Efficacy of posaconazole in a murine model of disseminated infection caused by Apophysomyces variabilis

Valentina Salas1, F. Javier Pastor1, Enrique Calvo1, Deanna A. Sutton2, Jagdish Chander3, Emilio Mayayo4, Eduardo Alvarez1 and Josep Guarro1*

1Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain; 2Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA; 3Department of Microbiology, Government Medical College Hospital, Chandigarh, India; 4Unitat de Anatomia Patològica, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain

*Corresponding author. Tel: +34-977-759359; Fax: +34-977-759322; E-mail: josep.guarro@urv.cat

Received 2 December 2011; returned 19 January 2012; revised 20 February 2012; accepted 25 February 2012

Objectives: We evaluated the in vitro activity of posaconazole and amphotericin B against several clinical strains of the mucoralean fungus Apophysomyces variabilis, and their efficacy in a murine model of disseminated infection caused by that fungus.

Methods: The in vitro susceptibility of seven strains of A. variabilis to posaconazole and amphotericin B was determined by using a broth microdilution method. The in vivo efficacy of both drugs, posaconazole at 20 mg/kg twice daily orally by gavage and amphotericin B at 0.8 mg/kg once daily intravenously, was evaluated against six of the strains previously tested in vitro using immunocompetent mice.

Results: In general, MICs of both drugs were within the range of susceptibility or intermediate susceptibility. Posaconazole and amphotericin B were able to significantly reduce the percentages of positive cultures in the affected tissues. However, in general, posaconazole significantly improved survival (median, 23 days; range, 7–30 days) compared with untreated controls (median, 6 days; range, 4–7 days) and, in some cases, with respect to the animals treated with amphotericin B (median, 15 days; range, 5–30 days).

Conclusions: Our results demonstrate the efficacy of posaconazole in the treatment of a disseminated murine infection caused by A. variabilis. However, further clinical studies are required to ascertain the potential use in human infections caused by this fungus.

Keywords: antifungal, animal models, fungal infections, A. variabilis

Introduction

Apophysomyces is a genus of the order Mucorales that typically causes necrotizing fasciitis in humans and primarily infects immunocompetent hosts.1,2 Recent physiological, genetic and morphological data have demonstrated that the genus Apophysomyces comprises at least four species, i.e. Apophysomyces elegans, Apophysomyces variabilis, Apophysomyces ossiformis and Apophysomyces trapeziformis.3 The clinical role of these new species is not yet known, but it seems that the prevalent species in clinical cases is A. variabilis,4 although A. trapeziformis recently infected 13 individuals injured in a tornado in the USA.5

Amphotericin B is the recommended drug for treating infections caused by members of Mucorales, but it has failed in an important number of infections.1 Posaconazole is a good alternative in the treatment of infections that are refractory or intolerant to polyenes.6,7 However, there have been few clinical data on the use of posaconazole in the management of Apophysomyces infections.7 Given the lack of clinical experience, animal models can be a predictive source of information on the efficacy of new therapeutic strategies.4 We tested the in vitro and in vivo activity of posaconazole and amphotericin B against a set of clinical strains of A. variabilis in a murine model of infection caused by this fungus.

Methods

Seven clinical strains of A. variabilis molecularly identified3 were used in the in vitro study (see Table 1). Their susceptibility to posaconazole and amphotericin B was evaluated using a reference method.6 The MIC (in mg/L) was read at 24 h.8
For the murine studies we randomly chose six of the strains previously tested in vitro.

On the day of infection, cultures on Czapek agar (CZA) were suspended in sterile saline and filtered through sterile gauze to remove clumps of sporangiospores or hyphae. The resulting suspensions were adjusted based on haemocytometer counts and by serial plating on CZA to confirm viability.

Male OF1 mice (Charles River) aged 6 weeks and weighing 30 g were used. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Immunocompetent mice were challenged with $1 \times 10^3$ cfu in 0.2 mL of sterile saline and injected via the lateral tail vein. Preliminary experiments testing several strains demonstrated that this inoculum was appropriate for producing an acute infection, with 100% of the animals dying within 7 days (data not shown).

