Combination antifungal therapy of murine aspergillosis: liposomal amphotericin B and micafungin

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Objectives: AmBisome and micafungin were used alone and in combination in a series of studies designed to identify any additive or antagonistic effects of combination antifungal therapy.

Methods: Immune-suppressed mice were infected either intravenously or intranasally with Aspergillus fumigatus. Micafungin, liposomal amphotericin B or both drugs together were administered for 7 days. Parameters of efficacy included survival and tissue burden of A. fumigatus.

Results: Whilst each drug was effective in murine aspergillosis, additive effects were observed only in reduction of tissue burden in limited experimental conditions. No antagonism was seen.

Conclusions: The present studies neither encourage nor discourage clinical use of combination therapy. Clinical trials are suggested before combined therapy is routinely adopted.

Keywords: antifungals, Aspergillus fumigatus, invasive aspergillosis

Introduction

Acute invasive aspergillosis (IA) is resistant to multiple antifungal drugs developed within the past few decades. The major determinant of treatment response appears to be the underlying condition rather than the antifungal drug used. In the largest open series yet reported, including patients treated after 1990, Patterson et al.¹¹ found that allogeneic bone marrow transplant recipients with IA have a failure rate of >85%. Better responses appear to occur in solid organ transplant patients and patients with chronic lung disease and corticosteroid therapy. However, these responses are only in the range of 50–60%. The most seriously immunocompromised patients were usually treated with amphotericin B, and overall amphotericin B failures were 65%. These discouraging findings were confirmed by Lin et al.² in a meta-analysis of 50 publications of case series of aspergillosis. Furthermore, Lin et al. found that the overall case fatality rate for lipid vehicle forms of amphotericin B was 51%, only modestly superior to the 67% for amphotericin B deoxycholate (AMBd).

In this environment, there has been a strong desire to introduce new forms of therapy, which might offer more hope for patients with IA. The echinocandins are a new class of antifungals targeted against β-D-glucan components in the fungal cell wall rather than ergosterol of the cell membrane of Aspergillus and other fungi.³⁻⁵ Of the echinocandins, caspofungin has been effective in murine aspergillosis.⁶,⁷ In the mouse model, protection has been demonstrated by prolongation of survival and reduction of tissue counts. Another echinocandin, micafungin, has been found effective in mice infected with itraconazole-resistant Aspergillus fumigatus and amphotericin B-resistant Aspergillus terreus.⁸ There is some discrepancy between mouse and rabbit models, in that the latter have shown prolonged survival but no reduction of tissue counts. This has been attributed to the selective activity of echinocandins on the actively growing hyphal tip, and fragmentation of older, sessile, but viable cells.⁹ Why the murine and rabbit models vary by tissue counts is unclear.

In clinical trials, the only available data on aspergillosis relate to caspofungin. An as yet unpublished open salvage trial of caspofungin has shown a 41% response in clinical IA.¹⁰ On the grounds of efficacy and good tolerance, caspofungin was licensed for salvage treatment of aspergillosis. However, the response rate was not dramatically superior to other antifungals used for salvage therapy.

The major sterol in fungal cell membranes is ergosterol, and this is the target of both polyenes and triazoles. There has been some interest in whether combinations of echinocandins (targeting the fungal cell wall) and other antifungals (targeting the fungal cell membrane) may be additive in their antifungal effects against A. fumigatus. Evaluation of echinocandins in animals has been somewhat dependent on the model used. Verweij et al.¹¹ found that in mice, caspofungin both

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prolonged survival and reduced lung and kidney tissue counts of *A. fumigatus*. Semi-quantitative cultures by tissue homogenization have been found to correlate well with PCR-based quantification of *A. fumigatus* in tissues.\(^9\) In the study by Bowman et al.,\(^9\) caspofungin also reduced kidney tissue counts of mice infected intravenously. Others have found that micafungin reduces lung counts of mice infected by the intranasal route.\(^9\)

In contrast, Roberts et al.\(^9\) found that anidulafungin prolonged survival but did not significantly reduce lung or kidney counts of *A. fumigatus* in rabbits infected intravenously. Petraitis et al.\(^9\) obtained similar results in chronic neutropenic rabbits with pulmonary aspergillosis: survival was prolonged and lung infarct scores were reduced; however, tissue counts were not reduced significantly. The same group found that micafungin and caspofungin gave similar results in rabbit pulmonary aspergillosis.\(^15,16\) This lack of effect on tissue burden was attributed to the selective activity of echinocandins at the hyphal tip, leaving older, non-reproducing segments of *Aspergillus* viable to resume growth when treatment was stopped.\(^7\) Studies such as this have concluded that echinocandins, while fungicidal for *Candida*, are fungistatic for *Aspergillus*. Thus, for reasons that are not yet clear, mouse models permit assessment of survival and tissue burden, while rabbits permit assessment of survival and lung infarct scores, but not tissue burden.

