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Efficacy of aerosolized amphotericin B desoxycholate and liposomal amphotericin B in the treatment of invasive pulmonary aspergillosis in severely immunocompromised rats

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The effects of treatment with aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B were evaluated in severely immunosuppressed rats with invasive pulmonary aspergillosis. Aerosol treatment with amphotericin B desoxycholate consisted of a single dose (60 min) with amphotericin B concentrations in the nebulizer reservoir of 1, 2 and 4 mg/mL, respectively. For liposomal amphotericin B, aerosol treatment consisted of single, double or quadruple doses with a nebulizer reservoir concentration of 4 mg/mL of amphotericin B. Treatment, started at 30 h after inoculation, with aerosolized amphotericin B desoxycholate (nebulizer reservoir concentration 2 mg/mL) significantly prolonged survival of rats as compared with placebo-treated rats, whereas treatment with aerosolized amphotericin B desoxycholate with nebulizer reservoir concentration of 1 or 4 mg/mL did not have a significant effect on survival. Treatment with aerosolized liposomal amphotericin B signifcantly prolonged survival with all treatment regimens when compared with placebo-treated animals. Aerosol treatment did not prevent dissemination of the infection. The effects of amphotericin B desoxycholate and liposomal amphotericin B on pulmonary surfactant function were also evaluated *in vitro*. Amphotericin B desoxycholate inhibited surfactant function in a dose-dependent fashion. Liposomal amphotericin B had no detrimental effect on surface activity of surfactant. These results indicate that aerosol administration of amphotericin B, especially the liposomal formulation, could be an additional approach to optimizing treatment of invasive pulmonary aspergillosis.

Introduction

An increase in the number of immunosuppressed patients at risk of developing fungal infections due to increasing advances in transplantation medicine has led to a substantial increase in the number of cases of invasive pulmonary aspergillosis (IPA) in the last few decades.[1] Standard treatment of IPA with intravenous amphotericin B desoxycholate (Fungizone) is often unsuccessful and complicated by severe, dose-limiting toxicity. Newly developed lipid-based modalities of amphotericin B have an increased therapeutic index as compared with conventional amphotericin B.[2] However, treatment of established infection with *Aspergillus* spp. in patients with persisting granulocytopenia is correlated with high failure rates. Thus, there is a continuing need for optimization of antifungal treatment in pulmonary aspergillosis.

The portal of entry of *Aspergillus* is often the respiratory tract, since the spores of this fungus are ubiquitous and easily inhaled. Spores descend to the lowest regions of the lungs and invasive disease subsequently develops. Further improvement in the treatment of IPA can therefore be sought in the administration of amphotericin B products via the pulmonary route. With this mode of administration, the therapeutic agent targets the lungs directly, and systemic toxicity is reduced. Improvement in efficacy as well as reduction of systemic side effects could be anticipated.

Few clinical studies exist on the use of aerosolized ampho-

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tericin B in the prophylaxis or treatment of fungal disease. These studies do not demonstrate a convincing beneficial effect of aerosolized amphotericin B, probably because of a lack of power or adequate study design.[3–5] Preclinical animal work therefore has to be performed in order to determine the value of aerosolized conventional and lipid preparations of amphotericin B.

Aerosol administration of different formulations of amphotericin B has been described and shown to be effective in the prophylaxis of pulmonary aspergillosis in animal models.[6–8] However, in these studies only temporary or mild immunosuppression was applied in the experimental models. In the clinical situation however, patients are generally persistently granulocytopenic and diagnosis of IPA is often late in the course of infection. An important feature of the experimental design of the present study is therefore the treatment of an established infection under severe, persisting immunosuppression.