The drugs assayed were posaconazole (Noxafil, Schering-Plough Ltd, Hertfordshire, UK) administered at 20 mg/kg of body weight orally by gavage twice daily and amphotericin B (amphotericin B deoxycholate, Xalabarder Pharmacy, Barcelona, Spain) administered at 0.8 mg/kg of body weight intravenously once daily. The doses were selected on the basis of previous experimental studies in which good efficacy was demonstrated against other species of Mucorales.9

**Figure 1.** Cumulative mortality of mice infected with *A. variabilis* (a) IMI 338332, (b) CBS 658.93, (c) GMHC 480/07, (d) UTHSC 06-4222, (e) GMCH 211/09 and (f) UTHSC 03-3644. IMI, International Mycological Institute, CABI-Bioscience, Egham, UK; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; GMCH, Government Medical College Hospital, Chandigarh, India; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA; POS 40, posaconazole administered at 20 mg/kg orally twice daily; AMB 0.8, amphotericin B administered at 0.8 mg/kg intravenously once daily. $^aP < 0.05$ versus control. $^bP < 0.05$ versus AMB 0.8.
The efficacy of posaconazole and amphotericin B was evaluated based on the results of survival, tissue burden reduction and histopathological studies. All treatments began 1 day after infection and lasted for 7 days. For survival studies, groups of 10 mice were randomly established for each strain and each treatment, and were checked daily for 30 days after challenge. Control groups received no treatment. For tissue burden studies, groups of 10 mice were also established and the animals were sacrificed on day 4 post-infection in order to compare the results with living controls.

Lungs, kidneys, liver, spleen and brain were aseptically removed, and then approximately half of each organ was cut using a sterile scalpel and five small pieces were put on the surface of CZA medium plates. To ensure the reproducibility of the experiments, samples were cultured in duplicate. The plates were incubated for 24 h at 40°C and were observed under a stereoscopic microscope to check for the presence of fungal growth. Statistical significance for the tissue culture study was estimated by the Mann–Whitney U-test. The mean survival time was estimated by the Kaplan–Meier method and compared among groups by using the log-rank test.

For the histopathology study, half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded and sliced into 2-μm sections, which were stained with haematoxylin and eosin, periodic acid–Schiff or Grocott’s methenamine silver and examined in a blinded fashion by light microscopy. Additionally, groups of five immunocompetent mice were challenged with the strain GMCH 211/09 and treated with 0.8 mg/kg amphotericin B once daily or 20 mg/kg posaconazole twice daily to determine the levels of these drugs in serum and brain by bioassay 4 h after administering the drug on day 4 of therapy.8

### Results

Following the suggested working breakpoints against Mucorales,8 86% of the strains tested were susceptible to posaconazole and 29% to amphotericin B (MIC = 1 mg/L), while the others showed intermediate susceptibility to both drugs (MIC = 2 mg/L). In vitro resistance was not observed in any case.

Posaconazole and amphotericin B were able to significantly prolong survival with respect to the control group for all the strains tested. Moreover, posaconazole also significantly prolonged survival compared with amphotericin B for two strains (Figure 1).

The organ cultures of the control animals were always positive with the exception of the lungs, which were the least affected organs. In general, both drugs were effective in reducing the percentages of positive cultures from the affected tissues with respect to the control group, with the exception of the strain GMCH 480/07, for which amphotericin B was only able to reduce the fungal load in the lungs and not in the other organs. In addition, posaconazole reduced the fungal load in the kidneys and in the other organs. In conclusion, posaconazole was able to reduce the fungal load in the kidneys of the infected mice with respect to amphotericin B.

At day 4 of treatment, posaconazole and amphotericin B levels in serum (7.15 ± 0.16 and 6.05 ± 0.62 mg/L, respectively) and brain (6.10 ± 0.86 and 4.95 ± 0.71 mg/g, respectively) were above the corresponding MICs for the isolates tested. Here, the limit of detection was found to be 0.125 mg/L for both of the drugs assayed.

The histological studies of controls revealed that the kidneys were the most affected organs. All organs tested showed abundant fungal elements, with infiltration by hyphae in the blood vessels, but fungal invasion was not observed in the lungs. Kidney sections showed glomerular and tubular invasion by hyphae, with signs of tubular necrosis and an inflammatory response. Fungal elements, necrosis and an inflammatory response were observed in kidney sections of mice treated with amphotericin B. Mice treated with posaconazole showed renal, hepatic and splenic congestion, but there were no signs of necrosis or inflammatory response and few fungal elements, which were only observed in the kidneys.

### Discussion

Infections caused by Apophysomyces are associated with severe morbidity and high mortality rates.1 In experimental studies, Apophysomyces has proven to be more virulent for mice than other species of Mucorales.10

To our knowledge, this is the first experimental study that has explored the efficacy of antifungal drugs against a relatively high number of strains of Apophysomyces. Previously, Dannaoui et al.15 showed a high efficacy of amphotericin B against one strain of A. elegans. In this study, the efficacy of that drug was more modest. However, our study differed in some relevant aspects, such as the treatment schedules, dosages and route of administration of amphotericin B, which makes any comparison difficult. Data on the efficacy of amphotericin B in the treatment of Apophysomyces infections is controversial. Of the four clinical cases reported by Chander et al.,10 only one of the two

