Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis

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Abstract

Oral azole antifungal therapy is used extensively for all forms of aspergillosis, including allergic bronchopulmonary aspergillosis (ABPA). However, long-term therapy may increase the risk of resistance. Here we report itraconazole and voriconazole resistance with reduced susceptibility to posaconazole in *Aspergillus fumigatus* in two patients exposed to itraconazole. Patients were diagnosed with ABPA and *Aspergillus* bronchitis related to innate immune defects. An azole susceptible strain was initially isolated from patient 1, but later a genetically different azole-resistant strain was cultured, possibly related to sub-therapeutic itraconazole levels, which could be a trigger for selection of resistance. The mechanism of resistance identified in this case was an L98H change in Cyp51A, accompanied by a tandem repeat in the promoter region of *cyp51A* leading to increased expression. No *cyp51A* mutation was found in azole-resistant isolates recovered from patient 2. Both patients responded to posaconazole, with plasma levels of >1.0 mg/L. Subsequently, susceptible strains of different molecular types were cultured from both patients, suggesting eradication and replacement.

Keywords: ABPA, antifungal, aspergillosis, *Aspergillus fumigatus*, azole, bronchitis, resistance
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Introduction

Allergic aspergillosis is a hypersensitivity disease manifesting in several clinical forms, including allergic bronchopulmonary aspergillosis (ABPA). ABPA is characterized by wheezing, bronchiectasis, pulmonary infiltrates and sputum containing brown plugs [1,2]. Asthmatics and cystic fibrosis patients are primarily at risk [2]. As ABPA is a long-term condition, its prevalence is higher than invasive aspergillosis (IA), but its annual incidence or rate of new diagnoses are probably lower. In addition to the conventional corticosteroid treatment, itraconazole (ITC) antifungal therapy has been shown to be advantageous in ~60% of cases [3].

Aspergillus bronchitis (or aspergillary bronchitis, as it was first called) [4] may occur in patients with underlying pulmonary or airway pathology [5], especially in the context of lung transplantation [6,7]. Antifungal therapy is probably effective if it has not evolved into pseudomembranous *Aspergillus* tracheobronchitis, which is usually fatal.

The azoles are the largest and most widely used class of antifungal drugs. Although voriconazole (VRC) is regarded as first-line therapy for invasive aspergillosis [5], ITC is still commonly used for chronic non-invasive forms of aspergillosis. Resistance to ITC in *Aspergillus fumigatus*, the species which causes the vast majority of cases of allergic aspergillosis, is well recognized. In our experience, it occurs mainly in patients with chronic forms of aspergillosis, particularly chronic cavitary pulmonary aspergillosis (CCPA) with aspergillomas. The frequency of ITC resistance in clinical *A. fumigatus* strains since the turn of the millennium (when most cases have been reported) is between 2% and 3%, and it can increase up to 6% depending on the area from which it is reported [8–10]. Cross-resistance between other azole drugs has also been reported [11,12]. To our knowledge, azole resistance has not been described among isolates caus-

ing allergic aspergillosis. In this report, we describe azole resistance in two patients with ABPA and *Aspergillus* bronchitis undergoing ITC therapy.

Case Reports

Patient 1

A 47 year-old asthmatic woman was diagnosed with ABPA. Her previous medical history was quite complex, including aortic valve replacement (twice), hypertension, hypermobility syndrome, perennial rhinitis and multiple allergies, including allergies to various houseplants, house dust, horses, dogs and pollen. The patient reported breathlessness especially on exercise. Cough was a predominant symptom, with discoloured brown sputum with hard brown plugs (which had a tree branch appearance). She had required several courses of oral corticosteroids for exacerbations. A computed tomography (CT) scan performed in January 2006 revealed some prominence and dilatation of the central bronchial tree consistent with mild bronchiectasis. There was no evidence of fibrosis or other parenchymal disease. She had normal immunoglobulin levels and eosinophil counts, with a total serum IgE of 3000 IU/mL and an anti-*Aspergillus* IgE of 94.5 IU/mL. A radioallergosorbent test (RAST) (IgE) was positive against several other fungi, including *Penicillium* (13.3), *Alternaria* (13.9), *Candida albicans* (4.4), *Saccharomyces cerevisiae* (1.3) and *Cladosporium* (1.0). Also, serum precipitins against *A. fumigatus* were at the limit of detection.