Results from our laboratory and from others show that after aerosol administration of liposomal amphotericin B, little or no drug was deposited in organs other than the lungs, which suggests that systemic toxicity following aerosol administration would be minimal.[6,9–11] Pulmonary toxicity of inhaled amphotericin B however, could be an important detrimental effect of inhalation therapy. There are many *in vivo* and *in vitro* parameters that could be indicative of damaged pulmonary tissue or impaired pulmonary function. In this study we chose pulmonary surfactant function as a toxicity indicator, since this is an important basic physiological function. Surface activity of pulmonary surfactant is relatively easily measured *in vitro*, and provides relevant information on the possible harmful influence of inhaled amphotericin B formulations.

In the present study, we describe the efficacy of aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B in the treatment of established IPA in rats with severe, persistent immunosuppression. Furthermore, we describe the influence of amphotericin B products on surfactant function *in vitro*.

Materials and methods

Materials

Amphotericin B desoxycholate (Fungizone, containing 50 mg amphotericin B and 41 mg desoxycholate per vial) was from Bristol Myers-Squibb (Woerden, The Netherlands) and liposomal amphotericin B (AmBisome) was from NeXstar (San Dimas, CA, USA). Hydrogenated soybean phosphatidylcholine (HSPC) and distearoylphosphatidylglycerol (DSPG) were from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol and cyclophosphamide were from Sigma Chemical Co. (St Louis, MO, USA). Sabouraud dextrose agar (SDA) was from Oxoid (Basingstoke, UK).

Animals

Female R-strain albino rats, specified pathogen free, 18–25 weeks old (own breed), weighing 185–225 g were used for all experiments. Animals received a normal, pathogen-free diet and water *ad libitum.* Experiments were approved by the animal experiments ethics committee of the Erasmus University Medical Center.

Aspergillus strain

A clinical isolate of *Aspergillus fumigatus* from an immunocompromised patient with IPA was used. The MIC and minimal fungicidal concentration (MFC) of amphotericin B for this strain were 0.4 and 0.8 mg/L, respectively.[12] This strain was stored under oil on SDA. At least once every 2 months, the strain was passed through a rat to maintain its virulence. For inoculation, conidia were harvested and suspended in sterile phosphate-buffered saline (PBS), as described previously.[12]

Immunosuppression and supportive care

Granulocytopenia was induced by ip administration of 90 mg/kg cyclophosphamide 5 days before fungal inoculation, followed by additional dosages of 60 mg/kg every 4 days throughout the study. This treatment resulted in a persistent granulocytopenia ($<0.5 \times 10^9$ cells/L) from the time of *A. fumigatus* inoculation up to the end of the study.[12] To prevent bacterial superinfection, strict hygienic care was applied, and animals received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water throughout the experiment. Furthermore, im administration of amoxicillin (40 mg/kg/day) was added to this regimen. Shortly before and after inoculation, gentamicin (40 mg/kg) was administered im.

Experimental lung infection

Infection of the lung was established according to the method described by Leenders *et al.*[12] Briefly, under general anaesthesia the left main bronchus was intubated. A canula was passed through the tube and the left lobe of the lung was inoculated with 0.02 mL of a suspension containing 2×10^4 conidia. This resulted in a left-sided pneumonia.

Antifungal treatment

Amphotericin B desoxycholate and liposomal amphotericin B were reconstituted according to the manufacturers' instructions and further diluted in 5% glucose up to an amphotericin B concentration in the nebulizer reservoir of 4 mg/mL in the case of liposomal amphotericin B or 1, 2 or 4 mg/mL in the case of amphotericin B desoxycholate. Since calculation of the actual dose delivered is a rather complicated measure, in this study nebulizer reservoir

concentration is given as an indirect dose indication. Treatment was started 30 h after fungal inoculation, at which time mycelial growth was established by histopathological examination (periodic acid–Schiff stain). The aerosolization procedure for liposomal amphotericin B and amphotericin B desoxycholate was as described previously.[13] In short: infected rats were constrained in cone-ended plastic tubes and placed in a nose-only inhalation apparatus (CH Technologies USA Inc., Westwood, NJ, USA). Aerosols were generated by a Collison six-jet nebulizer system (Model CN; BGI Inc., Waltham, MA, USA). The nebulizer operated at 20 L/min air flow. Under these conditions, >80% of the aerosol droplets that are generated are <5 μm mass diameter, which ensures substantial deposition of material in the alveobronchial region. Animals were exposed to aerosol treatment for one or more periods of 60 min.

