

Enhanced antifungal efficacy in experimental invasive pulmonary aspergillosis by combination of AmBisome with Fungizone as assessed by several parameters of antifungal response

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In common with a proportion of patients with invasive pulmonary aspergillosis (IPA), the efficacy of AmBisome treatment regimens in our rat model remains suboptimal. To investigate whether this might be the result of initially low antifungal activity of amphotericin B at the site of infection when administered in the liposomal form, Fungizone was added to AmBisome at the start of treatment. Groups of granulocytopenic rats with left-sided IPA received 10 day treatment regimens with either AmBisome 10 mg/kg/day ($n = 25$) or AmBisome 10 mg/kg/day combined with a single dose of Fungizone 1 mg/kg at day 1 ($n = 27$). Parameters of treatment response included survival, serum galactomannan (GM), size and quality of pulmonary macroscopic lesions, lung weight, viable fungal counts (cfu) and chitin content of the infected lung, and extrapulmonary disseminated fungal infection. In a separate experiment the significance of early start of treatment to obtain therapeutic efficacy was investigated. Compared with untreated controls, both treatment regimens showed a significant increase in survival and change in parameters of fungal infection except left lung cfu. The combination treatment showed a significant increase in survival compared with AmBisome monotherapy ($P = 0.02$) and a significant decrease in left lung chitin content ($P = 0.03$). Differences in circulating GM concentrations between the two treatment regimes approached significance ($P = 0.06$). Delay in the start of treatment from 16 to 24 h after fungal inoculation resulted in a significant decrease in therapeutic efficacy ($P = 0.02$). It is concluded that the efficacy of AmBisome therapy can be enhanced by the addition of Fungizone at the start of treatment. This is probably a result of active amphotericin B being immediately available in the lung at the start of treatment.

Introduction

During the past decade, invasive aspergillosis has become an increasingly common opportunistic infection appearing primarily in patients receiving cancer chemotherapy or immunosuppressants.¹ Amphotericin B (Fungizone) remains the first drug of choice in the treatment of patients with invasive pulmonary aspergillosis (IPA).² However, treatment with Fungizone is often unsuccessful^{3,4} and its use is limited by its dose-related nephrotoxicity.⁵ Several lipid-based formulations of amphotericin B have been developed to reduce the

toxicity associated with conventional amphotericin B.⁶ One of these lipid formulations is liposomal amphotericin B (AmBisome). AmBisome can be administered in higher dosages than Fungizone, which may result in a better therapeutic index. However, although the efficacy of high-dose AmBisome is equal to or better than Fungizone, failure rates still give cause for concern.^{7,8} One explanation for the limited success of AmBisome in a proportion of patients might be a substantial reduction in the immediate bioavailability of free amphotericin B when the drug is liposome encapsulated.⁹ Clinical studies have shown that early start of treatment with

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amphotericin B is important for a successful outcome.^{10,11} Therefore, low initial antifungal activity may cause a decrease in therapeutic efficacy. Evidence to support this hypothesis was found previously in our institution. Experimental studies showed that the addition of a single dose of Fungizone at the start of a 10 day AmBisome regimen significantly increased survival of granulocytopenic mice with disseminated candidiasis.¹²

In the present study, it was investigated whether the efficacy of AmBisome in our model of IPA in persistently granulocytopenic rats could be improved by the addition of Fungizone in the early phase of the disease. Several parameters for assessment of therapeutic response were used.

Materials and methods

Infection model of IPA

Our animal model, first described by Leenders *et al.*,¹³ was used with a few modifications. Specified pathogen-free female RP strain albino rats (18–25 weeks old, 185–225 g) were employed. Profound granulocytopenia was induced by intraperitoneal (ip) administration of 75 mg/kg cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 5 days before inoculation, followed by repeated doses of 60 mg/kg 1 day before and 3 and 7 days after fungal inoculation. This protocol resulted in granulocyte counts of $<0.1 \times 10^9/L$ on the day of fungal inoculation. To prevent bacterial superinfections, animals were given daily doses of 40 mg/kg amoxicillin intramuscularly (im) starting 1 day before inoculation and a 6 mg/kg dose of gentamicin im on the day of inoculation. In addition, rats received ciprofloxacin 660 mg/L and polymyxin B 100 mg/L in their drinking water throughout the experiment. Rats were inoculated with a clinical isolate of *Aspergillus fumigatus* originally isolated from an immunocompromised patient with IPA. In accordance with NCCLS procedures,¹⁴ the MIC of amphotericin B for this strain was 0.4 mg/L. Infection was established by intubation of the left main bronchus under general anaesthesia. A cannula was passed through the tube and the left lung was inoculated with 6×10^4 *A. fumigatus* conidia suspended in 20 μ L of phosphate-buffered saline (PBS).

