Evaluation of in vitro activity of voriconazole as predictive of in vivo outcome in a murine Aspergillus fumigatus infection model

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ABSTRACT
We have evaluated the in vitro activity of voriconazole against 61 strains of Aspergillus fumigatus by using broth microdilution, disk diffusion, and minimal fungicidal concentration procedures. We observed an excellent correlation between the results obtained with the three methods. Five percent of the strains showed MICs ≥ epidemiological cut-off value (ECV = 1 µg/ml). To assess if MICs were predictive of in vivo outcome, we tested the efficacy of voriconazole at 25 mg/kg daily in an immunosuppressed murine model of disseminated infection using ten strains representing various susceptibility patterns to the drug as determined by the in vitro study. Voriconazole prolonged survival and reduced fungal load in the kidneys and brain in those mice infected with strains with MICs ≤ 0.25 µg/ml, while in that with MICs of 0.5 - 2 µg/ml, the efficacy was variable and strain dependent, and in mice infected with the strain with MIC of 4 µg/ml the antifungal did not show efficacy Voriconazole reduced galactomannan antigenemia against practically all strains with an MIC < 4 µg/ml. Our results demonstrate that some relationship exists between voriconazole MICs and in vivo efficacy; however, further studies testing additional strains are needed to better ascertain which MIC values can predict clinical outcome.
Introduction

Invasive aspergillosis is an important cause of morbidity and mortality in the immunocompromised host, Aspergillus fumigatus being the leading cause of invasive aspergillosis worldwide (26). At present, voriconazole is the first choice in the treatment of such infections (32) and although A. fumigatus is generally susceptible to voriconazole, several studies have demonstrated an increasing number of azole-resistant isolates (14, 21, 31). This represents an important problem in the clinical management of invasive aspergillosis because therapeutic options are limited.

The development of clinical breakpoints of the most usual antifungal drugs might be useful for predicting the outcomes of fungal infections. However, the available antifungal susceptibility data are only based on in vitro and animal studies (13). A recent important step has been the proposal of epidemiological cut-off values (ECV) for voriconazole against several Aspergillus spp., including A. fumigatus (ECV = 1 μg/ml), and theoretically those isolates showing MICs higher than ECV will show resistance (9, 23).

We have evaluated the efficacy of voriconazole at 25 mg/kg (30) in a murine model of disseminated infection by A. fumigatus testing isolates with different MICs, in order to ascertain the role of the in vitro data as a predictor of infection outcome.

Materials and methods

Sixty one clinical strains of Aspergillus fumigatus were tested in the in vitro studies. Their susceptibility to voriconazole was evaluated using a broth microdilution method, carried out according to the CLSI guidelines for filamentous fungi (6) and a disk diffusion method that uses non-supplemented
Mueller-Hinton agar and 6 mm diameter paper disks containing 1 μg of voriconazole (8). The strain A. fumigatus ATCC MYA-3626 was used as quality control. The MICs (µg/ml) and IZDs (mm) were read at 48 and 24 h, respectively. Suggested ECVs of voriconazole for A. fumigatus are 1 µg/ml and ≥ 17 mm for microdilution and disk diffusion methods, respectively (8,9).

The minimal fungicidal concentration (MFC) was determined by subculturing 20 μl of each well that showed complete inhibition or an optically clear well relative to the last positive well and the growth control onto potato dextrose agar (PDA) plates. The plates were incubated at 35ºC until growth was observed in the control subculture. The MFC was the lowest drug concentration at which approximately 99.9% of the original inoculum was killed (11).

For in vivo studies, ten isolates with different in vitro susceptibilities were chosen (Table 1). Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain) weighing 30 g were used. Animals were housed under standard conditions. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Animals were immunosuppressed 1 day prior to infection by administering a single intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genoxal; Laboratories Funk S.A., Barcelona, Spain), plus a single intravenous (i.v.) injection of 150 mg/kg of body weight of 5-fluorouracil (Fluorouracilo; Ferrer Farma S.A., Barcelona, Spain). Previous studies with this immunosuppressive regimen have been demonstrated that the peripheral blood polymorphonuclear leukocyte (PMN) counts were < 100/µl from day 3 to 9 or later (20). Mice were challenged with $2 \times 10^3$ CFU in 0.2 ml of sterile saline,
injected via the lateral tail vein. Preliminary experiments demonstrated that this inoculum was appropriate for producing an acute infection, with 100% of the animals dying within 11 days (data not shown).

