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International Journal of Antimicrobial Agents 28 (2006) 450–453

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

Multi-azole resistance in *Aspergillus fumigatus*

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Received 30 May 2006; accepted 11 July 2006

Abstract

Azole resistance in *Aspergillus* spp. is unusual. We report a patient who received long-term treatment with itraconazole and voriconazole for bilateral chronic cavitary aspergillosis with aspergillomas whose isolates of *Aspergillus fumigatus* developed simultaneous resistance to itraconazole and voriconazole. A novel mutation (G138C) in the target gene (*cyp51A*) encoding 14α-demethylase was detected. The patient had some response to intravenous caspofungin, which he received six times weekly, without the development of resistance over 9 months.

Keywords: *Aspergillus*; Itraconazole; Resistance; Azole; Aspergillosis; *cyp51A*

1. Introduction

The use of azole compounds in the treatment of *Aspergillus* disease was established in vitro and in vivo in 1984 [1,2]. Itraconazole (ITC; R 51,211) was the first orally active compound for invasive aspergillosis. In 1996, a novel agent, voriconazole (VRC; UK109406), was shown to have anti-*Aspergillus* properties [3,4]. VRC is now the first-line therapy for invasive aspergillosis [5,6], and posaconazole (PSC; SCH 56592) has just been licensed. Ravuconazole (RVC; ER 30346 or BMS 207147) and BAL8557 are in clinical development.

Azole antifungals act by inhibiting the lanosterol 14α-demethylase, the gene product of *ERG11* (*cyp51* in *Aspergillus*), which catalyses an intermediate step in the biosynthetic manufacture of ergosterol from lanosterol in fungi. Absence of enzyme activity means that 14α sterols are not demethylated and C-14 methyl sterol intermediates accumulate. These insert into the plasma membrane of the fungus and cause growth arrest in *Saccharomyces cerevisiae* and *Candida albicans* [7,8] as well as increasing sensitivity to reactive oxygen species [9].

ITC resistance in *Aspergillus fumigatus* brought about by 14α-demethylase modifications has long been postulated [10]. In 2001, two 14α-demethylase genes (*cyp51A* and *cyp51B*) were identified in pathogenic *Aspergillus* spp. [11]. Alterations at glycine 54 (G54) in *cyp51A* have been described both in clinical ITC-resistant *A. fumigatus* strains and in laboratory mutants [12–15]. Interestingly, this alteration confers cross-resistance to ITC and PSC [16] but not VRC or RVC. Methionine 220 (M220) may also be a hot-spot for substitutions conferring resistance in *Aspergillus* [12,13], resulting in reduced susceptibility to ITC, PSC, RVC and VRC [17]. The relationships of G54 and M220 modifications with resistance have both been confirmed by transformation of the altered *cyp51A* genes into wild-type strains. Other point mutations reported include G138 and G448 and are associated with VRC and RVC cross-resistance, with a lesser reduction in susceptibility to ITC and PSC [16]. The patterns of cross-resistance with these point mutations in *Aspergillus*

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0924-8579/$ – see front matter
doi:10.1016/j.ijantimicag.2006.08.017

are presumably due to the relationship between the protein structure of *cyp51*A and characteristic drug-binding features, although exactly how is unclear at present. In this report, we describe the simultaneous development of ITC and VRC resistance in *A. fumigatus* in a patient with bilateral fungal balls complicating chronic cavitary pulmonary aspergillosis.

2. Case report

A 51-year-old man first presented in the late 1980s with a diagnosis of tissue biopsy-proven sarcoidosis for which he had been taking prednisolone. The referral request in 1999 indicated symptoms of fatigue and dyspnoea. Lung function was restrictive, with a forced expiratory volume in 1 s (FEV_1) of 1.78 L (45% predicted) and a forced vital capacity (FVC) of 1.92 L (110% predicted), giving a ratio of 93%. The carbon monoxide transfer factor (TL_{CO}) was 5.98 (55% predicted) and the carbon monoxide transfer coefficient (K_{CO}) was 1.68 (110% predicted). The patient's weight was 48.7 kg. Chest radiography showed severe bilateral upper zone fibrosis and lower zone emphysema (Fig. 1). Pleural thickening at the lung apex on the chest radiograph prompted concerns about a left-sided aspergilloma. Computed tomography (CT) images of the thorax showed a round soft tissue density lesion with an air crescent sign in the left apex, consistent with an aspergilloma. *A. fumigatus* precipitins were positive with a titre of 1/8.