Before experiments in infected rats, treatment regimes were administered to uninfected, granulocytopenic rats in which no noticeable toxicity was observed.

The experimental protocols adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus University Rotterdam.

Antifungal treatment

Antifungal agents were administered intravenously via the lateral tail vein to groups of rats daily. Treatment was started at 16 h after fungal inoculation, at which time hyphal growth was established. Fungizone was obtained from Bristol-Myers BV (Woerden, The Netherlands), and was diluted in 5% dextrose. AmBisome was obtained from NeXstar Pharmaceuticals (Wilrijk, Belgium) and diluted in 5% dextrose. The following treatment regimens were investigated: (i) AmBisome 10 mg/kg/day for 10 days; and (ii) AmBisome 10 mg/kg/day for 10 days combined with a single dose of Fungizone 1 mg/kg at day 1. When Fungizone and AmBisome were combined on the same day, AmBisome was administered first and Fungizone within 20 min thereafter. Controls received no treatment, since previous studies showed that placebo treatment with either 5% dextrose or empty liposomes did not influence survival, levels of circulating galactomannan (GM) or other parameters of fungal infection (data not shown).

Parameters for efficacy of antifungal treatment

The survival rate of rats was monitored twice daily until day 11 after fungal inoculation. GM was measured on days 3, 5, 7, 9 and 11 after fungal inoculation. At the end of the antifungal treatment rats had died or survived. Rats surviving on day 11 were killed and dissected. In rats that died and killed rats, the size of the pulmonary lesion of the left lung was measured, left lung weight was determined, organs were cultured for fungi, and chitin assays were carried out on the left lung homogenates.

GM detection

Blood for GM detection was sampled by puncture of the orbital venous plexus in the lateral canthus of the orbita. When cyclophosphamide, antifungal agents or antibiotics were administered to the rats on the same day as GM detection was carried out, blood samples for GM detection were taken within 2 h before injection of these agents. Concentrations of circulating GM were measured as described previously.¹⁵ Briefly, 300 μ L of each serum sample were used in a sandwich ELISA (Platelia *Aspergillus*; Sanofi Diagnostics Pasteur, Belgium). Each plate contained a calibration curve derived from rat serum samples containing 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/mL GM.

Pulmonary macroscopic lesions and left lung weight

Pulmonary macroscopic lesions were observed in both untreated and treated rats. Angio-invasive lesions, seen as macroscopic dark-red lesions, were histologically characterized by extensive hyphal broncho- and angio-invasion, and haemorrhagic infarction. Responsive lesions, seen as macroscopic light-red coloured lesions, were histologically charac-

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terized by the presence of relatively short hyphae and little angio- and bronchio-invasion with resulting less haemorrhagic infarction. The size of the two types of pulmonary lesion was expressed as a percentage of the total lung surface. The pulmonary lesion size was measured from photographs of the anterior of the lungs taken immediately after dissection.

Fungal cultures of organs

Left and right lungs were disrupted in 12 mL and livers in 20 mL of PBS using a tissue homogenizer (The Virtis Co. Inc., Gardiner, NY, USA) for 45 s at 10 000 rpm. Cfu in right lungs and livers were counted in 1:10 and undiluted homogenates. Cfu in left lungs were counted in 1:10 and 1:100 dilutions, and the remaining 10.68 mL of homogenate were used for the chitin assay. Dilutions of homogenates were spread on to Sabouraud agar plates. Undiluted homogenates were cultured according to the pour plate method. After incubation at 37°C for 36 h the cfu were counted.

