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Voriconazole MICs are predictive for the outcome of experimental disseminated scedosporiosis

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Background: Scedosporiosis is associated with a mortality rate of up to 90% in patients suffering from disseminated infections. Recommended first-line treatment is voriconazole, but epidemiological cut-off values and clinical breakpoints have not been determined.

Objectives: To correlate voriconazole treatment response in mice suffering from disseminated scedosporiosis with MIC values determined using CLSI broth microdilution, Etest (bioMérieux) and disc diffusion.

Methods: Voriconazole MICs for 31 *Scedosporium apiospermum* strains were determined using CLSI broth microdilution, Etest and disc diffusion. Groups of mice were challenged intravenously with 1 out of 16 *S. apiospermum* strains (voriconazole CLSI broth microdilution MIC range: 0.125–8.0 mg/L) and treated with 40 mg/kg voriconazole orally by gavage once daily. Efficacy of voriconazole was evaluated by a statistically significant ($P < 0.05$) reduction in fungal burden in brain.

Results: A categorical agreement of 90.4% was reached for CLSI broth microdilution and disc diffusion and of 93.6% for CLSI broth microdilution and Etest. Correlation of CLSI MICs and *in vivo* outcome was good, as mice challenged with strains with an MIC $\leq$2 mg/L responded to voriconazole therapy in 92.3% and those challenged with strains with an MIC $\geq$4 mg/L responded to voriconazole therapy in 33.3%.

Conclusions: CLSI broth microdilution and Etest deliver comparable results that enable a prediction of *in vivo* outcome. Our results suggest that voriconazole is able to reduce fungal burden in the brain of 92.3% of all mice challenged with strains with voriconazole CLSI MICs $\leq$2 mg/L, while mice challenged with strains with CLSI MICs $\geq$4 mg/L showed limited response to voriconazole treatment.

Introduction

Scedosporiosis and fusariosis are the most frequent mould infections, after aspergillosis.[1] In temperate climate zones, scedosporiosis is commonly caused by members of the '*Scedosporium apiospermum* species complex' (containing the former species: *Pseudallescheria ellipsoidea*, *Pseudallescheria fusoidea*, *Pseudallescheria angusta* and *Pseudallescheria boydii*).[2] These fungi cause a broad spectrum of diseases in both immunocompetent and immunocompromised patients.[3] Their special features are a pronounced neurotropism and resistance to most systemic antifungals (amphotericin B, itraconazole, flucytosine and echinocandins). In immunocompetent patients scedosporiosis is predominantly localized, but the majority of immunosuppressed patients (>50%) suffer from disseminated infections (mortality rates: 58%–75%). Particularly high mortality rates (>90%) are seen in patients with CNS involvement; in immunosuppressed patients, *Scedosporium* disseminates via haematogenous spread, while CNS scedosporiosis is seen in immunocompetent patients, exclusively after near-drowning.[3,4]

Although neither species-specific epidemiological cut-off values (ECVs) nor clinical breakpoints (CBPs) have been established for *Scedosporium* spp., case reports[3] and currently available treatment guidelines[5] suggest voriconazole (with or without surgery) as first-line treatment for scedosporiosis. Voriconazole reaches high CNS-substance levels.[4] However, a comprehensive evaluation of voriconazole treatment outcome in experimental disseminated scedosporiosis and a correlation with MIC values is lacking. Therefore, the objectives of this study were to determine if *in vivo* efficacy of voriconazole correlates with MIC values and to suggest a species-specific experimental tentative cut-off value.

In addition, we aimed to evaluate the performance of two commercial antifungal susceptibility tests [Etest and disc diffusion test (DDT)] in comparison with the gold standard method (CLSI broth microdilution), to offer alternatives for routine laboratory testing.