ITC 200 mg was started in October 1999. In November 1999 the patient received three installations of amphotericin (AMB) paste to the largest aspergilloma in the left apex. Despite this, CT of the thorax showed progressive disease with development of biapical aspergillomas and the patient continued to have haemoptysis and a raised C-reactive protein (CRP) (73 mg/L) and erythrocyte sedimentation rate. In October 2001 his haemoptysis increased again. His weight had dropped to 45.8 kg. His ITC levels were 0.89 mg/L (bioassay), reflecting antifungal-induced nausea and reduced drug compliance. He was admitted twice for systemic AMB and a further installation of intracavity AMB. By March 2002 his weight had dropped further to 44.4 kg and haemoptysis continued. *A. fumigatus* precipitins titre remained at 1/32. He

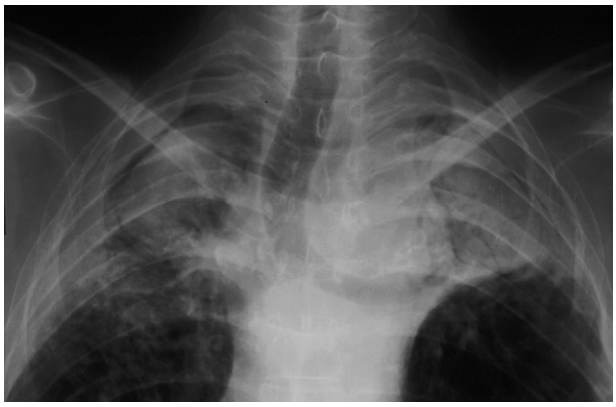

Fig. 1. Chest radiograph section showing complete bilateral upper lobe fibrosis and two large fungal balls each in a cavity.

was started on interferon-gamma (IFN-γ) 50 μg three times per week.

Throughout 2002 there was no clinical improvement despite ITC (at therapeutic levels), IFN-γ and steroids, on which he had become dependent. His weight drifted down to 42.3 kg. Precipitins remained elevated (1/32). His lung function deteriorated slightly to a FEV_1 of 1.67 L and a FVC of 1.71 L. In January 2003 his clinical state had deteriorated further with larger haemoptyses and increasing breathlessness. Sputum culture grew *A. fumigatus* persistently and his precipitins remained elevated (1/16). He was started on VRC and initially improved with weight gain to 57.5 kg, reduction in sputum volume and reduction of his CRP to 9 mg/L. Several moderate haemoptysis episodes in October 2003 were treated with bronchial embolization.

During early 2004 his weight improved further to 59.6 kg but in January 2004 *A. fumigatus* was cultured from his sputum with high minimum inhibitory concentrations (MICs) for ITC and VRC (Table 1). He gradually deteriorated and spent most of 2004 away from work owing to illness. He was then treated with intravenous caspofungin 50 mg six times weekly together with courses of antibiotics for >12 months. He died of respiratory failure due to infection in April 2006.

Table 1
Minimum inhibitory concentrations (MICs) of the patient's *Aspergillus fumigatus* isolates over the treatment period

Antifungal	MIC (mg/L)					
	23 January 2004	7 May 2004	26 November 2004	29 April 2005	17 June 2005[a]	17 June 2005[a]
Itraconazole	>8	>8	>8	>8	>8	>8
Amphotericin B	0.06	0.25	0.125	0.125	0.06	0.06
Voriconazole	8	>8	8	8	4	8
Posaconazole	4	4	N.T.	2	1	1
Ravuconazole	8	>8	N.T.	8	4	8
Caspofungin	0.25[b]	0.25[b]	0.5[b]	0.25[b]	0.25[b]	0.25[b]
Micafungin	≤0.015[b]	≤0.015[b]	N.T.	0.06[b]	0.06[b]	0.25[b]
Anidulafungin	≤0.015[b]	≤0.015[b]	N.T.	≤0.03[b]	0.03[b]	0.06[b]

N.T., not tested.
[a] Morphological variants.
[b] Minimum effective concentration (mg/L).

