Acquired itraconazole resistance in *Aspergillus fumigatus*

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The antifungal susceptibility profiles of four *Aspergillus fumigatus* isolates, recovered at different times from a patient treated with itraconazole for a pulmonary *Aspergillus* infection, were evaluated. Itraconazole MICs against two pre-treatment isolates were 0.5 mg/L, whilst two later isolates, recovered after at least 4 months of itraconazole therapy, had itraconazole MICs of >16 mg/L. In vivo susceptibilities to itraconazole and amphotericin B were tested in a murine model of disseminated aspergillosis. Treatment efficacy was evaluated by examining mortality rates and qualitative cultures of brain and kidneys. Itraconazole therapy significantly prolonged survival of mice infected with the initial isolates as compared with untreated controls. The third isolate was only partially susceptible to itraconazole in vivo, and the fourth isolate was highly resistant. The four isolates were typed by random amplified polymorphic DNA (RAPD) with four different primers. RAPD patterns obtained with each of them were identical, suggesting that the same strain was recovered over time and had acquired resistance to itraconazole.

In this study, we report the acquisition of resistance to itraconazole of *A. fumigatus* isolates in a patient following prolonged itraconazole therapy. The susceptibilities of these isolates to itraconazole were evaluated in vitro, and in vivo in a murine model of disseminated aspergillosis.

**Case report**

A 66-year-old man, with a long history of bronchiectasis accompanied by infection and productive cough, presented in mid-May 1997 for dyspnoea and weight loss. *A. fumigatus* (AF 1112) was isolated from a sputum sample. By the end of May the patient was hospitalized. A CT scan of the chest showed multiple cavities in the left lung, the largest being diagnosed as an aspergilloma, with fibrosis and bronchiectasis in the right lung. Surgical resection was not possible due to the poor respiratory function of the patient. A second *A. fumigatus* isolate (AF 1119) was recovered from a sputum specimen taken at this time. Treatment with itraconazole 400 mg daily was started in early June and continued for 5 months. In late September, the patient was hospitalized again and a culture of a bronchoalveolar lavage
Materials and methods

Organisms

The four isolates of *A. fumigatus* (AF 1112, AF 1119, AF 1237 and AF 1290) isolated in our laboratory were atypical isolates, which sporulated poorly. These strains have been deposited with the IHEM culture collection (Scientific Institute of Public Health, Brussels, Belgium) as IHEM 17905, 17906, 17907 and 17908, respectively. All four isolates sporulated equally poorly and there was no correlation between the degree of itraconazole resistance and the degree of sporulation. Moreover, the four isolates had the same growth rate. Identification was confirmed by E. Guého from the Pasteur Institute, Paris. Isolates were stored as conidial suspensions at –80°C in 10% glycerol until use.

In vitro susceptibility testing

Susceptibility testing was performed by means of a National Committee for Clinical Laboratory Standards (NCCLS)-based broth microdilution technique.\(^{10,11}\) RPMI 1640 (Gibco-BRL, Uxbridge, UK) with L-glutamine and without sodium bicarbonate, buffered at pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS), was used as the test medium. Amphotericin B (Sigma, St Louis, MO, USA) and itraconazole (Janssen Pharmaceutica, Beerse, Belgium) were provided by the manufacturers as powders. Both drugs were dissolved in dimethylsulphoxide (DMSO; Sigma) to a concentration of 1600 mg/L and stock solutions were stored frozen in aliquots at −80°C. The drug dilutions were prepared by following the standard additive two-fold drug dilution scheme described in the NCCLS reference method for yeasts\(^{12}\) with the medium as diluent. The final drug concentrations were 0.03–16 mg/L for amphotericin B and itraconazole. The isolates were grown on malt extract agar slants (Sanofi Diagnostics Pasteur, Marnes La Coquette, France) at 35°C for 5 days. The inoculum was prepared by washing the surface of the agar slants with 1 mL of sterile 0.9% saline containing 0.05% Tween 80. The resulting conidial suspensions were counted with a haemocytometer. Sterile microtitre plates with 96 U-shaped wells were used. The conidia were diluted in RPMI and each well of rows 2–12 was inoculated. The final inoculum concentration was 1 × 10⁴ conidia per mL. Row 1 was used as a sterility control and row 12 as a growth control. The determination of MICs for all isolates was performed in duplicate. Microplates were incubated at 35°C for 48 h and after this time the growth in each well was compared with that of the growth control with the aid of a microtitre reading mirror. Each well was given a numerical score: 0, no reduction in growth; 1, growth reduction of 25%; 2, growth reduction of 50%; 3, growth reduction of ≥75%; and 0, absence of growth (optically clear). MIC endpoints were defined as the lowest concentration that had a score of 0 (MIC-0) for amphotericin B. For itraconazole, both MIC-0 and the lowest drug concentration that had a score of 1 (MIC-1) were determined.