Materials and methods

In vitro antifungal susceptibility testing

Twenty-eight clinical isolates and three of environmental origin were used in the *in vitro* studies (Table 1). All isolates were previously identified by sequencing the internal transcribed spacer (ITS) region and the β-tubulin gene. The sequences were compared using blast against two in-house databases and the ISHAM ITS database (http://its.mycologylab.org/). The isolates were subcultured on potato dextrose agar (PDA) for 10 days at 30 °C and inocula were prepared as previously described.[6]

Voriconazole MIC values were determined using: (i) broth microdilution according to the CLSI M38-A2 document (international gold standard);[7] (ii) disc diffusion using non-supplemented Mueller–Hinton agar and 6 mm diameter paper discs containing 1.0 μg of voriconazole;[8] and (iii) Etest (bioMérieux SA, Spain), according to the manufacturer's instructions. The strains *Paecilomyces variotii* ATCC MYA-3630 and *Aspergillus fumigatus* ATCC MYA-3626 were used as quality controls.

All *in vitro* tests were incubated at 35°C for 72 h. Broth microdilution MICs were determined according to CLSI using an endpoint of complete growth inhibition.[7] For disc diffusion, inhibition zone diameters (IZDs) were measured to the nearest whole millimetre at the point at which there was a prominent reduction in growth (80%). Slight trailing around the edges was ignored. Etest MIC corresponded to the gradient concentration where the inhibition ellipse intersected the plastic strip. Etest MICs were rounded up to the next higher CLSI concentration for comparison. Strains were categorized as susceptible if the MIC was ≤2 mg/L and resistant if the MIC was ≥4 mg/L, on the basis of their response to voriconazole considering as endpoint a reduction in fungal burden in brain (see the next section). DDTs were read as follows: susceptible (IZD ≥17 mm), intermediate (IZD 14–16 mm) and resistant (IZD ≤13 mm).[9] The results of Etest and the CLSI broth microdilution method were analysed for their reproducibility by providing essential agreement values within ±1 and ±2 CLSI dilution steps, as previously described.[10] The minimal fungicidal concentration (MFC) was also determined by subculturing 20 μL from each well that showed complete growth inhibition on PDA plates. The MFC was the lowest drug concentration at which approximately 99.9% of the original inoculum was killed. Each isolate and method was tested in triplicate.

Murine model and ethics

Sixteen strains with different susceptibilities to voriconazole (based on CLSI MIC values) were selected for murine studies (Table 1). Groups of 16 OF-1 male mice (8 for fungal burden and 8 for survival analysis) (Charles River, Criffa SA, Barcelona, Spain) were immunosuppressed 2 days before the infection and every 5 days with 200 mg/kg cyclophosphamide. On the day of the infection, OF-1 mice were challenged intravenously with 5×10^3–1×10^4 conidia (inoculum size was adjusted to the virulence of each strain as tested in a previous study evaluating survival as endpoint; see Table 2) and treated with voriconazole at 40 mg/kg/day orally with grapefruit juice by gavage for 7 days.[11] The dose was selected on the basis of other studies evaluating the efficacy of 40 mg/kg voriconazole in OF-1 mice, where trough serum drug levels obtained were around 4.7–8.2 mg/L after 7–13 days of treatment.[12,13] Animals received grapefruit juice during and 2 days before initiation of treatment.[12] Brain fungal burden was the study endpoint, but survival and kidney fungal burden were also analysed. For comparability, all animals (control and treated groups) were euthanized after the first animal died in the control group (Table 2). Organs were worked up as previously described[11] for cfu determination/g of tissue. All animals were housed under standard conditions and care procedures were supervised and approved under the procedure number 8249, by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Statistical analysis

The fungal burdens of the control and treated groups were compared using the Mann–Whitney *U*-test. The mean survival time (MST) was estimated by the Kaplan–Meier method and compared among groups using the log rank test. All statistical analyses were performed with GraphPad Prism 6.0 for Windows. *P* values <0.05 were considered statistically significant.

Results

MICs were 0.125–8.0 mg/L for CLSI broth microdilution and 0.094 to >32.0 mg/L for Etest; IZDs ranged from 0 to 40.0 mm. The susceptibility results for each strain are presented in Table 1. Based on CLSI broth microdilution, 90.3% of the *S. apiospermum* strains were susceptible, 6.5% were intermediate and 3.2% were resistant; with Etest, 96.8% were susceptible and 3.2% were resistant. Using DDT, 87.1% of strains were susceptible and 12.9% were resistant. The essential levels of agreement between Etest and CLSI broth microdilution results (Etest MICs within ±1 and ±2 $\log_2$ dilutions of CLSI broth microdilution MICs) were 87.1% and 93.5%, respectively. We found 6.4% of minor errors between Etest and CLSI broth microdilution and 0% of major and very major errors, with a 93.6% categorical agreement between both methods, while agreement between CLSI broth microdilution and DDT was 90.4%. MFCs for all strains exceeded 16.0 mg/L, confirming the fungistatic activity of voriconazole against *S. apiospermum*.

