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Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rat model of pulmonary aspergillosis

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The efficacy of AmBisome, a liposomal formulation of amphotericin B, was compared with that of Fungizone (amphotericin B desoxycholate), in a rat model of unilateral, pulmonary aspergillosis. Repeated administration of cyclophosphamide resulted in persistent, severe, granulocytopenia. The left lung was inoculated with a conidial suspension of *Aspergillus fumigatus*, thus establishing an unilateral infection. Antifungal treatment was started 40 h after fungal inoculation, at which time mycelial disease was confirmed by histological examination. Both Fungizone 1 mg/kg and AmBisome 10 mg/kg resulted in increased survival in terms of delayed as well as reduced mortality. Quantitative cultures of lung tissue showed that only AmBisome 10 mg/kg resulted in reduction of the number of fungal cfus in the inoculated left lung. Compared with Fungizone, both AmBisome 1 mg/kg/day and AmBisome 10 mg/kg/day significantly prevented dissemination from the infected left lung to the right lung. In addition, both AmBisome regimens reduced hepatosplenic dissemination, and the 10 m/kg dosage fully prevented this complication. In conclusion, when compared with Fungizone, in this model AmBisome is more effective in reducing dissemination of unilateral, pulmonary aspergillosis, even when given in relatively low dosage. Such low dosages may have a place in prophylactic settings.

Introduction

Aspergillus spp. are opportunistic fungi, giving rise to pulmonary and other invasive infections in immunocompromised patients (Rinaldi, 1983; Gerson *et al.*, 1985; Ruutu *et al.*, 1987; Denning & Stevens, 1990; McWhinney *et al.*, 1993). The number of invasive aspergillus infections is steadily increasing (Denning *et al.*, 1991; McWhinney *et al.*, 1993; Khoo & Denning, 1994). This is due to growing numbers of susceptible patients, especially those with prolonged granulocytopenia after aggressive chemotherapy. Despite considerable toxicity amphotericin B is still the most effective agent to treat these infections. However, the efficacy of this drug is rather disappointing in persistently granulocytopenic patients in whom mortality rates up to 100% have been observed (Denning & Stevens, 1990).

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Encapsulated into liposomes amphotericin B has considerably diminished toxicity, and can be administered in much higher dosages (Lopez-Berestein *et al.*, 1985; Meunier, Prentice & Ringden, 1991; Proffitt *et al.*, 1991; de Marie, Janknegt & Bakker-Woudenberg, 1994; Mills *et al.*, 1994). The administration of higher dosages may result in improved efficacy, as the antifungal activity of amphotericin B is concentration dependent. We compared the efficacy of amphotericin B deoxycholate (Fungizone) with that of liposomal amphotericin B (AmBisome) in an animal model of pulmonary aspergillosis that closely mimics human disease.

Materials and methods

Animals

Female R strain albino rats, which were pathogen free, 18–25 weeks old and weighed 185–235 g (bred at REPGO-TNO, Rijswijk, The Netherlands), were used for all experiments. Both the control group and each of the three treatment groups consisted of 15 animals. Animals received a normal, pathogen free diet and water *ad libitum*.

Induction of granulocytopenia and supportive care

According to a slight modification of the method described by Roosendaal *et al.* (1991), persistent granulocytopenia was induced by one dose of cyclophosphamide 90 mg/kg intraperitoneally five days before fungal inoculation, followed by repeated doses of cyclophosphamide 60 mg/kg at 1 day before and 3, 7 and 11 days after inoculation. The doses of cyclophosphamide were adjusted for body weight changes during the experiment. Before the experiments with amphotericin B this scheme was tested in a group of six animals. Quantitation of blood leucocytes was done in blood samples obtained by orbital puncture under light CO_2 anaesthesia. Total leucocyte counts were determined by dilution of these samples in Türk solution in a haemacytometer. Total numbers of granulocytes were calculated after differential counts of leucocytes in cytospin preparations of buffy coats.

To prevent bacterial superinfections, strict hygienic care was maintained and animals received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water during the whole experiment. Starting 1 day before inoculation, daily im amoxycillin (40 mg/kg/day) was added to this regimen for the remainder of the experiment.

