Establishing In Vitro-In Vivo Correlations for Aspergillus fumigatus: the Challenge of Azoles versus Echinocandins

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Two clinical isolates of Aspergillus fumigatus, designated AT and DK, were recently obtained from patients failing caspofungin and itraconazole therapy, respectively. The isolates were tested by microdilution for susceptibility to itraconazole, voriconazole, posaconazole, ravuconazole, and caspofungin and by Etest for susceptibility to amphotericin B and caspofungin. Susceptibility testing documented that the DK isolate was azole resistant (itraconazole and posaconazole MICs, >4 μg/ml; voriconazole MIC, 2 μg/ml; ravuconazole MIC, 4 μg/ml), and the resistance was confirmed in a hematogenous mouse model, with mortality and the galactomannan index as the primary and secondary end points. Sequencing of the cp51A gene revealed the M220K mutation, conferring multiazole resistance. The Etest, but not microdilution, suggested that the AT isolate was resistant to caspofungin (MIC, >32 μg/ml). In the animal model, this isolate showed reduced susceptibility to caspofungin. Sequencing of the FKS1 gene revealed no mutations; the enzyme retained full sensitivity in vitro; and investigation of the polysaccharide composition showed that the β-(1,3)-glucan proportion was unchanged. However, gene expression profiling by Northern blotting and real-time PCR demonstrated that the FKS gene was expressed at a higher level in the AT isolate than in the susceptible control isolate. To our knowledge, this is the first report to document the presence of multiazole-resistant clinical isolates in Denmark and to demonstrate reduced susceptibility to caspofungin in a clinical A. fumigatus isolate with increased expression of the FKS gene. Further research to determine the prevalence of resistance in A. fumigatus worldwide, and to develop easier and reliable tools for the identification of such isolates in routine laboratories, is warranted.

The susceptibility testing of conidium-forming molds has recently been standardized (27, 36), but no breakpoints have yet been established, and susceptibility testing of molds is generally not performed in routine clinical microbiology laboratories. While the end point readings are straightforward for azoles and amphotericin B due to the growth-versus-no-growth pattern of inhibition, the end points for the echinocandins are more difficult to determine due to significant trailing growth. Recently, a number of reports have indicated that azole resistance is emerging in clinical Aspergillus isolates (7, 42, 44, 47). Azoles act by blocking the ergosterol (an essential cell membrane component) biosynthetic pathway through binding to and inhibition of the lanosterol 14α-demethylase enzyme, encoded by the erg11 (cp51A) gene. Azole resistance may be restricted to itraconazole or may involve cross-resistance to other triazoles as well and has been associated with a number of hot spots in the cp51A gene with or without a simultaneous tandem repeat in the cp51A promoter region (15, 25, 42). Resistance to caspofungin has been described for clinical Candida isolates, and recently, breakthrough infections with Aspergillus fumigatus isolates with elevated minimum effective concentrations (MECs) have been reported (18, 19). Finally, manipulated or laboratory-selected strains with various degrees of caspofungin resistance have been described (12, 31, 38). Some of these laboratory-manipulated strains have been found to have mutations in the ECM33 gene (afuEcm33), encoding cell wall proteins important for fungal cell wall organization, and in addition to possess caspofungin resistance to be hypervirulent in animal models (38). Others have mutations in the FKS1 gene, encoding a subunit of the β-1,3-α-glucan synthase enzyme involved in cell wall synthesis; this mechanism has been detected in clinical Candida isolates with reduced susceptibility to caspofungin (12). In still other resistant mutants, the glucan synthase enzyme itself exhibited a wild-type gene sequence, function, level, and caspofungin susceptibility (12).

Here we report the detection and confirmation in an animal model of the first Danish multiazole-resistant A. fumigatus isolate obtained from a patient on itraconazole treatment and the detection of an A. fumigatus isolate displaying decreased caspofungin susceptibility by the Etest, obtained from an Austrian patient failing caspofungin treatment. Our findings indicate a growing need for routine susceptibility testing of clinical A. fumigatus isolates in order to guide treatment and the need for surveillance of the susceptibility epidemiology of Aspergillus isolates in order to monitor changes in the susceptibility patterns of Aspergillus in general.