Voriconazole (Vfend; Pfizer S.A., Madrid, Spain) was administered at 25 mg/kg of body weight/dose once a day (QD) orally by gavage. We selected that dose based on a previous pharmacokinetic study in which this dose resulted in serum therapeutic concentrations against *Aspergillus flavus* in mice (33). On the other hand, although the authors did not measure serum concentration of voriconazole, lower doses such as 10 mg/kg were less effective in prolonging survival and reducing fungal load. In agreement with Warn et al. (33) we have obtained voriconazole serum concentrations higher than the corresponding MICs for all strains tested. In addition, this dosage does not exceed any doses usually used in similar mice experiments. Previous *in vivo* studies testing other fungi have used considerably higher doses of voriconazole such as 40, 60 and 80 mg/kg/day with good efficacy (18, 19, 24).

Due the rapid clearance of voriconazole observed in mice, from 3 days before infection, the animals that received this drug were given grapefruit juice instead of water on the basis of previous studies that demonstrated that grapefruit increases the voriconazole concentration in murine serum (12, 29). To prevent bacterial infection, all animals received ceftazidime at 5 mg/kg subcutaneously once daily. The efficacy of voriconazole was evaluated as prolonging survival of mice in comparison to controls, tissue burden reduction, and by determination of galactomannan serum levels by enzyme immunoassay. Treatments began one day after infection, and lasted for 7
days. For survival studies, groups of 8 mice were randomly established for each strain and each treatment and checked daily for 30 days after challenge. Controls received no treatment. For tissue burden studies, groups of 8 mice were also established and the animals were sacrificed on day 5 post-infection in order to compare the results with controls. Kidneys and brain were aseptically removed, weighed and homogenized in 1 ml of sterile saline. In previous murine studies these were the main target organs (6, 17). Serial 10-fold dilutions of the homogenates were plated on PDA and incubated 48 h at 35°C. The numbers of CFU/g of tissue were calculated. Additionally, before being sacrificed, approximately 1 ml of blood from each mouse belonging to the tissue burden groups was extracted by cardiac puncture. Pooled serum samples from mice of each treatment group were used to determine the drug concentration in serum by bioassay 4 h after drug administration (19, 24) and the galactomannan levels by enzyme immunoassay (Platelia Aspergillus®, Bio Rad, Marmes, la Coquette, France) as a marker of the treatment response (24). Values were expressed as a galactomannan index (GMI) defined as the optical density of a sample divided by the optical density of a threshold serum provided in the test kit.

For survival studies, the Kaplan-Meier method and log rank test were used. Differences were considered statistically significant at p < 0.05. When multiple comparisons were carried out, T-test with Welch’s correction and the Bonferroni correction was used to avoid an increase in type I error. The tissue burden studies were analyzed using the Mann-Whitney U test. The Kolmogorov-Smirnov test was carried out to determine the normal distribution
of galactomannan serum levels, and bioassay data, so that they could be analyzed using the T-test.

Results

The voriconazole MICs of the 61 A. fumigatus strains tested were in the range from 0.12 to 4 μg/ml with a modal MIC of 0.5 μg/ml and MIC50 and MIC90 values of 0.5 and 1μg/ml, respectively (data not shown). The IZD range was 0 – 34 mm (geometric mean = 26.85 mm, standard deviation ± 4.1 mm). The MFC ranged from 0.25 to 8 μg/ml (geometric mean = 0.77 μg/ml). Following the suggested ECV against Aspergillus fumigatus (9), most of the strains tested showed voriconazole MICs ≤ and ≥ IZDs ECV, i.e. 95 and 93 %, respectively. In general, a high correlation between the results obtained with the microdilution and the disk diffusion methods was observed. The differences between MICs and MFCs were never > 2 dilutions (data not shown).