Chitin assay

A 10.68 mL aliquot of left lung homogenate was used for the chitin assay, as described by Lehmann & White.¹⁶ In brief, the homogenate was centrifuged, resuspended in 4 mL of 3% sodium lauryl sulphate (SDS; Sigma Chemical Co., St Louis, MO, USA) and heated at 100°C for 15 min. After cooling, the pellet was washed once with distilled water, resuspended in 3 mL of 120% KOH solution and heated to 130°C for 1 h. Subsequently, 8 mL of ice-cold 75% ethanol were added, tubes were kept at 4°C for 15 min and 0.3 mL of Celite suspension (Celite 545; Sigma) were added. After centrifugation, the pellet was washed with cold ethanol (40%) and cold distilled water, and suspended in 0.5 mL of NaNO₂ (5%) and 0.5 mL of KHSO₄ (95%). After centrifugation, volumes of the supernatant were mixed with 12.5% NH₄SO₃NH₂ followed by 3-methyl-benzo-2-thiazolone hydrazone HCl monohydrate (MBTH; Sigma). After heating for 3 min, the supernatants were cooled, FeCl₃6H₂O (0.83%) was added and they were allowed to stand for 30 min. The optical density at 650 nm was read in a spectrophotometer. The chitin content of the organs was expressed as microgrammes of glucosamine per organ. Final measurements of chitin were corrected for the loss of volume of homogenate.

Toxicity

To check for the presence of toxicity following antifungal treatment, renal and hepatic functions were monitored in treated rats. Renal function was measured by serum creatinine and blood urea nitrogen (BUN) after 10 days of antifungal treatment. Hepatic function was measured by serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT).

Influence of treatment start time on survival

In a separate experiment the influence of start time of treatment on therapeutic efficacy was investigated. In this experiment, treatment was started at 16 h after fungal inoculation in one group, and at 24 h in the other, and survival of the rats was compared. All rats received the combination therapy.

Statistical methods

Differences in survival were assessed by the log rank test. Differences in proportions of animals with dissemination to the right lung and liver were examined by Fisher's exact test. Differences in parameters of fungal infection were assessed by Student's *t*-test with the exception of differences in chitin amounts, which were calculated using the Mann-Whitney *U*-test.

To analyse circulating GM concentrations, logarithmically transformed GM values were taken, showing an approximately normal distribution. The mean increase of log₁₀ GM concentration with time was determined for each group of rats by repeated measurements ANOVA (random coefficients regression model) using the SAS Proc Mixed statistical computer package. The correlation between the measured concentrations of GM and mortality was assessed using Cox regression with log₁₀ GM concentration as the time-dependent variable.

Results

Survival rate

Ninety-six per cent of untreated rats died <11 days after fungal inoculation. Both treatment regimens significantly improved survival compared with the untreated controls ($P < 0.0001$ for both treatment regimens), as shown in Figure 1. The addition of a single dose of Fungizone 1 mg/kg at the start of treatment (day 1) to the 10 day AmBisome regimen significantly improved survival compared with AmBisome monotherapy ($P = 0.02$).

Concentrations of circulating GM

In both the untreated and the treated groups, concentrations of circulating GM increased consistently over time (Table 1). The mean daily increases in log₁₀ GM concentrations for controls, AmBisome monotherapy and combination therapy were 0.33 [± 0.02 (S.E.M.)], 0.12 (± 0.02) and 0.08 (± 0.01), respectively. The mean increases in GM for the two treatment regimens were significantly lower than in controls ($P < 0.001$ for both regimens). The difference in log₁₀ GM increase between AmBisome monotherapy and combination therapy approached significance ($P = 0.06$).

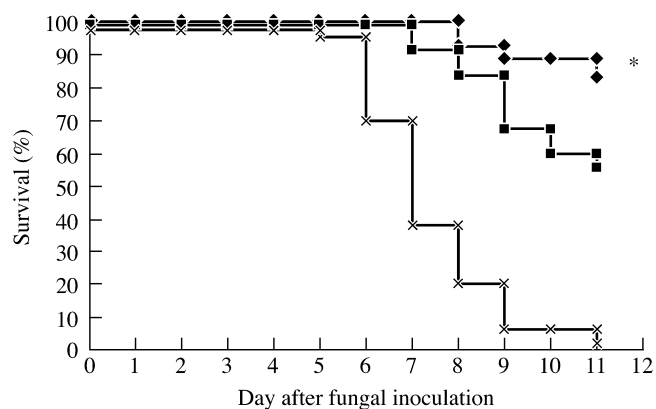


Figure 1. Survival of rats with IPA during antifungal treatment. Treatment was started 16 h after fungal inoculation and continued for 10 days. Diamonds, AmBisome 10 mg/kg/day + Fungizone 1 mg/kg on day 1; squares, AmBisome 10 mg/kg/day; ×, untreated controls. * $P = 0.02$ compared with AmBisome monotherapy.