3. Materials and methods

3.1. Susceptibility testing

MICs were determined for ITC, AMB, VRC, PSC and RVC, and minimum effective concentrations (MECs) for echinocandin drugs (caspofungin, micafungin and anidulafungin). Susceptibilities were determined by a modified National Committee for Clinical Laboratory Standards M38-A microtitre method [18], with RPMI-1640 supplemented with 2% glucose. Isolates were cultured for 48 h on Sabouraud glucose agar with chloramphenicol (Oxoid, Dorset, UK). Inocula were prepared in phosphate-buffered saline/Tween-80 and adjusted to 1×10^5 colony-forming units (CFU)/mL ($2 \times$ required final concentration, ca. 5×10^4 CFU/mL). Microtitre plates were incubated at 37 °C for 48 h. MICs were read by eye with a no growth end-point. MECs were determined microscopically by morphological change to hyphal elements.

3.2. Isolation of genomic DNA

Strains were grown at 37 °C and maintained in yeast–peptone–dextrose prior to DNA extraction using the MagNA Pure^{TM} DNA III kit (Roche Diagnostics, Branchburg, NJ), following the manufacturer's instructions.

3.3. Polymerase chain reaction (PCR) amplification and DNA sequencing of cyp51A

PCR and sequencing primers were designed to the coding region of *cyp51A* (GenBank accession number AF338659). The 50 μL PCR amplification mixture included 1× HighFi PCR Buffer (Brinkmann, Westbury, NY), 0.5 μM of each PCR primer, 0.5 mM dNTP mix (Brinkmann), 2.5 U of Triplemaster DNA polymerase (Brinkmann) and 10 ng of genomic DNA. PCR amplification was carried out at 94 °C for 1 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min 30 s and a final extension step of 72 °C for 5 min in a PTC100 96-well thermal cycler (MJ Research, Waltham, MA). PCR products were purified by vacuum filtration using the Montage PCR_{96} Cleanup Kit (Millipore, Bedford, MA). Nucleotide sequencing analysis was performed by automated DNA sequencing using the DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) and CEQ 8000 capillary electrophoresis DNA sequencer (Beckman Coulter).

4. Results

Susceptibility results for six sequential fungal isolates obtained from sputum specimens over a 17-month period (23 January 2004 to 17 June 2005) are shown in Table 1. Isolates showed elevated MICs to ITC and the newer triazole drugs VRC, PSC and RVC.

Given the azole-resistant phenotype of the strains, the initial two isolates were evaluated for mutations within the azole target gene *cyp51A*. DNA sequencing of the entire *cyp51A* gene from both strains revealed a single mutation at codon 138, which was altered from GGC to TGC, substituting the amino acid glycine for cysteine (G138C).

5. Discussion

The first published case of ITC-resistant *A. fumigatus* came in 1997 [19]. A review of azole use in *A. fumigatus* disease in 1998 concluded that in vitro resistance to azoles was uncommon and that the MICs of ITC were half that of VRC ($P < 0.01$) [20]. A study from The Netherlands reported 3 of 114 isolates of *A. fumigatus* resistant to ITC but all had low MICs for VRC [21]. Reports of VRC resistance are few and a review of the available literature suggests that VRC remains a useful agent particularly where resistance to ITC is reported [22]. Seven isolates with multiple azole resistances were recently described in a group of heavily immunosuppressed patients [23]. A single multidrug-resistant isolate was reported by Warris et al. [24], but the mechanism of resistance was not determined. We believe this is the first case of what appears to be multiple azole resistance in isolates of *A. fumigatus* from a single patient.

The evolution of this resistance mechanism likely reflected the strong selection pressure induced by multiple exposures to related but structurally distinct azole drugs. Mechanisms of azole resistance have been described in other fungi, especially *C. albicans*, although few have been elucidated in clinical isolates of *Aspergillus*. The G138C alteration found here has been identified in an azole-resistant laboratory mutant [16], but this is the first characterisation in a clinical isolate to our knowledge. This amino acid alteration is likely to disturb the heme environment, as the residue is situated near the heme cofactor of *cyp51A* [25], which should result in reduced drug-binding affinity. The nature of the resistance mechanism was expected to have no affect on echinocandin susceptibility, which was observed (Table 1).

The discovery left us with some difficulty regarding oral therapy for this patient. Until oral antifungals with novel methods of action are developed, azole resistance will become a considerable problem.

