Comparison of the dose-dependent activity and paradoxical effect of caspofungin and micafungin in a neutropenic murine model of invasive pulmonary aspergillosis

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Objectives: The safety and concentration-dependent pharmacodynamic characteristics of the echinocandins make them ideal candidates for dosage escalation in the treatment of aspergillosis. However, paradoxical attenuation of antifungal activity with increasing doses has been reported for some echinocandins in experimental models of invasive pulmonary aspergillosis (IPA).

Methods: We compared the activity of micafungin and caspofungin administered over a wide dosing range that encompasses clinical exposures (0.25–10 mg/kg) in a neutropenic murine model of IPA.

Results: Both echinocandins exhibited dose-dependent reductions in fungal burden; however, caspofungin displayed a relatively steeper dose–response curve with a modest paradoxical increase in fungal burden that was not seen in micafungin-treated animals. Equivalent activity was observed with both echinocandins at daily doses ranging from 4 to 10 mg/kg.

Conclusions: Both micafungin and caspofungin exhibit dose-dependent pharmacodynamic activity in vivo in the treatment of neutropenic IPA. Both echinocandins were equivalent at dosages ≥4 mg/kg day.

Keywords: Aspergillus, echinocandins, pharmacodynamics

Introduction

Invasive aspergillosis (IA) is a common mould infection in patients with haematological malignancies undergoing chemotherapy and/or haematopoietic stem cell transplantation. Pneumonia, with or without sinusitis, is the most common manifestation of aspergillosis in this population.¹ Although IA mortality rates have improved somewhat over the last decade due to changes in transplantation practices, earlier diagnosis and availability of new treatment options such as voriconazole and the echinocandins,² crude mortality rates still exceed 50%.³ Consequently, improved strategies for the management of IA are still required. Since the approval of caspofungin in 2001, echinocandins have become an important component of many front-line and salvage treatment strategies for IA.⁴,⁵ Echinocandins are non-competitive, concentration-dependent inhibitors of the (1,3)-β-D-glucan synthase enzyme complex, a target that impairs integrity of the fungal cell wall. Because β-1,3-glucan synthesis is present only in fungi, echinocandins can be administered at relatively high dosages with little toxicity or clinically significant drug interactions in humans.⁴,⁶

Unlike voriconazole and amphotericin B formulations, echinocandins display linear pharmacokinetics with increasing doses without evidence of dose-limiting toxicity in Phase II clinical studies.⁷ These properties, combined with their concentration-dependent pharmacodynamic characteristics, make echinocandins ideal candidates for dosage escalation in the treatment of IA.⁷ However, it is unclear whether dosage escalation improves the activity for all echinocandins in a similar fashion as no study has directly compared dose–response relationships for this antifungal class over a wide dosing range in experimental IA. Interestingly, dosage escalation has been associated with paradoxical attenuation of caspofungin⁷ and anidulafungin,⁸ but not micafungin⁹ activity in animal models of invasive pulmonary aspergillosis (IPA). The aim of this study was to directly
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Materials and methods

Reagents

Cortisone acetate and cyclophosphamide were obtained from Sigma Aldrich (St Louis, MO, USA). The human clinical formulations of micafungin (Astellas Inc., Deer Park, IL, USA) and caspofungin (Merck & Co., Inc., Rahway, NJ, USA) powder for injection were acquired from the hospital pharmacy and were freshly reconstituted according to the manufacturers’ directions prior to each experiment.

Inoculum preparation and in vitro testing

Aspergillus fumigatus 293 (AF 293) was grown on potato dextrose agar for 7 days prior to collection of conidia. Conidia were harvested from the slant with 0.1% Tween 20 in phosphate-buffered saline (PBS) and passed through a syringe with sterile glass wool to remove hyphal fragments. The resulting suspension was then centrifuged for 5 min at 15 000 g, the supernatant was discarded and the number of conidia was determined by counting on a haemocytometer. The final concentration was adjusted to 5 × 10⁷ conidia/mL. Harvested conidia were determined to be >98% viable based on plating of a serially diluted inoculum on Sabouraud dextrose agar. The minimum effective concentration (MEC) of micafungin and caspofungin was determined in RPMI growth media +2% glucose alone, and with the addition of 5% heat-inactivated mouse sera using methods described in the CLSI (formerly NCCLS) document M38-A.¹⁰

