ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1997, p. 1364–1368 Vol. 41, No. 6
0066-4804/97/$04.00+0

Itraconazole Resistance in *Aspergillus fumigatus*

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Received 8 July 1996/Returned for modification 19 November 1996/Accepted 3 March 1997

Invasive aspergillosis is an increasingly frequent opportunistic infection in immunocompromised patients. Only two agents, amphotericin B and itraconazole, are licensed for therapy. Itraconazole acts through inhibition of a P-450 enzyme undertaking sterol 14α demethylation. In vitro resistance in *Aspergillus fumigatus* to itraconazole correlated with in vivo outcome has not been previously described. For three isolates (AF72, AF90, and AF91) of *A. fumigatus* from two patients with invasive aspergillosis itraconazole MICs were elevated. A neutropenic murine model was used to establish the validity of the MICs. The isolates were typed by random amplification of polymorphic DNA. Analysis of sterols, inhibition of cell-free sterol biosynthesis from [^{14}C]mevalonate, quantitation of P-450 content, and [^{3}H]itraconazole concentration in mycelial pellets were used to determine the mechanisms of resistance. The MICs for the three resistant isolates were >16 μg/ml. In vitro resistance was confirmed in vivo for all three isolates. Molecular typing showed the isolates from the two patients to be genetically distinct. Compared to the susceptible isolate from patient 1, AF72 had a reduced ergosterol content, greater quantities of sterol intermediates, a similar susceptibility to itraconazole in cell-free ergosterol biosynthesis, and a reduced intracellular [^{3}H]itraconazole concentration. In contrast, AF91 and AF92 had slightly higher ergosterol and lower intermediate sterol concentrations, fivefold increased resistance in cell-free systems to the effect of itraconazole on sterol 14α demethylation, and intracellular [^{3}H]itraconazole concentrations found in susceptible isolates. Resistance to itraconazole in *A. fumigatus* is detectable in vitro and is present in wild-type isolates, and at least two mechanisms of resistance are responsible.

Invasive aspergillosis causes approximately 30% of fungal infections in patients dying with cancer (5). There has been a 14-fold increase in its incidence in the last 12 years as detected at autopsy (14). It affects between 10 and 25% of all patients with leukemia and between 5 and 25% of all patients following heart or lung transplantation. Until 1990 there was only one drug useful for treatment of *Aspergillus* disease, amphotericin B, which has to be given intravenously and has a number of serious toxicities. In 1990 itraconazole, which has the virtue of being able to be given orally, became available for the treatment of *Aspergillus* infection (12).

Aspergillus fumigatus is the most common species of *Aspergillus* causing pulmonary disease (3, 26). The vast majority of isolates are susceptible to both itraconazole and amphotericin B, although there are many different methods of determining in vitro susceptibility (10). Raised MICs of itraconazole have occasionally been noted but not correlated with in vivo outcome. In this report we document the occurrence of itraconazole resistance in *A. fumigatus* and provide evidence for two different resistance mechanisms.

CASE REPORTS

Patient 1. Patient 1, a 26-year-old woman from California cured of Hodgkin's disease, presented with constrictive pericarditis with large pleural effusions (12). Incomplete pericardiectomy showed the cause to be *A. fumigatus* (AF41) by culture of pericardial tissue and histology. She gradually responded to oral itraconazole at 400 mg daily over the following 4 months, with clearing of pleural effusions, pulmonary atelectasis, and anterior mediastinal aspergillosis. Her steady-state serum itraconazole concentration was 6.0 μg/ml, measured by bioassay. After 9 months of treatment, she developed a cough and *A. fumigatus* (AF72) was isolated from her sputum. No treatment change was instituted, and she went on to make a complete recovery during 10 months of therapy.

Patient 2. Patient 2, a 40-year-old man from San Francisco, Calif., with AIDS, developed *Pneumocystis* pneumonia in May 1987 and had two further episodes subsequently (8). He also developed disseminated *Mycobacterium avium-M. intracellulare* complex infection, which was treated with clofazamine, ethambutol, and rifampin, and cytomegalovirus retinitis which was complicated by a retinal detachment in the left eye in January 1988 leaving him almost blind in that eye. Despite ganciclovir treatment he suffered progressive visual loss in the right eye. He developed profound neutropenia (70 neutrophils/μl) and two episodes of probable sepsis in 1988.