Voriconazole resulted in a statistically significant reduction in fungal burden in brain, compared with the control, in 13 of the 16 strains tested, which coincided, with only one exception, with those showing MICs ≤2 mg/L by CLSI broth microdilution testing (Table 2). Mice challenged with strains of *S. apiospermum* with MICs >2 mg/L failed to respond to therapy in two (FMR 8869, MIC = 4 mg/L and HMM 12-09, MIC = 8 mg/L, respectively) out of three cases. Mice challenged with UANL-OC149 (MIC = 4 mg/L) showed a statistically significant reduction in brain burden when receiving voriconazole treatment. By using DDT, voriconazole showed efficacy against all the strains showing an IZD ≥18 mm with only one exception (HMM 10-35, IZD = 20 mm) and also against two strains considered resistant *in vitro* (UANL-OC149 and HMM 11-47, IZDs = 0 mm). With respect to the Etest, voriconazole showed efficacy against the strains with MICs between 0.094 and 1.5 mg/L with two exceptions (strains HMM 10-35, MIC = 0.38 mg/L and FMR 8869, MIC = 0.5 mg/L) and did not against HMM 12-09 (MIC >32 mg/L). Voriconazole treatment significantly reduced the fungal burden in the brain when mice were challenged with strains with CLSI MICs ≤2 mg/L compared with mice challenged with strains with CLSI MICs ≥4 mg/L ($P = 0.039$). Based on survival and fungal burden in kidney, a statistically significant difference between mice infected with strains with CLSI MICs ≤2 mg/L and mice infected with strains with CLSI MICs ≥4 mg/L was not found (Table 2).

Table 1. *In vitro* antifungal susceptibility results of 31 isolates of *S. apiospermum* using three antifungal susceptibility methods

	MIC (mg/L)[a]		
Strain	CLSI broth microdilution	Etest	IZD (mm)[b]
HMM 12-14	0.125	0.094	40.0
HMM 12-03	0.25	0.094	35.0
FMR 13015	0.25	0.125	30.0
FMR 13011	0.25	0.125	35.0
HMM 12-04	0.25	0.125	35.0
HMM 12-34	0.25	0.094	30.0
HMM 11-33	0.5	0.19	25.0
HMM 12-36	0.5	0.38	20.0
HMM 11-96	0.5	0.25	25.0
HMM 11-92	0.5	0.19	24.0
HMM 11-97	0.5	0.19	22.0
FMR 8856	0.5	0.25	25.0
HMM 11-93	0.5	0.25	20.0
HMM 11-87	0.5	0.25	25.0
HMM 12-33	0.5	0.25	22.0
HMM 11-36	0.5	0.38	25.0
HMM 10-35	1.0	0.38	20.0
HMM 11-86	1.0	0.75	25.0
HMM 10-34	1.0	1.5	30.0
HMM 11-34	1.0	0.25	25.0
HMM 12-06	1.0	0.75	22.0
FMR 9155	1.0	0.5	20.0
HMM 10-38	1.0	1.5	24.0
FMR 6922	2.0	1.0	20.0
HMM 10-31	2.0	1.0	18.0
HMM 11-47	2.0	1.5	0.0
UANL-PL071	2.0	1.0	20.0
FMR 9167	2.0	1.0	22.0
UANL-OC149	4.0	0.75	0.0
FMR 8869	4.0	0.5	0.0
HMM 12-09	8.0	>32.0	0.0

HMM, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Austria; FMR, Facultat de Medicina de Reus, Spain; UANL, Universidad Autónoma de Nuevo León, Mexico.

