Therapeutic and toxicologic studies in a murine model of invasive pulmonary aspergillosis

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Invasive pulmonary aspergillosis remains problematic in immunocompromised patient populations. We studied potential therapeutic options in a murine model of pulmonary aspergillosis in triamcinolone-suppressed DBA/2 mice infected intranasally with conidia from Aspergillus fumigatus. Mice were treated with liposomal-amphotericin B (AmBi; AmBisome), lipid-complexed amphotericin B (ABLC; Abelcet), voriconazole (VCZ), micafungin (MICA), caspofungin (CAS) or deoxycholate amphotericin B (AMBd) given alone or in combination. Monotherapy with AmBi, ABLC, AMBd, CAS or MICA had activity in prolonging survival; however, only AMBd or CAS reduced fungal burden in the lungs and kidneys. Combinations of AmBi plus CAS or MICA prolonged survival, but were not better than monotherapy. VCZ was ineffective and AMBd plus CAS showed a possible antagonism. AmBi or ABLC at higher dosages, or loading-doses of AmBi resulted in reduced survival. Histopathology showed increased incidence of serious renal and mild hepatic toxicity in triamcinolone-treated mice given an amphotericin B regimen compared to no or only triamcinolone (minimal renal changes occurred with CAS or VCZ with or without triamcinolone); suggestive of combined toxicity of triamcinolone and the amphotericin B in AmBi or ABLC. Infected treated mice showed progressive pulmonary disease including abscesses, angioinvasion and abundant intraleisonal fungi. High loading-doses of AmBi were associated with nephrosis and damage to other tissues. No monotherapy or combination regimen showed superiority for the treatment of pulmonary aspergillosis in corticosteroid-suppressed mice and the potential for combined drug toxicity was enhanced in these mice. High dosages of lipid-formulated amphotericin B also proved unsatisfactory. Additional studies are needed to evaluate improved treatment.

Keywords invasive pulmonary aspergillosis, Aspergillus fumigatus, amphotericin B, lipid-carried amphotericin B, murine models

Introduction

Invasive pulmonary aspergillosis remains a highly fatal complication in several immunosuppressed patient populations. Therapy is not optimal in spite of the recent addition of voriconazole and echinocandins to the therapeutic choices [1]. These results have spurred some clinicians to attempt the use of combination therapy, which has shown various degrees of success [1]. However, since controlled clinical trials are difficult to perform, studies involving animal models of aspergillosis have been performed to provide a rational basis for the use of combination therapy.

In recent studies we reported that a combination of lipid-formulated amphotericin B and voriconazole (VCZ) showed enhanced efficacy against central nervous system (CNS) aspergillosis in neutropenic mice [2–4] and that...
some combination therapies were active in a model of systemic aspergillosis in non-immunosuppressed mice [5]. However, similar studies have not been done in a model of invasive pulmonary aspergillosis. We undertook the current investigations to examine the comparative efficacies of several antifungal agents, as well as their toxicologic and histologic aspects, to determine whether there was a basis for the use of combination therapy.

We used a murine model of pulmonary aspergillosis in DBA/2 mice that were immunosuppressed with the glucocorticoid triamcinolone [6]. The C5 deficiency in DBA/2 mice combined with steroids allows a wide range of challenge inocula to be explored [6]. This model simulates patients on high or prolonged dose glucocorticoid therapy, which is a risk factor for the development of invasive pulmonary aspergillosis [1,7]. The model is a rigorous test for antifungal efficacy and has demonstrated a reduced activity of various antifungals in comparison with their activity and effectiveness in nonsuppressed or neutropenic animals (reviewed in [8,9]). Specifically, we have found using this model that conventional deoxycholate amphotericin B (AMBd) in combination with itraconazole was ineffective and possibly antagonistic [10], as well as poor results with micafungin (MICA) alone or in combination with nikkomycin Z [6].

We report here our results examining echinocandins, voriconazole and lipid-formulated amphotericin B preparations for dose-escalating efficacy, combination efficacy and subacute toxicities.