MATERIALS AND METHODS

Isolates. The “A. fumigatus DK” isolate was recovered from a 21-year-old cystic fibrosis patient on long-term itraconazole treatment (200 mg twice daily for 14 months). This isolate was multiazole resistant (itraconazole MIC, >4 μg/ml;...
posaconazole MIC, >4 µg/ml; voriconazole MIC, 2 µg/ml; ravuconazole MIC, 4 µg/ml) but susceptible to amphotericin B (MIC, 0.5 µg/ml), and caspofungin (MIC, 0.125 µg/ml). The other isolate, the “A. fumigatus AT1” isolate, was recovered from a patient with invasive pulmonary aspergillosis who failed caspofungin monotherapy. This isolate was susceptible to azoles and amphotericin B (MICs, 0.25 µg/ml for itraconazole, 0.06 µg/ml for posaconazole, 0.125 µg/ml for voriconazole, and 0.5 µg/ml for amphotericin B) but resistant by the Etest to caspofungin (MIC, >32 µg/ml).

Susceptibility testing. Susceptibility was determined independently (i) at two laboratories by the EUCAST antifungal MIC method for spore-forming molds using Candida krusei ATCC 6258 as a control and (ii) at one laboratory using a modified EUCAST method with a slightly lower inoculum of 5 × 10^5 cells/ml (36) and (iii) susceptibility tested to caspofungin by the CLSI (formerly NCCLS) method, using Aspergillus flavus ATCC 29340 and Aspergillus fumigatus ATCC 29340 as susceptibility testing procedure controls and A. fumigatus EMFR-6786P as an echinocandin-resistant control (27). Microtiter plates were inoculated with a spore suspension, incubated at 35°C for 24 to 48 h, and read visually and with a spectrophotometer. The azole MIC was defined as the lowest drug dilution yielding no growth (single colonies on the surface are ignored), and the MEC of echinocandin was defined as the lowest drug concentration resulting in aberrant growth. Finally, susceptibilities to amphotericin B and caspofungin were determined by the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer’s recommendations.

DNA sequencing. The entire coding region of the cyp51A gene from the A. fumigatus DK isolate was amplified and both strands were sequenced as previously described (15). Consensus sequences were aligned and mismatches identified using MegAlign (DNASTAR, Madison, Wis.). Two housekeeping genes were used because differences in expression were observed at different time points in A. fumigatus (unpublished data). No significant differences were observed when these two normalizing genes were used (dabcyl) at the 3′ ends. 5′-inositol as the high-dose posaconazole given orally (50 mg/kg), or glucose given i.p. (control group). Colony diameters were measured in triplicate every 24 h for 96 h. The average diameter was used to determine the radial growth rate (Kr). Kr was calculated using the linear regression of the radius versus time by a method described previously (34).

Aspergillus SUSCEPTIBILITY TO CASPOFUNGIN AND AZOLES 3505

Vol. 52, 2008

Aspergillus fumigatus conidia (10^7 per petri dish) were inoculated into the centers of 90-mm-diameter minimal medium agar and YPD (1% yeast extract, 2% Bacto peptone, 2% dextrose) petri dishes. Colony diameters were measured in triplicate every 24 h for 96 h. The average diameter was used to determine the radial growth rate (Kr). Kr was calculated using the linear regression of the radius versus time by a method described previously (34).

Animal model. A total of 138 NMRI mice (weight, 26 to 30 g; Harlan Scandina, Allerød, Denmark) were injected intraperitoneally (i.p.) with 200 mg of cyclophosphamide/kg of body weight on day −3 and 100 mg/kg on day zero to produce prolonged immunosuppression. Mice were kept five to seven to a cage and were allowed free access to food and water. Mice in groups of 10 to 13 were challenged on day zero by intravenous injection of an inoculum of 1.5 × 10^7 CFU of A. fumigatus in 200 µl with a 25-gauge syringe. Forty-nine mice received the A. fumigatus DK isolate, and 49 and 40 mice received the A. fumigatus AT isolate in two separate experiments. Mice were treated on days 1 to 4 (Monday to Friday) and 7 to 10 (Monday to Thursday) with 0.25 ml of either caspofungin administered i.p. (0.225 mg/kg), low-dose posaconazole given orally (5 mg/kg), high-dose posaconazole given orally (20 mg/kg), or glucanase given i.p. (control mice) (29, 40, 46, 48). Mice were observed daily and evaluated by assigning one of the following scores, from 0 to 4: 0, healthy; 1, minor clinical signs of infection and inflammation (e.g., observations of minor signs of distress and pain, changed activity, and social withdrawal); 2, severe signs of infection, such as stiff movements, lack of curiosity, forced ventilation, changed body position, piloerection on the skin, or changes in the pattern of movement (animals scored 2 were reevaluated later the same day); 3, severe suffering and pain (the mouse was obviously out of energy, placed in a 45° angle position and died). Susceptible mice were sacrificed on day 11 after a total bleed. The experiments were approved by the Danish Animal Experimentation Committee under the Ministry of Justice (number 2004/561–835).