In vivo experiments

Mice. Female OF-1 outbred mice (IFCA CREDO, l’Arbresle, France), 5–7 weeks old and weighing 20–22 g, were used throughout the experiments. Mice were housed in groups of 10 and were given food and water *ad libitum*. Animal studies were conducted in accordance with recommendations of the European Community (Directive no. 86/609/EEC, 24 November 1986).

Infection. The inoculum was prepared by culturing the strains on potato carrot agar in cell culture flasks for 10 days at 35°C. Spores were harvested by washing the agar surface with sterile saline containing 0.05% Tween 80, and the resulting spore suspension was counted in a haemocytometer and stored at 4°C until infection. Viability was determined by plating serial 10-fold dilutions prepared in saline with 0.05% Tween 80. Plates were incubated at 35°C and cfu counted at 24 h. The spore suspension was adjusted to the required concentration in saline before administration. Preliminary studies were performed to determine the LD₉₀ (90% lethal dose) for each isolate by testing three to four inoculum concentrations. The LD₉₀ results were between 5 × 10⁸ and 8 × 10⁷ cfu/mouse for the four isolates. Mice were infected by injection of 0.1 mL of the conidial suspension into a lateral tail vein. After infection, mice were randomized into the different treatment groups.

Drugs and therapy. Amphotericin B desoxycholate (Fungizone, Bristol-Myers Squibb, Paris, France) was given intraperitoneally in 5% glucose and itraconazole (Sporanox oral solution, Janssen Pharmaceutica) was diluted in sterile water and given by gavage. Treatment with both drugs was commenced 2 h after infection and was continued for 10 days. For each isolate, four groups of 10 mice were used. One group was treated with 4.5 mg/kg/day od of amphotericin B by intraperitoneal injection, and one group was treated with 100 mg/kg/day of itraconazole administered bd by gavage. Control mice were infected but received only 5% glucose, one group by intraperitoneal injection and one group by gavage. Animals were checked twice daily for mortality. The mice were observed for a further 12 days after the end of treatment.
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*Organ cultures.* Qualitative organ cultures were performed on mice that died before the end of the experiment and on all mice on day 22 post-infection. Surviving mice were killed by cervical dislocation. Brain and kidneys were removed and homogenized with 3 mL of saline in a tissue grinder. A 0.1 mL aliquot of the suspension was plated on to Sabouraud’s agar and plates were incubated at 35°C for 3 days for control groups and 5 days for treated groups.

*Itraconazole assay.* Twelve uninfected mice were given itraconazole by gavage at 100 mg/kg/day. Six mice were killed after 4 days of treatment and six after 6 days of treatment, 6 h after the last dose. Blood was obtained by cardiac puncture and itraconazole serum concentrations were determined by high performance liquid chromatography (HPLC).13,14

**Data analysis.** In each experiment, data for the two control groups were pooled for the analysis. Mortality data were compared by the Kruskal–Wallis test. Qualitative organ cultures were compared by Fisher’s exact test.