Materials and methods

Murine model of pulmonary aspergillosis

A murine model of pulmonary aspergillosis was studied as described previously [6]. In brief, 8-week-old male DBA/2 mice were immunosuppressed by using triamcinolone acetonide (1 mg/mouse) given subcutaneously 1 day prior to infection (day -1). On day 0, mice were infected with conidia of Aspergillus fumigatus (10AF) given intranasally. Therapy began on day 1 following infection, and was given for 10 consecutive days. Groups of 10 mice received D5W, CAS or MICA at 8 mg/kg, VCZ at 16 mg/kg, AMBd at 0.8 mg/kg, AmBi or ABLC at 16 mg/kg, 8 mg/kg, or 0.8 mg/kg. Based on our prior data on half-lives, CAS and MICA were given in all studies twice daily by intraperitoneal injection (split dose), VCZ was provided once daily by gavage, and D5W, AMBd, AmBi and ABLC were given intravenously (i.v.) once daily [2–6,10]. CAS and MICA were given in 0.1 ml saline per dose, VCZ was provided in 0.1 ml in 4% PEG 400 and AmBi and AMBd were given in 0.2 ml in D5W. The dosage regimens selected for the echinocandins and VCZ were those previously studied and were efficacious and nontoxic in the CNS and systemic aspergillosis models [2–5], whereas the amphotericin B drugs were explored in various dose-finding regimens.

Combination therapy studies

Using drug dosages chosen from the monotherapy studies, the efficacy of various combinations was examined. On day 0, mice were infected with 2.52 × 10⁴ viable conidia/mouse of A. fumigatus (10AF) given intranasally. Therapy began on day 1 following infection, and was given for 10 consecutive days. Groups of 10 mice received D5W, CAS or MICA at 8 mg/kg, VCZ at 16 mg/kg, AMBd at 0.8 mg/kg, AmBi at 8 mg/kg, AmBi + MICA, AmBi + CAS, AMBd + CAS, AmBi + VCZ, or AmBi for 3 d followed by VCZ for 7 d.

Rescue of early progression of experimental pulmonary aspergillosis

We examined the potential of using initially high loading doses of AmBi as a way to enhance efficacy compared to a constant daily dosage of AmBi, or AMBd. On day 0, mice were infected by intranasal instillation with 2.2 × 10⁴ viable conidia/mouse of A. fumigatus (10AF). Therapy was begun on day 1 following infection, and given for 10 consecutive days. Groups of 10 mice received D5W, AmBi at 4 or 8 mg/kg, or AmBi at 24 mg/kg or 32 mg/kg given for 1, 2 or 3 doses followed by AmBi at 8 mg/kg for the remaining doses.

On day 12 postinfection, all surviving mice were bled while under anesthesia and then euthanatized. The liver
and heart, as well as a sample of the lungs and kidneys were removed for histological studies.

**CFU determinations**

To further assess treatment efficacy, the fungal burden remaining in the tissues was determined in some studies. In brief, surviving mice were euthanized by CO₂ anoxia and colony forming units (CFU) remaining in the kidneys and lungs were determined by quantitative plating of organ homogenates on Sabouraud dextrose agar (SDA) with 50 mg chloramphenicol per liter as described previously [6,11–13].

**Subacute toxicity**

The comparative toxicities of AmBi, ABLC, CAS and VCZ in uninfected mice pretreated with triamcinolone to emulate the immunosuppressed status of the mice used in the pulmonary model of aspergillosis were examined. MICA-treated mice were not studied for toxicity. Groups contained five 8-week-old male DBA/2 mice for each treatment arm. Eleven groups of mice received 1 mg of triamcinolone subcutaneously and another 11 groups of mice received no glucocorticoid pretreatment. Treatment began 2 days after the triamcinolone treatment. Mice were given one of the following regimens, by the routes stated, with or without triamcinolone treatment: no treatment control; AMBd at 0.8 mg/kg; AmBi 0.8, 8 or 16 mg/kg; ABLC 0.8, 8 or 16 mg/kg; VCZ at 40 mg/kg (another dose studied previously [2,3]); or CAS at 0.8 or 8 mg/kg.

Two days after the cessation of therapy mice were anesthetized, exsanguinated for serum collection and the liver, kidney and brain placed in 10% buffered formalin for histological examination. Serum chemistry determinations were performed at Idexx Laboratories, West Sacramento, CA.