In July 2006, ITC treatment was started (capsules, 200 mg daily) while the patient was treated with omeprazole, which led to reduced cough. In October 2006, culture of her sputum yielded *A. fumigatus* susceptible to ITC (laboratory number F15767). Random serum ITC levels measured using a bioassay [13,14] in October 2006 were below the level of detection (<0.8 mg/L). She was also found to have very low mannose-binding lectin levels (0.3 mg/L; normal >4 mg/L). The patient was then treated with ITC oral solution (400 mg daily) which she found very difficult to tolerate as a result of nausea and diarrhoea. Nevertheless, an adequate, randomly tested serum ITC level (5.2 mg/L) was observed in November 2006, as determined with a bioassay [13,14].

Sputum culture performed in January 2007 revealed *Mycobacterium xenopi*. Because of drug interactions between the rifamycins and ITC, the patient was treated with ethambutol and ciprofloxacin in combination with ITC. Previous severe cholestasis under erythromycin therapy as a child precluded the use of a macrolide.

In February 2007, while being treated with a ITC solution, her sputum was abundantly yellow and thick, and the patient reported feeling very tired. Sputum culture revealed heavy growth of *A. fumigatus* (F16216), which was azole resistant (Table 1). In June 2007, posaconazole (PSC) was prescribed as salvage therapy. Over the next 6 months, her health was reasonable under PSC treatment. PSC serum levels were randomly measured using a bioassay (18th European Congress of Clinical Microbiology and Infectious Diseases, abstract P1351) between July 2007 and December 2008 and ranged from 1.01 to 1.36 mg/L. After a stormy course involving a lung abscess and a probably non-cardiogenic pulmonary oedema requiring mechanical ventilation in early and late 2008, respectively, during which VRC, amphotericin B (AMB) and PSC were used sequentially, an azole-susceptible *A. fumigatus* was isolated (F18830).

Patient 2

A 41 year-old female engineer who visited building sites suffered from intermittent haemoptysis and was diagnosed with bilateral lower-lobe bronchiectasis in 1999. She was a non-smoker and denied any constitutional upset, chest pain or shortness of breath. Since 1999 she had required antibiotics

TABLE 1. Minimum inhibitory concentration results (MIC, mg/L) and *cyp51A* mutations found in *Aspergillus fumigatus* isolates. Minimum fungicidal concentrations are also shown, unless identical to MIC values

	Susceptibility (mg/L)					
	ITC	VRC	PSC	RVC	AMB	*cyp51A*/Cyp51A alteration
Patient 1						
F15767	0.25 (0.5)	0.5	0.06	0.5	0.25 (1)	None
F16216	>8	8	2	8	0.125 (0.5)	L98H + promoter alteration
F18830	0.25	0.5	0.125	ND	0.5 (2)	ND
Patient 2						
F16311	>8	8	1 (2)	4	0.25 (0.5)	None
F16351	>8	4 (8)	0.5 (2)	4	0.25 (1)	None
F18718	1	2	0.125	ND	0.5 (>8)	ND

ITC, itraconazole; VRC, voriconazole; PSC, posaconazole, RVC, ravuconazole; AMB, amphotericin B; ND, not determined.

approximately three to four times per year, with little sputum production between episodes of infection. Additional past medical history included perennial rhinitis for which she took no medication; there was no history of asthma. She had bilateral extensive foot onychomycosis for which she had been prescribed ITC capsules, 1 week course per month for 3 months, without success. Plasma ITC levels were not determined. She had a normal sinus CT scan and her lung function was normal.

Since January 2007, the patient reported a chronic cough with variable amounts of sputum, yellow to green in colour. She did not improve despite two courses of amoxicillin-clavulanate. She reported feeling increasingly unwell and more breathless, without any wheezing or chest pain. Blood analyses showed positive *Aspergillus* precipitins (titre 1/8). IgE levels were within the normal range and RAST against *A. fumigatus* was negative. There was no eosinophilia, and the serum C-reactive protein level was low at 2 mg/L. She was deficient in mannose-binding lectin (0.65 mg/L) with a non-functional genotype (HYPA LYPB). Her anti-*Haemophilus influenzae* and anti-*Clostridium tetanus* antibody levels were within the normal range but anti-pneumococcal antibody levels were low. She was subsequently vaccinated with pneumococcal vaccine. In March 2007, treatment was started with ITC capsules (200 mg twice per day), but as sputum showed heavy growth of ITC-resistant *A. fumigatus* (F16311 and F16351), ITC was discontinued. Her α1-antitrypsin level was also low (0.2 g/L) with a ZZ genotype, and her cystic fibrosis genotype was wild-type homozygous. Treatment with PSC solution (400 mg twice daily) was started in June 2007. A random serum PSC level in July was 3.03 mg/L. The patient developed no side effects and over 3 months of PSC therapy had a complete resolution of her cough and generalized symptoms. Unfortunately, she relapsed 3 weeks after discontinuing therapy (with haemoptysis and dyspnoea) but responded to re-introduction of PSC which was continued for 6 weeks. A random serum PSC level measured during December 2007 was 1.07 mg/L. In May 2008, her sputum culture yielded *A. fumigatus*, resistant to ITC and VRC and susceptible to PSC, after another episode of mild haemoptysis, which was not treated. In September 2008, an azole-susceptible *A. fumigatus* was isolated (F18718). Throughout 2007 and 2008, *Aspergillus* precipitins were detectable at a titre of 1/8.