*Molecular typing* Conidia were inoculated into 30 mL of liquid Sabouraud’s medium and cultured for 24 h at 35°C. After this time the mycelium was harvested and washed twice with sterile saline. It was then transferred to a 50 mL polypropylene tube containing six glass beads (4 mm diameter). The tube was immersed in liquid nitrogen for 10 s and vortexed vigorously for 1 min. Thereafter, DNA extraction was performed using a QIAmp Tissue Kit (Qiagen, Los Angeles, CA, USA) according to the manufacturer’s manual.

Random amplified polymorphic DNA (RAPD)-PCR was performed with primers 2 and 5 (5'H11032-GCTGGTGG and GCGCACGG, respectively)15 and primers R108 and R151 (5'H11032-GTATTGCCCT and GCTGTAGTGT, respectively).16 Amplification reactions were performed in 50 μL volumes containing 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 1.5 mM MgCl2, 100 μM (each) dNTPs, 0.2 μM primer, 50 ng of genomic DNA and 2.5 U of Taq DNA polymerase (Perkin Elmer, NJ, USA). Amplifications were carried out in a Minicycler (MJ Research, MA, USA) for 1 cycle of 5 min at 94°C, followed by 45 cycles of 1 min at 94°C, 2 min at 35°C and 2 min at 72°C. A final extension was performed at 72°C for 10 min. A blank control tube containing all reagents except the template DNA was also processed. Amplification products were separated by electrophoresis through 1.8% agarose gels containing ethidium bromide. RAPD profiles were compared visually. Both faint and intense bands were included in the interpretation.

**Results**

**In vitro susceptibility testing**

The results of susceptibility testing for the four *A. fumigatus* isolates used in the study are shown in Table I. Susceptibility tests were performed at least three times for each strain and MICs were reproducible on re-testing. For isolates AF 1112 and AF 1119, itraconazole was active with an MIC-1 of 0.5 mg/L. Isolates AF 1237 and AF 1290 exhibited high itraconazole MICs of >16 mg/L. For the four isolates, itraconazole MIC-0 and MIC-1 were similar. All four isolates were susceptible *in vitro* to amphotericin B with MICs ≈ 1 mg/L.

**In vivo experiments**

*Survival.* Survival curves (Figure 1) demonstrate that each of the four isolates caused an acute and lethal infection in control mice. Median survival time of mice infected with each of the four isolates is shown in Table II.

With strains AF 1112 (Figure 1a) and AF 1119 (Figure 1b) all control mice died within 10 days. Treatment with amphotericin B or itraconazole resulted in 100 and 90% survival, respectively. This was significantly higher than for mice receiving no treatment ($P < 0.001$). Figure 1c shows the survival of mice infected with strain AF 1237. There was 100% mortality in untreated control mice. No mice treated with amphotericin B died, which was significantly lower than for mice treated with itraconazole.

**Table I. In vitro susceptibility to amphotericin B and itraconazole of four *A. fumigatus* isolates used for *in vivo* studies**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Amphotericin B (MIC-0 mg/L)</th>
<th>Itraconazole (MIC-0 mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF 1112</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>AF 1119</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>AF 1237*</td>
<td>1</td>
<td>&gt;16</td>
</tr>
<tr>
<td>AF 1290*</td>
<td>0.5</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>

MICs were determined after 48 h incubation. For itraconazole, MIC-1 (75% inhibition) and MIC-0 (100% inhibition) were determined.

*Isolates cultured after at least 4 months of itraconazole therapy.
better than no therapy \( (P < 0.001) \). In contrast, 50% of mice treated with itraconazole died, which was still better than no therapy \( (P < 0.01) \).

The survival curves for mice infected with strain AF 1290 are shown in Figure 1 (d). All mice given glucose either by gavage or intraperitoneally died. No mice treated with amphotericin B died, which was significantly better than no therapy \( (P < 0.001) \). All mice treated with itraconazole died by day 8, which was not significantly different from control mice.