**Statistical analyses**

Survival of the various treatment groups was compared by log rank test. CFU comparisons were done by Mann-Whitney U test with an arbitrary value of 5 assigned to data points missing due to the death of the animals [14,15], which assures that death is considered as a worse outcome than is survival with any burden; no correction was applied to account for multiple comparisons [16].

**Results**

**Monotherapy studies**

These studies were performed to assess the comparative efficacies of AmBi, ABLC, AMBd, MICA, CAS and VCZ for the treatment of experimental pulmonary aspergillosis. Assessment of survival shows that 70% of control mice succumbed to infection. In comparison, 40–80% of treated mice succumbed to infection (Fig. 1). Possible toxicity was observed with ABLC at 16 mg/kg, as these animals died sooner than did D5W-treated controls (Fig. 1). No significantly efficacious dose was found for any of the formulations tested. However, 16 mg/kg of AmBi, 8 mg/kg of ABLC, 0.8 mg/kg of AMBd, 8 mg/kg of MICA or CAS and 16 mg/kg of VCZ appeared to be modestly active.

No clear trend in reduction of fungal burden in the lungs or the kidneys of surviving animals was noted (data not shown); the lack of efficacy in the prolongation of survival left too few survivors to give meaningful data. It should be noted that both echinocandins at 8 mg/kg cleared five of five surviving mice of CFU in the kidney and three or four of five surviving mice of CFU in the lungs. VCZ at 16 or 32 mg/kg cleared four of six and three of four surviving mice of CFU in the kidney and four of six and two of four surviving mice of CFU in the lungs, respectively. Overall, none of the formulations had significant efficacy at the doses tested against pulmonary aspergillosis in this model.

**Subacute toxicity studies**

Although the initial study of monotherapy showed no significant efficacy for any of the drugs tested, a favorable effect was suggested from the survival data. Thus, we sought to determine whether subclinical drug toxicity was occurring and whether there was a possible cost to the tissues to attain the modest efficacy that was observed. This was studied in uninfected animals that were or were not pretreated with triamcinolone to judge potential combined effects of steroid pretreatment and antifungal therapy. Liver, kidney, and lung sections were evaluated microscopically from animals that survived to the scheduled sacrifice. Mortality occurred in some groups of triamcinolone-treated animals, which limited the histopathology evaluation (Table 1). This was particularly evident with groups given 8 or 16 mg/kg of AmBi or ABLC, with three to five of five animals dying prior to the end of the study; no mice given the 0.8 mg/kg doses of AmBi or ABLC died. Two or fewer mice died in groups given other regimens. In contrast, no mice died in any of the groups that had not been pretreated with triamcinolone.