Using Cox regression, it was found that there was a significant correlation between GM concentration and mortality ($P < 0.001$). A 10-fold increase in GM concentration was associated with an approximate six-fold increase in mortality (relative death rate: 5.6; 95% CI: 3.0–10.3). This correlation between GM concentration and mortality was not significantly influenced by the type of treatment administered ($P = 0.12$).

Pulmonary macroscopic lesions and left lung weight

Antifungal treatment had an effect on the weight and macroscopic appearance of the left lung (Table 2). A shift was observed from angio-invasive lesions in untreated animals to responsive lesions in both treatment groups. Histologically, angio-invasive lesions showed extensive fungal broncho- and

angio-invasion, and tissue haemorrhagia, all of which in responsive lesions were clearly reduced. Compared with controls, left lung weight was significantly lower in both treatment groups ($P < 0.001$ for both regimens). Compared with the AmBisome monotherapy group, the number of angio-invasive lesions was lower in the combination therapy group, but this difference was not significant ($P = 0.31$).

Fungal cultures of organs

AmBisome monotherapy did not reduce cfu cultured from the left lung (Table 2). In contrast, the combination therapy significantly reduced the cfu in the left lung compared with both the untreated controls ($P < 0.001$) and the AmBisome monotherapy group ($P = 0.04$). Both treatment regimens completely prevented dissemination to the liver.

Chitin content of the infected lung

Both the AmBisome monotherapy and the combination therapy reduced chitin content in the left lung compared with untreated controls ($P = 0.03$ and < 0.01 , respectively). Compared with AmBisome monotherapy, the combination therapy significantly reduced left-lung chitin content ($P = 0.03$).

Toxicity

There were no significant differences between the treatment groups in serum creatinine, BUN, ALAT and ASAT concentrations after 10 days of treatment (data not shown).

Influence of treatment start time on survival

In a separate experiment, it was investigated whether a delay in the start of treatment would have an effect on the therapeutic efficacy in our model. We compared the efficacy of the combination therapy when started at 16 h ($n = 10$) or at 24 h after fungal inoculation ($n = 12$) (Figure 2). A significant

Table 1. GM serum concentrations in rats with IPA during different antifungal treatment regimens^a

		Day 3 ^b	Day 5	Day 7	Day 9	Day 11
Untreated controls	no. of rats ^c	20	19	11	5	1
	log ₁₀ GM concentration	0.83 ± 0.10	1.42 ± 0.12	1.95 ± 0.12	2.55 ± 0.22	2.65
AmBisome 10 mg/kg/day	no. of rats	20	20	19	16	13
	log ₁₀ GM concentration	0.32 ± 0.12	0.67 ± 0.14	1.00 ± 0.11	0.87 ± 0.17	0.78 ± 0.12
AmBisome 10 mg/kg/day + Fungizone 1 mg/kg on day 1	no. of rats	17	17	16	16	16
	log ₁₀ GM concentration	0.26 ± 0.06	0.51 ± 0.12	0.85 ± 0.14	0.69 ± 0.12	0.93 ± 0.14

Data are shown as means ± S.E.M.

^aTreatment was started 16 h after fungal inoculation and continued until day 10.

^bDay after fungal inoculation.

^cGM measurements were carried out on all rats that survived at the indicated days after fungal inoculation.

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Table 2. Parameters of fungal infection in rats with IPA receiving different treatment regimens^a

	Untreated controls	AmBisome 10 mg/kg/day	AmBisome 10 mg/kg/day + Fungizone 1 mg/kg on day 1
No. of rats ^b	28	25	26
Angio-invasive lesion left lung (%)	78 ± 5.8	27 ± 6.7*	17.6 ± 6.3*
Responsive lesion left lung (%)	5.7 ± 2.7	47 ± 7.2*	50 ± 7.7*
Weight left lung (g)	1.16 ± 0.07	0.74 ± 0.05*	0.73 ± 0.05*
log ₁₀ cfu in left lung	3.06 ± 0.08	3.05 ± 0.09	2.80 ± 0.07*†
Culture positive right lung (%)	79 ± 8	24 ± 9*	12 ± 6*
log ₁₀ cfu in right lung	1.62 ± 0.19	0.47 ± 0.18*	0.19 ± 0.12*
Culture positive liver (%)	75 ± 8	0 ± 0.0*	0 ± 0.0*
log ₁₀ cfu in liver	1.01 ± 0.09	0 ± 0.0*	0 ± 0.0*
Left lung chitin content (µg glucosamine)	15.4 (0–36.0)	4.7 (0–42.7)*	0.5 (0–57.5)*†

Data are shown as means or percentages ± S.E.M., except chitin contents, which are shown as medians (range).