Liposome preparation

Placebo liposomes with similar lipid composition (HSPC: DSPG:cholesterol, 2:1:0.8) and similar liposomal diameter to liposomal amphotericin B were prepared by the film hydration method. Lipids were dissolved in 2 mL chloroform:methanol (1:1, v/v). The lipid mixture was evaporated to dryness in a round bottom flask at 65°C. The lipid film was hydrated by vortex mixing with a buffer solution containing 10 mM sodium succinate, 10% (w/v) sucrose (pH 5.5). Liposomes were sonicated, which resulted in vesicles with an average particle size of 100 nm, as measured by dynamic light scattering. The liposome suspension was concentrated by ultracentrifugation at 280 000**g** for 2 h at 4°C in a Beckman ultracentrifuge L-70 (Beckman, Palo Alto, CA, USA) and further diluted in buffer to a concentration of 40 μmol/mL of lipid (similar to the lipid concentration in aerosolized liposomal amphotericin B). Phospholipid content was determined by phosphorus assay.[14] Placebo liposomes were aerosolized as described above during a single treatment period of 60 min.

Efficacy of aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B

Groups comprised of 15 infected rats each were treated with aerosolized amphotericin B desoxycholate or aerosolized liposomal amphotericin B. For amphotericin B desoxycholate, treatment consisted of a single nebulization period of 60 min with a nebulizer reservoir concentration of 1, 2 or 4 mg/mL amphotericin B. For liposomal amphotericin B, treatment consisted of a single, double (q 24 h) or quadruple (q 24 or q 12 h) nebulization period. Controls received aerosolized glucose 5% or placebo liposomes. In one experiment, pure amphotericin B suspended in glucose 5% (nebulizer reservoir concentration 4 mg/mL) was nebulized in order to evaluate survival after treatment with this agent alone. Animals were checked twice daily and mortality was recorded for the 12 days following fungal inoculation, after which time post-mortem studies were conducted on all animals. The left lung, right lung and liver were dissected and homogenized in 20 mL PBS for 45 s at 20 000 rpm in a VirTis homogenizer (VirTis, Gardiner, NY, USA). Volumes of 0.2 and 2 mL and the remainder of each homogenate were spread on to or poured into SDA plates. Plates were incubated for 24 h at 37°C followed by 24 h at 25°C.

Surfactant function experiments

Freeze-dried natural surfactant prepared from bovine lavages was a gift from the Department of Anaesthesiology of the Erasmus University Medical Center Rotterdam. It consisted of *c.* 90–95% phospholipids, 1% hydrophobic proteins (surfactant proteins B and C) and 1% free fatty acids. This surfactant is highly surface active at low concentrations. Influence of different amphotericin B formulations on surfactant function was determined by means of a modified Wilhelmy balance system (E. Biegler GmbH, Mauerbach, Austria). This system records the surface tension of an air–liquid film over several cycles of mechanical compression and expansion of this film. The lower the surface tension at minimal surface area, the higher the surface activity of the applied film.

The trough of the Wilhelmy balance was filled with warm saline (37°C) and calibrated. After calibration, 100 μL of surfactant (1 mg/mL total lipids) alone or in the presence of various concentrations of amphotericin B formulations was applied on to the saline hypophase and allowed to spread for 2 min. The surface area was compressed and expanded with a cycling speed of one cycle per 3 min and an area reduction from 100% to 20%. Minimal surface tension (γ_{min}) was measured after three cycles at 20% surface area, and is expressed as mN/m. Inhibition of surfactant function of natural surfactant by amphotericin B formulations resulted in increased surface tension at minimal surface area.