Fungal strain

A strain of *Aspergillus fumigatus* isolated from an immunocompromised patient with invasive pulmonary aspergillosis was used. This strain was stored under oil on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, England) slopes during the study. At least once every two months, the strain was passed through a rat to maintain its virulence. The MIC and MFC of amphotericin B for this *A. fumigatus* strain (Schmitt *et al.* 1988) were 0.4 and 0.8 mg/L, respectively. There were no differences in MIC and MFC values if a high inoculum (1×10^5 conidia/mL) versus a low inoculum (5×10^3 conidia/mL) was used.

Experimental lung infection

A fumigatus was cultured at 37°C for 96 h on SDA until a dense mycelium with abundant conidia had formed. These conidia were harvested in 10 mL sterile saline. Conidia were removed from the thallus by rubbing the colony-surface gently with a glass pipette. This resulted in a conidial suspension without hyphal elements (confirmed by microscopy). The suspension was washed twice in sterile phosphate buffered saline (PBS). Subsequently, conidia were resuspended in sterile PBS (pH 7.4) and counted in a haemacytometer. In addition, the viability of the conidia was determined by culturing ten-fold serial dilutions on SDA.

Infection of the left lung was established according to the methods described by Bakker-Woudenberg, Van den Berg & Michel (1982). Briefly, under general anaesthesia the left main bronchus was intubated. A cannula was passed through the tube and the left lobe of the lung was inoculated with 0.02 mL of the conidial suspension. This resulted in a left-sided pneumonia. Sixty minutes before this procedure animals received a prophylactic im dose of gentamicin (6 mg/kg) and an extra im dose of amoxycillin (40 mg/kg).

Antifungal treatment

Amphotericin B deoxycholate (Fungizone, Bristol Myers-Squibb, The Netherlands) and liposomal amphotericin B (AmBisome, Vestar, San Dimas, CA) were reconstituted and further diluted in 5% glucose in water. Daily doses were administered iv into the tail vein over at least 30 sec, in a volume of 1 mL or less. Animals received one of the following regimens: 1 mg/kg Fungizone, 1 mg/kg AmBisome or 10 mg/kg AmBisome (these dosages were calculated *a priori* and not adjusted for weight-changes during the experiment). A comparison dosage of 10 mg/kg of Fungizone was not investigated as the LD_{50} of Fungizone in rats is 1.6 mg/kg (Proffitt *et al.*, 1991). Treatment was started 40 h after fungal inoculation, at which time mycelial disease could be confirmed by histological examination of lung tissue, and was continued for 10 consecutive days.

Toxicity

Our study was not designed to investigate in detail the toxicity associated with the treatment regimens. However, to check for the occurrence of gross toxicity, renal and hepatic functions were monitored. Abnormalities of renal function were detected by measuring serum creatinine and blood urea nitrogen (BUN) after ten days of administration of Fungizone (1 mg/kg/day and AmBisome 10 mg/kg/day. Abnormalities of hepatic function were detected by measuring serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT).

Therapeutic end points

Several end points were used to evaluate the efficacy of antifungal treatment. Animals were checked twice every day and mortality was recorded for 12 days after fungal inoculation. In addition, surviving animals were sacrificed 24 h after the last dose of antifungal agent to determine the extent of fungal disease present. From all animals that died or were killed the left and right lung, liver and spleen were aseptically removed and disrupted in 20 mL distilled water in a homogenizer (The VirTis Co. Inc., Gardiner,

NY, USA) for 45 sec at 10,000 rpm. Liver and spleen of each rat were homogenized together. Serial ten-fold dilutions of the homogenates were prepared in distilled water and 0.2 mL samples of each dilution, as well as 2 mL samples of the undiluted homogenates, were spread on to SDA plates which were incubated at 37°C for 48 h. Cfus were counted after 24 and 48 h of incubation. The remainders of the organ homogenates were poured into SDA plates and incubated at 37°C for 48 h, in order to count very low numbers of cfus or to prove sterility of the organs.

Statistical analysis

Differences in survival curves were assessed using Wilcoxon two-tailed test of life tables. This test examines the decrease in survival with time as well as the final percentage of survival. Differences in proportions of animals with dissemination to the right lung, liver and/or spleen were examined by Fisher's exact test. Differences in mean log cfu were examined by Mann-Whitney test. P values <0.05 were considered significant in these analyses.