Comparison of the four experiments showed that for groups treated with itraconazole, there was a significant difference in median survival \( (P < 0.05) \) between mice infected with AF 1290 and those infected with AF 1112 or AF 1119 (Table II).

**Culture results.** Results of qualitative organ cultures are shown in Table III. For all strains, infection occurred in the kidney tissues of 100% of untreated controls and in the brain of 75–90% of controls. Amphotericin B treatment eradicated or prevented *A. fumigatus* infection in both kidney and brain tissues, whatever the strain tested. For strains AF 1112 and AF 1119, itraconazole significantly reduced kidney and brain infections as compared with controls \( (P < 0.0001 \text{ to } P < 0.0005) \). In contrast, itraconazole did not significantly reduce the number of infected kidneys in mice infected with strain AF 1237 or AF 1290.

**Itraconazole assay.** Serum itraconazole concentrations were assessed to ensure adequate absorption of the antifungal agent. Mean itraconazole levels in serum as detected by HPLC are shown in Table IV. The concentration of itraconazole and the active metabolite, hydroxy-itraconazole, in serum was >8 mg/L after 4 days of treatment and only a slight increase was observed after 6 days of treatment.

**Molecular typing**

The four isolates were typed by means of each of the four primers (primer 2, primer 5, R108 and R151) used separately. The RAPD patterns obtained are shown in Figure 2. Identical patterns were obtained for the four strains, while five unrelated strains, tested in the same conditions, exhibited different RAPD patterns (data not shown). Molecular typing was performed at least twice, with reproducible results.

**Discussion**

In this study, we tested the antifungal susceptibilities of four isolates of *A. fumigatus* recovered from a patient, both *in vitro* and in an animal model. Two isolates were obtained before treatment with itraconazole and two were post-treatment isolates. The results suggested that the strain acquired resistance to itraconazole during treatment with this antifungal agent.
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**Table II.** Survival times for mice infected with four different *A. fumigatus* isolates and treated with amphotericin B 4.5 mg/kg/day or itraconazole 100 mg/kg/day

<table>
<thead>
<tr>
<th>Group</th>
<th>AF 1112</th>
<th>AF 1119</th>
<th>AF 1237</th>
<th>AF 1290</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>5 (3–10)</td>
<td>7 (3–10)</td>
<td>5 (3–8)</td>
<td>5 (3–8)</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>23 (23)</td>
<td>23 (23)</td>
<td>23 (23)</td>
<td>23 (23)</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>23 (20–23)</td>
<td>23 (18–23)</td>
<td>16 (6–23)</td>
<td>7 (4–8)</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with controls.

**Table III.** Qualitative culture results for kidney and brain for all mice

<table>
<thead>
<tr>
<th>Strain and group (no. of mice in group)</th>
<th>No. of survivors</th>
<th>No. of kidney positive</th>
<th>No. of brain positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF 1112 controls (n = 20)</td>
<td>0</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>amphotericin B (n = 10)</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>itraconazole (n = 10)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AF 1119 controls (n = 20)</td>
<td>0</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>amphotericin B (n = 10)</td>
<td>9</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>itraconazole (n = 10)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AF 1237 controls (n = 20)</td>
<td>0</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>amphotericin B (n = 10)</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>itraconazole (n = 10)</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AF 1290 controls (n = 20)</td>
<td>0</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>amphotericin B (n = 10)</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>itraconazole (n = 10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*P < 0.0001 compared with controls.