Statistical analysis

Survival curves were generated by the method of Kaplan and Meier. Statistical evaluation of differences in the survival curves was performed by the log rank test. This test examines the decrease in survival with time as well as the final percentage survival. Differences in proportions of animals with *A. fumigatus* in the left lung and dissemination to the right lung and liver at the time of death were examined by Fisher's exact test. Data of surface activity measurements were compared using the two-sided *t*-test. *P* values of ≤ 0.05 were considered significant.

Results

Effect of aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B on survival

The effect of aerosol treatment on survival of rats with pulmonary aspergillosis is shown in Figures 1 and 2. In this model of pulmonary aspergillosis placebo-treated rats died between days 4 and 9 after inoculation. At a nebulizer reservoir concentration of 2 mg/mL administration of aerosolized amphotericin B desoxycholate 30 h after inoculation led to a significantly prolonged survival ($P = 0.006$) as compared with controls (Figure 1). Administration of aerosolized amphotericin B desoxycholate with a nebulizer reservoir concentration of 1 or 4 mg/mL however, showed no beneficial effect on the survival of rats as compared with controls. Administration of a single dose of aerosolized liposomal amphotericin B (nebulizer reservoir concentration 4 mg/mL) resulted in significantly prolonged survival ($P = 0.002$) as compared with glucose controls as well as empty liposome controls (Figure 2a). Aerosolized liposomal amphotericin B adminstered as a double (q = 24 h) or quadruple (q = 24 and 12 h) dose also significantly prolonged survival ($P \leqslant 0.005$) as compared with glucose-treated animals (Figure 2b). Intensification of treatment yielded no further improvement in survival as compared with single dose treatment. No statistically significant differences were observed between aerosolized amphotericin B desoxycholate and the four different regimens of aerosolized liposomal amphotericin B.

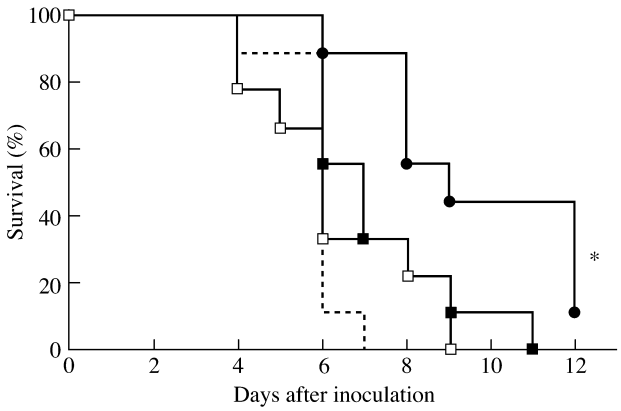

Figure 1. Effect of a single dose of aerosolized amphotericin B desoxycholate with a reservoir concentration of 1 mg/mL (□), 2 mg/mL (●) or 4 mg/mL (■) on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan–Meier plot). Each group consisted of 15 animals. Control animals received aerosolized 5% glucose (----). Treatment was started 30 h after inoculation, at which time mycelial growth was established. *$P = 0.006$ versus control rats.