### Table 1. Results of microscopic examination expressed as a percentage of positive cultures

<table>
<thead>
<tr>
<th>Strains</th>
<th>Treatments</th>
<th>kidney</th>
<th>spleen</th>
<th>liver</th>
<th>lung</th>
<th>brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMI 338332</td>
<td>none</td>
<td>100</td>
<td>92</td>
<td>96</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>POS 40</td>
<td>22ab</td>
<td>72a</td>
<td>68a</td>
<td>0a</td>
<td>18a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>80</td>
<td>52a</td>
<td>50a</td>
<td>18a</td>
<td>20a</td>
<td></td>
</tr>
<tr>
<td>CBS 658.93</td>
<td>none</td>
<td>90</td>
<td>90</td>
<td>100</td>
<td>34</td>
<td>58</td>
</tr>
<tr>
<td>POS 40</td>
<td>10a</td>
<td>28a</td>
<td>44a</td>
<td>0a</td>
<td>6a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>6a</td>
<td>36a</td>
<td>66a</td>
<td>0a</td>
<td>8a</td>
<td></td>
</tr>
<tr>
<td>GMCH 480/07</td>
<td>none</td>
<td>96</td>
<td>92</td>
<td>84</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td>POS 40</td>
<td>44a</td>
<td>38a</td>
<td>36a</td>
<td>0a</td>
<td>40a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>68</td>
<td>66</td>
<td>82</td>
<td>8a</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>UTHSC 06-4222</td>
<td>none</td>
<td>92</td>
<td>84</td>
<td>96</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>POS 40</td>
<td>12a</td>
<td>16a</td>
<td>40a</td>
<td>0a</td>
<td>8a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>14a</td>
<td>28a</td>
<td>14a</td>
<td>0a</td>
<td>6a</td>
<td></td>
</tr>
<tr>
<td>GMCH 211/09</td>
<td>none</td>
<td>90</td>
<td>90</td>
<td>92</td>
<td>38</td>
<td>82</td>
</tr>
<tr>
<td>POS 40</td>
<td>28a</td>
<td>26a</td>
<td>48a</td>
<td>0a</td>
<td>6a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>12a</td>
<td>50a</td>
<td>78</td>
<td>0a</td>
<td>16a</td>
<td></td>
</tr>
<tr>
<td>UTHSC 03-3644</td>
<td>none</td>
<td>88</td>
<td>86</td>
<td>88</td>
<td>40</td>
<td>76</td>
</tr>
<tr>
<td>POS 40</td>
<td>6a</td>
<td>30a</td>
<td>40a</td>
<td>0a</td>
<td>12a</td>
<td></td>
</tr>
<tr>
<td>AMB 0.8</td>
<td>12a</td>
<td>38a</td>
<td>74a</td>
<td>0a</td>
<td>10a</td>
<td></td>
</tr>
</tbody>
</table>

IMI, International Mycological Institute, CABI-Bioscience, Egham, UK; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; GMCH, Government Medical College Hospital, Chandigarh, India; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX, USA; POS 40, posaconazole administered at 20 mg/kg orally twice daily; AMB 0.8, amphotericin B administered at 0.8 mg/kg intravenously once daily. 

For survival studies, groups of 10 mice were randomly established for each strain and each treatment, and were checked daily for 30 days after challenge. Control groups received no treatment. For tissue burden studies, groups of 10 mice were also established and the animals were sacrificed on day 4 post-infection in order to compare the results with living controls.
treated with amphotericin B showed a successful outcome. In another retrospective study, a favourable outcome was obtained in 9 of the 13 patients treated with amphotericin B, regardless of the MIC values. The identity of the species of Apophysomyces involved in those infections is unknown, although they were attributed to A. elegans. It is likely that they were caused by A. variabilis, which seems to be a more prevalent species of the genus. We expect now that as more reliable methods for the identification of the species of Apophysomyces are available, our understanding of the clinical role of the different species of the genus will improve.

In our study, although the two drugs tested significantly reduced the presence of hyphae in infected tissues, posaconazole was more effective than amphotericin B in improving survival. Up to now, posaconazole has been successfully used in one patient with an Apophysomyces infection that was resistant to amphotericin B.

In this study, most of the posaconazole MICs were within the range of susceptibility (1 mg/L) or intermediate susceptibility (2 mg/L), and correlation between the outcome and MICs was not observed. This was probably due to the difference of only one dilution, which is not enough to categorize different degrees of susceptibility.

However, in any case, it would be interesting in future animal studies to refine these results with posaconazole MICs <1 mg/L, if they are available, in order to define as accurately as possible the MICs that better correlate with in vivo outcome.

In summary, posaconazole showed good efficacy in our experimental model of disseminated infection and merits further investigation in order to develop effective treatments for human infections caused by A. variabilis.

Acknowledgements
We thank the curators of CBS (Utrecht, The Netherlands) and CABI-Bioscience (Egham, UK) culture collections for providing some of the strains used in this study.

Funding
This study was supported by internal funding.

Transparency declarations
None to declare.

References