The concentration-dependent activity of micafungin and caspofungin was tested in vitro using the optimized XTT assay as described by Antachopoulos et al.¹¹ Briefly, 2-fold serial dilutions of micafungin or caspofungin (final concentrations, 0.125–256 mg/L) were prepared in the RPMI assay media ±5% mouse sera and dispensed into 96-well microtitre plates (Costar 3596; Corning Inc., Corning, NY, USA). After inoculation of trays with AF 293 test isolate (final inoculum 2.5 × 10⁴ conidia/mL) and incubation at 37°C for 48 h, 0.05 mL of an XTT reaction solution (0.1 mg/mL XTT + 6.25 μM menadione) was added to each well, a drug free control and media control wells, and incubated for an additional hour before absorbance was determined at 450 and 630 nm using a microplate spectrophotometer (Powerwave X Select, Corning Inc., Corning, NY, USA). After inoculation of trays with AF 293 test isolate (final inoculum 2.5 × 10⁴ conidia/mL) and incubation at 37°C for 48 h, 0.05 mL of an XTT reaction solution (0.1 mg/mL XTT + 6.25 μM menadione) was added to each well, a drug free control and media control wells, and incubated for an additional hour before absorbance was determined at 450 and 630 nm using a microplate spectrophotometer (Powerwave X Select, Corning Inc., Corning, NY, USA). Absorbance readings were standardized in relation to unconverted XTT in the media control wells and plate absorbance (692 nm) to determine ΔOD at 492 nm. The per cent metabolic activity was calculated after subtraction of the background absorbance using the following formula: % metabolic activity = (Absdrug well − Abbackground)/Abscontrol − Abbackground control).¹¹ Experiments were performed in four replicates on separate occasions.

Statistical analysis

All data were expressed as means ± SD of the means and compared by Mann–Whitney test or one-way analysis of variance with Tukey’s post-test for multiple comparisons where appropriate. Differences were considered statistically significant when P values were <0.05. In vivo dose–response data were evaluated by fitting a four-parameter logistic model (Hill equation) to experimental data using computer curve-fitting software (Prism 4, GraphPad Software, Inc, San Diego, CA, USA). Goodness-of-fit was assessed by R² and the standard error of the EC₅₀ value.

Results

In vitro concentration-dependent activity

Susceptibility testing of AF 293 revealed a micafungin MEC of 0.25 mg/L in RPMI alone and 0.5 mg/L in RPMI + 5% mouse serum. The caspofungin MEC was 0.25 mg/L in RPMI alone and did not change in the presence of serum. Both echinocandins reduced hyphal metabolic activity in a concentration-dependent fashion that reached a plateau within a couple

Mice were immunosuppressed with intraperitoneal (ip) injections of cyclophosphamide 150 mg/kg at 4 days and 1 day prior to infection. This regimen results in total polymorphonuclear neutrophil depletion until 96 h after inoculation.¹² In addition, animals received a single 300 mg/kg intraperitoneal dose of cortisone acetate suspension prepared in PBS with 0.2% Tween 20, 1 day prior to infection. Mice were inoculated intranasally under 6% isoflurane: oxygen anaesthesia with 0.03 mL suspension of 5 × 10⁷ A. fumigatus conidia/mL prepared in PBS as described previously.¹³ Following inoculation, animals were returned to the cages and observed until they regained consciousness. This protocol results in reproducible infection of the lungs with >50% of untreated animals succumbing to the infection 96–120 h after inoculation.¹²

Antifungal treatment

Starting 12 h after inoculation, immunosuppressed mice (10 per treatment arm) were administered either micafungin or caspofungin, 0.25, 0.5, 1, 4 or 10 mg/kg prepared in sterile saline as daily intraperitoneal injection (0.2 mL). Control animals were administered saline alone. Treatment was continued for 4 days before mice were euthanized by CO₂ narcosis and lungs were aseptically removed and stored at −80°C until analysis of fungal burden.

