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# Aerosol Delivery of Amphotericin B Desoxycholate (Fungizone) and Liposomal Amphotericin B (AmBisome): Aerosol Characteristics and In-vivo Amphotericin B Deposition in Rats

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**Abstract**

In the treatment or prophylaxis of invasive pulmonary aspergillosis, it may be attractive to administer the antifungal agent amphotericin directly to the pulmonary route via aerosol inhalation. In this study, we describe the aerosol characteristics of aerosolized non-liposomal amphotericin B (Fungizone) and liposomal amphotericin B (AmBisome), and the in-vivo aerosol deposition.

Aerosols were generated with a Collison nebulizer. Aerosol amphotericin concentrations and mass median diameters were measured. In-vivo pulmonary deposition was evaluated by measuring amphotericin concentrations in lungs of treated rats. Whole body aerosol deposition was determined by measuring radioactivity in tissues of rats after treatment with radiolabelled liposomes. For Fungizone and AmBisome, aerosol amphotericin concentrations were $24{\cdot}5 \pm 4{\cdot}9$ and $23{\cdot}8 \pm 3{\cdot}0\,\mu g\,L^{-1}$, respectively. The values for the median mass diameter were 1·38 and 2·26 $\mu$m for Fungizone and 2.43 and 1.97 $\mu$m for AmBisome. Amphotericin concentrations in lungs after 60-min nebulization of Fungizone or AmBisome were $24{\cdot}2 \pm 6{\cdot}4$ and $21{\cdot}7 \pm 2{\cdot}6\,\mu g\,g^{-1}$, respectively. After nebulization of radiolabelled liposomes, no radioactivity was retrieved from tissues other than the lungs or the gastrointestinal tract. Nebulization of either Fungizone or AmBisome leads to respirable aerosols and results in a substantial lung tissue concentration of amphotericin and low systemic exposure of amphotericin B. Aerosol administration of either Fungizone or AmBisome may be an attractive approach to prevent or treat pulmonary aspergillosis.

*Aspergillus* spp., and especially *Aspergillus fumigatus*, are increasingly recognized as major fungal pathogens in severely immunosuppressed or neutropenic patients (Denning 1998). The most encountered form of disease due to *Aspergillus* is invasive pulmonary aspergillosis. For many years the polyene antifungal agent amphotericin B has been the drug of choice in the treatment of many fungal infections, including invasive pulmonary aspergillosis (Denning 1998). However, use of this drug is hampered by considerable toxicity following intravenous injection, such as renal toxicity, anaemia and hypokalaemia (Gallis et al 1990). New (lipid based) formulations of amphotericin B, which have an increased therapeutic index as compared with conventional amphotericin B, are currently available (Hiemenz & Walsh 1996). However, the treatment of immunocompromised patients who have an established pulmonary infection of *Aspergillus* spp. is often unsuccessful, and optimization of an antifungal treatment for invasive pulmonary aspergillosis remains a challenge. *Aspergillus* spp. are respiratory pathogens and pulmonary infections leading to invasive pulmonary aspergillosis are usually acquired through inhalation of *Aspergillus* conidia. However, concentrations of amphotericin after intravenous injection of non-liposomal and liposomal amphotericin are seen mainly in liver and spleen (Christiansen et al 1985; Janknegt et al 1992). This may be the cause of treatment failure often observed in

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invasive pulmonary aspergillosis. Improvement in the treatment of invasive pulmonary aspergillosis can therefore be sought in the aerosol administration of amphotericin B or of lipid formulations of amphotericin B. Aerosol inhalation is an important method of drug delivery in pulmonary disease since it targets the lungs and reduces exposure of other organs to the drug, thus minimizing side effects (Lambros et al 1997; Ramsey et al 1999). Aerosol administration of non-liposomal as well as liposomal amphotericin in the treatment or prophylaxis of fungal infections has been reported by others and appeared to be promising (Kilburn 1959; Allen et al 1994; Beyer et al 1994; Hertenstein et al 1994; Reichenspurner et al 1997; Trigg et al 1997).