In January 1989, the patient developed pain and numbness in his right scapula and shoulder which advanced down the right arm. A mass in the right apical area was seen in chest X-ray and confirmed on computer tomography scan. Needle aspiration in February revealed hyphae on smear, and the sample grew *A. fumigatus*. The patient was treated with 560 mg of amphotericin B over 3 weeks. Despite resolution of his

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symptoms, the mass remained unchanged radiographically. He tolerated the amphotericin B poorly and refused further therapy.

Three months after the amphotericin B was stopped, the radicular symptoms recurred, and a repeat percutaneous lung aspiration again grew *A. fumigatus* (AF90). Itraconazole, 200 mg twice daily, was initiated with improvement. However, the symptoms then worsened, and serum itraconazole concentrations were undetectable. Rifampin was discontinued, and itraconazole was increased to 200 mg three times daily. At this time, the patient's CD4 cell count was 2/μl (normal > 800/μl) in blood. He received the increased dose of itraconazole for 6 weeks, and his radicular symptoms markedly improved. The lesion remained stable radiographically. A repeat percutaneous aspiration of the cavitary area in the right apex after 4 weeks on the higher dose showed necrotic material with some hyphae. The culture was sterile. A repeat serum itraconazole concentration was 11.0 μg/ml. Occasional neutropenia required interruption of ganciclovir. He remained blind and decided to discontinue all medication on 27 December 1989. His sputum grew *A. fumigatus* (AF91) on 29 December 1989. He died on 3 January 1990.

MATERIALS AND METHODS

Organisms. Seven clinical isolates of *A. fumigatus* were studied: H06-03 (Pfizer Central Research, Sandwich, Kent, United Kingdom), AF6, AF41, AF72 (NCPF 7099; National Collection of Pathogenic Fungi at Mycology Reference Laboratory, Bristol, United Kingdom), AF90, AF91 (NCPF 7100), and AF210 (NCPF 7101). H06-03 is of clinical origin, but the details were not available. AF6 was isolated from a renal transplant patient with disseminated aspergillosis, unsuccessfully treated with itraconazole (11). AF210 was isolated from the surface of the liver in a patient with a laparostomy (6).

Antifungal agents. Itraconazole powder (obtained from Janssen Research Foundation, Beerse, Belgium) was dissolved in 50% acetone–50% 0.2 M HCl in a glass test tube and stored at −20°C for susceptibility testing. For model work, itraconazole was solubilized in hydroxy-β-cyclodextrin (16). This produced a stock of 25 mg/ml which was then further diluted and stored at 4°C. Amphotericin B (Fungizone) was purchased from E. R. Squibb, Hounslow, Middlesex, United Kingdom. For work on mechanisms of resistance, ketoconazole and [^{3}H]itraconazole were kindly supplied by Janssen Research Foundation and fluconazole was kindly supplied by Pfizer Central Research.

In vitro susceptibility testing. The isolates were stored at −70°C in 15% glycerol, and for each experiment fresh subcultures on Sabouraud dextrose agar from the frozen stock were used. Testing was performed using a macrobroth dilution technique that is similar to that described by Moore et al. (22). RPMI 1640 (Sigma, Poole, United Kingdom) supplemented with 2% glucose buffered to pH 7.0 with 10 M NaOH was used as the medium. The final range of itraconazole dilutions was 0.03 to 16 μg/ml.

The inoculum of *Aspergillus* conidia was prepared in sterile phosphate-buffered saline with Tween 80 (0.05%) initially, and then dilutions in RPMI 1640 were made to 2×10^3/ml. Equal volumes (1 ml) of inoculum and drug dilution in 5-ml tubes comprised the test. The tubes were incubated with loose caps at 37°C on a gyratory shaker at 30°C to the horizontal for 40 to 42 h. The MIC was read visually and was defined as the concentration of drug in the first tube that showed no growth.

Murine model experiments. Virus-free, male CD-1 mice were purchased from Charles River UK Ltd. The animal model was a modification of a prior temporarily neutropenic model (9). The inoculum (0.15 ml) was given intravenously instead of intranasally on day 0, 3 days after cyclophosphamide (200 mg/kg of body weight).