Tissue fungal burden

Pulmonary fungal burden was determined by real-time quantitative PCR using previously reported methods.¹⁴ Briefly, DNA samples isolated from homogenized lungs were assayed in duplicate using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), primers and a dual-labelled fluorescent hybridization probe specific for the Aspergillus 18S rRNA gene.¹⁴

The threshold cycle (Ct) of each sample was interpolated from a seven-point standard curve of Ct values prepared by spiking uninected mouse lungs with known amounts of conidia (10¹—10⁷) from AF 293. An internal standard was amplified in separate reactions to correct for the per cent difference in DNA recovery.¹⁵ Results are reported as conidial equivalents (CEs) of A. fumigatus DNA.

Eight-week-old female BALB/c mice (18–25 g) (Charles River Laboratories) were used in all experiments. Mice were housed in sterilized filter top cages and had access to sterile food and water ad libitum. Animals were cared for in accordance with the highest standards for humane and ethical care as approved by the Institutional Animal Care and Use Committee.
dilutions of the MEC (Figure 1). For micafungin, the concentration-response curve shifted slightly when the drug was tested in assay medium containing 5% mouse sera (Figure 1a). However, a paradoxical increase in hyphal viability at supra-MECs of micafungin was not observed.

Exposure to caspofungin produced a similar concentration-dependent reduction in hyphal metabolic activity that maximized once concentrations approached the MEC (Figure 1b). A paradoxical increase in fungal burden was not evident in micafungin treatment groups. For caspofungin, a modest increase in fungal burden (Δ +0.43 log_{10}) was observed as daily doses increased from 1 and 2 mg/kg/day; but the difference in the mean fungal burden of the treatment groups was not statistically significant (P = 0.35).

Pharmacodynamic parameters were determined by fitting a four-parameter logistic Emax model to experimental data. Data were initially fitted to the combined sigmoid concentration effect models and Gaussian models proposed by Antachapoulos et al.11 for describing echinocandin paradoxical effect in vitro. However, this model was found to be inferior to a standard Emax model excluding the 2 mg/kg outlier in the caspofungin group based on Akaike’s information criterion and unacceptably large 95% confidence intervals (CIs) for the estimate of the ED50.17 This poor fit was probably due to the fewer doses tested in vivo (n = 6–7) compared with the number of in vitro concentrations used by Antachapoulos et al. (n = 12) to develop their model for characterizing the dual sigmoidal Emax curves associated with paradoxical activity. In vivo pharmacodynamic parameters estimated by the fit of the experimental data to Emax model are presented in Table 1. Goodness-of-fit was good with an $R^2$ of 0.93 for micafungin and 0.98 for caspofungin. Compared with caspofungin, the in vivo dose–response relationship of micafungin was characterized by a relatively shallower dose–response curve (Hillslope $-2.71$; 95% CI $-3.2$ to $-2.1$ versus Hillslope $-1.38$; 95% CI $-1.9$ to $-0.9$, respectively; $P < 0.05$), resulting in higher effective doses (ED50 and ED90) compared with caspofungin (Table 1).

**Discussion**

To our knowledge, this is the first head-to-head study of echinocandin pharmacodynamics in an experimental model of IPA.
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Table 1. In vivo pharmacodynamic parameters determined by non-linear regression

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<tr>
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<th>Micafungin</th>
<th>Caspofungin</th>
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<tbody>
<tr>
<td>Coefficient of determination ($R^2$)</td>
<td>0.93</td>
<td>0.98</td>
</tr>
<tr>
<td>ED$_{50}$ (± 95% CI)</td>
<td>1.03 mg/kg (0.56–1.50)</td>
<td>0.79 mg/kg (0.63–0.95)</td>
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<tr>
<td>ED$_{90}$ (± 95% CI)</td>
<td>2.34 mg/kg (1.78–2.54)</td>
<td>1.42 mg/kg (1.24–1.69)</td>
</tr>
<tr>
<td>Hillslope (± 95% CI)</td>
<td>$-1.38 (-1.9$ to $-0.9)$</td>
<td>$-2.71 (-3.2$ to $-2.1)$</td>
</tr>
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ED$_{50}$, 50% effective dose; ED$_{90}$, 90% effective dose; 95% CI, 95% confidence interval of estimate. Estimates were derived by fitting a four-parameter logistic Emax model (sigmoidal curve with variable slope) to fungal burden data; $Y = Y_{max} + (Y_{max} - Y_{min})[1 + 10^{(log ED_{50} - X)/slope}]$.