Histological assessment of the kidneys from mice treated with AMBd, AmBi, or ABLC, alone or with triamcinolone treatment, showed an association with minimal to moderate nephrotoxicity. ABLC-treated animals had the highest incidence of renal tubular changes and the most severe findings, AMBd-treated animals had a slightly lower incidence and severity, while AmBi was associated with the least (Table 2). The microscopic findings were confined
to the cortex and affected only the convoluted tubules. Tubular changes included one or more of the following: tubular regeneration, degeneration/dilation, mineralization, cysts, protein and granular cast formation. The most extensive renal tubular damage was identified in the 16 mg ABLC per kg treatment group and involved large areas of the cortex. The term nephrosis was used to describe these more extensive tubular lesions in which abundant regenerative and lesser numbers of dilated degenerative tubules replaced normal tubules (Fig. 2A). Dilated tubules were often filled with protein and granular casts. Rare individual necrotic tubular epithelial cells and mineral deposition were present. Similar tubular findings, in low incidence and minimal severity, were identified sporadically in AmBi treatment groups. Significant renal tubular damage was identified in one control animal treated with triamcinolone alone. This was possibly due to a subclinical infectious process, yet organisms were not observed in the tissues.
only damaged, regenerating tubules remained. Correlating with the histopathology, clinical chemistry changes were identified in the ABLC 16 mg/kg treatment group only, i.e., an increase in average blood urea nitrogen (BUN) and creatinine, as compared to controls, consistent with decreased renal function (Table 1), with all other treatment group values are considered to fall within normal ranges. In the liver, AmBi or ABLC treatment, with or without triamcinolone immunosuppression, was associated with minimal to mild hepatocellular necrosis and inflammation and diffuse Kupffer cell hyperplasia and vacuolization (Fig. 2B). At low dose (0.8 mg/kg/day), hepatocellular necrosis and inflammation was identified in greater incidence in AmBi versus ABLC treatment groups, but the severity was similar (minimal to mild). Hepatocellular necrosis/inflammation was considered an adverse effect, whereas Kupffer cell hyperplasia/vacuolization was believed to be an adaptive response due to activation and accumulation of scavenged drug [17] in the hepatic reticuloendothelial system and not a toxic effect of drug treatment. The Kupffer cell hyperplasia/vacuolization increased in incidence and severity in a dose-dependent fashion with both AmBi and ABLC treatment. AmBi and ABLC were also associated with rare vascular changes identified in the liver including microthrombi and histiocytic emboli in central veins; the significance of which was unclear. Subtle increases in serum activities of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) levels were detected in several groups. However, a direct correlation between individual animals with increased liver enzymes and those with histological findings was not evident (data not shown). Additional vascular findings were identified in the lung in animals given AmBi at 0.8, 8.0 or 16 mg/kg, ABLC at 16.0 mg/kg, or CAS at 0.8, 8.0 or 16 mg/kg (see Table 2). Overall by histology, AMBd, AmBi and ABLC treatment alone or with triamcinolone treatment were associated with nephrotoxicity and hepatotoxicity. Hepatotoxicity was identified in relatively equal incidence and severity in treated mice with or without triamcinolone pretreatment. A finding associated with triamcinolone treatment was the presence of random, sporadic, chronic inflammatory lesions in the liver, kidney or lung, which often contained intra-lesional bacterial colonies, consistent with secondary opportunistic bacterial infections. The secondary infections were identified in relatively low incidence, in one third of the mice examined and were considered to be incidental findings; no mouse was noted to have these lesions in more than a single organ. However, only usual target tissue samples were collected for histopathological evaluation and serum chemistry analysis. Since no tissues

Table 1  Survival and serum chemistries of surviving uninfected DBA/2 mice given no triamcinolone (triam) or pretreated with triamcinolone followed by 10 consecutive days of antifungal therapy.

<table>
<thead>
<tr>
<th>Group – DBA/2 mice</th>
<th>Day of death</th>
<th>No. mice alive/no. mice total</th>
<th>BUN2 mg/dl mean ± SD</th>
<th>Creatinine3 mg/dl mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment control</td>
<td>None</td>
<td>5/5</td>
<td>29.8 ± 2.3</td>
<td>0.38 ± 0.1</td>
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<tr>
<td>AMBd 0.8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>28.8 ± 4.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>AmBi 0.8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>25.4 ± 1.9</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>AmBi 8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>28.8 ± 5.0</td>
<td>0.36 ± 0.1</td>
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<tr>
<td>AmBi 16 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>29 ± 4.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>ABLC 0.8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>23 ± 3.7</td>
<td>0.44 ± 0.2</td>
</tr>
<tr>
<td>ABLC 8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>20 ± 2.8</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>ABLC 16 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>42.8 ± 14.2</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>VCZ 40 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>22.2 ± 1.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>CAS 0.8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>22.4 ± 5.7</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>CAS 8 mg/kg</td>
<td>None</td>
<td>5/5</td>
<td>22.5 ± 6.2</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>No treatment control + triam</td>
<td>12</td>
<td>4/5</td>
<td>21.3 ± 4.5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>AMBd 0.8 mg/kg + triam</td>
<td>9</td>
<td>4/5</td>
<td>14.3 ± 6.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>AmBi 0.8 mg/kg + triam</td>
<td>None</td>
<td>5/5</td>
<td>15 ± 5.6</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>AmBi 8 mg/kg + triam</td>
<td>8, 12, 12</td>
<td>2/5</td>
<td>22.5</td>
<td>0.3</td>
</tr>
<tr>
<td>AmBi 16 mg/kg + triam</td>
<td>4, 4, 5, 6</td>
<td>1/5</td>
<td>28</td>
<td>0.4</td>
</tr>
<tr>
<td>ABLC 0.8 mg/kg + triam</td>
<td>None</td>
<td>5/5</td>
<td>13 ± 4.5</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>ABLC 8 mg/kg + triam</td>
<td>4, 5, 5, 5, 8</td>
<td>0/5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ABLC 16 mg/kg + triam</td>
<td>3, 3, 4, 4, 5</td>
<td>0/5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VCZ 40 mg/kg + triam</td>
<td>8, 12</td>
<td>3/5</td>
<td>29.7 ± 3.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CAS 0.8 mg/kg + triam</td>
<td>9, 10</td>
<td>3/5</td>
<td>22.6 ± 1.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CAS 8 mg/kg + triam</td>
<td>12</td>
<td>4/5</td>
<td>19.7 ± 2.6</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