Galactomannan (GM) antigen detection was performed on serum samples downloaded from http://aac.asm.org on May 7, 2021 by guest
obtained on day 11 from surviving mice by use of the commercially available Platelia Aspergillus kit for immunoenzymatic detection of GM antigen (Bio-Rad, France).

**Statistics.** Survival was compared by a Mantel-Cox log rank test with a P value of \( <0.05 \) as the level of significance. GM levels were compared among the various treatment groups by using the Mann-Whitney test.

**RESULTS**

Testing of the susceptibility of the *A. fumigatus* DK isolate showed it to be azole resistant (Fig. 1a) but caspofungin susceptible, with a microdilution MEC of 0.25 \( \mu g/ml \) and an Etest end point of 0.125 \( \mu g/ml \) (Fig. 2a). The isolate was lethal in the mouse model at inoculum sizes described previously, with a mortality rate of 93% on day 5 and 100% mortality on day 9 (Fig. 3). The survival of mice was 100% in the caspofungin group, in contrast to 16.7 and 25% survival in the two posaconazole-treated groups, respectively (\( P <0.001 \) and \( P =0.0002 \) for comparisons of caspofungin-treated animals with animals receiving low- and high-dose posaconazole, respectively). The survival of treated animals receiving caspofungin or high-dose posaconazole was significantly better than that of control animals (\( P <0.0001 \) and \( P =0.005 \), respectively). *Aspergillus* GM index values in the sera of mice surviving on day 11 were determined (Fig. 4). All surviving animals challenged with the *A. fumigatus* DK isolate were positive for GM antigen, and no statistical difference was found between the index levels for caspofungin-treated and posaconazole-treated animals (\( P =0.0887 \)). In order to characterize the mechanism of resistance, the entire coding region of the cyp51A gene was amplified, and both strands were sequenced; revealing an M220K mutation (an alteration from methionine to lysine at codon 220).

The Etest suggested that the *A. fumigatus* AT isolate was caspofungin resistant (Fig. 2b). The microdilution assay, however, showed partial growth inhibition of both the AT and DK isolates by caspofungin, and attempts to determine MEC end points by evaluation of micromorphology did not demonstrate differences in susceptibility to caspofungin; both isolates showed aberrant growth at concentrations of 0.25 \( \mu g/ml \) or higher (Fig. 5). In vivo susceptibility tests in the mouse model were performed twice for the *A. fumigatus* AT isolate, because initially the animals in the caspofungin group accidentally received one dose of posaconazole on day 3 instead of caspofungin, which might have improved their outcome. In both experiments, the survival of animals receiving caspofungin was low (25 to 40%) compared to that of animals receiving posaconazole (70 to 83% survival in the low-dose groups and 75 to 100% survival in the high-dose groups [Fig. 3]). This lower survival for caspofungin-treated animals was statistically significant compared to the survival rates for both posaconazole groups in the first experiment (\( P \) values, 0.097 and 0.0115, respectively) and compared to that of the high-dose posaconazole group in the second experiment (\( P =0.0039 \)). However, for all treatment groups, survival was better than that for the control groups (\( P =0.001 \) to \( <0.0001 \)). Animals treated with posaconazole had a significant dose-dependent decrease in the GM indices relative to those of control and caspofungin-treated animals (Fig. 4).