In this report, a system for aerosol administration of the commercially available products amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome) has been described to evaluate several aspects of the stability of nebulized suspensions, output of mass and volume of amphotericin B suspensions and delivered dose of amphotericin B. The in-vivo deposition in the lungs and the biodistribution in the other organs of the rat have been evaluated also.

## Materials and Methods

*Materials*

Amphotericin B desoxycholate (Fungizone, Bristol Myers-Squibb, Woerden, The Netherlands) and liposomal amphotericin B (AmBisome, NeXstar, San Dimas, CA) were reconstituted in distilled water according to the manufacturer's instructions and further diluted in 5% glucose in water up to a concentration of $4\,mg\,mL^{-1}$ amphotericin B. Amphotericin B (activity $916\,\mu g\,g^{-1}$) for calibration was from Bristol Myers-Squibb (Woerden, The Netherlands). Hydrogenated soy phosphatidylcholine (HSPC) and distearoyl phosphatidylglycerol (DSPG) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, dimethylsulphoxide (DMSO) and 8-hydroxyquinone sulphate were from Sigma Chemical Co (St Louis, MO). $^{67}$Gallium-citrate was from Mallinckrodt Medical BV (Petten, The Netherlands). Desferoxamine mesylate (Desferal) was from Novartis (Basel, Switzerland). All other reagents were of analytical grade.

*Animals*

Female RP strain rats (specified pathogen free, 18–25 weeks, 185–225 g) were used. The experiments were approved by the animal experiments ethical committee of the Erasmus University Medical Center.

*Nebulization apparatus and procedure*

The aerosol inhalation system is schematically depicted in Figure 1. Compressed air was supplied to the nebulizer with a flow rate of $20\,L\,min^{-1}$ and 37·5 psi as measured by a flow indicator (Shorate, Brooks Instrument BV, Veenendaal, The Netherlands) and a pressure indicator (ITT Fluid Technology, St George, SC). Aerosols were generated by a Collison six jet nebulizer (Model CN, BGI Inc., Waltham, MA). A glass nebulizer reservoir with a small fill hole at the jar bottom ensured high efficiency nebulization of fluids. Generated aerosols were led through a nose-only exposure inhalation chamber (CH Technologies USA Inc., Westwood, NJ). This inhalation chamber is suitable for aerosol treatment of 24 individual animals. A continuous flow was present inside the system, due to a vacuum pump at the end, operating with a flow rate of $23\,L\,min^{-1}$. In connection with this vacuum pump was a filter house containing two filters, one main filter to extract aerosol droplets from outgoing air, the other filter to compensate for over or under pressure developing during nebulization. In the in-vivo experiments, rats were constrained in cone-ended plastic animal holders such that only their nose was in contact with the aerosol.

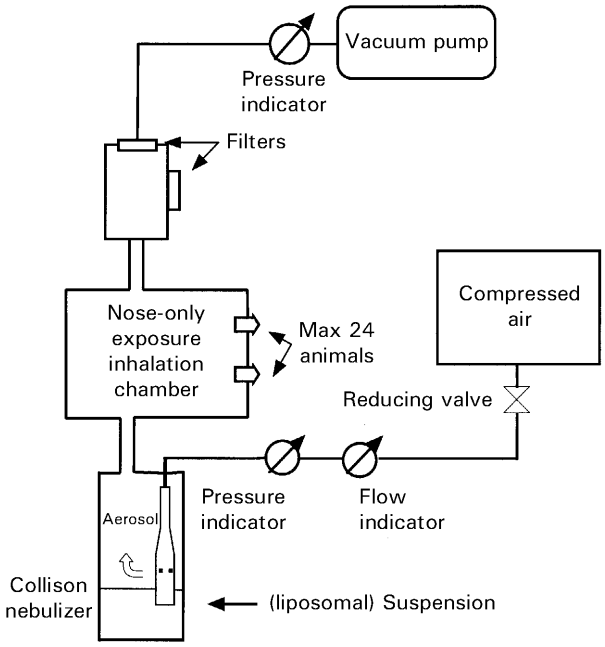


Figure 1. Schematic representation of the aerosol inhalation system.