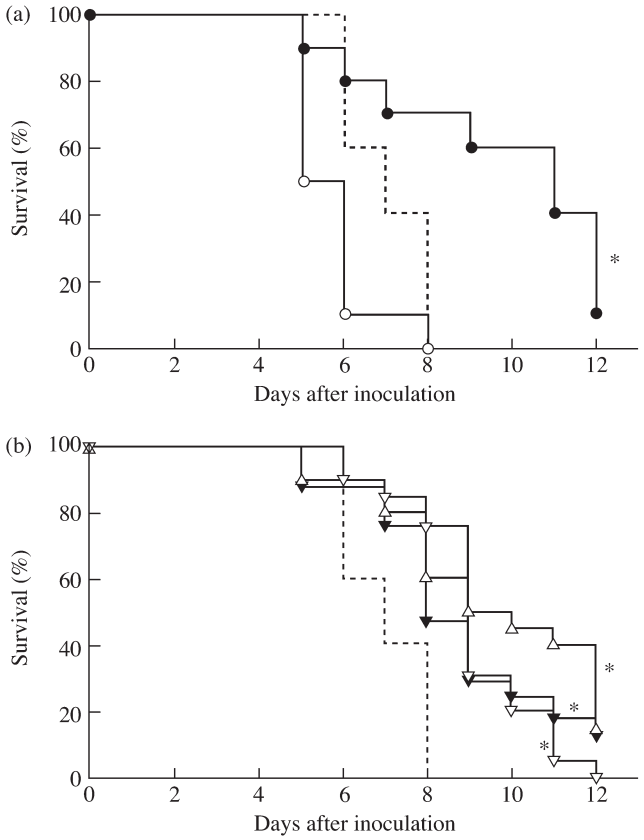

Figure 2. Effect of single (a) or multiple (b) dose aerosolized liposomal amphotericin B (L-AMB) versus glucose or empty liposome controls on survival of severely immunosuppressed rats with pulmonary aspergillosis (Kaplan–Meier plot). Groups of 15 animals each were treated with aerosolized L-AMB (reservoir concentration 4 mg/mL) as a single dose (●), as a double dose (q 24 h) (△) or as a quadruple dose, either delivered every 24 h (▽) or every 12 h (▼). Controls were treated with placebo liposomes (○), or 5% glucose (----). *$P < 0.05$ versus placebo liposomes or glucose controls.

Effect of aerosolized amphotericin B desoxycholate and aerosolized liposomal amphotericin B on the presence of viable A. fumigatus in the left lung and on dissemination to the right lung and liver

The results of post-mortem quantitative cultures of *A. fumigatus* from lungs and liver after treatment are shown in Table I. Cultures revealed that at the time of death the infection had disseminated to the right lung and liver in the majority of rats. Aerosol treatment had no significant beneficial effect on prevention of dissemination to the right lung or the liver.

Influence of amphotericin B products on surfactant function

Table II shows the mean γ_{min} of surfactant alone or in combination with different concentrations of amphotericin

Table I. Effect of aerosolized amphotericin B desoxycholate (AMB-DOC) and liposomal amphotericin B (L-AMB) versus aerosolized glucose 5% controls on the presence of viable *A. fumigatus* in the left lung and dissemination to the right lung and liver at the time of death[a]

Aerosol treatment	AMB nebulizer concentration (mg/mL)	Dosind frequency and interval (min)	% culture-positive organs		
			left lung	right lung	liver
Glucose 5%		1 × 60	100	60	53
AMB-DOC	1	1 × 60	100	53	40
AMB-DOC	2	1 × 60	80	40	60
AMB-DOC	4	1 × 60	100	87	87
Placebo liposomes		1 × 60	100	53	53
L-AMB	4	1 × 60	100	40	40
L-AMB	4	2 × 60, q = 24 h	100	47	73
L-AMB	4	4 × 60, q = 24 h	100	47	67
L-AMB	4	4 × 60, q = 12 h	100	33	53

[a]Leucopenic rats ($n = 15$) were inoculated in the left lung at time zero with 2×10^4 conidia of *A. fumigatus*.
Start of treatment was at 30 h.

B alone, amphotericin B desoxycholate, liposomal amphotericin B and desoxycholate. The natural surfactant was highly surface active at the concentrations examined (γ_{min} 1.97 ± 1.23). Minimal surface tensions after mixing of surfactant with amphotericin B or liposomal amphotericin B in different concentrations yielded similar low values. Addition of increasing concentrations of amphotericin B desoxycholate to natural surfactant resulted in significantly increased γ_{min}, indicating a loss of surface activity of the mixtures. An increase in γ_{min} of natural surfactant was also seen with addition of increased concentrations of desoxycholate.

Discussion

The importance of invasive aspergillosis has progressively increased and it is now a major direct or contributory cause of death at leukaemia treatment centres and bone marrow and solid organ transplantation centres.[1] In the present study, the efficacy of aerosolized conventional amphotericin B (amphotericin B desoxycholate) and aerosolized liposomal amphotericin B were evaluated in a rat model of severe pulmonary aspergillosis. In this aspergillosis model, rats received cyclophosphamide injections before fungal inoculation and throughout the whole experiment. This resulted in persistent deep granulocytopenia, in order to closely mimic a highly relevant, difficult to treat clinical situation. Treatment was started at 30 h after fungal inoculation, at which time hyphal formation was confirmed by histological examination of pulmonary tissue.