Animal models have also been used to study antifungal drug combinations in aspergillosis. One murine study with micafungin has shown an additive effect of echinocandins and amphotericin B, while another did not.\(^18,19\) Some studies have also found toxic interactions between echinocandins and the corticosteroids used to predispose mice to aspergillosis.\(^20\)

We considered that some of the conflicting results of combination studies might be based on experimental conditions, such as inoculum size, route of infection, timing of initiation of therapy and drug doses. We wished to use a model that could use both survival and tissue burden for protection. Accordingly, we conducted a series of studies exploring these parameters in murine IA.

Materials and methods

Pathogen

*A. fumigatus* clinical isolate 94-2766 was maintained in water. The MIC at 48 h for this isolate is 0.05 mg/L of amphotericin B and 1 mg/L of liposomal amphotericin B and micafungin using the NCCLS method adapted for moulds.\(^21\) The minimum effective concentration (MEC; according to the method of Kurtz et al.)\(^3\) is <1 mg/L for micafungin. For *in vivo* studies, potato flake agar plates were inoculated with conidia, sealed with paraffin and allowed to grow in an incubator at 37°C for 1 week. At this time saline was added to the surface. Conidia and small mycelial fragments were then harvested by scraping the surface with an inoculum loop. The suspension was passed through sterile glass wool to remove mycelial fragments. The conidia were suspended in isotonic saline and counted in a haemocytometer. Conidia were diluted to achieve the desired inoculum size. Colony counts were also made by serial dilution. Inocula are reported as viable colony forming units (cfu) per mouse. Counts were generally within 70% of the anticipated inoculum.

Animal models for infection

Outbred ICR male mice were used, in groups of 8–12 mice/treatment regimen. We used two models. The first, an intravenous infection, was used because many studies of experimental aspergillosis have used this model, although we believe it is not very relevant to naturally occurring infection. In this model, mice were rendered neutropenic by 5-fluorouracil at 150 mg/kg intravenously (iv) and cyclophosphamide at 200 mg/kg intraperitoneally. These drugs were given 1 day before intravenous infection. In prior studies, similar regimens reduced the absolute neutrophil count to <100/µL for 10+ days. The primary infectious targets of this model include the spleen, kidneys and liver. The lungs are much less heavily infected. This model is easy to study, but suffers the major disadvantage of being totally unlike human disease, which almost invariably commences in the lungs and sinuses, and spares the kidneys. The second model used an intranasal infection of mice, which were treated with cortisone acetate at 100 mg/kg, given subcutaneously on the day preceding, the day of and the day after infection. As in clinical aspergillosis, the lungs are the primary site of infection, with spleen and other organ involvement coming later in the course of disease, presumably from haematogenous dissemination.

Treatment regimens

Treatment began on day 1 or 2 after infection, and continued through day 7 after infection. Micafungin was obtained from Fujisawa Healthcare, Inc. (Deerfield, IL, USA), as lyophilized powder for injection. It was diluted in saline and given intraperitoneally in a 0.2 mL volume once daily. Liposomal amphotericin B was obtained commercially as AmBisome (manufactured for Fujisawa Healthcare, Inc. by Gilead Sciences, Inc., San Dimas, CA, USA) for injection. It was diluted in 5% dextrose and given iv in a 0.2 mL volume once a day. Following completion of treatment mice were either observed up to 30 days after infection for survival, or were sacrificed for tissue burden studies 1 day after completion of treatment. Mice succumbing at earlier points in tissue burden studies were similarly cultured. For tissue burdens, the organs were removed and homogenized, using a uniform time and speed setting on a homogenizer to cause a consistent disruption of fungal microcolonies, in 2 mL of isotonic saline. Aliquots of tissue were semi-quantitatively cultured on potato dextrose agar plates using serial 10-fold colony count dilutions. Plates were placed in an incubator at 37°C until colonies could be counted. The undiluted homogenate was saved at 4°C until initial cultures were counted. When the undiluted homogenate had no counts, the entire organ homogenate was cultured. Counts were expressed as cfu/organ. Additive effects could thus be demonstrated by increased survival and reduction of tissue burden.

