatus* was recovered from an immunocompetent patient receiving long-term antifungal therapy for cerebral aspergillosis. A G448S amino acid substitution in the azole target (Cyp51A) was identified as the cause of the resistance phenotype. This article describes the first isolation of a voriconazole-resistant *A. fumigatus* isolate from an immunocompetent patient in Spain.

CASE REPORT

A 45-year-old woman with a recurrent brain abscess was referred to the Hospital General Universitario Gregorio Marañón (HGUGM), Madrid, Spain, in July 2011. She was immunocompetent with no comorbid conditions and had presented with chronic otitis media with cholesteatoma. Two years before (in 2009), the patient had undergone open tympanoplasty, which was unsuccessful, and she required a radical mastoidectomy. In November 2010, the patient had several postsurgical complications, namely, persistent otorrhea, gradual facial palsy, and an abscess in the right temporal lobe that was successfully removed. Culturing of the resected tissue yielded a fungal isolate identified as *Aspergillus fumigatus* by standard mycological procedures and molecular methods (see below). Treatment with voriconazole at 200 mg (4 mg/kg) twice a day was thus initiated, and monitoring of serum drug levels for 8 weeks revealed good tolerance and apparent efficacy of the antifungal. However, 2 months later, the patient experienced a continuous and intense earache. Magnetic resonance imaging (MRI) revealed a new abscess at the cerebellopontine angle and signs of petrosal bone osteomyelitis. The abscess was drained, and a petrosectomy was performed. Resection was extensive, with cochleostomy and removal of granulation tissue and the whole facial nerve, whose second and third portions were completely infiltrated by the fungus. *A. fumigatus* was again isolated from mucus and bone biopsy specimens. Treatment with voriconazole was continued, but the patient's condition deteriorated. Eventually, voriconazole was replaced with liposomal amphotericin B.

Upon admission to the HGUGM (in July 2011), the patient again underwent a petrosectomy. Aspergillomas were removed from cerebral tissue, and biopsy specimens once more yielded *A. fumigatus*. Two months later, the patient was still receiving liposomal amphotericin B (3 mg/kg/day) with no further complications. She had no apparent symptoms, and MRI showed that the lesions had disappeared.

Mycological procedures. Culturing of clinical samples was performed on Sabouraud-chloramphenicol medium (Oxoid, Madrid, Spain). The incubation temperature was 37°C. Fungal isolates were macro- and microscopically examined according to standard procedures.

The *A. fumigatus* isolate recovered from biopsy samples taken at the HGUGM after the second petrosectomy (see above), which was named TP1362, was used in subsequent detailed phenotypic and molecular studies. Two *A. fumigatus* strains from a previous study (21), CM-237 (azole susceptible) and CR019 (multiazole resistant), were used as controls throughout this work.

Molecular characterization of isolates. Conidia from each of the aforementioned strains were inoculated into 3 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and grown overnight at 37°C, after which mycelium mats were harvested and DNA was extracted as previously described (9). Species-specific identification was performed by partial PCR amplification and subsequent sequencing of the β -tubulin gene as described elsewhere (1). After the identity of isolate TP1362 was confirmed as *A. fumigatus*, the full coding sequences of the *cyp51B* and *cyp51A* genes, including the *cyp51A* promoter, were amplified using the PCR conditions described by Mellado et al. (21). To rule out the possibility that any sequence changes identified were due to PCR-induced errors, each isolate was independently analyzed twice. No differences were found among the *cyp51B* sequences of the three strains. However, the *cyp51A* gene from isolate TP1362 carried a G1413A mutation that was responsible for a G448S amino acid substitution. No further changes were found in the sequence of the remainder of the *cyp51A* gene or in the promoter region.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed by using the broth microdilution (BMD) method described in a 2008 EUCAST technical note (30) and the Etest in accordance with the manufacturer's recommendations. The results of these assays are shown in Tables 1 and 2 and Fig. 1, respectively. The antifungal agents tested were

Received 9 February 2012 Returned for modification 9 April 2012

Accepted 27 April 2012

Published ahead of print 9 May 2012

Address correspondence to Maricela Valerio, maricela.valerio@hotmail.com.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.00329-12

TABLE 1 EUCAST microdilution susceptibility test results of the three clinical strains analyzed

Strain	MIC range (mg/liter)			
	Amphotericin B	Itraconazole	Voriconazole	Posaconazole
CM-237	0.125	0.125	0.25	0.03–0.06
TP1362	0.25	0.50	4.0–8.0	0.125
CR019	0.25	>8.0	4.0	1.0

itraconazole (Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole (Pfizer, S.A., Madrid, Spain), posaconazole (Schering-Plough, Madrid, Spain), and liposomal amphotericin B (Sigma, Madrid, Spain). Susceptibility profiles were determined at least three times for each isolate on different days. *Aspergillus flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains. *In vitro* susceptibility and resistance were defined according to the epidemiological cutoff values (ECVs) recently published for *A. fumigatus*. Isolates with itraconazole and voriconazole MICs of ≤ 1 $\mu\text{g/ml}$ were considered to belong to wild-type populations; for posaconazole, this value was ≤ 0.25 $\mu\text{g/ml}$ (10, 26).