Preliminary studies were carried out to determine the 90% lethal dose of each of the four *Aspergillus* isolates used for these experiments. The conidial inocula yielding 90% lethal doses for each mouse were as follows: AF72, 3.75×10^5/ml; AF90, 1.5×10^6/ml; AF91, 1.8×10^6/ml; and AF210, 2.55×10^5/ml.

Amphotericin B (5.0 mg/kg) or a 5% dextrose control was given intraperitoneally at 18 h, after a further 24 h, and at 4 and 7 days. Two different doses of itraconazole were given: 25 and 75 mg/kg, administered in 0.25 ml by gavage. Three doses were given on days 1 and 2, and two doses were given on days 3 to 10. A control group was given 5% dextrose by gavage.

Uninfected but cyclophosphamide-treated mice were treated according to the dosing regimen and sacrificed at intervals. They were bled by cardiac puncture, and serum itraconazole levels were measured by bioassay (12).

Mortality and quantitative culture were compared by using the Mann-Whitney ranked-sum test or the Kruskal-Wallis test if the Mann-Whitney test was not possible. All analyses were done with the computer package Minitab (Minitab Data Analysis Software, Philadelphia, Pa.).

Molecular typing. Each patient isolate was DNA typed by random amplification of polymorphic DNA (RAPD) using primer R108 as previously described (1).

Biochemical studies. The isolates of *A. fumigatus* were grown on Czapek Dox agar plates (Oxoid, Basingstoke, Hants, United Kingdom) at 37°C for 3 days. Conidia were collected by washing the cultures with sterile Sabouraud liquid medium (Oxoid) containing 2 to 3 drops of Tween 80. Cultures were initiated with 5×10^5 conidia/ml and grown for 18 h at 37°C in Sabouraud dextrose medium on an orbital shaker (120 rpm). Preparation of cell extracts was done according to the method of Ballard et al. (2).

In vitro sterol biosynthesis and identification were as reported by Joseph-Horne et al. and Venkateswarlu et al. in previous studies of ergosterol biosynthesis (17, 31). The reaction mixture consisted of cell extract (924 μl; protein concentration, 1.5 to 2.0 mg/ml), cofactor solution (50 μl; containing 1 μmol of NADP, 1 μmol of NADPH, 1 μmol of NAD, 3 μmol of glucose-6-phosphate, 5 μmol of ATP, and 3 μmol of reduced glutathione in distilled water; pH adjusted to 7.0 with 10 M KOH), divalent cation solution (10 μl of 0.5 M $MgCl_2$ and 5 μl of 0.4 M $MnCl_2$), dimethyl sulfoxide or solution of azole antifungal compound in dimethyl sulfoxide (1 μl), and [2-^{14}C]mevalonate (10 μl; 0.25 μCi [9.3 kBq]; 4.7 nmol). The mixture was incubated at 37°C for 2 h with shaking (120 rpm), after which the reaction was stopped by adding 1 ml of freshly prepared saponification reagent (15% [wt/vol] KOH in 90% [vol/vol] ethanol) and heating for 1 h at 80°C for saponification.

Nonsaponifiable lipids (sterols and sterol precursors) were extracted twice with 3 ml of petroleum ether (bp, 40 to 60°C) and dried under nitrogen. The nonsaponifiable lipid was suspended in 100 μl of petroleum ether and applied to silica gel thin-layer chromatography plates (ART 573; Merck), and the plates were developed with toluene-diethyl ether (9:1, vol/vol). Radioactive sterols were located by autoradiography and excised for scintillation counting. The production of C14,4-desmethyl sterol was assessed for inhibition as described previously (31), and 50% inhibitory concentrations (IC_{50}s) for ergosterol biosynthesis were calculated.

Samples for gas chromatography and mass spectrometry were prepared from 100 ml of 24-h-old cultures. The mycelia were saponified in 15% (wt/vol) KOH in 90% (vol/vol) ethanol at 80°C for 1 h. Nonsaponifiable lipids (sterols and sterol precursors) were extracted three times with 5 ml of hexane and dried under nitrogen. Following silylation for 1 h at 60°C with bis(trimethylsilyl)trifluoroacetamide (20 μl) in 100 μl of toluene, sterols were analyzed by gas chromatography-mass spectrometry (VG 12-250; VG Biotech) using split injections with a split ratio of 20:1. Sterol identification was done by reference to relative retention time and mass spectra as reported previously (17, 31).