**Table IV.** Itraconazole concentration in serum, as measured by HPLC, in non-infected mice after 4 and 6 days of treatment at 100 mg/kg/day

<table>
<thead>
<tr>
<th>Time (no. of mice)</th>
<th>Itraconazole concentration ± s.D. (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>native</td>
</tr>
<tr>
<td>Day 4 (6)</td>
<td>3.13 ± 0.76</td>
</tr>
<tr>
<td>Day 6 (6)</td>
<td>3.37 ± 0.60</td>
</tr>
</tbody>
</table>
Although a broth microdilution adaptation of the NCCLS reference method for yeasts has shown good intra- and inter-laboratory reproducibility, there is no standardized method for susceptibility testing of filamentous fungi. For this reason we used an animal model of disseminated aspergillosis in immunocompetent mice to confirm the resistance detected in vitro. The results of susceptibility testing in vitro showed a clear increase in MIC from 0.5 mg/L for isolates recovered before itraconazole therapy, to 16 mg/L for isolates cultured after 4–5 months of therapy. In vitro data were confirmed for each of the four isolates in an animal model of disseminated aspergillosis. This model has been used previously to confirm de novo resistance of A. fumigatus to itraconazole. Although there was good correlation between in vitro results and the animal model data, evaluation of the therapeutic efficacy of itraconazole in the patient reported here is difficult. Absence of clinical improvement during itraconazole therapy could be attributed to the resistance of the fungus; however, it could also have been due to the severe underlying respiratory disease.

Previous studies have demonstrated itraconazole resistance of A. fumigatus in vitro and the resistance was confirmed for some strains in vivo in animal models of aspergillosis. In one study, four itraconazole-resistant strains of A. fumigatus were isolated from three patients during long-term itraconazole therapy. However, genotypic analysis of the strains was not performed so it was not possible to determine whether the strains acquired resistance or if the patients were subsequently infected by a resistant strain from the environment. Denning et al. reported two patients from whom itraconazole-resistant strains were isolated. In one case, a resistant strain was obtained in culture, although the patient had not been treated with itraconazole. In the other case, a strain was isolated during treatment with itraconazole but was genotypically distinct from the initial strain cultured before treatment.

To our knowledge, the present study is the first to document the acquisition of itraconazole resistance in an A. fumigatus strain during prolonged therapy with this antifungal agent. The genetic diversity of A. fumigatus is very important and genetic variations of A. fumigatus isolates cultured from respiratory specimens in patients with aspergillosis or cystic fibrosis have been demonstrated. Nevertheless, in patients with aspergillosis, although several types could be recovered in the same patient, one predominant genotype is usually found. In the case reported here, the diagnosis of chronic invasive pulmonary aspergillosis was possible. In this context, the disease is probably produced by a single strain of Aspergillus, rather than there being multiple strains involved.

Microevolutionary changes within an infecting strain over time have been demonstrated in Candida albicans. It is possible, due to microevolution, that some substrains in a colonizing population present altered phenotypes, including changes in susceptibility to antifungal drugs. One can suggest, in a patient with chronic aspergillosis and/or aspergilloma, that the high population density of A. fumigatus is a critical factor for strain microevolution or clonal selection of resistant substrains under the drug selection pressure. The situation is probably different in immunosuppressed patients with invasive aspergillosis who have a shorter course and less exposure to the antifungal drug.

Several molecular mechanisms ofazole resistance in Candida spp. have been reported, but very little is known about the mechanisms of resistance in Aspergillus spp. A reduced intracellular accumulation of itraconazole was observed in some strains, possibly due to an efflux pump or to a reduced penetration of the drug. In two other strains, the mechanism of itraconazole resistance has been related to a possible modification of the target enzyme 14-α-demethylase.

In the present study we used RAPD to type the isolates.
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The primers used (2, 5, R108 and R151) have been shown to provide a high level of discrimination between strains of *A. fumigatus*.

Further studies have to be done to confirm that *A. fumigatus* could acquire resistance to itraconazole as a result of treatment with this antifungal. Susceptibility studies of *A. fumigatus* strains repeatedly isolated from colonized patients under long-term therapy with itraconazole are needed to evaluate the frequency of this phenomenon.

Acknowledgements

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References


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