*Amphotericin B spectrophotometrical analysis and liposomal characterization*

Amphotericin B concentrations in the nebulizer reservoir and in the inhalation chamber sample were determined spectrophotometrically. After diluting the samples in DMSO–methanol 1:1 v/v the observed extinctions at 410 nm could be interpolated in a standard calibration curve.

Mean particle size of liposomes was determined by dynamic light scattering (4700 system, Malvern Instruments, Malvern, UK) (Chong & Colbow 1976).

Phospholipid content was determined by phosphorus assay (Bartlett 1959).

*Measurement of volume and mass output*

Volume output of nebulized liquid was determined gravimetrically by subtracting the weight of the complete reservoir containing the residue after nebulization from the weight of the reservoir containing the liquid to be nebulized.

Mass output of amphotericin B was determined by subtracting the amount of amphotericin (mg) in the reservoir after nebulization from the amount of amphotericin in the reservoir before nebulization.

*Aerosol concentration measurement*

Aerosol samples were extracted from the inhalation chamber at the breathing level of rats as depicted in Figure 2. During a 60-min nebulization period a total sample volume of 1 L was extracted, at frequent intervals, using a 10-mL syringe. This sampling volume provided sufficient drug for spectrophotometrical analysis. The sampled air was led through 10-mL collection medium consisting of a mixture of DMSO–methanol 1:1 v/v, which could thereafter directly be measured according to the method described above.

*Droplet size measurements*

Droplet size distribution was measured using a laser velocity particle sizer (Aerosol Particle Sizer 3320A, TSI Inc., St Paul, MN). Aerosols were generated with compressed air at a flow rate of 10 L $min^{-1}$. Distribution of the number of generated aerosol particles was directly measured with this technique. For extrapolation of mass distribution from the number distribution, specific gravity was set at that of the solvent (1 g $cm^{-3}$) and relative humidity at 60%. From the mass distribution, the parameters mass median diameter, geometric standard deviation (g.s.d.) and percentage of particles with a mass diameter $< 5\ \mu m$ were calculated.

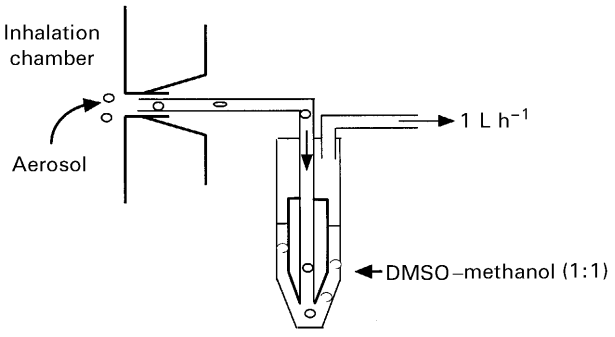


Figure 2. Inhalation chamber sampling procedure.

*Preparation of radiolabelled liposomes*

Liposomes were labelled with $^{67}Ga$ according to the method described by Gabizon et al (1988). Liposomes were prepared using the film hydration method. Liposomal membranes consisted of HSPC–DSPG–cholesterol in a molar ratio of 2:1:0·8, similar to the lipid composition of AmBisome. 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) and 5 mM desferoxamine. Liposomes were sonicated which resulted in vesicles with an average particle size of 100 nm, as measured by dynamic light scattering. Unencapsulated desferoxamine was removed by eluting the suspension over a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), followed by ultracentrifugation at 280 000 *g* for 2 h at 4°C. Phospholipid content was determined by phosphorus assay (Bartlett 1959). [$^{67}Ga$]Citrate (370 MBq $mL^{-1}$) was diluted 1:9 in a 0·5 mg $mL^{-1}$ 8-hydroxyquinone sulphate solution and incubated for 1 h at 50°C to yield [$^{67}Ga$]oxine. The liposome suspension was incubated overnight at 4°C with $3{\cdot}7 \times 10^{-2}$ MBq [$^{67}Ga$]oxine ($\mu$mol lipid)$^{-1}$. Unencapsulated [$^{67}Ga$]oxine was removed by gel-filtration (Sephadex G-50 column, Pharmacia, Uppsala, Sweden), and liposomes were concentrated by ultracentrifugation as described above.