Microsomal fractions were obtained as described by Masaphy et al. (21). P-450 content was measured according to the method of Omura and Sato (23).

Drug uptake experiments were carried out as described previously (19), in 100 ml of Sabouraud dextrose medium. *A. fumigatus* spores were inoculated (5×10^5/ml) and allowed to grow at 37°C at 150 rpm for 24 h, and [^{3}H]itraconazole was added to a final concentration of 5×10^{-8} M (36.7 mCi/mmol). After 1 h of incubation mycelia were collected and washed five times with saline (0.15 M NaCl) containing 5 μM unlabelled itraconazole. Then the mycelia were filtered onto preweighed Whatman 3MM filter paper and kept at 80°C for 24 h to measure dry cell weight. One hundred milligrams (dry cell weight) of mycelia was digested with a 2:1 mixture of H_2O_2 and $HClO_4$ (1 ml) at 70°C for 3 h and mixed with 10 ml of scintillation solution for scintillation counts. Results are expressed as picomoles of [^{3}H]itraconazole per 100 mg (dry weight) of mycelium.

RESULTS

We have used a macrodilution test format for susceptibility testing that is reproducible with itraconazole and consistently yields high MICs for the resistant isolates. The MICs were 0.25 μg/ml for H06-03, AF41, and AF210; 0.5 μg/ml for AF6; and >16 μg/ml for AF72, AF90, and AF91. This test has been compared with several other different in vitro susceptibility tests, and most have confirmed much higher MICs for AF72, AF90, and AF91 compared with those for susceptible isolates such as AF210, AF41, and H06-03 (data not shown).

The animal model data in the four experiments and others (data not shown) confirm the in vitro findings of resistance. Experiments with AF72 and AF90 have been conducted at least twice, and the results were reproducible. Concentrations in serum of itraconazole at 25 and 75 mg/kg in these models range from, respectively, 1.0 to 4.1 μg/ml (mean and standard error, 2.71 and 0.87 μg/ml, respectively) and 7.6 to 15.0 (mean and standard error, 11.8 and 2.19 μg/ml, respectively). The upper dose groups mirror those found in patients who usually respond to therapy. With the susceptible isolate (AF210) used, treated animals survived (90 to 100%) whereas the controls