Materials and Methods

Isolates

Aspergilli were identified as *A. fumigatus*, according to macro- and micro-morphological characteristics, and subcultured at 50°C to exclude *A. lentulus*. Isolates are held in the Regional Mycology Laboratory, Manchester culture collection.

Susceptibility testing

Susceptibilities were determined in triplicate using a modified EUCAST method [15]. ITC (Research Diagnostics Inc., Concord, CA, USA), VRC (Pfizer Ltd, Sandwich, UK), PSC (Schering-Plough, NJ, Kennilworth, USA), ravuconazole (RVC; Eisai, Woodcliff Lake, NJ, USA) and AMB (Sigma, Poole, UK) were serially diluted to provide a final drug concentration range of 8–0.015 mg/L. RPMI-1640 (Sigma) was supplemented with glucose (2%) (Sigma). Inocula were prepared in phosphate-buffered saline with 0.05% Tween 80 (Sigma), quantified using a haemocytometer, and adjusted to give a final concentration of 5×10^4 CFU/mL. Spore suspensions were loaded into flat bottomed microtitre plates (Costar Corning, Lowell, MA, USA) and incubated at 37°C for 48 h. Minimum inhibitory concentrations (MICs) were deduced visually, with a no-growth endpoint. A putative resistance breakpoint of >2 mg/L was used for ITC, VRC and RVC, and >0.5 mg/L for PSC. Minimum fungicidal concentrations were also determined [16].

Molecular techniques

DNA was extracted using the FastDNA SPIN kit (Q-Biogene, Carlsbad, CA, USA) according to the manufacturers' instructions. The promoter and the entire coding region of the *cyp51A* gene were amplified. Reaction mixes (25 μL) were prepared consisting of PCR Master Mix (Promega, Southampton, UK; providing final concentrations of 1.5 mM $MgCl_2$, 200 μM each dNTP and 0.625 U *Taq* DNA polymerase), 500 nM of each primer and approximately 15 ng DNA. PCR amplification was conducted on a QB-96 thermal cycler (Quanta Biotech, Surrey, UK), conditions and primers were as described previously [12]. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, West Sussex, UK). Both strands were sequenced with eight primers in total [12] using the BigDye Terminator Ready Reaction Mix version 1.1, on an ABI 3730 Genetic Analyser (Applied Biosystems, Warrington, UK).

Microsatellite typing was conducted by amplification of six loci (3A, 3B, 3C, 4A, 4B and 4C) as previously described [17], except that PCR reactions were not multiplexed. Capillary electrophoresis was carried out on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems).

Sequences were aligned and mismatches identified using AlignX (Invitrogen, Paisley, UK) by comparison to an azole-susceptible strain (Genbank accession number AF338659). Mutations were confirmed by repeating PCR and sequencing of both strands using the closest forward and reverse prim-

ers. Microsatellite data were analysed using PEAK SCANNER Software v1.0 (Applied Biosystems) and adjusted using a correction factor from sequenced alleles [18] to determine the size of the allele.

Results

Susceptibility results are shown in Table 1. *A. fumigatus* was first isolated from patient 1 in October 2006 (laboratory number F15767), which was azole-sensitive. Four months later (February 2007), a resistant isolate was cultured (F16216). Cross-resistance was seen between all four azoles that were tested (Table 1). Subsequently, a susceptible isolate (F18830) was cultured in October 2008. Two azole cross-resistant isolates of *A. fumigatus* were cultured from patient 2 in February and March 2007 (F16311 and F16351), and in September 2008 a susceptible isolate (F18718) was recovered. Susceptibility to AMB was retained in all isolates.

