Combined therapy in treatment of murine infection by *Fusarium solani*

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**Objectives:** We evaluated the efficacy of voriconazole, amphotericin B and micafungin alone and combined in a murine model of disseminated infection by *Fusarium solani*.

**Methods:** Immunosuppressed mice were treated with voriconazole at 60 mg/kg/day, amphotericin B at 3 mg/kg/day, micafungin at 10 mg/kg/day or combinations of these antifungal drugs. For survival studies, treatment began 1 day after infection and continued for 10 days. For tissue burden studies, animals were sacrificed on day 6 of the treatment and the fungal load in the kidneys and spleens was measured. The experiments were carried out using two different clinical strains of *F. solani*.

**Results:** Only the combination of voriconazole plus amphotericin B prolonged the survival of the mice versus the controls for the two strains tested. However, this combination only reduced tissue burden in the kidney and spleen of mice infected by one strain. The other treatments were clearly less effective.

**Conclusions:** Voriconazole plus amphotericin B may have a role in the treatment of fusariosis.

Keywords: voriconazole, amphotericin B, micafungin, murine model

**Introduction**

Several species of *Fusarium* can cause invasive fungal infections, mainly in patients with underlying haematological malignancies or haematopoietic stem cell transplantation. These infections are commonly refractory to treatment and show a high mortality rate. Most of the cases of fusariosis are due to *Fusarium solani*, which seems to be the most virulent species of the genus, at least for mice, and also the most resistant to antifungals. Although *F. solani* showed universal resistance to antifungal drugs in an *in vitro* study, primary therapies with voriconazole or with lipid formulations of amphotericin B are currently recommended to treat fusariosis. Some clinical trials have shown that these drugs are effective, although against which species of *Fusarium* is unknown. In two studies that treated 11 and 21 patients with voriconazole, the overall response was 43% to 45%. In another clinical trial, of 11 patients with fusariosis treated with amphotericin B lipid complex, 9 had a complete or partial response. On the other hand, treatment with these drugs, especially different formulations of amphotericin B, failed in many other cases of fusariosis. Interestingly, in two recent cases, the combination of voriconazole and amphotericin B led to clinical improvement before neutropenia resolved. In order to assess whether this limited success was merely anecdotal or not, since in both cases the species involved were different, we have evaluated the efficacy of this combined therapy. Combinations of each of the two drugs with an echinocandin were also tested.

**Materials and methods**

**Fungi**

Two clinical isolates, FMR 7995 and FMR 4391, belonging to two different genetic clades of the *F. solani* species complex were used in this study. The isolates were stored at −80°C in potato dextrose broth with glycerol, and prior to testing they were subcultured on potato dextrose agar at 30°C. On the day of infection, 5 day cultures on potato dextrose agar were suspended in sterile saline and filtered through sterile gauze to remove hyphae. The resulting suspensions were adjusted to the desired inoculum size with a haemocytometer. Dilutions of the original suspension were cultured on potato dextrose agar plates to confirm the counts determined with the haemocytometer.
Animals

Male OF1 mice (Charles River, Crifka S.A., Barcelona, Spain) with a mean weight of 30 g were used. The animals were housed in standard boxes with corn cob bedding and had free access to food and water. All experiments were performed in accordance with the highest standards for care and treatment of research animals, as approved by the Animal Welfare Committee of the Rovira i Virgili University.

Drugs

The drugs tested were amphotericin B, purchased as Fungizona (Bristol-Myers Squibb, S.L., Madrid, Spain); micafungin, provided by Astellas Pharma Inc., Tokyo, Japan; and voriconazole, purchased as Vfend (Pfizer S.A., Madrid, Spain).

Immunosuppression

Mice were immunosuppressed by a single intraperitoneal (ip) injection of 200 mg of cyclophosphamide (Genoxal; Laboratorios Funk S.A., Barcelona, Spain) per kilogram of body weight plus a single intravenous injection of 150 mg of 5-fluorouracil (Fluoro-uracil; Productos Roche S.A., Madrid, Spain) per kilogram on the same day of challenge.\(^{14,15}\)

Infection and therapy

Mice were challenged with 5 \(\times 10^3\) or 6.6 \(\times 10^4\) conidia/mL of the strain FMR 7995 or FMR 4391, respectively, in 0.2 mL of sterile saline, injected into the lateral tail vein. These inocula were chosen on the basis of a preliminary study because they caused the death of 90\% of animals 8 or 9 days after the challenge (data not shown).

For survival studies, groups of 10 mice were randomly established for each strain, treatment and as a control group before starting. Treatment began 1 day after infection and continued for 10 days. Amphotericin B was given at 3 mg/kg of body weight intraperitoneally in a volume of 0.2 mL; micafungin at 10 mg/kg of body weight was given subcutaneously in a volume of 0.1 mL; and voriconazole at 60 mg/kg of body weight was given orally by gavage in a volume of 0.2 mL. Mice also received the following drug combinations: amphotericin B/voriconazole, voriconazole/micafungin and amphotericin B/micafungin at the doses described above. Control animals received no treatment. From day 3 prior to infection, the mice that received voriconazole and the control mice were given 100\% grapefruit juice (Hero, Spain) instead of water.\(^{16}\) The mice in each experiment were checked daily for 30 days after the challenge.