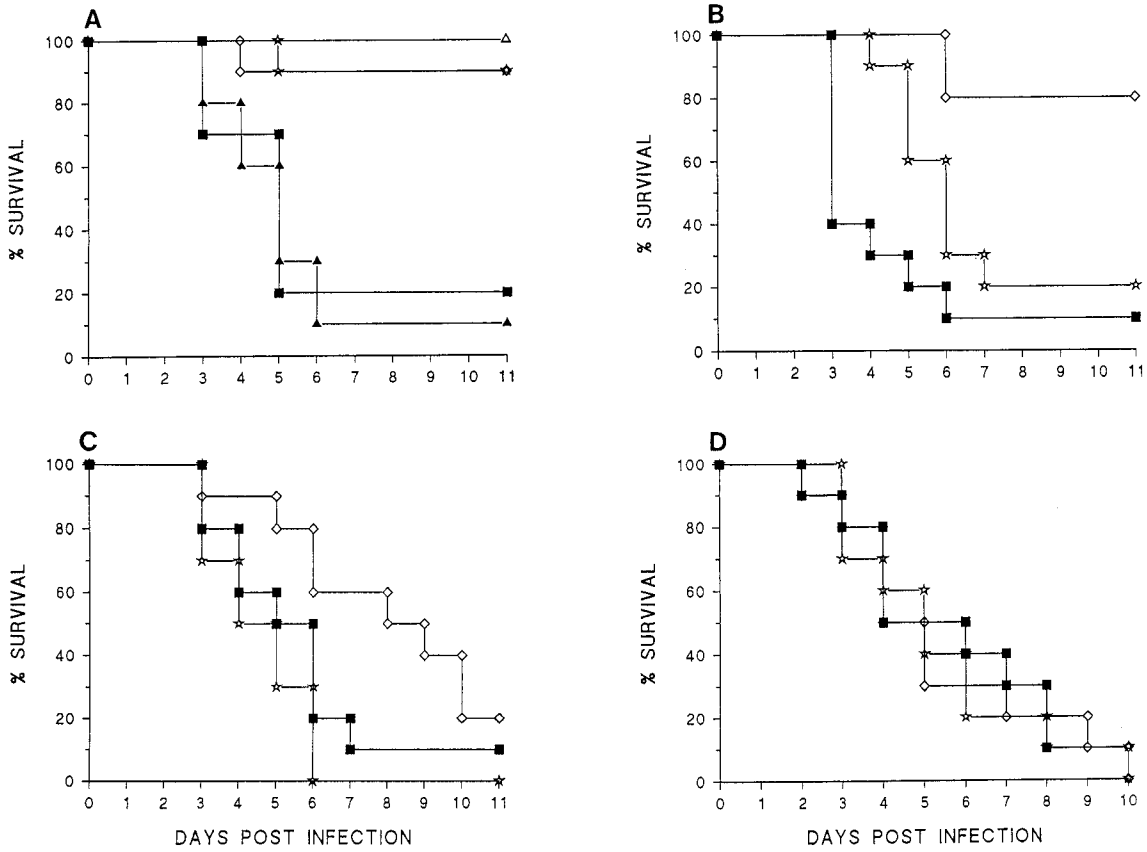

FIG. 1. Survival curves of a murine model with AF210 (a susceptible isolate) (a), AF72 (b), AF90 (c), and AF91 (d). Infection was initiated on day 1, and treatment was initiated after 18 h on day 2. The experiment was terminated on day 11. Treatment groups: ▲, intraperitoneal 5% dextrose control; ■, 5% dextrose gavage control; ⋆, itraconazole (25 mg/kg) by gavage; ◇, itraconazole (75 mg/kg) by gavage; △, amphotericin B (5 mg/kg) intraperitoneally.

succumbed (10 to 20% survival) ($P = 0.001$ to 0.005) (Fig. 1A). Both amphotericin B and itraconazole were efficacious against AF210. With AF72, the control animals had a 90% mortality, compared with an 80% mortality in those treated with itraconazole at 25 mg/kg ($P = 0.01$ to 0.04) (Fig. 1B). The median survival in the controls was 3 days, compared with 6 days in the itraconazole (25 mg/kg)-treated group. However, those treated with the higher dose of itraconazole (75 mg/kg) responded better to treatment (20% mortality; $P = 0.001$ to 0.005). With AF90, the control mice had a 90% mortality, compared with 80% mortality for those treated with 75 mg/kg ($P = 0.07$ to 0.1) and 100% mortality for those treated with 25 mg/kg ($P = 0.07$ to 0.3) (Fig. 1C). There were no discernible differences in mortality (100%) between the control group and those mice treated with itraconazole (25 and 75 mg/kg) and those infected with AF91 ($P = 0.53$) (Fig. 1D).

A. fumigatus AF90 and AF91 were indistinguishable by RAPD with primer R108 and three other primers and Southern hybridization with M13 (1) and were distinct from AF72 (Fig. 2). AF72 was also distinct from AF41, the original isolate causing disease in patient 1. Our findings for these two isolates with RAPD are consistent with prior data generated by restriction endonuclease analysis (7). As AF90 and AF72 were primary isolates from the patients, this finding implies that de novo itraconazole resistance in *A. fumigatus* can be acquired by nonhospitalized patients in the United States.

There were slight differences in the sterol profiles of the two susceptible isolates H06-03 and AF6, with ergosta-5,7-dienol

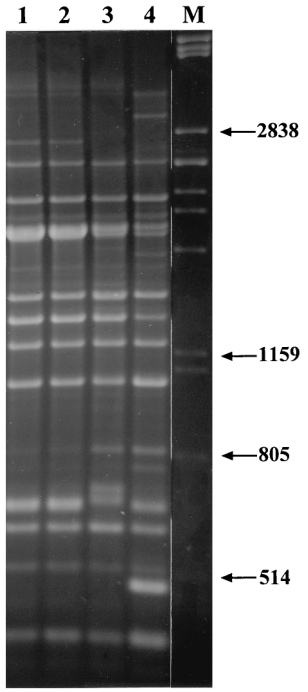

FIG. 2. RAPD typing with primer R108 of clinical isolates from the two patients. The markers (lane M) are lambda DNA digested with *Pst*I, and selected bands are indicated with sizes in base pairs. Lane 1, AF90; lane 2, AF91; lane 3, AF41; lane 4, AF72.