Sequencing of the FKS1 gene of the *A. fumigatus* AT isolate did not reveal any mutations. Susceptibility profiling of the glucan synthase showed full susceptibility (data not shown), and when the carbohydrate composition was investigated, the
total hexose content was decreased in both the AT isolate and the control isolate upon caspofungin pretreatment, but the proportion of β-1,3-α-glucan was unchanged (Table 1). However, Northern blot analysis demonstrated expression of the FKS gene in susceptible and resistant A. fumigatus isolates, yet with marked differences in the expression level, as shown in Fig. 6. Caspofungin treatment did not reduce FKS gene expression in the A. fumigatus AT isolate, in contrast to the reduction in the susceptible control isolate (Fig. 6). This overexpression of the FKS gene was confirmed by real-time PCR. Table 2 compares the FKS1 expression ratios of the A. fumigatus AT isolate and two wild-type strains. The AT isolate showed constitutively higher FKS1 expression (3.09-fold on average) than the wild-type strains. In the presence of a low concentration of caspofungin, the AT isolate showed higher expression ratios than the wild-type strains (3.15-fold on average). These experiments were also performed at higher caspofungin concentrations. When ≥0.5 μg/ml of caspofungin was used, no A. fumigatus total RNA was obtained for the AT isolate, ATCC 13073, or A. fumigatus 293. However, RNA of strain EMFR-S678P (35) was obtained at high caspofungin concentrations (>4 μg/ml).

Finally, we observed a higher growth rate for the AT isolate. In order to evaluate these preliminary observations objectively,
the radial growth rate was determined. Two different variables were measured: diameter and Kr. The latter variable is independent of time and is related to the specific growth rate when the culture conditions are constant (34). Thus, Kr variations represent metabolic differences between different strains more accurately than diameter. Figure 7 shows the results of measurement of these two variables for 4 days. All the strains reached the exponential, stationary, and death phases at the same times (24 to 48 h, 48 to 72 h, and 72 to 96 h, respectively) independently of the solid medium used. The AT isolate showed a larger colony diameter and a higher Kr but the same slope as the wild type and the caspofungin-resistant FKS1 mutant (Fig. 7). These results may suggest a higher metabolic rate for the AT isolate with no alteration in the cell cycle (higher Kr and no differences in growth curves).

DISCUSSION

A decade ago, amphotericin B formulations and itraconazole were the only options for antifungal treatment of invasive aspergillosis, but since then, voriconazole has become the first choice for primary therapy due to its superior efficacy in comparison with conventional treatment, and caspofungin has been licensed for salvage therapy (13, 21, 45). With the increasing rates of invasive aspergillosis and candidiasis, the use of expanded-spectrum azoles and echinocandins has increased, and accordingly the potential selection pressure has also increased. Azole resistance, including voriconazole and/or posaconazole resistance, has been reported for clinical *Aspergillus* isolates in the United Kingdom, Spain, the United States, and, with increasing frequency, The Netherlands (3, 15, 24, 25, 44). Recently, breakthrough infections with *A. fumigatus* isolates, with MECs in the range of 0.125 to 8 μg/ml, in patients receiving caspofungin prophylaxis/empirical treatment have been reported (19). Finally, slow-sporulating *A. fumigatus* isolates with multi-drug class resistance have been isolated from patients who received prior fluconazole treatment (3).

The DK isolate described here was resistant to posaconazole...
TABLE 1. Polysaccharide composition of the AT isolate with and without caspofungin pretreatment in comparison with the control

<table>
<thead>
<tr>
<th>Isolate and pretreatment</th>
<th>Caspofungin susceptibility</th>
<th>Dry wt (mg) of mycelium</th>
<th>Wt (% of AI)</th>
<th>% of dry wt (mg) of AI that is AI</th>
<th>% of the AI wt (mg) that is hexosesa</th>
<th>% of total amt of hexoses in the AI that isb</th>
<th>% of total amt of hexoses that is a β-(1,3)-glucanc</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>Reduced</td>
<td>30</td>
<td>4.7</td>
<td>15.7</td>
<td>6.5</td>
<td>10</td>
<td>74</td>
</tr>
<tr>
<td>Cas</td>
<td></td>
<td>60</td>
<td>12.5</td>
<td>20.8</td>
<td></td>
<td>7</td>
<td>86</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>142</td>
<td>22.6</td>
<td>15.9</td>
<td>12</td>
<td>7</td>
<td>86</td>
</tr>
<tr>
<td>Control</td>
<td>Normal</td>
<td>15</td>
<td>1.5</td>
<td>10.0</td>
<td>6</td>
<td>10</td>
<td>74</td>
</tr>
<tr>
<td>Cas</td>
<td></td>
<td>50</td>
<td>8.4</td>
<td>16.8</td>
<td></td>
<td>10</td>
<td>74</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>108</td>
<td>20.6</td>
<td>19.1</td>
<td>12</td>
<td>5</td>
<td>91</td>
</tr>
</tbody>
</table>

a AI, alkali-insoluble fraction.