A change from CTC to CAC at codon 98 in *cyp51A* resulting in an L98H change was found in the azole-resistant isolate F16216 from patient 1. In addition, F16216 was found to have a 34-base tandem repeat (5′-GAATCACGCGGTCC GGATGTGTGCTGAGCCGAAT-3′) in the promoter region of the *cyp51A* gene. No *cyp51A* mutations were found in the azole-resistant isolates from patient 2, or the initial susceptible isolate from patient 1, compared with the wild type (AF338659). Genbank accession numbers for *cyp51A* sequences are as follows: EU807919 (F15767), EU807920 (F16216), EU807921 (F16311) and EU807922 (F16351). The *cyp51A* gene of the later azole-susceptible isolates from either patient was not sequenced.

Microsatellite typing (data not shown) revealed that all three isolates recovered from patient 1 (F15767, F16216 and F18830) were genetically unrelated, whereas typing of azole-resistant isolates from patient 2 (F16311 and F16351) very strongly suggested that the two isolates are of the same molecular type. The subsequent susceptible isolate (F18718) from this patient was genetically distinct.

Discussion

Here we describe the occurrence of azole resistance in two patients with allergic aspergillosis treated with ITC. Of major concern is the reduction in susceptiblity to VRC and PSC in both cases (Table 1), given the limited number of oral agents curently available for the treatment of aspergillosis.

Currently, the primary mechanism of azole resistance reported in *Aspergilli* is related to alterations of the Cyp51 enzyme. Azoles act by disrupting the ergosterol biosynthetic pathway, specifically by inhibiting lanosterol 14α-demethylase (Cyp51), resulting in fungal cell instability. Amino acid changes may alter protein structure and as a result affect drug binding and thereby conferring resistance. In patient 1, *cyp51A* sequencing of a resistant isolate (F16216) revealed a mutation at codon 98 (resulting in a L98H change). Thus far this mutation has always been accompanied by a 34-bp tandem repeat in the promoter region (as is the case here), resulting in an eight-fold increase in expression of *cyp51A* [19]. The combination of the two alterations has been shown to be crucial to yield this level of resistance [19].

No *cyp51A* mutations were found in the azole-resistant isolate from patient 2, suggesting the action of another as yet ill-defined mechanism. Patient 2 had previously been exposed to ITC (for onychomycosis), although drug levels were not determined. The prior use of low-dose and intermittent ITC (pulse therapy) may have precipitated the development of azole resistance in this case.

We performed molecular typing of the isolates recovered from both patients. Microsatellites are short tandem repeats of DNA which are highly polymorphic, and consequently can be used in a highly discriminatory test to type strains. Six microsatellite loci were tested, which provides a high discriminatory power. The results strongly suggest that the three isolates from patient 1 are all different strains. Patient 1 had evidence of subtherapeutic serum ITC levels, as a result of poor bioavailability, which probably increased the risk of drug resistance.

Interestingly, genetically distinct susceptible *A. fumigatus* isolates were subsequently obtained from both patients. It would have been opportune to analyse several colonies in these cases, as it is possible that the patients had mixed infections, but unfortunately only single colonies were referred for testing. Eradication of azole-resistant strains has not previously been demonstrated. PSC levels in both patients were adequate. In patient 1, VRC and/or AMB may have played a part in the eradication of the resistant *A. fumigatus* isolate.

The cases reported here suggest that exposure to subtherapeutic levels of ITC is a potential trigger for the development of azole resistance. The ideal lower limit of ITC plasma (and tissue) concentrations has not been determined, either for response to therapy or prevention of resistance. ITC plasma levels are highly variable, reflecting the dose used, oral bioavailability (which is often compromised with capsules), rate of metabolism primarily by CYP3A4 and key drug interactions. We utilize an ideal range of 5–15 mg/L for bioassay, based on a higher frequency of failure at lower concentrations and more adverse

events at higher concentrations. Long-term exposure to ITC [20] and VRC may also lead to resistance [12,19–22]. Acquisition of a resistant isolate from the environment, potentially mediated by agrochemical azole exposure, is also a possibility [10].

Since the first report of ITC resistance in 1997 [20], several further cases have been documented in patients with invasive and chronic pulmonary aspergillosis. However, this is the first report of azole resistance in ABPA and in another superficial disease. (Outline clinical information reported as patients 8 and 10 respectively in another report [23]). Given the frequent use of ITC for allergic aspergillosis, in both asthmatic and cystic fibrosis patients, this finding is of concern and suggests that azole susceptibility testing should be become an integral part of the management of these patients, especially if they are poorly responsive to therapy, or deteriorate after an initial response. Limited therapy options, in particular oral choices, require increased vigilance for azole cross-resistance.

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