TABLE 1. Relative sterol compositions of 24-h-old mycelia of wild-type (H06-03 and AF6) and itraconazole-resistant (AF72, AF90, and AF91) isolates of *A. fumigatus* grown without itraconazole

Sterol	% of total sterols[a] in:				
	H06-03	AF6	AF72	AF90	AF91
Ergosta-tetraenol	5.6	6.9	6.8	ND	ND
Ergosterol	81.1	66.5	67.2	88.9	87.8
Ergosta-5,7-dienol	ND	4.6	6.3	ND	ND
14α-Methyl fecosterol	4.1	3.6	3.0	ND	ND
4-Methyl-ergosta-8-enol	ND	4.9	3.8	ND	ND
Ergosta-7,22-dienol	3.0	ND	ND	ND	ND
Unidentified	6.2	13.5	12.9	11.1	12.2

[a] ND, not detected.

and 4-methyl-ergosta-8-enol being present in AF6 (Table 1). The pattern of sterols seen in AF6 is very similar to that seen in the resistant isolate AF72. In contrast, both AF90 and AF91 had a higher percentage of ergosterol (Table 1) and no ergosta-tetraenol or 14α-methylfecosterol, which distinguishes them from other isolates.

There was an up to twofold difference in the overall P-450 content of the isolates (Table 2), with no distinct pattern related to susceptibility. AF72 had about 50% of the P-450 content of one of the susceptible isolates (H06-03), but the content of the other susceptible isolate, AF6, fell between the values of the other resistant isolates, AF90 and AF91. However, the relative activity of the three azoles tested at the subcellular level was markedly different in AF90 and AF91. Substantially larger concentrations of itraconazole (4.5- to 6-fold), fluconazole (4.5- to 5.5-fold), and ketoconazole (4- to 6-fold) were required to inhibit the incorporation of labelled mevalonate into desmethylated sterols compared with the two susceptible isolates and with AF72.

The intracellular concentrations of [^{3}H]itraconazole in isolates H06-03, AF90, and AF91 ranged from 316.8 to 523.2 pmol/100 mg (dry weight) (Table 3). These data are consistent with an about twofold variation in intracellular concentrations of itraconazole in susceptible isolates. In contrast, the intracellular concentration of [^{3}H]itraconazole in AF72 was reduced about 7.5-fold to 82.8 pmol/100 mg (dry weight).

Thus, resistant isolate AF72 had low intracellular concentrations of itraconazole accounting for resistance, whereas resistant isolates AF90 and AF91 required much more itraconazole (and ketoconazole and fluconazole) to inhibit the ergosterol synthetic pathway.

TABLE 2. Specific contents of cytochrome P-450 in microsomal function and antifungal IC_{50}s for incorporation of [2-^{14}C]mevalonate into C14,4-desmethylated sterols in cell-free bioassays of wild-type (H06-03 and AF6) and itraconazole-resistant (AF72, AF90, and AF91) isolates of *A. fumigatus*

Isolate	P-450 content (pmol/mg of protein [mean ± SD])	IC_{50} (nM [mean ± SD]) of:		
		Itraconazole	Fluconazole	Ketoconazole
H06-03	18.9 ± 3.8	52.5 ± 7.5	1,500 ± 300	74.6 ± 6.3
AF6	14.0 ± 3.2	38.3 ± 14.3	1,240 ± 250	59.2 ± 4.5
AF72	9.3 ± 0.8	36.3 ± 14.3	1,170 ± 330	52.8 ± 7.8
AF90	13.5 ± 1.7	243.4 ± 21.6	6,879 ± 467	324.5 ± 14.8
AF91	15.4 ± 2.9	296.7 ± 18.5	8,321 ± 358	412.8 ± 12.4

TABLE 3. Intracellular concentrations of [^{3}H]itraconazole in wild-type (H06-03 and AF6) and itraconazole-resistant (AF72, AF90, and AF91) isolates of *A. fumigatus*

Isolate	Mean intracellular [^{3}H]itraconazole concn ± SD (pmol/100 mg [dry wt])
H06-03	523.2 ± 20.4
AF6	316.8 ± 25.2
AF72	82.8 ± 25.2
AF90	470.4 ± 30.0
AF91	440.6 ± 22.8