b Data are averages for two samples for the caspofungin-pretreated isolates.

c Cas, caspofungin pretreatment by culturing in the presence of 0.04 μg/ml caspofungin.

in vivo, in an animal model, with mortality and the GM index at the end of therapy as end points. Posaconazole was chosen as the compound because it is metabolized less rapidly in mice than voriconazole (28, 37, 41). The clinical context was prior itraconazole treatment for 14 months. However, infections in azole-naïve patients with resistant strains have been described elsewhere, suggesting the possible presence of azole-resistant isolates in the environment (44). Azoles act by inhibiting lanosterol 14α-demethylase (Cyp51), an enzyme in the ergosterol biosynthetic pathway, resulting in fungal cell instability. The most commonly reported mechanism of azole resistance in Aspergillus is single nucleotide repeats in the *cyp51A* gene (5, 6, 8, 22, 24, 26). These substituted amino acids may alter drug binding, thus conferring resistance. In the present case, an M220K mutation was detected in the *cyp51A* gene. Codon 220 is a well-characterized hot spot, for which a replacement of methionine with isoleucine, associated with itraconazole resistance and reduced susceptibility to other azoles, is most commonly reported (5, 6, 24). The effect on cross-resistance is dependent on the specific amino acid substitution. In the present case, the mutation involved the replacement of methionine with lysine, and the isolate also showed cross-resistance to voriconazole, posaconazole, and ravuconazole, in agreement with the findings for a similar clinical isolate from the United Kingdom (14, 24). Despite successful outcomes for the caspofungin-treated mice, the GM levels were not significantly decreased. Several previous studies have reported correlations between GM levels and outcome during caspofungin treatment (20, 43). However, a paradoxical increase in the GM index or in quantitative PCR results despite a histopathological effect and/or clinical improvement has been observed in a number of other studies of animal models and of humans (16, 32, 39, 48). In vitro, higher doses of caspofungin but not voriconazole have been shown to be associated with an increased release of GM into the culture medium (16), and one may speculate that the primarily static inhibition exhibited by caspofungin in comparison to the more fungicidal anti-Aspergillus activity of azoles may contribute to the prolonged circulation of GM despite clinical success.

The results regarding the caspofungin susceptibility of the *A. fumigatus* AT isolate are less clear. The Etest and animal model results suggested reduced susceptibility, but in our hands the microdilution MEC determination did not identify this isolate as unusual. The studies of the possible mechanism showed that changes at the enzyme level were not relevant, because there were no *FKS1* mutations and the semipurified enzyme retained full susceptibility in vitro. The isolate was characterized by rapid growth, and gene expression profiling upon exposure to subinhibitory concentrations of caspofungin demonstrated overexpression of the *FKS* gene, suggesting that...
this played a role. Thus, even though the exact mechanism has not yet been determined, it is likely that a conditional resistance mechanism is operational. The clinical context was failure despite caspofungin treatment. However, other factors may have contributed to the poor outcome, and our animal experiments cannot predict if human infection may be treated with or without elevated doses of caspofungin. The EUCAST and CLSI microdilution MEC tests did not categorize this isolate as unusual, and recently, Candida albicans isolates with mutations in the hot spots and markedly elevated Etest MICs were shown to have only slightly elevated caspofungin MECs by microdilution (reference 2 and personal observation). These findings indicate that further studies are needed to define the best methodological parameters for in vitro testing of susceptibility to caspofungin and to determine the true rate of reduced susceptibility in Candida and Aspergillus.

In summary, this is, to our knowledge, the first report to describe and confirm in an animal model a clinical A. fumigatus isolate from Denmark with multiazole resistance and a clinical isolate for which the Etest endpoint and in vivo susceptibility testing in an animal model suggest decreased susceptibility to caspofungin, possibly due to upregulation of the target enzyme level. Our detection of these isolates illustrates the necessity to be aware of reduced susceptibility among clinical A. fumigatus isolates and the need for monitoring susceptibility not only for epidemiological purposes but also for the guidance of clinical treatment.

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