*Deposition of radiolabelled liposomes*

Rats were treated with nebulized $^{67}Ga$-labelled liposomal suspension according to the nebulization method described above. Groups of two rats were killed with pentobarbital (100 mg $kg^{-1}$, i.v.) after 10, 20, 30, 40, 50 and 60 min of nebulization. Directly afterwards approximately 0·3 mL blood was sampled via an orbital puncture and left lung, right lung, trachea, tongue, oral cavity, lower jaw, snout, stomach and oesophagus, intestines, spleen,

liver and kidney were dissected. Organs, tissues and blood were weighed and analysed for $\gamma$-irradiation with a Minaxi autogamma 5000 gamma counter (Packard Instrument Company, Meriden, CT).

*Calculation of percentage deposition of radioactivity*

Measured counts of $^{67}$Ga-labelled liposomal suspension before and after 60-min nebulization were $6{\cdot}5 \times 10^6$ and $1{\cdot}2 \times 10^7$ counts min$^{-1}$ mL$^{-1}$, respectively. From these values, total counts min$^{-1}$ nebulized and the inhalation chamber concentration can be calculated. Delivered doses at the breathing point of rats can subsequently be derived as follows:

$$\begin{aligned}\text{delivered dose (counts min}^{-1}\text{)} &= \text{minute volume (L min}^{-1}\text{)} \\ &\times \text{aerosol chamber concentration (counts min}^{-1}\text{ L}^{-1}\text{)} \\ &\times \text{duration of nebulization (min)}\end{aligned} \quad (1)$$

Minute volume of rats was calculated according to Guyton (1947):

$$\text{minute volume (L min}^{-1}\text{)} = (\text{body weight (g)})^{0.75} \times 0.0021 \quad (2)$$

Measured counts min$^{-1}$ in organs and tissues divided by the delivered dose gives the relative deposition.

*Maximum pulmonary concentration of amphotericin after nebulization of Fungizone or AmBisome*

Directly after 60-min nebulization of either Fungizone or AmBisome, rats were killed and lungs were removed. Right and left lung lobes were weighed and homogenized in 20 mL 5% glucose. Amphotericin B was extracted from the homogenate with ethanol in a 4:6 (v/v) ratio. The extracts were centrifuged for 5 min at 13 000 *g* and concentrations of amphotericin in the supernatants were determined by HPLC with a UV detector operating at 382 nm. The mobile phase consisted of a 0·1 M sodium acetate solution (pH 7·2) containing 0·2% (v/v) triethylamine and 60% (v/v) acetonitrile and was pumped through a Guard pre-column (Chromguard, 10 × 3 mm; Chrompack, Middelburg, The Netherlands) followed by a reverse-phase $C_{18}$ column (Chromspher, 100 × 3 mm, Chrompack, Middelburg, The Netherlands) at a flow rate of 0·5 mL min$^{-1}$. The lower quantity of determination of this assay was 0·2 mg L$^{-1}$.

## Results

*Solute concentration during the process of nebulization*

Table 1 gives mean concentrations of amphotericin in the nebulizer reservoir before and after nebulization of Fungizone and AmBisome. For AmBisome, total lipid concentration and amphotericin-lipid ratio ($\mu$g amphotericin B ($\mu$mol total lipid)$^{-1}$) are given. After 60-min nebulization, concentrations of amphotericin B in the reservoir were increased with both Fungizone and AmBisome. Increase in concentration of amphotericin B with nebulization of Fungizone was more pronounced than with AmBisome ($P = 0{\cdot}006$). Total lipid concentration in the AmBisome suspension increased linearly with concentration of amphotericin in AmBisome (Table 1). As a result, the amphotericin/lipid ratio of the liposomal suspension did not change during nebulization.