Results

Granulocytopenia

The dosaging scheme of cyclophosphamide used was sufficient to keep rats persistently granulocytopenic (granulocytes $< 0.1 \times 10^9$/L) from the onset of infection (time zero) throughout the whole experiment. The numbers of total leucocytes and granulocytes are shown in Figure 1. Differential counts showed that less than 10% of the leucocytes consisted of granulocytes.

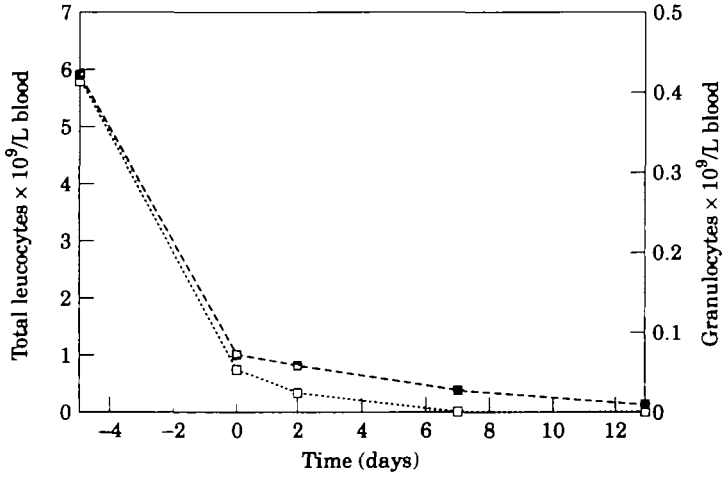

Figure 1. Number of total leucocytes (– – –) and neurophil granulocytes (....) in the blood of rats ($n = 5$) treated intraperitoneally with cyclophosphamide 90 mg/kg at day −5, and repeated doses of 60 mg/kg on days −1, 3, 7 and 11.

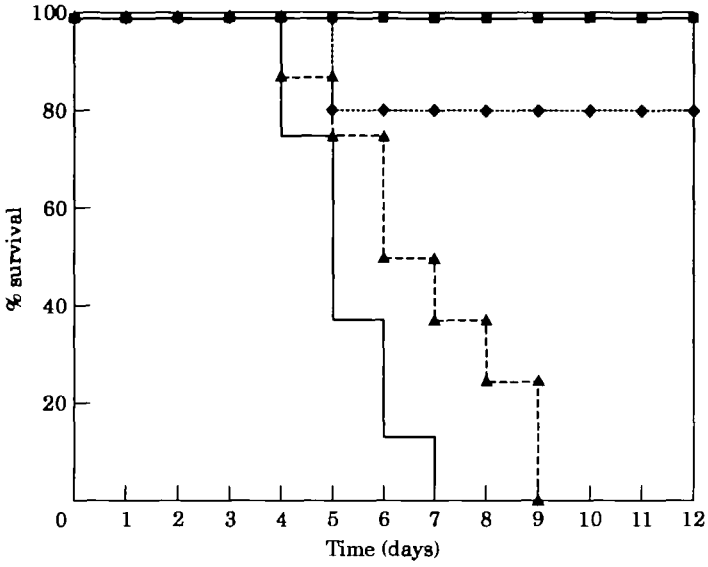

Figure 2. Survival of granulocytopenic rats after inoculation of the left lung with various inocula of *A. fumigatus* conidia; 1×10^2 (■) or $1 - 10^3$ (◆) ($n = 5$), 1×10^4 (▲) or 5×10^4 (——) ($n = 8$)

Experimental infection

Inoculation of relatively low numbers of conidia (1×10^2 or 1×10^3) resulted in survival of all or 80%, respectively, of the animals for at least 12 days. Inoculation of higher numbers of conidia (1×10^4 or 5×10^4) resulted in 100% mortality, the first rats dying