1The day of death is given, with the day counted as if this was an infection model. Antifungal therapy began on day 1 and was given through day 10. All surviving mice were exsanguinated under anesthesia and euthanatized on day 14. Serum was collected for assay of BUN and creatinine.
2BUN = blood urea nitrogen.
3Creatinine in serum.
were collected for bacteriology, the incidence of secondary bacterial infections may be higher than identified.

**Combination therapy**

Because the monotherapy study showed that no treatment tested provided significant protection, the doses chosen for this study were the high doses from the previous study, except for VCZ, for which the 16 mg/kg rather than 32 mg/kg dosage was used, due to better survival found in the monotherapy study with the lower dosage.

The survival of the treated animals is presented in Fig. 3. These results showed that among the various treatment regimens only AMBd, MICA, CAS and AmBi/Hi11001 MICA or AmBi/Hi11001 CAS significantly prolonged survival ($P < 0.03–0.0003$, dependent on comparison) (Fig. 3). AMBd or CAS alone proved the most effective regimens. Analysis of the survival curves indicates the evolution of disease with this challenge was somewhat less severe than in the previous study (e.g., 50% death in the control group occurred one day earlier in the first study, in what already is a rapidly progressive infection model), and this difference may explain the improved performance of AMBd and the echinocandins. AmBi alone was ineffective and inferior to AMBd or CAS alone ($P < 0.02–0.05$, dependent on comparison). No combination regimen was superior to both of the respective monotherapy regimens. Interestingly, AmBi + CAS was superior to AMBd + CAS ($P < 0.05$). The combination of AMBd + CAS appeared antagonistic as AMBd or CAS alone was superior to the combination ($P < 0.005–0.002$, dependent on comparison). No regimen with VCZ showed significant efficacy.

The recovery of CFU from the organs of surviving mice showed that only AMBd or CAS alone caused significant reduction of the fungus in lungs and kidneys ($P < 0.03$).
No significantly enhanced efficacy was found with any combination regimen. Overall, the results of the combination therapy indicate that no combination regimen tested showed any significantly enhanced efficacy compared to the monotherapy. AMBd + CAS appeared worse than either drug alone. VCZ was ineffective alone or in combination with AmBi, in contrast to results in the CNS model [2,3].

**Rescue from early progression**

The premise of this experiment was to determine whether a monotherapy regimen of AmBi could be defined that was significantly efficacious, unlike the previously tested monotherapy regimens. This was based on the observation made as part of a combination therapy study that all surviving mice given AmBi at 8 mg/kg were free of detectable infection in the lungs. This suggested that the initial dosage of AmBi was inadequate to protect some animals, but that if the animal survived through several days of dosing it was possible to attain cure in the lungs. This potential was determined by assessing very high-dose loading regimens of differing durations followed by a constant dose monotherapy regimen.

None of the regimen configurations of dose and duration were found to be superior to the diluent control treatment for the prolongation of survival (see Fig. 4). As shown in the survival curves, no AmBi regimen prolonged survival over that of the D5W-treated controls ($P > 0.05$). Mice given D5W survived significantly longer than did those given AmBi at 32 mg/kg for 1 or 3 days followed by AmBi at 8 mg/kg for the remaining time of treatment ($P = 0.013$, and 0.014, respectively). In the two prior experiments, AmBi at 8 mg/kg was not significantly different from the control, whereas in the present investigation it was slightly worse ($P = 0.04$). In comparison with AmBi at 8 mg/kg there were no differences in survival versus groups receiving one of the loading dose regimens that began with AmBi at 24 or 32 mg/kg followed by AmBi at 8 mg/kg. However, in comparison with AmBi at 4 mg/kg, mice given AmBi at 32 mg/kg for 1 day followed by 9 days of AmBi at 8 mg/kg, AmBi at 32 mg/kg for 3 days followed by AmBi at 8 mg/kg for 7 days, and AmBi at 8 mg/kg only, died significantly earlier ($P = 0.007$, 0.003 and 0.024, respectively). These data were indicative that higher doses of AmBi were inducing toxicity, since AmBi-treated mice died sooner than did D5W-treated controls or lower dose AmBi regimens.