Discussion. Invasive aspergillosis (IA) is an important cause of morbidity and mortality, and *A. fumigatus* is among the most prevalent airborne fungal pathogens worldwide (12, 23). Cerebral aspergillosis is one of the most severe clinical forms of IA (29), usually presenting a poor prognosis and resulting in a fatal outcome. In fact, the risk of death associated to cerebral aspergillosis usually exceeds 90%.

Resistance to triazoles in *A. fumigatus* was first described in the United Kingdom in the 1980s (8). Since then, reports of clinical isolates showing reduced susceptibility to different triazole antifungals have multiplied (8, 13, 22, 28, 32) and azole-resistant IA, whether a primary or a breakthrough infection, is well documented (2, 4–7, 13, 22). Development of resistance in azole-treated patients and an environmental route of resistance have also been reported (16, 22, 31, 32, 33). The main mechanisms accounting for triazole resistance in *A. fumigatus* are point mutations in the *cyp51A* gene encoding 14- α sterol demethylase, which is the target of azole drugs (9, 13, 14, 20, 21). Specific mutations in *cyp51A* may result in resistance to one, two, or all three triazoles (9, 13, 14, 19, 21, 24). However, only a few mutations have been clearly associated with *in vitro* and *in vivo* resistance to voriconazole (31).

In this article, we report a case of cerebral aspergillosis caused by a voriconazole-resistant isolate of *A. fumigatus* in an immunocompetent patient treated with that antifungal. The

TABLE 2 Etest susceptibility range values of the three clinical strains analyzed at 24 and 48 h

Strain	MIC range (mg/liter)					
	Itraconazole		Voriconazole		Posaconazole	
	24h	48h	24h	48h	24h	48h
CM-237	0.12	0.75	0.12	0.25	0.03	0.12
TP1362	0.75–1.5	2.0–4.0	0.50	2.0–4.0	0.06–0.12	0.25
CR019	>32	>32	1.5	2.0	0.5	0.75–2.0

FIG 1 Susceptibilities, determined by Etest, of an-azole susceptible strain of *A. fumigatus* (CM-237), a multiresistant strain (CR019), and the clinical isolate recovered from our patient (TP1362) to voriconazole (VOR), itraconazole (ITC), and posaconazole (POS).

cyp51A gene sequencing revealed a G1413A mutation causing a G448S amino acid substitution. According to the azole MICs obtained and considering the ECVs (10, 26), this *A. fumigatus* isolate should be considered resistant to voriconazole but not to itraconazole or posaconazole. Unfortunately, we did not have the previous isolate obtained from the abscess in the right temporal lobe to compare it with the isolate obtained later from the abscess drained by petrosectomy. We assume that the *A. fumigatus* strain acquired voriconazole resistance under voriconazole pressure, but we cannot rule out reinfection with another strain.

Azole resistance due to a G1413A mutation has been reported in the United Kingdom (13) and France (3). In both cases, the strains carrying the mutation were considered resistant to multiple azoles. It is notable that we found that the Etest showed a lack of correlation with BMD methods, especially in the case of itraconazole, as has been described elsewhere (11). It should be noted that ECVs determined using BMD may not be applicable to the results of Etest. Surprisingly, this mutation was previously reported by Manavathu et al. (17, 18), and it was then defined as moderately resistant to itraconazole and posaconazole. The lack of agreement between the MICs for strains of different origins carrying the same single mutation (G448S) could be due to differences in the methods used for antifungal susceptibility testing. Alternatively, the *A. fumigatus* strains described by Bellete et al. (3) and Howard et al. (13) might rely on more than one mechanism that would render them resistant to multiple azoles. So far, resistance to itraconazole appears to be common to all azole-resistant *A. fumigatus* strains, and thus, this trait is commonly used as a marker in screening for azole resistance. The emergence of strains resistant to only voriconazole should necessarily change the latter approach and voriconazole will have to be included in antifungal susceptibility testing methods.

Residue G448 forms part of the conserved heme-binding

domain, and it is conserved in all of the *ERG11/cyp51*-encoded cytochrome P450s of yeasts and filamentous fungi. The G448S amino acid substitution described here corresponds to the G464S substitution in *Candida albicans* *ERG11* and the G484S substitution in *Cryptococcus neoformans*, both of which are involved in fluconazole resistance (25, 27). Several studies have demonstrated that this amino acid substitution detected in *C. albicans* *ERG11* confers a change in the orientation of the P450 heme-binding domain, leading to decreased azole binding and decreased catalytic activity of the enzyme (15, 27). Although it is known that this mutation also alters susceptibility to voriconazole (and, to a lesser extent, to other azole drugs), the exact mechanism accounting for the resistance phenotype remains to be studied in more detail.