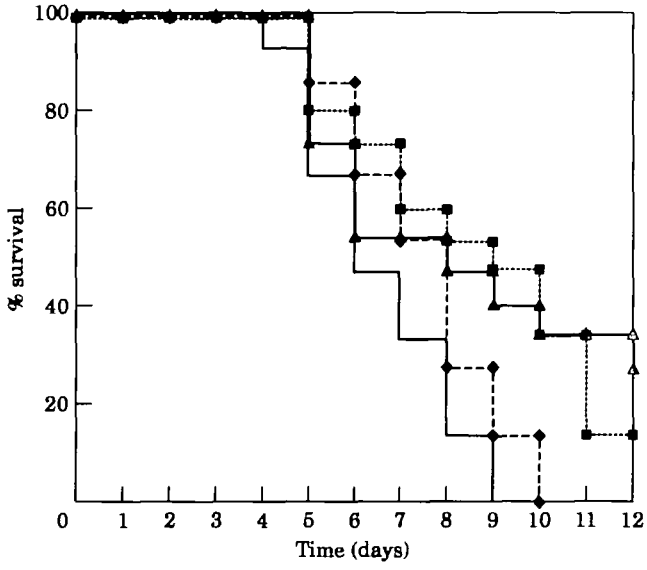

Figure 3. Effect of AmBisome versus Fungizone on survival of granulocytopenic rats inoculated with 1×10^4 *A. fumigatus* conidia. Groups of 15 animals each received Fungizone 1 mg/kg (■) AmBisome 1 mg/kg (◆) or AmBisome 10 mg/kg (▲) on each of 10 consecutive days. Treatment was started 40 h after fungal inoculation, at what time mycelial growth was firmly established. Control rats survival is also indicated (——).

on day four after fungal inoculation (Figure 2). In all experiments on efficacy of antifungal treatment an inoculum of 1×10^4 conidia was used. The size of the inoculum also influenced the time at which histologically proven mycelial disease was observed. Inocula of 1×10^2, 1×10^4 and 5×10^4 resulted in mycelia disease at ≥ 48 h, 40 h and 30 h after inoculation, respectively. Histological examination revealed abundant septate branching hyphae invading blood-vessels and lung tissue. Often gross haemorrhagic infarctions were macroscopically visible, even at a time when infective lesions were still small. If left untreated, inoculation with 1×10^4 conidia, initially resulted in a left-sided, invasive, pulmonary infection. After several days the infection disseminated and *A. fumigatus* could be detected in the right lung, liver and spleen.

Comparative efficacies of AmBisome and Fungizone

Following fungal inoculation all untreated rats died (Figure 3). Mortality was observed starting from day 5 after inoculation; by day 9 all rats had died. AmBisome 1 mg/kg/day did not influence survival significantly. Both Fungizone 1 mg/kg/day and AmBisome 10 mg/kg/day resulted in increased survival ($P = 0.006$ and 0.027, respectively) in terms of delayed as well as reduced mortality. Only 2/15 (13%) rats treated with Fungizone 1 mg/kg and 4/15 (27%) of those treated with AmBisome 10 mg/kg survived the study period of 12 days. Survival patterns between the animals treated with these regimens did not differ significantly ($P = 0.75$).

The results of the quantitative culture of internal organs are shown in the Table. The number of cfus found in the left lung was not significantly reduced following treatment with Fungizone 1 mg/kg/day or AmBisome (1 mg/kg/day). In contrast, treatment with AmBisome 10 mg/kg/day resulted in a significant reduction of the number of cfus in the left lung ($P = 0.003$).

Fungizone 1 mg/kg/day had no effect on the dissemination of the infection to the right lung, the number of rats with infected right lungs and the number of cfus found in these lungs being the same as in the untreated animals. Dissemination to the right lung after treatment with AmBisome both at 1 mg/kg/day and 10 mg/kg/day did not differ significantly from that occuring in untreated animals. However, both regimens reduced dissemination to the right lungs when compared with treatment with Fungizone ($P = 0.014$). In addition, the numbers of cfus found in the right lung, when dissemination had occurred, were significantly smaller than those found in untreated rats ($P = 0.0008$ and 0.0012, respectively).

All treatment regimens reduced dissemination of the infection to the liver and spleen. However, only with AmBisome both at 1 and 10 mg/kg/day did these differences reach statistical significance ($P = 0.0017$ and 0.0002, respectively). AmBisome 10 mg/kg/day completely prevented dissemination of infection to these organs. During these experiments we did not study dissemination to the brains of the rats. However, viable fungal elements were not found in the brains of any of the untreated animals ($n = 10$).