Significant prolongation of survival was observed after aerosol treatment with amphotericin B desoxycholate with a nebulizer reservoir concentration of 2 mg/mL. Lack of efficacy with a lower dose of amphotericin B desoxycholate was probably due to inadequate concentrations of amphotericin B in the lung tissue, whereas lack of efficacy with the high dose could be a consequence of pulmonary toxicity that overshadowed antifungal efficacy. Prolonged survival was also seen in animals treated with a single dose of aerosolized liposomal amphotericin B (nebulizer reservoir concentration 4 mg/mL). Similar results were obtained after double or quadruple dosing with aerosolized liposomal amphotericin B, therefore it appears that intensifying the aerosol regimens does not lead to improvement in efficacy in terms of survival. The lack of a dose–effect relationship after aerosol administration is probably due to inadequate deposition of bioactive amphotericin B at the site of the infection, which is not ameliorated by giving extra doses. The survival of rats after treatment with the optimal dose of aerosolized amphotericin B desoxycholate and with aerosolized liposomal amphotericin B seems to be similar to survival seen after 10 day treatment with iv liposomal amphotericin B (10 mg/kg) in the same animal model.[12]

The observations in terms of survival in the present study resemble those found in clinical practice, where, despite intravenous antifungal treatment, survival is <10–20% in persistently immunosuppressed patients with an established infection. However, the observed prolongation of survival after aerosol treatment may be useful in cases of temporary granulocytopenia. When leucocyte counts are restored early enough in the course of infection, improvements in the outcome of treatment can be expected, since it is known from clinical experience that bone marrow recovery and increasing numbers of circulating leucocytes are crucial factors in the favourable outcome of treatment.[1] Aerosol treatment with amphotericin B could be an alternative to current intravenous regimens, which offer good prognosis with neutrophil recovery.

Table II. Mean minimal surface tension (γ_{min}) of surfactant (1 mg/mL) together with saline, AMB, AMB-DOC, L-AMB or DOC

	Concentration (mg/mL)		
	AMB	DOC	γ_{min} (mN/m)[a]
Saline	–	–	1.97 ± 1.23
AMB	0.02		2.81 ± 0.63
	0.2		2.65 ± 0.47
	2		2.70 ± 0.53
AMB-DOC[b]	0.02	0.016	7.53 ± 1.51[c]
	0.2	0.16	14.76 ± 1.85[c]
	2	1.6	47.53 ± 0.89[c]
L-AMB	0.02		2.44 ± 0.41
	0.2		1.11 ± 0.72
	2		1.42 ± 0.83
DOC		0.008	1.94 ± 0.77
		0.08	5.59 ± 1.80[c]
		2.1	23.13 ± 1.16[c]
		21	45.21 ± 2.08[c]

[a]Each value represents the mean ± S.D. of three individual experiments.
[b]In AMB-DOC, 54% (g/g) is AMB, 46% is DOC. Therefore, 0.02 mg/mL AMB correlates with 0.016 mg/mL DOC.
[c]$P \leqslant 0.05$.

Treatment of animals with a suspension of 4 mg/mL pure amphotericin B in glucose 5% had no effect on survival as compared with controls (data not shown). The lack of efficacy may be the result of poor aerosolization characteristics and/or poor aqueous solubility and therefore poor availability of the drug at the site of deposition.