Voriconazole significantly prolonged survival and reduced fungal load in kidneys and brain with respect to the control group in those animals infected with isolates having MICs ≤ 0.25 μg/ml. In mice challenged with strains with MICs from 0.5 to 2 μg/ml the results were variable, and in those infected with the only strain with MIC = 4 μg/ml the drug did not show efficacy. (Table 1, Figures 1 and 2). Significant differences on survival were observed when results obtained with strains with MICs < 0.5 μg/ml were compared with those for strains with MICs from 0.5 to 4 μg/ml (p < 0.02).

At day 5 of the experiment, the serum concentration of voriconazole was 8.09 ± 3.05 μg/ml. Galactomannan concentration in serum at day 5 of the experiment was significantly lower (p <0.05) in animals treated with
voriconazole than in control animals, with the exception of mice challenged
with one strain for which the MIC was 0.5 µg/ml and those infected with the
strain with voriconazole MIC of 4 µg/ml (data not shown).

Discussion

In this study, we have selected several *A. fumigatus* strains with different *in
vitro* susceptibilities to voriconazole in order to assess if there is any
relationship between the *in vitro* activity and its *in vivo* efficacy. Only 5% of the
61 isolates tested showed MICs > ECV. In previous similar studies the values
were between 1.4 - 4.1% (9, 22, 30). We observed an excellent correlation
between the *in vitro* results obtained with microdilution, disk diffusion and
MFC. In agreement with other authors, our *in vitro* results suggest that the
disk diffusion method could be a good option for determining voriconazole
susceptibility (10, 25), due to its simplicity, low cost and high reproducibility.
Our MFC data (MFC/MIC ≤ 2) confirmed the fungicidal activity of this drug
against *A. fumigatus* (15, 16).

Mavridou *et al.* (2010) (18) demonstrated a good efficacy of voriconazole at
40 mg/kg in prolonging the survival of non-immunosuppressed mice infected
with three *A. fumigatus* isolates with voriconazole MICs from 0.12 to 0.25
µg/ml, but this dosage was ineffective in mice infected with a strain with MIC
of 2 µg/ml. In our study voriconazole also showed efficacy in those animals
infected with *A. fumigatus* isolates having MICs ≤ 0.25 µg/ml, but in those with
MIC > 0.25 µg/ml their efficacy was variable and strain dependent.

Nevertheless, it should be noted that our study differs in many important
aspects from that of Mavridou *et al.* (2010) (18) such as the voriconazole
dosage, the immune status of animals, and the endpoints used to evaluate
the *in vivo* efficacy of the treatment, which makes any comparison difficult. In
the present study only one dose of voriconazole was evaluated because the
use of an additional dose for the same strains would have required more than
40 groups of animals. The dose tested demonstrated efficacy seen in previous
similar studies (5, 33) and using this dose, the serum levels of that drug were
always greater than the MICs for the strains tested. A possible limitation of
the present study is the use of a systemic infection model, which is not the
most common clinical presentation of *A. fumigatus* infection. However
systemic infections are not uncommon, especially in immunocompromised
patients, and are often fatal (3, 4). Another potential weakness could be the
use of mice to evaluate voriconazole efficacy, bearing in mind that the
metabolism and clearance of the drug is very rapid in the mouse. However,
the use of grapefruit juice to increase voriconazole serum concentration
demonstrated the effectiveness of its oral administration in several
experimental murine mycoses (12, 29, 33). In contrast, one of the most
important aspects of this study was the large number of strains used which,
although important, is atypical in this type of study (13). It is also well known,
as has been demonstrated in this study, that different isolates of a given
species frequently show a different response to the same antifungal agent.
Therefore, the use of only one strain to infer antifungal susceptibility of a
species can produce erroneous results.

Our results demonstrate that *A. fumigatus* strains show important variability in
their *in vivo* responses to voriconazole, particularly for those isolates with MIC
d values > 0.25 µg/ml, and without any clear relationship between increasing
MIC and *in vivo* response to voriconazole. These results agree with those of
Baddley et al (2009) (2) who investigated the relationship between the *in vitro* susceptibility of 115 *A. fumigatus* isolates recovered from patients with invasive aspergillosis who received voriconazole, and the clinical outcome. They observed that MICs ≥ ECV were not statistically associated with increased mortality.