For tissue burden studies, groups of five mice were randomly established for each strain, treatment and as a control group. All five animals from each group and each treatment were sacrificed by CO\(_2\) inhalation on day 6 post-infection. The spleens and kidneys were aseptically removed and homogenized in 1 mL of sterile saline. Serial 10-fold dilutions of the homogenates were plated on potato dextrose agar, incubated for 48 h at 30°C and examined daily for 3 days. The number of cfu per gram of tissue was calculated. This experiment was repeated and the data of the two independent experiments were combined.

Statistics

Survival rates were evaluated by the Kaplan–Meier method and compared among groups using the log-rank test. Colony counts in tissue burden studies were analysed using the Mann–Whitney U-test. Calculations were made using SPSS 14.0 and GraphPad 4.0 for Windows. A \(P\) value of \(\leq 0.01\) was considered statistically significant.

Results

The \textit{in vitro} antifungal activities of amphotericin B, voriconazole and micafungin against the strains FMR 7995 and FMR 4391 of \textit{F. solani}, determined in a previous study, were 2, 8 and \(>16\) mg/L, and 4, 4 and 64 mg/L, respectively. Figure 1 shows the effects of the different treatments on mice survival. For strain FMR 7995, only amphotericin B/voriconazole and amphotericin B significantly prolonged survival versus the control group \((P = 0.0004\ and \ P = 0.004)\). For strain 4391, only treatment with the combination of voriconazole/micafungin \((P = 0.0001)\) and amphotericin B/voriconazole \((P = 0.01)\) significantly prolonged survival versus the control group.

Figure 2 shows the effects of the treatments on the fungal load of mice. We tested amphotericin B, voriconazole, micafungin, amphotericin B/voriconazole and voriconazole/micafungin. None of the five treatments tested reduced the tissue burden in any of the organs studied for strain FMR 7995. However, for strain 4391, amphotericin B/voriconazole significantly reduced tissue burden in kidneys and spleen \((P = 0.002\ and \ P = 0.006\), respectively).

Discussion

Although the results were not impressive, the present study demonstrated some efficacy of the amphotericin B/voriconazole combination in a murine model of \textit{F. solani} infection. Despite the high doses of both compounds, the results are only modest: the tissue burden decreased, but minimally so, and only in
one strain. In a separate experiment that used animals that were not infected but treated with the same drugs and doses as here, none of them died (data not shown). Several authors have already evaluated the efficacy of each of the two drugs in murine models. Graybill et al. demonstrated that voriconazole showed some efficacy in a murine-disseminated infection by a strain of \textit{F. solani}, although they studied only doses of 10 and 20 mg/kg/day and the degree of protection was modest. The other combination using voriconazole tested in our study, voriconazole plus micafungin, also performed poorly but demonstrated some degree of effectiveness in one strain. Therapy with amphotericin B has been more widely investigated and several animal studies have shown that the efficacy of this drug and its lipid formulations is limited or null. A few clinical cases have been successfully treated with voriconazole, however, in all the cases that resolved, with one exception, the species involved was not \textit{F. solani}. The combination of voriconazole with amphotericin B has already demonstrated synergy against three species of \textit{Fusarium} in an \textit{in vitro} study.

One of the difficulties of the present study was to develop a suitable model for evaluating the efficacy of the compounds tested. Since \textit{Fusarium} is not highly pathogenic for immunocompetent individuals, the inocula had to be extremely high. Thus, in previous studies that tested immunocompetent mice, we had to use inocula of 10\(^8\) conidia/mL for \textit{Fusarium verticillioides} or 10\(^7\) conidia/mL for \textit{F. solani} to obtain an acute infection. Despite the fact that the animals were immunosuppressed, the fungal load in the various organs was very low in the present study. In the animals that were immunosuppressed 1 day before the challenge, the fungal load in the various organs was very low and not enough to reveal any improvement due to the different treatments (data not shown). Only when the animals were immunosuppressed on the same day as the challenge was the degree of infection considered to be useful for the experiment.

The difficulties in obtaining a suitable experimental murine model of fusariosis have already been indicated by Odds et al. Very few of the mice infected with \textit{Fusarium oxysporum} and immunosuppressed with cyclophosphamide at doses of 150 mg/kg on day −1 died, even with an inoculum of 10\(^8\) cfu/mL.

Although we have demonstrated that \textit{in vitro} antifungal resistance is generalized in the cryptic species of the \textit{F. solani} complex, here we wanted to study \textit{in vivo} two strains belonging to different genetic clades in order to obtain more conclusive results. The results obtained with both strains were similar and demonstrated that we are still a long way from developing an effective treatment against \textit{F. solani} infections.

**Figure 2. Effects of the antifungal treatments on tissue burden of \textit{F. solani} for strains FMR 7995 (a) and FMR 4391 (b) in kidneys and spleen of mice.** AMB, amphotericin B at 3 mg/kg; VRC, voriconazole at 60 mg/kg; MFG, micafungin at 10 mg/kg. *P* \(\leq 0.01\) versus control. The horizontal lines of scatter plots indicate mean values.
In conclusion, a combination of voriconazole and amphotericin B showed some degree of effectiveness in this murine model. Further studies are needed to assess whether adjusting the doses and the duration of the treatment can improve the modest efficacy of the combination. However, it should be taken into account that in invasive fusariosis, regardless of the treatment used, resolution of myelosuppression is crucial if the infection is to be cured.

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**Transparency declarations**

None to declare.

References