*Stability of liposomes to nebulization*

Diameters of liposomes in the AmBisome suspension before nebulization, in the remnant volume in the reservoir after nebulization, and of the inhalation chamber sample taken during 60-min

Table 1. Amphotericin concentration, total lipid concentration and amphotericin/lipid ratio in the reservoir suspension before and after 60-min nebulization of Fungizone and AmBisome.

| | Fungizone Amphotericin concn (mg mL$^{-1}$) | AmBisome Amphotericin concn (mg mL$^{-1}$) | Total lipid concn ($\mu$mol mL$^{-1}$) | Amphotericin/lipid ($\mu$g $\mu$mol$^{-1}$) |
|---|---|---|---|---|
| Before nebulization | 4·1 ± 0·6[a] | 4·1 ± 0·2 | 38·6 ± 2·2 | 104·1 ± 9·0 |
| After nebulization | 12·9 ± 1·0 | 8·4 ± 2·2 | 81·6 ± 13·9 | 106·5 ± 11·6 |
| Ratio concn$_{after}$/concn$_{before}$ | 3·1 ± 0·2[b] | 2·1 ± 0·6 | 2·3 ± 0·4 | 1·0 ± 0·0 |

[a]Each value represents the mean ± s.d. of three individual experiments. [b]$P = 0{\cdot}006$ vs AmBisome.

Table 2. Liposomal diameters of AmBisome before and after nebulization in the nebulizer reservoir and in the inhalation chamber sample.

| | Liposomal diameter (nm) |
|---|---|
| Before nebulization | 74·0 ± 6·1[a,b] |
| After nebulization | 84·1 ± 5·2 |
| Inhalation chamber sample | 86·1 ± 1·4 |

[a]Each value represents the mean ± s.d. of at least three individual experiments. [b]$P = 0{\cdot}004$ and 0·013 vs liposomal diameter after nebulization and of the chamber sample, respectively.

nebulization are shown in Table 2. A significant increase in mean liposomal size was observed after nebulization in the residue suspension after nebulization ($P = 0{\cdot}004$) as well as in the inhalation chamber sample ($P = 0{\cdot}013$) as compared with liposomal diameter of the suspension to be nebulized.

*Volume and mass output*

The nebulised volumes of glucose 5%, Fungizone or AmBisome were similar during a 60-min nebulization period (Table 3). Mass output in terms of mg amphotericin B was 35·40 ± 6·20 mg for Fungizone and 57·13 ± 10·17 mg for AmBisome. Amphotericin B output after nebulization of Fungizone was significantly lower than after nebulization of AmBisome ($P = 0{\cdot}031$).

*Aerosol concentration measurement*

Aerosol concentrations measured at the breathing-points of rats were the same for Fungizone and AmBisome (L collected air)$^{-1}$ over a 1-h period (24·53 ± 4·93 and 23·75 ± 2·97 $\mu$g L$^{-1}$, respectively).

*Aerosol size distribution*

Figure 3 shows the droplet size distribution of aerosol droplets for Fungizone, AmBisome and for 5% glucose in water. A similar homogenous distribution for all formulations was observed (median droplet size for Fungizone 0·743 $\mu$m and 0·758 $\mu$m

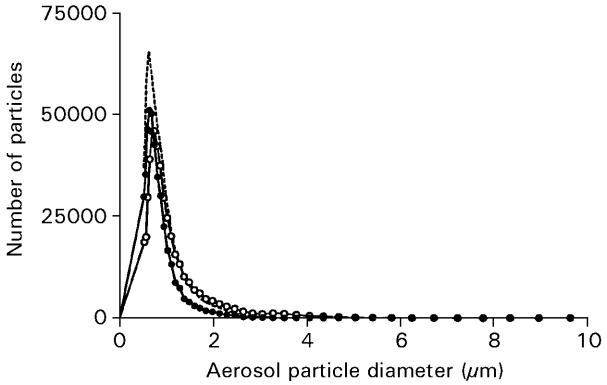


Figure 3. Aerosol particle number distribution of aerosolized Fungizone (●), AmBisome (○) and 5% glucose in water (- - -) as determined by laser diffraction.

for AmBisome). Figure 4 shows the calculated cumulative mass distribution. Percentages of particles < 5-$\mu$m mass diameter were 83·20 and 86·60 for nebulized Fungizone and AmBisome, respectively. Derived mass median diameter and geometric standard deviation (g.s.d.) for nebulized Fungizone were 1·38 $\mu$m and 2·26 $\mu$m, respectively. The values for AmBisome were 2·43 $\mu$m and 1·97 $\mu$m, respectively.