Although several clinical studies have shown that voriconazole serum concentrations ≤ 1 µg/ml have been generally associated with therapeutic failure (1,27), in the present study we did not observe this relationship, because voriconazole showed low efficacy against different isolates of *A. fumigatus*, although all drug serum levels were ≥ 4 µg/ml.

Taking into account all the parameters of treatment response used in this study, our results revealed that only a MIC = 4 µg/ml was associated with treatment failure, however only one such strain was evaluated, whereby it is not possible to draw conclusions about these results. Nevertheless, only 0.9% of *A. fumigatus* strains showed voriconazole MICs ≥ 4 µg/ml (9).

In summary, using this MIC model, voriconazole significantly prolonged survival and reduced fungal load in kidneys and brain with respect to the control group in those animals infected with isolates having MICs ≤ 0.25 µg/ml. In those mice challenged with strains with MICs from 0.5 to 4 µg/ml the results were variable and poorly predictive of *in vivo* response. .

Further studies testing additional strains with varying voriconazole MICs are necessary to more accurately define the clinical significance of *in vitro* data.
References


BAL 4815, the active component of the prodrug BAL8557, in a neutropenic murine model of disseminated *Aspergillus flavus*. J. Antimicrob. Chemother. 58: 1198-1207
Table 1: Mean survival time of mice infected with different strains of *A. fumigatus*

<table>
<thead>
<tr>
<th>Group infected with the strains</th>
<th>Mean survival time in days (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VRC 25 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>FMR 10220 (0.12)</td>
<td>21.5 (7-30)ª,ª,ª</td>
</tr>
<tr>
<td>FMR 10536 (0.25)</td>
<td>21.8 (7-30)ª,ª</td>
</tr>
<tr>
<td>FMR 10513 (0.25)</td>
<td>17.5 (6-30)ª,ª</td>
</tr>
<tr>
<td>FMR 10528 (0.5)</td>
<td>9.1 (6-17)</td>
</tr>
<tr>
<td>FMR 10505 (0.5)</td>
<td>13 (5-30)ª</td>
</tr>
<tr>
<td>FMR 7738 (1)</td>
<td>14.2 (6-30)ª</td>
</tr>
<tr>
<td>FMR 10512 (1)</td>
<td>11.4 (6-30)</td>
</tr>
<tr>
<td>UTHSC 10-3338 (2)</td>
<td>20.3 (13-30)ª,ª</td>
</tr>
<tr>
<td>UTHSC 10-246 (2)</td>
<td>17.4 (5-30)ª,ª</td>
</tr>
<tr>
<td>UTHSC 10-448 (4)</td>
<td>9.3 (6-30)</td>
</tr>
</tbody>
</table>

ª *P* < 0.05 versus control; ª *P* < 0.05 versus FMR 10528; ª¢ *P* < 0.05 versus UTHSC 10-448.
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Figure 1. Box-plot of changes in fungal load of mice infected with $2 \times 10^3$ CFU of *A. fumigatus* with respect to the respective control in kidneys of mice treated with voriconazole at 25 mg/kg orally once a day.

- a $P < 0.05$ versus control;
- b $P < 0.05$ versus FMR 10505;
- c $P < 0.05$ versus UTHSC 10-448.
Figure 2. Box-plot of changes in fungal load of mice infected with $2 \times 10^3$ CFU of *A. fumigatus* with respect to the respective control in brain of mice treated with voriconazole at 25 mg/kg orally once a day.

a $P < 0.05$ versus control;
b $P < 0.05$ versus FMR 10528;
c $P < 0.05$ versus FMR 7738;
d $P < 0.05$ versus FMR 10512;
e $P < 0.05$ versus UTHSC 10-3338;
f $P < 0.05$ versus UTHSC 10-246;
g $P < 0.05$ versus UTHSC 10-448.