Toxicity

Serum creatinine, BUN, ALAT and ASAT concentrations after 10 days of treatment were not significantly increased (data not shown).

Table. Effects of AmBisome and Fungizone on the dissemination of *A. fumigatus* in granulocytopenic rats

Treatment	Left lung		Right lung		Liver and spleen	
	% positive	median $\log_{10}$ cfu	% positive	median $\log_{10}$ cfu	% positive	median $\log_{10}$ cfu
None	100	3.7 (1.8–4.3)	80	2.5 (1.6–2.5)	66	1.9 (0.7–2.5)
Fungizone 1 mg/kg/day	100	3.5 (2.7–3.9)	93	2.0 (0–2.5)	27	1.4 (0.6–1.7)
AmBisome 1 mg/kg/day	100	3.3 (2.6–3.7)	47[a]	0.9 (0.7–2.0)[b]	7[c]	0.5 (0–0.9)
AmBisome 10 mg/kg/day	100	3.1 (1.9–3.9)[b]	47[a]	1.1 (0–2.3)[b]	0[c]	0

Note: All animals were challenged with 1×10^4 *A. fumigatus* conidia in the left lung and followed for up to 12 days.
[a] $P < 0.02$ compared with Fungizone treated animals (not significant compared with untreated animals), Fisher's exact test.
[b] $P < 0.01$ compared with untreated animals, Mann-Whitney test.
[c] $P < 0.01$ compared with untreated animals, Fisher's exact test.

Discussion

Currently, amphotericin B deoxycholate (Fungizone) is the drug of choice for treating invasive pulmonary aspergillosis. Newly developed antifungals should, therefore, be compared with Fungizone to evaluate their efficacy. Because administration of Fungizone is associated with a number of side effects, the development of new antifungals with the same efficacy but less toxicity would be a major step forward. Liposomal formulations of amphotericin B are proven to be considerably less toxic than amphotericin B deoxycholate, thus allowing much higher dosages (up to ten-fold) (Lopez-Berestein *et al.*, 1985; Meunier *et al.*, 1991; Proffitt *et al.*, 1991; Mills *et al.*, 1994). AmBisome (Vestar, San Dimas, California, USA) is a commercially available liposomal formulation (small unilamellar vesicles) containing amphotericin B (Proffitt *et al.*, 1991).

Invasive pulmonary aspergillosis is an opportunistic infection usually diagnosed in patients who have been treated with corticosteroids and/or chemotherapeutic agents. In various animal models of aspergillosis corticosteroids are administered (Graybill, Kaster & Drutz, 1983; Van Cutsen, Van Gerven & Janssen, 1987; Schmitt *et al.*, 1988; Patterson *et al.*, 1989; Niki *et al.*, 1991; Dixon, Polak & Walsh, 1994). However, corticosteroids have been reported to cause combined toxicity with amphotericin B (Graybill *et al.*, 1983; Graybill & Kaster, 1984). To avoid the possible influence of this combined toxicity on survival rates of animals, in the present study cyclophosphamide was used to obtain persistent, severe, granulocytopenia. Because with this regimen the viability of pulmonary macrophages is not affected, we examined the histology of the left lung at different times after fungal inoculation to ascertain that conidia had germinated when antifungal treatment was started. It was found that the time to germination depended on the inoculum size. Antifungal treatment was started when invasive lesions had already developed, which is comparable with the clinical situation; most other published models ignore this pathophysiological state. It was observed that the size of macroscopic lesions did not correlate with the numbers of viable *A. fumigatus* cultured from those lungs. Francis *et al.* (1994) reported similar observations. The invasion of blood vessels by *Aspergillus* spp. and the consequent extensive haemorrhagic infarction of lung tissue might help explain these findings. The left lung of a rat consists of a single lobe, so hyphal invasion of the vessels of this lung might cause alteration of the macroscopic aspect of the entire lung at a relatively early stage.