^aTreatment was started 16 h after fungal inoculation and continued until day 10.

^bMeasurements were carried out on rats that died during treatment and on rats that were killed at the end of treatment.

**P* < 0.05 compared with untreated controls.

†*P* < 0.05 compared with rats receiving AmBisome monotherapy.

reduction in survival was seen when start of treatment was delayed from 16 to 24 h (*P* = 0.02).

Correlation between parameters of fungal infection and survival in treated rats

To investigate which parameters of fungal infection were associated with survival in rats receiving antifungal treatment (either AmBisome monotherapy or combination therapy), we compared the results of rats that died during treatment with those of rats that survived until the end of treatment (Table 3). In the surviving animals the size of the responsive lesion was significantly larger, whereas the angio-invasive lesion was

smaller. In addition, the parameters of fungal load were lower in surviving animals, with the left lungs containing significantly less cfu and chitin compared with animals that died. The number of cfu in the right lungs was also lower in surviving rats and the difference in the percentage of rats with dissemination approached significance.

Discussion

In this study, we compared the therapeutic efficacy of two treatment schedules using a clinically relevant animal model of IPA during persistent neutropenia. In this model, Leenders *et al.*¹³ showed that a 10 day treatment with high doses (10 mg/kg) of AmBisome did not result in increased survival compared with a 10 day treatment with Fungizone 1 mg/kg. Clinical studies in neutropenic patients have also shown that AmBisome treatment is not always superior to standard Fungizone therapy.^{7,8,17}

The aim of our study was to investigate whether the efficacy of high doses of AmBisome could be enhanced by increasing the immediate availability of the biologically active drug in the early phase of treatment. This hypothesis was based on studies carried out in our laboratory by van Etten *et al.*^{9,12,18} in a mouse model of invasive candidiasis. In biodistribution studies that measured the levels of amphotericin B in the lung at various time points after a single injection of Fungizone 0.3 mg/kg, they found that amphotericin B could be detected in the lung at all time points from 5 min to 12 h. In contrast, after a single injection with AmBisome 7 mg/kg, concentrations of amphotericin B in the lung could not be detected until 12 h and later. In addition, they reported that the *in vitro* antifungal activity of AmBisome during short-term

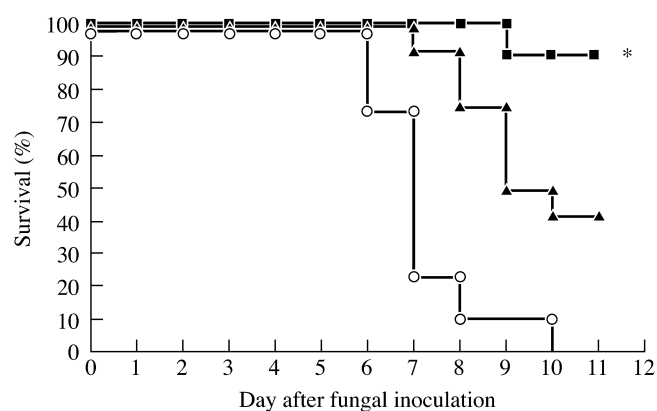


Figure 2. Survival of rats with IPA during antifungal treatment with AmBisome 10 mg/kg/day plus Fungizone 1 mg/kg on day 1. Treatment was started 16 h after fungal inoculation in one group of rats and at 24 h in the other. Squares, start of treatment at 16 h; triangles, start of treatment at 24 h; circles, untreated controls. **P* = 0.02 compared with rats in which therapy was started at 24 h.

Table 3. Comparison of the parameters of fungal infection in rats that died during treatment^a with rats that were killed at the end of treatment

	Treated rats that died before end of treatment	Treated rats that survived until end of treatment	<i>P</i> value
No. of rats	15	35	
Angio-invasive lesion left lung (%)	41 ± 9	14 ± 5	0.008
Responsive lesion left lung (%)	15 ± 5	35 ± 6	<0.001
Weight left lung (g)	0.74 ± 0.06	0.74 ± 0.04	0.982
log ₁₀ cfu in left lung	3.1 ± 0.09	2.83 ± 0.07	0.02
Culture positive right lung (%)	33 ± 12	11 ± 5	0.06
log ₁₀ cfu in right lung	0.66 ± 0.25	0.18 ± 0.09	0.04
Left lung chitin content (µg glucosamine)	20.4 (0–42.7)	5.1 (0–57.5)	0.004

Data are shown as means or percentages ± S.E.M., except chitin contents that are shown as medians (range).