*In-vivo deposition of $^{67}$Ga-labelled liposomes*

Distribution of delivered dose in relevant organs and tissues after 60-min nebulization calculated as percentage of the delivered dose is shown in Table 4. The largest part was retrieved from the intestines (26%). From the left and right lung only 2% and 4%, respectively, of the delivered dose was retrieved. Less than 1% was observed in blood, kidneys, liver and spleen. During the 60-min nebulization period, linear accumulation of radioactivity in the lungs of rats was observed (Figure 5).

*Deposition of amphotericin in lungs after nebulization of Fungizone and AmBisome*

Table 5 shows the absolute amount of amphotericin B ($\mu$g) and the concentration of amphotericin ($\mu$g amphotericin B (g tissue)$^{-1}$) retrieved from both

Table 3. Volume output and mass output of amphotericin after 60 min nebulization of Fungizone or AmBisome.

| | Glucose (5%) | Fungizone (4 mg mL$^{-1}$) | AmBisome (4 mg mL$^{-1}$) |
|---|---|---|---|
| Volume (mL) | 23·7 ± 1·0[a] | 23·4 ± 1·2 | 23·2 ± 1·3 |
| Mass (mg amphotericin) | – | 35·4 ± 6·2[b] | 57·1 ± 10·2 |

[a]Each value represents mean ± s.d. of three individual experiments. [b]$P = 0{\cdot}031$ vs AmBisome.

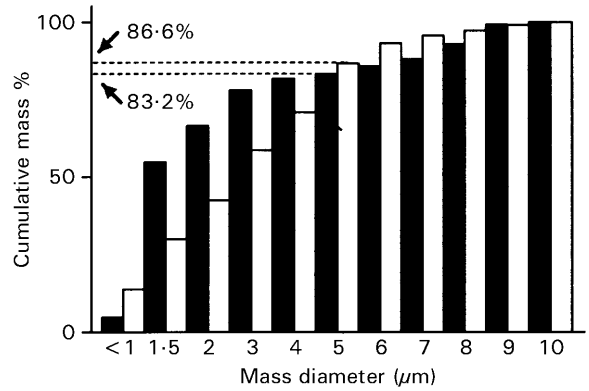


Figure 4. Cumulative mass distribution of aerosol droplets after aerosolization of Fungizone (■) or AmBisome (□).

Table 4. Recovery of radioactivity (% of delivered dose) in different organs and tissues of rats after aerosolization (60 min) of $^{67}$Ga-labelled liposomes.

| Organ/tissue | % deposition |
|---|---|
| Left lung | 2·06 ± 0·22 |
| Right lung | 3·84 ± 0·58 |
| Trachea | 1·40 ± 0·25 |
| Tongue | 1·06 ± 0·14 |
| Lower jaw | 1·37 ± 0·82 |
| Snout | 13·36 ± 1·42 |
| Oral cavity | 0·32 ± 0·23 |
| Stomach/oesophagus | 2·69 ± 0·01 |
| Intestines | 25·30 ± 3·82 |
| Liver | 0·27 ± 0·10 |
| Spleen | 0·01 ± 0·00 |
| Kidney | 0·31 ± 0·08 |
| Blood | 0·03 ± 0·02 |

Each value represents the mean ± s.d. of three individual experiments.

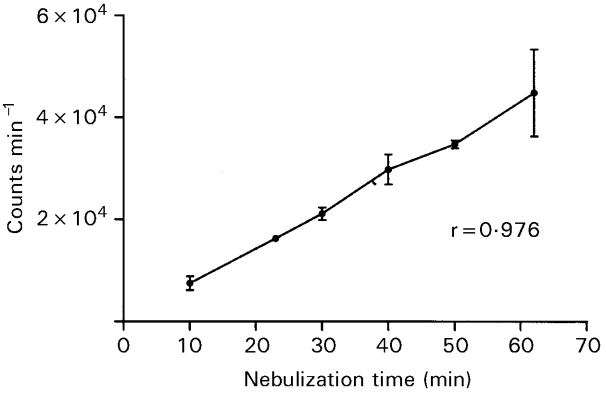


Figure 5. Radioactivity in lungs of rats (n = 2) at different times during aerosolization (60 min) of $^{67}$Ga-labelled liposomes. Each point represents the mean and range.

left and right lung lobes of rats treated with aerosols of Fungizone or AmBisome. The absolute deposited dose of amphotericin B (μg) and the concentration of amphotericin B in lung tissue reached directly after nebulization were similar for Fungizone and AmBisome. Furthermore, concentrations of amphotericin B were similar in both lung lobes in all rats.