In man pulmonary aspergillosis is often initially detected in one of both lungs. In the rat, by intubating the left main bronchus we were able to inoculate only the left lung with conidia of *A. fumigatus*, which allowed us to study progression of the infection to the right lung, a phenomenon often seen in clinical situations. Furthermore, this study allows one to study drug pharmacokinetics in infected lung tissue versus uninfected lung tissue in the same animal. By intubating the left main bronchus it is possible to inoculate rats in a standardized manner, as demonstrated by the relatively small range of days at which untreated animals died. The same observations were made by Schmitt *et al.* (1988) who inoculated fungi into the trachea.

The efficacy of antifungal agents in invasive aspergillosis is very much influenced by the duration of deep granulocytopenia (Denning & Stevens, 1990). In man persistent, severe granulocytopenia is associated with mortality rates up to 100% despite treatment with amphotericin B. In the present animal model, rats were granulocytopenic during the whole experiment to mimic human conditions. The

mortality rate was dependent on the size of the inoculum. This inoculum-dependent-mortality was described by Dixon *et al.* (1989), and might be explained by the ability of pulmonary macrophages to clear a certain amount of viable conidia from the lung. By increasing the number of conidia administered it was possible to increase the number of animals dying as well as to shorten the time to death. The inoculum which resulted in 100% mortality with the first animals dying at day five, was chosen to study the efficacy of antifungal treatment. The onset of mortality is of importance in clinical settings. A delay in the progression of infection which can be achieved by antifungal treatment will provide additional time to repopulate the patient's bone marrow. From clinical experience it is known, that bone marrow recovery is a crucial factor predicting a favorable outcome. In the present model all untreated rats died early. AmBisome 1 mg/kg/day did not significantly influence survival, in contrast, both Fungizone 1 mg/kg/day and AmBisome 10 mg/kg/day increased survival by almost 2 days; both treatment regimens resulted in the survival of some animals (13% and 27%, respectively). These percentages are relatively low when compared with those reported by Francis *et al.* (1994). We believe this to be largely due to differences between the respective experimental models; for example, antifungal therapy was started earlier by Francis *et al.* (1994) than in our study. The importance of these differences are underscored by the fact that not all of their control animals died during the study period. The observations in the present study resemble those found in clinical practice where, despite antifungal treatment, survival percentages are less than 10–20% in patients with persistent granulocytopenia.

Clear differences were found in the number of organs which were infected when rats died or were sacrificed. AmBisome in both the low and the high dosage prevented dissemination from the infected left lung to the right lung in more than 50% of the animals, while Fungizone did not prevent such dissemination. These findings were rather surprising, as van Etten *et al.* (1995) found, in uninfected mice, that after administering AmBisome, lung tissue concentrations were lower as compared with levels achieved after equivalent doses of Fungizone. It is possible that AmBisome is distributed more directly to the fungal elements and surrounds these (Adler-Moore & Proffitt, 1993), or, if liposomes are taken up by macrophages, this might alter local concentration of the drug, possibly resulting in higher concentrations at sites of infection. Furthermore, the relatively prolonged blood circulation of AmBisome, which results in increased serum concentrations compared with those obtained after administration of Fungizone, may help to prevent haematological fungal dissemination (Proffitt *et al.*, 1991). These results suggest a role for relatively low dosages of AmBisome in a prophylactic setting. The significant reduction of viable *A. fumigatus* after treatment with 10 mg/kg/day AmBisome, in both the infected left lung and the organs involved in dissemination, may indicate that this regimen prevents progression of fungal lesions. In clinical settings reduction of the number and size of lesions may improve success rates, notably in cases when bone marrow recovery occurs.

Both AmBisome regimens significantly reduced hepatosplenic dissemination. However, only the higher dosage of AmBisome was able to fully prevent this dissemination. This may be due to the fact that high concentrations of AmBisome accumulate in these organs (Francis *et al.*, 1994). In the present study mortality did not seem to directly correlate with the degree of dissemination of infection. This might be due to the relatively acute and progressive type of pneumonia which was induced. Respiratory insufficiency resulting from vascular involvement of the left lung, rather

than dissemination in itself, may thus be the direct cause of death in the present model. This hypothesis, however, needs to be confirmed.

We conclude that in this rat model AmBisome is more effective than Fungizone in reducing dissemination of pulmonary aspergillosis. Relatively low dosages of AmBisome may have a place in prophylactic settings. The optimal treatment dosages of AmBisome need to be further defined.

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