Consistent with prior studies, both echinocandins exhibited concentration-dependent effects on Aspergillus hyphal metabolic activity in vitro and dose-dependent reductions in pulmonary fungal burden in vivo.$^{11}$ However, subtle pharmacodynamic differences were observed between caspofungin and micafungin. Despite similar in vitro potency, caspofungin exhibited a steeper dose–response curve in vivo with a modestly lower ED$_{50}$ and ED$_{90}$ than micafungin over a similar dosing range. However, the ED$_{50}$ and ED$_{90}$ were not significantly different for the two echinocandins (Table 1). Differential patterns of protein binding may explain, in part, the shallower dose–response curve for micafungin compared with caspofungin in vivo.$^{18}$

A modest attenuation of echinocandin activity at doses above the ED$_{90}$ was observed in caspofungin, but not micafungin-treated animals, and was overcome at doses >4 mg/kg daily. In contrast to bona-fide resistance mechanism that renders a strain untreatable with echinocandin therapy, the paradoxical effect has been characterized as a phenomenon of dose-dependent drug tolerance resulting from adaptive fungal cell physiology in response to drug-induced cell wall damage.$^{19}$ Experiments using genome-wide profiling approaches and forward genetic screens with a library of Saccharomyces cerevisiae knockout mutants have demonstrated the influence of multiple, complex networks of pathways involved in cell wall biosynthesis, signal transduction, and cellular vacuole and transport function on echinocandin susceptibility.$^{19}$ When these pathways are blocked in Candida species, resistance is typically modest and has not been shown to conclusively result in therapeutic failure in animal models of infection.$^{19}$

Similar to Candida species, evolutionarily conserved homeostatic stress-response pathways are present in A. fumigatus and have been reported to be differentially expressed at caspofungin concentrations associated with paradoxical increases of in hyphal metabolic activity. Wiederhold et al.$^{20}$ reported that a paradoxical increase in AF 293 viability following caspofungin exposure was associated with increased expression of mitogen-activated protein-kinase A and compensatory increases of cell wall chitin. Our study is the first to directly address the question of whether there are inherent differences between the echinocandins in vivo to induce a paradoxical effect. Although the paradoxical phenotype was more evident for caspofungin compared with micafungin, it is also clear that the paradoxical attenuation of caspofungin activity may be more obvious in vivo compared with micafungin due to the relatively steeper dose–response curve, which may allow for clearer visualization of the paradoxical tolerance at higher caspofungin doses.

Our study does have several important limitations. We were only able to test a single isolate of A. fumigatus. The degree of paradoxical activity can vary from one isolate to the next among various fungal species.$^{11}$ Additionally, patterns of antifungal activity in experimental IPA can vary depending on the types of underlying immunosuppression, which was not tested in our study.$^{21}$ Finally, we did not perform extensive pharmacokinetic analysis although drug exposures achieved with doses used in this murine model have been previously verified. Specifically, work from our laboratory$^{13}$ and Gumbo et al.$^{22}$ demonstrated peak plasma exposures range from 0.7 to 25 mg/L over the range of dosing regimens tested in this model. Nevertheless, extrapolation of our results to other animal models and fungal species must be made with caution.

Taken as a whole, our data support the notion of equivalency of micafungin and caspofungin in experimental IPA. The clinical significance of pharmacodynamic differences or propensity for attenuation with increasing doses should be the focus of studies that test larger numbers of isolates, as well as careful analysis of human data in IPA treated with echinocandin monotherapy.

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Transparency declarations

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