The results of the CFU determinations were difficult to interpret since so few animals survived. However, a trend toward fewer CFU in animals given AmBi at 4 mg/kg as compared with those given D5W was noted and may be indicative of some efficacy, but requires additional study (data not shown).

Various tissues from mice given one of the high-dose regimens were examined histologically to determine whether toxicities due to the loading doses of AmBi might be manifest in the tissues. Examination of tissues from D5W-treated controls showed invasive fungal pneumonia with vascular invasion, thrombosis, infarction, hemorrhage, edema, and abscess formation in the lungs (Fig. 5). Nephrosis was not observed in control animals. Interestingly, all D5W-treated animals showed evidence of epicardial mineralization and some renal dissemination of the fungal disease. Similarly, the majority of the animals treated with one of the AmBi regimens, except those given 32 mg/kg for 2 days followed by 8 mg/kg, showed fungal pneumonia with invasive vascular disease of the lungs (Fig. 5). The lack of fungal pneumonia in the lungs of the group given AmBi at 32 mg/kg for 2 days followed by AmBi at 8 mg/kg was corroborated by the CFU data (not shown).
Fig. 3  Cumulative survival of treated DBA/2 mice infected with pulmonary aspergillosis and given mono or combination therapy (indicated, e.g., as ‘AmBi/MICA’). The top panel shows survival of mice given combination therapy of AmBi with MICA in comparison with each individual therapy. The middle panel shows survival of mice given AMBd or AmBi combination therapy with CAS, as well as the individual therapies. The bottom panel shows survival of mice given AmBi combination therapy with VCZ; AmBi(3)VCZ(7) refers to sequential therapy consisting of 3 days of treatment with AmBi at 8 mg/kg followed by 7 days of therapy with VCZ at 16 mg/kg. Drug abbreviations are the same as those in the legend for Fig. 1.
Minimal to moderate nephrosis was observed in all treatment groups (Table 3), except in animals given AmBi at 4 mg/kg. Histologically, all treatment regimens inhibited dissemination of aspergillosis to the kidneys. Minimal to mild hepatotoxicity manifested as random areas of necrosis and inflammation was observed only in

Mineralization of the epicardium was found in all treated animals, comparable to that identified in the controls.
Fig. 5   Invasive fungal pneumonia in control and treated animals. Panel (A): Acute necrotizing pulmonary inflammation, hemorrhage, edema, vascular thrombosis (T), and abscess formation (A) in a control animal, bar = 300 μm, H&E stain. Panel (B): Higher magnification of peribronchiolar abscess (A) involving blood vessel (arrows delineate vascular wall), Bar = 60 μm, H&E stain. Panel (C): Higher magnification of vascular thrombosis (T), Bar = 60 μm, H&E stain. Panel (D): Gridley stain demonstrates abundant intravascular fungal hyphae. Arrows delineate vascular wall. Internal elastic lamina stains magenta with Gridley stain, Bar = 60 μm. Panel (E): Animal from high dose treatment group (AmBi 24 mg/kg (days 1, 2, and 3) + 8 mg/kg (days 4–10) with similar necrotizing pulmonary inflammation and focal abscess formation to that in controls in Panel 5A), Bar = 300 μm, H&E stain. Panel (F): Higher magnification of vascular thrombosis (T) and marked tissue necrosis within fungal abscess, Bar = 60 μm H&E stain. Panel (G): Gridley stain demonstrates vascular fungal invasion. Arrows delineate vascular wall, Bar = 60 μm.