DISCUSSION

Very little work has been published concerning azole resistance in pathogenic *Aspergillus* species. We have previously reviewed the methodology for determining in vitro susceptibility in *Aspergillus* (10). Many methods, most of which are in accord with the relative activities of different agents in vivo, have been used. It is likely that a small number of itraconazole-resistant isolates have previously been isolated among 221 tested (10–12, 28). However, no systematic attempt to correlate either experimental in vivo or clinical outcome with in vitro susceptibility has been undertaken, and therefore, the veracity of in vitro testing has been in doubt. Breakpoints for resistance have not been determined. However, given the ≥32- to 64-fold increase in MICs for our three resistant isolates compared with those for the susceptible wild-type isolates and confirmation in a reproducible animal model, we are confident that the three isolates that we have studied here are genuinely resistant.

Patient 1 was infected with a susceptible isolate and responded to therapy. While on therapy her sputum grew AF72, a resistant isolate almost certainly acquired by inhalation in or around her home in California. Patient 2 developed invasive aspergillosis in the context of AIDS due to the primarily resistant isolate AF90 (and AF91). He had a partial clinical response to therapy but no radiographic improvement. His serum itraconazole concentration (11.0 mg/liter) was substantially higher than in most patients and presages an excellent response to therapy which was not observed. Thus, the clinical data are in accord with the in vitro, murine model, and biochemical data.

Azole antifungal resistance has been studied in several other species of fungi, and a number of recurring themes emerge. These include changes in cellular content of azole (altered uptake or efflux mechanisms), mutations in sterol $\Delta^{5,6}$ desaturation during ergosterol biosynthesis, and mutations in or elevated levels of sterol 14α-demethylase. Perhaps the most frequent finding to date has been low intracellular fluconazole concentrations in *Candida albicans*, *Candida glabrata*, and *Candida krusei* (13, 15, 24, 27, 29, 31). This appears to be the result of an energy-dependent efflux mechanism (24, 27). We have found reduced intracellular concentrations in one of our isolates (AF72) which may be mediated by a related efflux pump. A similar efflux mechanism has been observed for fenarimol resistance in *Aspergillus nidulans* (13).

Ergosterol is a major and essential component of the cell membrane in most fungi. The minor changes in sterol pattern seen in our isolates rule out a $\Delta^{5,6}$ desaturase mutation being responsible for resistance, even though this has been shown to be a mechanism of resistance in *Saccharomyces cerevisiae* (19), *Ustilago maydis* (18), and *C. albicans*. The absence of intermediate sterols in AF90 and AF91 is entirely consistent with

resistance mediated by either increased expression of the sterol 14α-demethylase or altered enzyme affinity for azoles.

There is also some evidence for the overexpression of cytochrome P-450 mediating sterol 14α-demethylase in azole resistance. This was postulated as the major mechanism underlying resistance in a pair of isolates of *C. glabrata* (30). However, overexpression of enzyme from <3 to 100 pmol/mg of microsomal protein in *S. cerevisiae* transformants expressing *C. albicans* 14α-demethylase alters the MIC only fivefold (19). Thus, data on resistant mutants do not currently provide strong evidence for overexpression of sterol 14α-demethylase as a mechanism of resistance, and it was not the mechanism of resistance in our isolates.

Alteration of the target enzyme, sterol 14α-demethylase, has been shown to be a mechanism for resistance for three azole-resistant mutants of *U. maydis* generated recently (17). These mutants had unaltered azole and P-450-specific contents but exhibited ergosterol biosynthesis which was resistant to azole treatment in vivo and in vitro. In our isolates AF90 and AF91, this appears to be the primary mode of resistance.

Clearly further work on the molecular mechanisms of resistance is called for. For AF90 and AF91 the focus will be on changes in the target enzyme, sterol 14α-demethylase, while for AF72 an alteration in a membrane transporter may be involved, as has been observed in *C. albicans* (4, 25, 27). However, our work to date establishes that azole-resistant isolates of *Aspergillus* occur and may cause disease and that at least two mechanisms of resistance are responsible.

ACKNOWLEDGMENTS

We are indebted to the expert technical assistance from Linda Hall, Graham Morrissey, and Caroline Moore.

K. Oakley was funded from grants from the British Society for Antimicrobial Chemotherapy and the Fungal Research Trust. K. Venkateswarlu was the recipient of a Commonwealth Scholarship. David A. Stevens was supported by the NIAID Mycoses Study Group.

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