[a]The broth microdilution method was performed according to CLSI document M38-A2,[7] and Etest (bioMérieux) was performed according to the manufacturer's instructions. Strains were considered susceptible if MIC was ≤2 mg/L, intermediate if MIC was 4 mg/L and resistant if MIC was >4 mg/L.

[b]The disc diffusion method was performed following the recommendations of CLSI document M51-A.[8] Strains were classified as susceptible if IZD was ≥17 mm, intermediate if IZD was between 14 and 16 mm, and resistant if IZD was ≤13 mm.

Discussion

Mortality rates among patients suffering from disseminated scedosporiosis remain high depending on the underlying disease (highest in HSCT recipients) and the site of infection (CNS) and can be up to 90%.[3,14] ECVs and CBPs have not been established for the interpretation of voriconazole MICs, the currently suggested drug of first-line treatment. Due to the rareness of the disease, these will remain difficult to establish. In contrast to *Scedosporium*, ECVs for amphotericin B and some azoles have been established for most clinically relevant moulds such as *Aspergillus* spp., *Fusarium* spp. and some Mucorales.[15,16]

The present study has some limitations that mean that careful interpretation of the results is required. MIC values of voriconazole for *S. apiospermum* >1 mg/L are rare, as MIC_{90} is <2 mg/L.[17] Therefore, only a limited set of voriconazole-resistant strains was tested. There is an absence of pharmacokinetic data to confirm that voriconazole exposure in the present model is clinically relevant, and the use of grapefruit may increase the efficacy due to the inhibition of voriconazole metabolism by the mucosal cytochrome enzymes, as previously shown by Liu and Sugar.[12]

Our results and the results of other murine models of scedosporiosis demonstrated the efficacy of voriconazole against *S. apiospermum*, *Scedosporium boydii* and *Scedosporium aurantiacum* strains with voriconazole MICs between 0.25 and 1 mg/L.[11,18,19] Similar to our results, in a guinea pig model, azole did not show efficacy against one *S. apiospermum* strain with an MIC of 8.0 mg/L.[20]

In the clinical setting, the relevance of susceptibility determination is largely unknown. Most *S. apiospermum* isolates have voriconazole MICs <2 mg/L. Considering our results, a therapeutic failure for patients infected with *S. apiospermum* strains with MIC >2 mg/L is likely when there is CNS involvement. However, alternative therapeutics are lacking due to the multiresistance of these fungi. Posaconazole is one alternative that demonstrated efficacy in treating brain abscesses due to *S. apiospermum*.[21] Surgical debridement should be considered whenever possible.[5]

Overall, our results suggest that voriconazole was able to reduce fungal burden in the brain of 92.3% of all mice challenged with *S. apiospermum* strains with voriconazole CLSI broth microdilution MICs ≤2 mg/L and in 33.3% of mice challenged with strains with MICs ≥4 mg/L. When MICs were determined by Etest, voriconazole showed efficacy against 86.7% of strains with an MIC ≤2 mg/L, while the single strain with a higher MIC failed to respond. Finally, 91.7% of the strains with an IZD >17 mm and 50% of strains with an IZD <13 mm responded to the voriconazole therapy when fungal burden in brain was used as the endpoint criterion. In addition, statistical differences ($P = 0.039$) in brain fungal burden were observed between strains with CLSI MICs ≤2 mg/L and ≥4 mg/L, suggesting a reduced treatment response for voriconazole and strains with MICs ≥4 mg/L. However, significant differences were not found when kidney fungal burden and survival were studied ($P = 0.189$ and $P = 0.133$, respectively). This might be due to an organ-specific drug level effect, with higher voriconazole concentrations being reached in the kidneys. Survival differences failed to reach statistical significance between mice challenged with strains of either category (≤2 mg/L or ≥4 mg/L), but were significant when compared with the control; this might be due to the high standard deviations observed within the groups.

In conclusion, our results suggest that voriconazole might show efficacy against strains with voriconazole CLSI MICs ≤2 mg/L. In addition, Etest and CLSI broth microdilution were found to be suitable methods to generate reliable voriconazole MICs for *S. apiospermum* strains.