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animals given an AmBi regimen (Table 3) except those given AmBi at 24 mg/kg for 1 day followed by AmBi at 8 mg/kg. Additional findings associated with AmBi treatment included rare venous thrombi within central veins, the significance of which was unclear. Kupffer cell hyperplasia and vacuolization was identified consistently in all treated animals and increased in severity in a dose-dependent fashion with increased loading dose. This finding was consistent with that seen in the subacute toxicity study. Of note, AmBi is readily phagocytosed and accumulates in hepatic reticuloendothelial cells [18]. Some tissue samples had localized sites of secondary infection due to bacteria observed in the kidneys, liver or heart.

**Discussion**

The current studies were performed to evaluate dose-escalating monotherapies, as well as various combination therapies as to their efficacy in treating experimental murine pulmonary aspergillosis in a glucocorticoid suppressed host. Because high-dose steroid use is considered a significant risk factor for development of invasive aspergillosis, particularly for hematopoetic cell transplant patients [19], we felt it important to study drug efficacies in a model reflecting immunosuppression using a glucocorticoid. Overall, our results indicate that none of the antifungal drugs tested as monotherapy consistently proved to be significantly efficacious. This includes VCZ, which is suggested to be a favored drug for aspergillosis based on a randomized clinical trial [1]. However, most of the patients in that trial had neutropenia, usually transient, as their major risk factor, and aggressive disease in steroid-treated mice (who are also complement-deficient) or humans may represent an even more formidable obstacle.

Combination therapy using AmBi plus CAS or MICA proved effective to some extent, but no combination proved significantly better than both of the respective monotherapies. These data are similar to our previous studies using this model [6], as well as those of other investigators examining aspergillosis in different animal models, where only modest or no benefit was observed using a combination approach [20–24]; other studies have demonstrated antagonism in the use of some combinations [10,25,26]. In contrast, we and others have shown a significant benefit to combination therapy in some models of experimental aspergillosis [3,5,8,27,28]. Thus, the benefit of combination therapy may be reliant on the site of infection and type of model used.

The outcomes of our monotherapy studies, although they did not demonstrate significant differences, were suggestive that higher dosages of AmBi, CAS or MICA did have some effectiveness and that echinocandin monotherapy regimens were effective in the combination therapy studies. However, we questioned whether there was a cost to the animal in
terms of subclinical toxicity in the tissues that could have a deleterious effect. All three amphotericin B preparations were associated with renal and hepatic damage. The histopathology in the rescue studies further suggested the renal and hepatic toxicity associated with increasing the amphotericin B dose explains the accelerated mortality seen with most of these regimens. When comparing toxicity of these drugs in rodents to those in humans, some consideration should be given to the therapy duration relative to the total lifetime of a mouse, which is much greater than the relative lifetime of a human with ordinary human dosing of these agents. In addition, some of the doses used exceeded, in mg/kg, doses used in humans.

The combination of rapidly progressing acute infection and drug toxicities make interpretation of the efficacy results difficult. We have noted in studies with blastomycosis that an increased frequency of lethal AMB toxicity occurred in mice (without steroid treatment) that had severe disease, but no lethal toxicity occurred in animals that were uninfected or had milder infection [29]. Furthermore, we have previously reported the unexpected toxicity of an echinocandin, LY303366 (anidulafungin) in glucocorticoid-treated mice [30]. We earlier reported similar results with MICA [6], and our data here suggest a similar interaction for CAS, apparently making this combination toxicity a drug class effect, at least in mice. Thus, the apparent variations in the efficacy of each antifungal we tested may be due, in part, to toxicities, which may be influenced further by the severe nature of infection.

The renal and hepatic damage we described was not associated with triamcinolone-treatment. However, lethal toxicity occurred in uninfected triamcinolone-treated DBA/2 mice given either AmBi or ABLC at 8 or 16 mg/kg, indicative of a synergistic toxicity between the glucocorticoid and amphotericin B which possibly lead to increased nephrotoxicity. Whether this is similar to the synergistic renal toxicity of cortisone and amphotericin B reported by Kisch et al. [31], or is related to cardiac effects, similar to the reversible cardiac enlargement reported in patients [32], remains to be determined. It is possible that the mineralization observed histologically is also related. However, this is a common observation in the tissues of DBA/2 mice and thus could be a confounding factor [33,34]. The observation of inflammatory lesions, presumed to be possible secondary bacterial infection in some mice, could lead one to consider that deaths were a result of these infections. However, as noted in the results, uninfected triamcinolone-treated mice given AmBi or ABLC at the highest dose died earlier than did those given a lower dose, which would be indicative of a dose-responsive toxicity rather than death due to infection.