Aerosol treatments with either amphotericin B desoxycholate or liposomal amphotericin B had no effect on dissemination of *A. fumigatus* to the right lung or liver. It was shown previously that intravenous administration of liposomal amphotericin B in the same rat model of IPA does reduce dissemination.[12] This difference can be explained by the different pharmacokinetic profile of aerosol administration as compared with intravenous administration. Previous work in our laboratory has shown that aerosol treatment results in negligible systemic exposure to amphotericin B and subsequent negligible tissue concentrations in organs other than the lungs in contrast to the extensive tissue distribution following intravenous administration. Considering this, it would be interesting to study the efficacy of high dose aerosol treatment in combination with low dose systemic administration of amphotericin B in order to optimize drug delivery to the lungs and reduce dissemination at the same time.

Although limited data are available, inhaled liposomal amphotericin B seems to be well-tolerated by humans.[15] With inhaled amphotericin B desoxycholate side effects such as cough, nausea and mild bronchospasms are reported. These pulmonary side effects were in some cases dose limiting or even necessitated discontinuation of inhalation therapy.[16–18] The major side effect, which is cough, is presumed to be caused by desoxycholate rather than by amphotericin B.[18] In our study, no acute toxicities in terms of death or increased breathing frequencies were observed in animals during aerosol treatment with either amphotericin B desoxycholate or liposomal amphotericin B. *In vitro* surfactant function tests were performed in order to evaluate the disturbance of surfactant function after disposition of the amphotericin B desoxycholate and liposomal amphotericin B. The lowest amphotericin B concentrations and therefore the corresponding desoxycholate concentrations were based upon our observed lung deposition results, which were *c.* 20 μg/g lung tissue for amphotericin B desoxycholate and for liposomal amphotericin B. Liposomal amphotericin B alone (data not shown) showed high surface activity, similar to that of the surfactant, which can be explained by the presence in the formulation of phospholipids that are surface active components. As expected, liposomal amphotericin B did not influence surface activity of the surfactant. However, there was a dose-dependent inhibition of surfactant function by amphotericin B desoxycholate. This was due to the detrimental effects of desoxycholate on surfactant function, since this agent alone showed high influence on surface activity of natural surfactant in comparison with amphotericin B without desoxycholate. These results are in

accordance with those found by Griese *et al.*[19] Our results suggest that inhalation of amphotericin B desoxycholate but not liposomal amphotericin B may lead to surfactant dysfunction and this should be considered when choosing a formulation to be nebulized.

In this refractory model of IPA, survival of infected animals was prolonged after even a single dose of aerosolized amphotericin B desoxycholate or liposomal amphotericin B. Despite the uncertainties about pathogenesis and the precise antifungal activities of administered agents, these results encourage further investigations of the potential of aerosolized amphotericin B products in other settings such as prophylaxis or empirical treatment. Furthermore, it would be interesting to determine efficacy for other lipid formulations of amphotericin B that are currently on the market, since these products have different structural properties.

In summary, both aerosolized amphotericin B desoxycholate and liposomal amphotericin B are effective in prolonging survival of persistently leucopenic rats infected with *A. fumigatus*. Liposomal amphotericin B demonstrated no negative effect on pulmonary surfactant, as opposed to amphotericin B desoxycholate, and therefore would be the formulation of choice for aerosol administration in patients suffering from invasive pulmonary aspergillosis.

References

1. Denning, D. W. (1998). Invasive aspergillosis. *Clinical Infectious Diseases* **26**, 781–803.

2. Hiemenz, J. W. & Walsh, T. J. (1998). Lipid formulations of amphotericin B. *Journal of Liposome Research* **8**, 443–67.

3. Erjavec, Z., Woolthuis, G. M., de Vries-Hospers, H. G., Sluiter, W. J., Daenen, S. M., de Pauw, B. *et al.* (1997). Tolerance and efficacy of amphotericin B inhalations for prevention of invasive pulmonary aspergillosis in haematological patients. *European Journal of Clinical Microbiology and Infectious Diseases* **16**, 364–8.

4. Hertenstein, B., Kern, W. V., Schmeiser, T., Stefanic, M., Bunjes, D., Wiesneth, M. *et al.* (1994). Low incidence of invasive fungal infections after bone marrow transplantation in patients receiving amphotericin B inhalations during neutropenia. *Annals of Hematology* **68**, 21–6.