Table 2. Results of *in vivo* efficacy of voriconazole in mice

			Fungal burden studies ($N = 8$)					Survival studies ($N=8$)	
Strain	MIC (mg/L)	Inoculum (cfu/animal)	day of euthanasia	$\log_{10}$ cfu/g of brain of control mice	$\log_{10}$ cfu/g of brain of treated mice	$\log_{10}$ cfu/g of kidney of control mice	$\log_{10}$ cfu/g of kidney of treated mice	mortality (range in days) (control group) (MST)	mortality (range in days) (treated group) (MST)
HMM 12-14	0.125	1×10^4	+7	4.287 ± 0.12	1.493 ± 0.51*	5.27 ± 0.51	3.45 ± 1.1*	5–13 (9.5)	11–15 (12.33)*
HMM 12-03	0.25	1×10^4	+7	4.37 ± 1.12	1.63 ± 1.25*	5.321 ± 0.34	2.7 ± 1.02*	7–14 (9.38)	8–20 (13.63)*
FMR 13015	0.25	7×10^3	+5	6.83 ± 0.05	5.29 ± 0.53*	5.77 ± 0.35	4.55 ± 0.5*	5–7 (5.33)	7–10 (8.66)*
FMR 13011	0.25	1×10^4	+7	6.11 ± 0.61	2.68 ± 1.01*	5.46 ± 0.28	4.13 ± 0.28*	6–17 (9)	6–20 (14.6)*
HMM 11-33	0.5	5×10^3	+6	5.25 ± 1.25	2.66 ± 0.65*	5.12 ± 0.73	3.54 ± 1.1*	6–17 (9.25)	12–20 (15.63)*
HMM 12-36	0.5	1×10^4	+7	4.99 ± 0.6	2.94 ± 0.33*	4.64 ± 0.91	3.425 ± 0.55*	5–8 (6.17)	7–11 (8.6)*
HMM 11-96	0.5	7×10^3	+5	3.33 ± 1.8	0.87 ± 0.58*	5.547 ± 0.56	2.37 ± 0.64*	5–10 (6.67)	10–13 (11.8)*
HMM 10-35	1.0	5×10^3	+5	4.14 ± 1.01	2.82 ± 1.5	4.54 ± 0.62	4.07 ± 0.83	6–15 (10.88)	8–20 (13)
HMM 11-86	1.0	7×10^3	+5	5.43 ± 0.68	2.69 ± 1.45*	4.85 ± 0.82	3.38 ± 1.11	5–11 (8.33)	11–13 (11.5)*
HMM 10-34	1.0	5×10^3	+7	3.14 ± 0.49	0.68 ± 0.15*	5.15 ± 0.69	4.28 ± 1.1*	7–12 (10.38)	13–20 (17.63)*
FMR 6922	2.0	5×10^3	+5	5.18 ± 0.68	3.85 ± 0.61*	5.194 ± 0.6	3.8 ± 0.93*	6–20 (7)	6–20 (11.5)
HMM 10-31	2.0	5×10^3	+5	5.75 ± 0.92	3.49 ± 1.4*	5.27 ± 0.75	4.83 ± 0.5	5–14 (6.5)	6–20 (11.63)
HMM 11-47	2.0	7×10^3	+7	4.2 ± 0.33	3.5 ± 0.35*	4.11 ± 0.22	3.79 ± 0.56	5–11 (9)	10–18 (13.63)*
UANL-OC149	4.0	5×10^3	+5	6.3 ± 0.22	5.13 ± 0.13*	5.24 ± 0.94	4.01 ± 0.55*	5–7 (5.66)	6–11 (8)*
FMR 8869	4.0	5×10^3	+7	4.64 ± 0.64	3.55 ± 0.88	5.48 ± 0.36	4.5 ± 0.7*	7–11 (8.83)	5–20 (14.6)*
HMM 12-09	8.0	5×10^3	+5	5.47 ± 0.67	5.1 ± 0.84	5.22 ± 0.72	4.48 ± 0.5	5–8 (7.6)	4–12 (8.28)

*Reduction in organ fungal burden and survival increase of treated mice was statistically significant in comparison with the control group ($P \leq 0.05$).

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