To our knowledge, this is the first report of an *A. fumigatus* clinical isolate carrying the specific G448S mutation in Spain. The observed *in vitro* resistance of the isolate to voriconazole led to treatment failure, and the patient was cured only after therapy with liposomal amphotericin B. Therefore, the data presented here confirm the role of this mutation in the development of decreased *in vivo* susceptibility to voriconazole, as well as the importance of antifungal resistance as a factor determining the clinical outcome of IA not only in immunocompromised patients but also in immunocompetent hosts.

ACKNOWLEDGMENTS

We thank Thomas O'Boyle for editing and proofreading the manuscript. The useful comments provided by Sergio Álvarez-Pérez on a previous version of the manuscript are also gratefully acknowledged.

E.M. was supported by research projects from the Spanish Ministry of Science and Innovation (SAF2008-04143).

In the past 5 years, M.C.-E. has received grant support from Astellas Pharma, bioMérieux, Gilead Sciences, MSD, Pfizer, Schering-Plough, Soria Melguizo S.A., the European Union ALBAN program, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, the Spanish Health Research Fund, the Instituto de Salud Carlos III, the Ramon Areces Foundation, and the Mutua Madrileña Foundation. M.C.-E. has also been an advisor/consultant to the Pan-American Health Organization, Gilead Sciences, MSD, Pfizer, and Schering-Plough and been paid for talks on behalf of Gilead Sciences, MSD, Pfizer, and Schering-Plough. The rest of us have no potential conflicts of interest.

REFERENCES

- Alcazar-Fuoli L, et al. 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. *Antimicrob. Agents Chemother.* 52:1244–1251.
- Arendrup MC, et al. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One* 5:e10080.
- Bellef B, et al. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med. Mycol.* 48:197–200.
- Bueid A, et al. 2010. Azole antifungal resistance in *Aspergillus fumigatus* 2008 and 2009. *J. Antimicrob. Chemother.* 65:2116–2118.
- 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.
- Chrysanthou E. 1997. In vitro susceptibility of respiratory isolates of *Aspergillus* species to itraconazole and amphotericin B. Acquired resistance to itraconazole. *Scand. J. Infect. Dis.* 29:509–512.
- Dannaoui E, et al. 2001. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* 47:333–340.
- Denning DW, et al. 1997. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 41:1364–1368.
- 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.
- Espinel-Ingroff A, et al. 2010. Wild-Type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). *J. Clin. Microbiol.* 48:3251–3257.
- Espinel-Ingroff A, Rezusta A. 2002. E-test method for testing susceptibilities of *Aspergillus* spp. to the new triazoles voriconazole and posaconazole and to established antifungal agents: comparison with NCCLS broth microdilution method. *J. Clin. Microbiol.* 40:2101–2107.
- Herbrecht R, et al. 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N. Engl. J. Med.* 347:408–415.
- Howard SJ, et al. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* 15:1068–1076.
- Howard SJ, et al. 2006. Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* 28:450–453.
- Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. 1999. The G464S amino acid substitution in *Candida albicans* sterol 14 α -demethylase causes fluconazole resistance in the clinic through reduced affinity. *Biochem. Biophys. Res. Commun.* 262:174–179.
- Lionakis MS, et al. 2005. Increased frequency of non-*fumigatus* *Aspergillus* species in amphotericin B or triazole-pre-exposed cancer patients with positive cultures for aspergilli. *Diagn. Microbiol. Infect. Dis.* 52:15–20.
- Manavathu E, Espinel-Ingroff A, Alangaden G, Chandrasekar P. 2003. Molecular studies on voriconazole resistance in a clinical isolate of *Aspergillus fumigatus*, abstr M-392. Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., Chicago IL. American Society for Microbiology, Washington, DC.
- Manavathu E, Baskaran I, Krishnan S, Alangaden G, Chandrasekar P. 2003. Molecular cytochrome P450 14 α -sterol demethylase mutation-dependent triazole cross-resistance in *Aspergillus fumigatus*, abstr M-471. Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., Chicago IL. American Society for Microbiology, Washington, DC.
- 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.
- Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14 α -sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob. Agents Chemother.* 48:2747–2750.
- Mellado E, et al. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob. Agents Chemother.* 51:1897–1904.
- Mortensen KL, et al. 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents Chemother.* 54:4545–4549.
- Pappas PG, et al. 2010. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin. Infect. Dis.* 50:1101–1111.
- 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.
- Rodero L, et al. 2003. G484S amino acid substitution in lanosterol 14 α -demethylase (*ERG11*) is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate. *Antimicrob. Agents Chemother.* 47:3653–3656.
- Rodriguez-Tudela JL, et al. 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 52:2468–2472.
- Sanglard D, Ischer F, Koymans L, Bille J. 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14 α -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* 42:241–253.
- Snelders E, et al. 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* 5:e219.

29. Stevens DA, Melikian GL. 2011. Aspergillosis in the 'nonimmunocompromised' host. *Immunol. Invest.* **40**:751–766.
30. 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.
31. 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.
32. Verweij PE, Mellado E, Melchers WJ. 2007. Multiple-triazole-resistant aspergillosis. *N. Engl. J. Med.* **356**:1481–1483.
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.