Statistics

For survival studies, the Wilcoxon test of life tables was used. For tissue burden studies the Mann–Whitney *U*-test was used. For all comparisons, *P*≤0.05 was considered significant.

Results

Neutropenic mice infected intravenously

Survival response. Figure 1 shows two studies of mice infected with \(7 \times 10^4\) conidia/mouse. In Figure 1(a), treatment was given from day 1–7 with liposomal amphotericin B, micafungin or both. Doses were picked to reflect regimens close to the minimum protective dose we had identified in prior studies. Micafungin at 0.125 mg/kg and liposomal amphotericin B at 0.25 or 0.5 mg/kg were not protective, nor was the combination of micafungin and liposomal amphotericin B at 0.25 or 0.5 mg/kg. This study indicated that there was no clear additive effect when subeffective doses were combined. In Figure 1(b), the dose of micafungin was raised modestly to 1 mg/kg, just in the protective range, and liposomal amphotericin B was lowered slightly to 0.15 mg/kg. Micafungin treatment was delayed until 24 h after treatment had begun with liposomal amphotericin B. In this study,
both individual drugs were significantly protective alone ($P \leq 0.05$). The combination was superior to controls, but not to micafungin or liposomal amphotericin B alone.

Tissue burden. In Tables 1 and 2, tissue burdens are shown for mice infected iv with $1.7 \times 10^5$ conidia/mouse. Both liposomal amphotericin B and micafungin were started 1 day after infection (concurrent initiation of therapy). Micafungin at 0.5 mg/kg (study 1) did not significantly reduce tissue counts. Liposomal amphotericin B at 0.5 mg/kg significantly reduced spleen and lung counts. The combination was not superior to liposomal amphotericin B alone. This study was repeated, and lungs were harvested for quantitative cultures. Neither drug was effective, although micafungin had a $P$-value of 0.08 compared with controls (data not shown). These drug doses are close to the margin of efficacy.

The effect of sequencing drugs is also presented in Tables 1 and 2. The inoculum was $1.6 \times 10^5$ conidia/mouse. Drug doses were decreased slightly for liposomal amphotericin B, to 0.3 mg/kg, and
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Table 1. Fungal burdens in spleen of mice infected with A. fumigatus [median (range) log_{10} cfu/spleen]

<table>
<thead>
<tr>
<th>Treatment group (drug doses in mg/kg)</th>
<th>Study 1 (iv): 1.7×10^5 conidia/mouse</th>
<th>Study 2 (iv): 1.6×10^5 conidia/mouse</th>
<th>Study 3 (iv): 2.0×10^5 conidia/mouse</th>
<th>Study 4 (in): 7.2×10^5 conidia/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>3.06 (2.71–4.08)</td>
<td>3.11 (0–5.72)</td>
<td>3.03 (2.41–3.46)</td>
<td>0 (0–0.30)</td>
</tr>
<tr>
<td>Mica 0.5</td>
<td>2.77 (0.30–3.91)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmBs 0.5</td>
<td>0.30 (0.1–0.78)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mica 0.5 + AmBs 0.5</td>
<td>0.84 (0–1.62)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1</td>
<td>–</td>
<td>2.37 (0–3.93)</td>
<td>2.44 (0–4.32)</td>
<td></td>
</tr>
<tr>
<td>AmBs 0.3 D2</td>
<td>–</td>
<td>2.01 (0–2.88)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1 + AmBs 0.3 D2</td>
<td>–</td>
<td>0.30 (0–1.72)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2</td>
<td>–</td>
<td>2.62 (1.38–3.11)</td>
<td>2.65 (1.26–3.89)</td>
<td>0 (0–1.15)</td>
</tr>
<tr>
<td>AmBs 0.3 D1</td>
<td>–</td>
<td>1.65 (0–1.59)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2 + AmBs 0.3 D1</td>
<td>–</td>
<td>0.30 (0–2.08)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>AmBs 0.15 D1</td>
<td>–</td>
<td>–</td>
<td>2.11 (1–4.92)</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1 + AmBs 0.15 D2</td>
<td>–</td>
<td>0.30 (0–1.72)</td>
<td>0 (0–0.90)</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2 + AmBs 0.15 D1</td>
<td>–</td>
<td>1.80 (0–2.32)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2 + Amgs 0.15 D1</td>
<td>–</td>
<td>0.69 (0–2.45)</td>
<td>0 (0–0.90)</td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.05, significant reduction compared with controls.
iv, intravenous infection; in, intranasal infection; Mica, micafungin; AmBs, liposomal amphotericin B; D1, therapy began on day 1; D2, therapy began on day 2.