## Discussion

Before assessing the efficacy of nebulized products in an experimental model of pulmonary aspergillosis, it is important to gain insight into several aspects of nebulizer function. This is important because parameters such as design, operating conditions and ancillary equipment are pivotal for the final delivered dose and product stability (Niven et al 1992; Waldrep et al 1994). The Collison nebulizer has been widely used in aerosol research for many years and is documented in detail (May 1973; Young et al 1977).

Volume output of the Collison nebulizer under the experimental conditions used was the same for both amphotericin B preparations examined. For Fungizone and AmBisome, concentration of amphotericin B in the nebulizer reservoir occurred during the nebulization process. The phenomenon of concentration of nebulized solution or dispersed material was previously described as a consequence of evaporative loss of water needed for humidification of inflowing air (Ip & Niven 1994; Roth et al 1996). This indicates that determinations of mass output should not be calculated from initial reservoir concentrations. Increase in amphotericin B concentration after nebulization of Fungizone was higher compared with AmBisome, which was directly correlated with a lower mass output of amphotericin for nebulized Fungizone. Sorensen et al (1994) showed that inhalation chamber air amphotericin B concentrations decreased as a function of time during nebulization of Fungizone, which could explain the difference in aerosol concentration between Fungizone and AmBisome. Those authors explained the phenomenon as due to the foaming of Fungizone due to the presence of the detergent desoxycholate. It is an option to calculate amphotericin B aerosol concentrations from solute concentrations before and after nebulization (mass output). However, without considering inertial impaction and gravimentational sedimentation of aerosol droplets inside the aerosol inhalation system this method will result in an inaccurate measurement (overestimation) of delivered dose. Direct measuring of aerosol concentrations would

Table 5. Amount and concentration of amphotericin B in left and right lungs of rats after 60-min nebulization of Fungizone or AmBisome.

| Formulation | Left lung | | Right lung | |
|---|---|---|---|---|
| | Amphotericin B ($\mu$g) | Amphotericin B concn ($\mu$g g$^{-1}$) | Amphotericin B ($\mu$g) | Amphotericin B concn ($\mu$g g$^{-1}$) |
| Fungizone | 15·3 ± 1·1 | 24·2 ± 6·4 | 21·5 ± 0·2 | 19·9 ± 4·8 |
| AmBisome | 12·5 ± 2·5 | 21·7 ± 2·6 | 20·8 ± 3·8 | 21·6 ± 0·9 |

Each value represents mean ± sd of three individual experiments.

give more precise estimations of amphotericin B output. Despite the observed differences in mass outputs of amphotericin B, we have seen a similar measured aerosol concentration for nebulized Fungizone and nebulized AmBisome. This is in agreement with Allen et al (1994).

The amphotericin B-lipid ratio of AmBisome was not influenced by nebulization. It can be concluded from this that amphotericin B remains associated with the liposomal membrane during nebulization. Liposomal size was, however, affected by the nebulization process. The mean liposome size increased slightly during 60-min nebulization, probably because of fusion of a small population of vesicles as result of air pressure upon passage through the nebulizer orifices. This size increase was however small, which indicates that liposomal integrity was hardly influenced. A formulation with similar lipid composition and liposomal size as AmBisome (26 mol% cholesterol) has been shown to be relatively stable to nebulization (Niven & Schreier 1990; Niven et al 1991). In addition, an increase in liposomal size will not influence the deposition pattern of nebulized AmBisome, since liposomes are still several-times smaller than average aerosol droplet size.