There have been several reports that the pathogenesis of pulmonary aspergillosis is influenced by whether the host is neutropenic or has been immunosuppressed with glucocorticoids. It has been suggested that glucocorticoid-treatment of animals results in an over-exuberant cellular response composed primarily by PMNs, which are responsible for tissue damage, and few fungal elements despite inocula of up to $10^7$ conidia. These results suggest mortality is, in part, due to the host’s own response, rather than the infecting Aspergillus [35–38]. In contrast, neutropenic animals have been reported to have necrotizing pulmonary lesions with a limited cellular infiltrate, extensive hyphal growth, angioinvasion and tissue damage [35–37].

Histologic examination of tissues from infected animals with or without antifungal therapy (rescue from early progression) does not fit the hypothesis that mortality of the steroid-treated mice is due to inflammatory response rather than infection. We demonstrate both extensive severe inflammation and necrosis, and abundant intra-lesional fungi. The lesions were quite impressive with abundant necrosis, thrombosis, vascular damage, supplicative inflammation and numerous elongated branching fungal hyphae. Abscesses containing fungal hyphae were forming, but without much of a phagocytic cell effect. Any of the above findings could have killed the animals. There are primary differences in experimental design from the published studies of others, as we use DBA/2 mice (C5- deficient), single-dose immunosuppression with triamcinolone, which has less mineralocorticoid activity than other corticosteroids used by other investigators, and an inoculum of only $2 \times 10^4$ conidia.

The studies done in mice given high multiple dosages of cortisone acetate (10 mg per mouse per dose) by Balloy et al. [35], do not address the possible contribution of the mineralocorticoid effects of the cortisone, which could cause hypokalemia and subsequent arrhythmias and contribute to the early mortality of the infected animals; nor were preventative measures taken against secondary bacterial infections. The mineralocorticoid effects of cortisone may be quite important as 4- to 20-fold lower doses (0.5–2.5 mg) of cortisone caused a 22–41% rise in systolic blood pressure within 24 h, as well as myocardial and renal lesions, liver necrosis and lipodisosis, and pulmonary congestion with a diffuse pneumonia in mice [39]. Thus, it is possible that the previous investigators’ use of cortisone acetate influenced the outcome and overall histological picture of the models performed and could be a topic of further study.

Overall, the results of our various studies indicate that therapy of pulmonary aspergillosis in glucocorticoid-suppressed DBA/2 mice is a difficult task. All of the compounds tested showed at least some activity, yet combinations were not beneficial. Dose-escalation or the use of loading doses of AmBi or ABLC were not beneficial, further corroborating our previous work in a CNS model.
of murine aspergillosis indicating that equivalent results can be attained with lower and potentially less toxic doses [2,3]. These conclusions are similar to those reached in clinical trials of low versus high dose AmBi for aspergillosis [40]. The rapid progression to death in the model, and the initiation of therapy when the infection is already aggressively advancing, may make demonstration of clear-cut efficacy more difficult. Another, future, approach may be to initiate treatment at an earlier time, analogous to that in the slower disease evolution that occurs in many patients. Many combinations were studied by us, but it was not feasible to test all possible combinations. For example, azole-echinocandin combinations could be a subject of future study. The histological assessment of the animals showed that unlike other steroid-suppressed pulmonary models of murine aspergillosis [35,38], our model resulted in demonstrable invasive pulmonary disease, thus showing its utility. Response rates reported for the various antifungal drugs in various patient populations that included pulmonary disease were as low as 20% for AMBd, 35% for lipid-formulated amphotericin B’s, 45% for echinocandins, and 50% for voriconazole in some series [41–44]. Interestingly, the efficacy shown by these antifungal drugs in our model gave similar results, suggesting that this model is reflective of drug efficacy in patients.

Acknowledgments

We thank M. Martinez and J. Capilla for their assistance during the course of these studies.

Funding

These studies were funded in part by a grant from Gilead Sciences, and also received partial support from the Foundation for Research in Infectious Diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the manuscript.

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This paper was first published online on Early Online on 9 May 2011.