Table 2. Fungal burdens in lungs of mice infected with A. fumigatus [median (range) log_{10} cfu/lungs]

<table>
<thead>
<tr>
<th>Treatment group (drug doses in mg/kg)</th>
<th>Study 1 (iv): 1.7×10^5 conidia/mouse</th>
<th>Study 2 (iv): 1.6×10^5 conidia/mouse</th>
<th>Study 3 (iv): 2.0×10^5 conidia/mouse</th>
<th>Study 4 (in): 7.2×10^5 conidia/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.59 (0.90–2.32)</td>
<td>2.88 (1.60–3.46)</td>
<td>2.20 (1.48–2.61)</td>
<td>4.07 (0–4.66)</td>
</tr>
<tr>
<td>Mica 0.5</td>
<td>1.04 (0.30–3.11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmBs 0.5</td>
<td>0.15 (0–1.30)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mica 0.5 + Ambs 0.5</td>
<td>0 (0–0.78)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1</td>
<td>–</td>
<td>0 (0–1.30)</td>
<td>1.11 (0–1.95)</td>
<td></td>
</tr>
<tr>
<td>AmBs 0.3 D2</td>
<td>–</td>
<td>0.30 (0–1.90)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1 + AmBs 0.3 D2</td>
<td>–</td>
<td>0 (0–0.30)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2</td>
<td>–</td>
<td>1.87 (0–2.60)</td>
<td>1.19 (0–2.54)</td>
<td>4.08 (3.23–4.58)</td>
</tr>
<tr>
<td>AmBs 0.3 D1</td>
<td>–</td>
<td>0 (0–1.59)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2 + AmBs 0.3 D1</td>
<td>–</td>
<td>0.30 (0–1.15)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>AmBs 0.15 D1</td>
<td>–</td>
<td>0.80 (0–3.34)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>AmBs 0.15 D2</td>
<td>–</td>
<td>0.15 (0–1.71)</td>
<td>1.82 (0–4.95)</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D1 + AmBs 0.15 D2</td>
<td>–</td>
<td>1.33 (0–1.72)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mica 1.0 D2 + AmBs 0.15 D1</td>
<td>–</td>
<td>0.30 (0–2.60)</td>
<td>3.38 (1.46–4.49)</td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.05, significant reduction compared with controls.
iv, intravenous infection; in, intranasal infection; Mica, micafungin; AmBs, liposomal amphotericin B; D1, therapy began on day 1; D2, therapy began on day 2.

increased slightly for micafungin, to 1 mg/kg. Results for spleen are shown in Table 1 (study 2) and results for lungs in Table 2 (study 2). Compared with controls, neither micafungin nor liposomal amphotericin B significantly reduced counts in the spleen. The combination of drugs reduced counts significantly in the spleen (P ≤ 0.05, compared with controls). There appeared to be no advantage when drugs were begun on day 1 compared with day 2 after infection. Lung counts are shown in Table 2. Both micafungin and liposomal amphotericin B were effective in reducing counts ~2 logs (P ≤ 0.05 compared with controls). Liposomal amphotericin B appeared to be equipotent whether begun on day 1 or day 2, but micafungin was significantly more effective when begun on day 1 (study 2). The most potent regimens appeared to be the combinations, but they were not significantly more effective than liposomal amphotericin B alone (P > 0.05). Because of the relatively great potency of liposomal amphotericin B used alone, a further study was conducted with the dose of liposomal amphotericin B reduced to 0.15 mg/kg. The effects of the dose reduction are shown in Tables 1 and 2. Mice were infected with 2×10^5 conidia/mouse (study 3). As shown in Table 1, spleen tissue counts were not reduced by micafungin alone or by liposomal amphotericin B when begun on day 1. Liposomal amphotericin B begun on day 2 did reduce spleen counts significantly (P ≤ 0.05 compared with controls). It is not clear why this was superior to liposomal amphotericin B begun on day 1. Of the combinations, both were
superior to controls, but the combination treatment with liposomal amphotericin B initially, followed by addition of micafungin 1 day later, was superior to micafungin alone and liposomal amphotericin B alone ($P \leq 0.05$ compared with controls or either drug used alone). As shown in Table 2, lung tissue counts were reduced by micafungin and by liposomal amphotericin B when begun on either day 1 or day 2 ($P \leq 0.05$ compared with controls). The combination with micafungin begun on day 1 and liposomal amphotericin B begun on day 2 was significantly more effective than controls, but no more effective than either drug used alone ($P > 0.05$).