Droplet size and shape play a significant role in the drug deposition in the respiratory passageways. Preferably, more than 90% of the generated droplet population should be in the 0·5–10 $\mu$m range to maximize delivery in pulmonary tissues and fluid. In general, droplets of 1–5 $\mu$m are considered optimal for penetrating the lower respiratory tract (Newman & Clarke 1983; Barry & O'Callaghan 1996). Particles of this size have been demonstrated to deposit in the lung via gravimentational sedimentation, inertial impaction or by diffusion into terminal alveoli by Brownian motion (Hickey 1996).

Aerosol droplet sizing by laser based analysis offers a few advantages over the impaction approach frequently used to characterize aerosol droplets. The nebulizer cloud can be measured directly after leaving the nebulizer reservoir, and therefore information is collected on the 'true' droplet size that enters the respiratory tract of laboratory rats. Moreover, this technique is much faster than the impaction technique and no chemical analysis of solute is required. Laser diffraction has proven to be robust and reliable for measuring droplet sizes directly after nebulization (Clark 1995). However, care must be taken with interpreting number distribution results from laser scattering data. When using this method, extrapolation of number distribution to mass distribution must be made in order to predict the final mass deposition in pulmonary regions of interest. For this, the specific gravity of droplets should be known.

As seen from the number distribution, a very homogenous aerosol cloud was generated by the Collison nebulizer under the experimental conditions of this research. With Fungizone and AmBisome a large number ($>80\%$) of droplets was below 5-$\mu$m mass diameter. Therefore, a substantial deposition in the alveobronchial region could be expected.

To evaluate the biodistribution of nebulized materials in rats, we have used $^{67}$Ga-labelled liposomes. This indirect measure of biodistribution was chosen because radiolabelled AmBisome is not available and analysis of amphotericin B in all organs and tissues is difficult to measure accurately. The radiolabelled liposomes however, had a liposome composition similar to AmBisome and therefore results could be correlated directly to the biodistribution of nebulized AmBisome. The percentage deposition was calculated on the basis of the aerosol concentration. Total in-vivo counts only accounted for 52% of the calculated delivered dose. Two important factors could attribute to the relatively low in-vivo counts compared with the calculated delivered dose. Impaction and subsequent loss of nebulized material inside the system was not taken into account. Furthermore, the exhaled fraction of aerosol clouds could add up to loss of material. The highest deposition of radioactivity was seen in the gastrointestinal tract (28·0%), due to deposition of inhaled particles inside the oral region and on the snout. Swallowing of deposited

material would lead to high intestinal drug levels. Gastrointestinal absorption of amphotericin B is however very small ($< 5\%$) and biodistribution through this route would thus not lead to significant systemic drug levels. Deposition of radioactivity in lungs accumulated linearly in time and was 11·3% of total in-vivo counts. Very little ($< 1\%$) radioactivity was found in the internal organs.

As could be expected from the similar aerosol concentrations measured, amphotericin B concentrations reached in lung tissues of treated rats directly after 60-min nebulization were similar with Fungizone and AmBisome. For *Aspergillus fumigatus*, the pathogen of interest in our animal model, the in-vitro minimal inhibitory concentration is $0{\cdot}4-0{\cdot}8\ \mathrm{mg\,L^{-1}}$. Final concentrations of amphotericin B in pulmonary tissues reached with both formulations should be sufficient for in-vivo killing. The results of this study were obtained in healthy rats. Whether deposition in diseased lungs would be equivalent remains to be examined.

In summary, we have reported on the nebulization of non-liposomal and liposomal amphotericin B with a Collison nebulizer. Fungizone was less efficiently nebulized than AmBisome, when mass output of amphotericin B was considered. However, aerosol concentration of amphotericin B was similar with the two products. The delivered dose of amphotericin B could therefore be considered the same. Aerosols of Fungizone and AmBisome were highly respirable and resulted in sufficient lung tissue concentrations of amphotericin B. Distribution of inhaled amphotericin B to organs other than the lungs or the gastrointestinal tract is expected to be negligible. Furthermore, liposomes containing amphotericin B are physically stable to nebulization. It appears that aerosol administration of amphotericin B formulations is a promising approach to target the lungs and may offer a valuable strategy in the treatment or prevention of invasive pulmonary aspergillosis.

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