Acknowledgment

This work was supported by Professor David W. Denning's endowment fund.

References

[1] Heeres J, Backx LJ, Van Cutsem J. Antimycotic azoles. 7. Synthesis and antifungal properties of a series of novel triazol-3-ones. J Med Chem 1984;27:894–900.

[2] Van Cutsem J, Van Gerven F, Van de Ven MA, Borgers M, Jannsen PA. Itraconazole, a new triazole that is orally active in aspergillosis. Antimicrob Agents Chemother 1984;26:527–34.
[3] George D, Miniter P, Andriole VT. Efficacy of UK-109496, a new azole antifungal agent, in an experimental model of invasive aspergillosis. Antimicrob Agents Chemother 1996;40:86–91.
[4] Oakley KL, Moore CB, Denning DW. The in vitro activity of voriconazole against *Aspergillus* spp. and comparison with itraconazole and amphotericin B. J Antimicrob Chemother 1998;42:91–4.
[5] Denning DW, Ribaud P, Milpied N, et al. Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. Clin Infect Dis 2002;34:563–71.
[6] Herbrecht R, Denning DW, Patterson TF, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 2002;347:408–15.
[7] Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE. Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem Biophys Res Commun 1995;207:910–5.
[8] Kelly SL, Lamb DC, Kelly DE, et al. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett 1997;400:80–2.
[9] Shimokawa O, Nakayama H. Increased sensitivity of *Candida albicans* cells accumulating 14 alpha-methylated sterols to active oxygen: possible relevance to in vivo efficacies of azole antifungal agents. Antimicrob Agents Chemother 1992;36:1626–9.
[10] Moore CB, Sayers N, Mosquera J, Slaven J, Denning DW. Antifungal drug resistance in *Aspergillus*. J Infect 2000;41:203–20.
[11] Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 2001;39:2431–8.
[12] Chen J, Li H, Li R, Bu D, Wan Z. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. J Antimicrob Chemother 2005;55:31–7.
[13] da Silva Ferreira ME, Capellaro JL, dos Reis Marques E, et al. In vitro evolution of itraconazole resistance in *Aspergillus fumigatus* involves multiple mechanisms of resistance. Antimicrob Agents Chemother 2004;48:4405–13.
[14] Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. A point mutation in the 14alpha-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 2003;47:1120–4.
[15] Nascimento AM, Goldman GH, Park S, et al. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother 2003;47: 1719–26.
[16] Manavathu EK, Baskaran I, Krishnan S, Alangaden G, Chandrasekar PH. Cytochrome P450 14-alpha-sterol demethylase mutation dependent triazole cross-resistance in *Aspergillus fumigatus*. In: 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy. Chicago, IL: American Society for Microbiology; 2003 [abstract M-471].
[17] Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 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 2004;48:2747–50.
[18] National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard. Document M38-A. Wayne, PA: NCCLS; 1998.
[19] Denning DW, Venkateswarlu K, Oakley KL, et al. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 1997;41:1364–8.
[20] Verweij PE, Mensink M, Rijs AJ, Donnelly JP, Meis JF, Denning DW. In vitro activities of amphotericin B, itraconazole and voriconazole against 150 clinical and environmental *Aspergillus fumigatus* isolates. J Antimicrob Chemother 1998;42:389–92.
[21] Verweij PE, Te Dorsthorst DT, Rijs AJ, De Vries-Hospers HG, Meis JF. Nationwide survey of in vitro activities of itraconazole and voriconazole against clinical *Aspergillus fumigatus* isolates cultured between 1945 and 1998. J Clin Microbiol 2002;40:2648–50.
[22] Manavathu EK, Abraham OC, Chandrasekar PH. Isolation and in vitro susceptibility to amphotericin B, itraconazole and posaconazole of voriconazole-resistant laboratory isolates of *Aspergillus fumigatus*. Clin Microbiol Infect 2001;7:130–7.
[23] Balajee SA, Weaver M, Imhof A, Gribskov J, Marr KA. *Aspergillus fumigatus* variant with decreased susceptibility to multiple antifungals. Antimicrob Agents Chemother 2004;48:1197–203.
[24] Warris A, Weemaes CM, Verweij PE. Multidrug resistance in *Aspergillus fumigatus*. N Engl J Med 2002;347:2173–4.
[25] Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM. 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 2004;48:568–74.