5. Schwartz, S., Behre, G., Heinemann, V., Wandt, H., Schilling, E., Arning, M. *et al.* (1999). Aerosolized amphotericin B inhalations as prophylaxis of invasive aspergillus infections during prolonged neutropenia: results of a prospective randomized multicenter trial. *Blood* **93**, 3654–61.

6. Allen, S. D., Sorensen, K. N., Nejdl, M. J., Durrant, C. & Proffit, R. T. (1994). Prophylactic efficacy of aerosolized liposomal (AmBisome) and non-liposomal (Fungizone) amphotericin B in murine pulmonary aspergillosis. *Journal of Antimicrobial Chemotherapy* **34**, 1001–13.

7. Cicogna, C. E., White, M. H., Bernard, E. M., Ishimura, T., Sun, M., Tong, W. P. *et al.* (1997). Efficacy of prophylactic aerosol amphotericin B lipid complex in a rat model of pulmonary aspergillosis. *Antimicrobial Agents and Chemotherapy* **41**, 259–61.

8. Schmitt, H. J., Bernard, E. M., Hauser, M. & Armstrong, D. (1988). Aerosol amphotericin B is effective for prophylaxis and therapy in a rat model of pulmonary aspergillosis. *Antimicrobial Agents and Chemotherapy* **32**, 1676–9.

9. Koizumi, T., Kubo, K., Kaneki, T., Hanaoka, M., Hayano, T., Miyahara, T. *et al.* (1998). Pharmacokinetic evaluation of amphotericin B in lung tissue: lung lymph distribution after intravenous injection and airspace distribution after aerosolization and inhalation of amphotericin B. *Antimicrobial Agents and Chemotherapy* **42**, 1597–600.

10. Lambros, M. P., Bourne, D. W. A., Abbas, S. A. & Johnson, D. L. (1997). Disposition of aerosolized liposomal amphotericin B. *Journal of Pharmaceutical Sciences* **86**, 1066–9.

11. Niki, Y., Bernard, E. M., Schmitt, H. J., Tong, W. P., Edwards, F. F. & Armstrong, D. (1990). Pharmacokinetics of aerosol amphotericin B in rats. *Antimicrobial Agents and Chemotherapy* **34**, 29–32.

12. Leenders, A. C. A. P., de Marie, S., ten Kate, M. T., Bakker-Woudenberg, I. A. & Verbrugh, H. A. (1996). Liposomal amphotericin B (Ambisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rat model of pulmonary aspergillosis. *Journal of Antimicrobial Chemotherapy* **38**, 215–25.

13. Ruijgrok, E. J., Vulto, A. G. & van Etten, E. W. M. (2000). Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats. *Journal of Pharmacy and Pharmacology* **52**, 619–27.

14. Bartlett, G. R. (1959). Phosphorus assay in column chromatography. *Journal of Biological Chemistry* **234**, 466–8.

15. Purcell, I. F. & Corris P. A. (1995). Use of nebulised liposomal amphotericin B in the treatment of *Aspergillus fumigatus* empyema. *Thorax* **50**, 1321–3.

16. Diot, P., Rivoire, B., Le Pape, A., Lemarie, E., Dire, D., Furet, Y. *et al.* (1995). Deposition of amphotericin B aerosols in pulmonary aspergilloma. *European Respiratory Journal* **8**, 1263–8.

17. Dubois, J., Bartter, T., Gryn, J. & Pratter, M. R. (1995). The physiologic effects of inhaled amphotericin B. *Chest* **108**, 750–3.

18. Gryn, J., Goldberg, J., Johnson, E., Siegel, J. & Inzerillo, J. (1993). The toxicity of daily inhaled amphotericin B. *American Journal of Clinical Oncology* **16**, 43–6.

19. Griese, M., Schams, A. & Lohmeier, K. P. (1998). Amphotericin B and pulmonary surfactant. *European Journal of Medical Research* **3**, 383–6.

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