^aTreatment was started 16 h after fungal inoculation and continued until day 10.

exposure (6 h) of *Candida albicans* was significantly less than that of Fungizone. The latter finding was explained by a relatively slow release of amphotericin B from the liposomes. Furthermore, they found that addition of a single dose of Fungizone to a 5 day regimen of AmBisome at the start of treatment significantly increased survival and decreased fungal load in the kidney of granulocytopenic mice with disseminated candidiasis.

Our findings are in accordance with these studies. In our rat model of IPA we have also observed that rat survival increased significantly when a single dose of Fungizone (on day 1) was added to a 10 day AmBisome regimen. When higher doses of AmBisome (up to 30 mg/kg) were administered on day 1 and AmBisome 10 mg/kg on days 2–10, no improvement compared with AmBisome monotherapy was seen (data not shown), indicating that changing the absolute amount of AmBisome on day 1 had no significant impact. Multiple doses of Fungizone 1 mg/kg (at day 1, 3 and 5, or at day 1 and 6, or twice at day 1) combined with the 10 day AmBisome regimen did not further enhance survival compared with a single addition of Fungizone 1 mg/kg at day 1 (data not shown). This indicates that only addition of Fungizone at day 1 is relevant for improving survival. In addition, early antifungal activity was shown to be a highly important factor, since an 8 h delay in starting antifungal treatment resulted in significant reduction in survival. Therefore, it is likely that the improved antifungal efficacy resulting from the addition of Fungizone to the AmBisome regimen at day 1 is caused by increased bio-availability of amphotericin B in the lung in the early phase of the disease.

In addition to survival, we investigated the value of several parameters of fungal infection as markers for treatment response. Antifungal treatment appeared to have an effect on most of these parameters. Concentrations of serially sampled serum GM were significantly lower in both treatment groups

compared with untreated controls, whereas the difference between treatment groups approached significance. Other investigators have also described a suppressive effect of antifungal treatment on circulating GM concentrations in both animal models and patients with IPA.^{19,20} In addition, increases in GM concentration in our model were associated with a significantly increased mortality. This indicates that serum GM concentrations are a relevant parameter for measuring treatment response.

Antifungal treatment resulted in a reduction in lung weight and size of the dark-red coloured haemorrhagic angio-invasive lesion, and an increase in the size of the light-red coloured responsive lesion. The size of this responsive lesion was also associated with increased survival, as opposed to the angio-invasive lesion. Others have also described decreased haemorrhagic lesions in a rabbit model of IPA and increased lighter coloured 'resolving' lesions under antifungal treatment.²¹ These findings indicate that these lesions reflect an important aspect of the pathogenesis of IPA. Since responsive lesions represented areas with reduced broncho-angio invasion and haemorrhagia, inhibition of the invasive spread of the fungus is probably important for successful antifungal treatment.

The numbers of cfu cultured from the left lungs of rats receiving combination therapy were lower than those of rats receiving AmBisome monotherapy. However, compared with untreated controls the cfu of the AmBisome monotherapy group were not significantly decreased. This may be explained by the fact that the number of cfu does not represent the real viable fungal load: larger hyphae represent a larger fungal load but not necessarily a larger number of cfu.²² In accordance with this, several studies reported that the numbers of cfu cultured from the organs in their animal models of IPA did not significantly increase over time,^{16,23,24} in contrast with quantitatively measured fungal DNA in one

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of these studies.²⁴ Chitin may be a more reliable indicator for fungal load than cfu, since it is a constituent from the fungal cell wall and the amount of chitin increases with the growth of the hyphae.¹⁶ Indeed, we found a significant decrease in chitin amount in the left lung in both treatment groups compared with untreated rats, as well as significant differences between the two treatment groups.

In conclusion, we have demonstrated that the addition of a single dose of Fungizone at the start of AmBisome treatment enhanced the therapeutic efficacy of AmBisome monotherapy. Apart from survival, parameters of fungal disease that best represented increased treatment response were left-lung chitin content and serum GM concentration.

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