A follow-up study was carried out using the same doses of liposomal amphotericin B and micafungin, with initiation of liposomal amphotericin B on day 1, and micafungin delayed until day 2. Mice were infected with $1.7 \times 10^5$ conidia/mouse. In this study, neither liposomal amphotericin B nor micafungin nor combined therapy significantly reduced lung counts below controls (data not shown). In the spleen counts, neither liposomal amphotericin B nor micafungin significantly reduced tissue burden below controls, but combined therapy reduced spleen counts by 2 logs ($P = 0.007$), confirming the results shown in Tables 1 and 2.

**Steroid-treated mice infected intranasally**

**Survival.** Figure 2 presents results of mice pretreated with steroids and challenged intranasally with $2.1 \times 10^7$ conidia/mouse. In this study, initial therapy was liposomal amphotericin B at 0.15 mg/kg beginning day 1, followed by micafungin on day 2 at 1 mg/kg. Liposomal amphotericin B and combined therapy significantly prolonged survival over controls ($P \leq 0.05$) and were equipotent. Micafungin alone was ineffective ($P > 0.05$).

**Tissue burden.** Tables 1 and 2 show study 4 tissue burden results for spleen (Table 1) and lungs (Table 2). Mice were infected with $7.2 \times 10^5$ conidia/mouse, and were treated with either liposomal amphotericin B or micafungin in the same regimens as shown in Figure 2. In this model, tissue burden tends to be high in the lungs and low in the spleen (reflecting delayed dissemination). Neither individual drugs nor combination regimens significantly reduced tissue counts in the lungs or in the spleens. Counts in the spleens were essentially too low to identify any differences. Finally, a tissue burden study was repeated with the same doses of drugs and a slightly higher inoculum of $1.2 \times 10^5$ conidia/mouse. Results were similar to those presented in Tables 1 and 2, and identified no effective regimen (data not shown).

**Discussion**

The studies presented above showed that protection was dependent on multiple variables. First, prolongation of survival could be demonstrated, both in neutropenic animals infected intravenously, and in mice pretreated with corticosteroids, infected intranasally and then treated with liposomal amphotericin B or micafungin and liposomal amphotericin B (Figures 1 and 2). However, in the present study, we showed no survival advantage of combined treatment over liposomal amphotericin B used alone. One limitation of survival studies, looking for additive effect, is the need to use individual drug treatment regimens just at the margin of single drug efficacy. This is required to show that combined therapy is superior to each component. If a single drug gives as much as 50% protection, it is difficult to show improvement of combined therapy over single drugs. Likewise, if more than a few controls survive it is difficult to show protection of treatment groups. This limitation is imposed in part by the limitation of a 2 log difference between 0% and 100% survival. Some of the variability of our studies was the consequence of inoculum doses just a little above or below the optimal dose. Thus, while we found each drug to be effective in prolonging survival in iv-challenged mice, and liposomal amphotericin B effective in intranasally challenged mice, we did not show additive effects.

This is one reason why we chose a model in which we could also use tissue burden as a measure of protection. We chose to measure tissue burdens in the spleen (a target for iv infection) and the lungs (a target for pulmonary infection). When drug therapy was sequenced...
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as shown in Table 1 for iv infection, combined liposomal amphotericin B and micafungin reduced spleen tissue burden significantly compared with controls, whereas neither drug used alone was superior to controls. Initiation of either liposomal amphotericin B or micafungin 1 day before the other drug did not affect results. Liposomal amphotericin B was similarly effective in reducing spleen counts when begun on day 1 or day 2, but micafungin was more effective when begun on day 1. Again, combined therapy was superior to controls, but combinations were not superior to liposomal amphotericin B alone. Sequencing liposomal amphotericin B on day 1 and micafungin on day 2 showed efficacy of the combination over either drug used alone (P ≤ 0.05). However, the benefit was apparent only in spleen tissue counts. Using somewhat different doses of drugs, as shown in Table 1, the best effect was again seen in spleen counts, and again with liposomal amphotericin B begun on day 1 and micafungin on day 2.

Finally, we tried to reproduce these results in mice infected intranasally, and treated with sequenced therapy of liposomal amphotericin B followed by micafungin. At the doses used, liposomal amphotericin B alone showed significant prolongation of survival, but micafungin added nothing. In contrast to studies of intravenously infected mice, tissue burden studies showed no effect of either liposomal amphotericin B or micafungin alone or both drugs together.

The above studies illustrate both the potential and the limitations of in vivo studies. Our goal was to elucidate the conditions, if any, under which additive effects of liposomal amphotericin B and micafungin could be demonstrated. In our hands, the iv model of infection ultimately showed additive protection of combined therapy over both micafungin and liposomal amphotericin B alone, but under restricted circumstances, namely reduction of spleen tissue counts when liposomal amphotericin B was initiated before micafungin. These studies also show the complexity of combination therapy models in vivo, as well as some of the peculiarities of working with echinocandins in filamentous fungal infections. In prior in vitro studies, Kurtz et al. have shown that echinocandins act against the growing hyphal tip of Aspergillus. This activity tends to break each microcolony of fungus into smaller fragments. Bowman et al. have confirmed this with supravital staining of Aspergillus colonies. The bulk of Aspergillus may be killed, but there is always a remnant of viable organisms. These tend to be the ones where no active growth is occurring at the time of drug exposure. This is in contrast to Candida, in which killing is almost complete.

In this setting, in vivo studies of efficacy of echinocandins in aspergillosis take on a relatively greater importance. Petraitiene et al. found that micafungin significantly prolongs survival of pulmonary aspergillosis in neutropenic rabbits, but that tissue colony counts are not reduced by echinocandin therapy. In their rabbit studies, survival was a better indicator of protection than measurement of tissue burden in the lungs.

In the murine model, we have found marked evidence that micafungin prolongs survival in neutropenic mice infected by the iv route (to cause disseminated disease), or steroid treated mice infected by the intranasal route (to cause local pulmonary disease). In this model tissue burden is more useful than in the rabbit. However, tissue burden was reduced only in those mice infected by the iv route. Whether the key determinant is the neutropenia, the route of infection (which targets different organs), or tissue processing is unclear as yet. Quantification of mycelial fungal tissue burden has always been less exact than for yeasts, and the echinocandins may further complicate this in the way that they act. However, we have found that our semi-quantitative cultures correlate well with quantitative PCR for measurement of fungal burden, and believe this method is valid for determining the density of infection.

It is clear that the route of infection (and possibly neutropenia) are not the only factors required to demonstrate additive effects. In our model, additive antifungal drug effect was also dependent upon sequencing of therapy, and was evident only when liposomal amphotericin B was initiated prior to micafungin. Concurrent initiation of both drugs, or beginning micafungin before liposomal amphotericin B, showed no additive effects, but also showed no antagonism. It is not clear whether this sequence predicts optimal clinical responses to combined therapy.

As an additional note, we were encouraged but puzzled to find that our results differ from those of Clemens et al., who found that anidulafungin, given to steroid-pretreated mice, appeared to be antagonized by the steroids. The meaning of this difference may again lie in the methods, or in differences between anidulafungin and micafungin, but we are encouraged that toxicity was not a problem in our intranasal model.

In summary, this study shows some benefit of micafungin and liposomal amphotericin B, but also sounds a note of caution. On the positive side, there were no studies that showed antagonism, and additive effects were confirmed in two studies. On the negative side, the relevance of these studies to clinical, almost always pulmonary, aspergillosis is undefined. Careful sequencing of drugs, beginning with liposomal amphotericin B and later adding micafungin, may not be a realistic clinical strategy. In addition, it is not clear whether our demonstration of additive effects in spleen counts has any relevance to pulmonary disease, which is present in virtually all patients with acute IA. Defining the additive benefits of combination therapy with echinocandins and liposomal amphotericin B is likely to require clinical trials. The present studies show no reason not to conduct such trials, and suggest that there may be a benefit. Some physicians have already begun to use combinations of echinocandins and triazoles or polyenes for treatment of IA. The present study suggests that a more cautious approach should be adopted before this practice is widely adopted.

References

8. Warn, P., Morrissey, G., Morrissey, J. et al. (2003). Activity of micafungin (FK463) against an itraconazole-resistant strain of Aspergil-
J. R. Graybill et al.


