

Deposition of amphotericin B aerosols in pulmonary aspergilloma

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ABSTRACT: The aim of the present study was to characterize amphotericin B aerosols nebulized by ultrasonic and jet nebulizers and to study their deposition and pharmacokinetics in patients with pulmonary mycetoma.

The aerodynamic behaviour and pulmonary deposition of amphotericin B particles were measured using a direct isotopic method based on stable labelling of the drug with ^{99m}Tc. Each nebulizer was bench tested for inhaled mass and particle size distribution. Three patients suffering from pulmonary aspergilloma were enrolled for a 4 week clinical study. They received 5 mg of amphotericin B daily delivered by either Fisoneb® or DP100® (ultrasonic) or Respirgard II® (jet) nebulizers. Deposition of radiolabelled amphotericin B was measured once with each nebulizer using a gamma-camera. In two patients, amphotericin B serum concentration was monitored over a 330 min period after the nebulization had been completed.

Inhaled masses of the three nebulizers, assessed as % of labelled drug caught in inspiratory filter in duplicate experiments, were: 5.8 and 3.6% for Respirgard II®; 26.5 and 28.3% with Fisoneb®; 5.9 and 6.3% for DP100®. Mass median aerodynamic diameter (mean±SD) results were: 0.28±0.04 µm with Respirgard II®; 4.82±0.78 µm with Fisoneb®; and 2.27±1.14 µm with DP100®. Because of larger particles and significantly greater inhaled mass, Fisoneb® delivered more amphotericin B to the central airways, the lung periphery and in the mycetoma lung regions. Amphotericin B serum concentrations correlated with pulmonary deposition and remained below 25 ng·mL⁻¹. No untoward effects were reported by the patients during the 4 week trial.

This study demonstrates that amphotericin B suspension can be accurately radio-labelled, is effectively nebulized by a variety of nebulizers, and is well-tolerated by human subjects.

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Aerosolized treatment of pulmonary aspergillosis has been proposed as an adjunctive local therapy for invasive fungal disease [1] and a potential alternative measure in patients with mycetomas who are not candidates for surgery [2]. Topical therapy to the lung *via* nebulization may decrease systemic side-effects and increase local effectiveness in immunocompromised hosts with invasive disease by providing high pulmonary levels, thus, allowing reduction of usual intravenous doses. Pulmonary mycetomas, whilst less threatening than invasive disease, often bleed and become superinfected, causing clinical problems that are difficult to treat if they cannot be definitively removed at surgery. Whilst topical therapy with amphotericin B aerosols may be theoretically advantageous, there are important questions regarding practical aspects of delivery that should be addressed before large clinical studies are performed. What is the best method to generate these aerosols? Can currently available preparations be nebulized? Will patients tolerate the aerosol?

The aim of the present study was to determine whether amphotericin B suspension can be effectively nebulized and deposited in the human respiratory tract. This was achieved in several steps. A method for direct labelling of amphotericin B was validated and the aerosol, nebulized from various types of nebulizer, was characterized both *in vitro* and *in vivo*. To demonstrate the effective deposition in the human respiratory tract, three subjects with clinically stable pulmonary mycetomas served as a human model of *Aspergillus* infection.

Materials and methods

Nebulizers

Three nebulizers (one jet and two ultrasonic nebulizers) were compared. The jet nebulizer was the Respirgard II® (Europe Medical, France) which was used at a

9 L·min⁻¹ continuous airflow. The ultrasonic nebulizers were the Fisoneb® (Fisons, France) and the DP100® (DP Medical, France). Fisoneb® operates at 1.3 MHz frequency and was used at the maximum flow rate. DP100® operates at 2.4 MHz frequency and was used for nebulization at the maximum flow rate and ventilation at the medium flow rate.

Amphotericin B labelling process and test procedures

Pure amphotericin B powder was used (control number 240447, provided by Bristol Co., Les Collines de l'Arche-Cedex 24-92057 Paris La Défense, France) for both *in vitro* bench experiments (inhaled mass, particle size distribution) and *in vivo* deposition studies.

Labelling method. Five milligrams of amphotericin B were mixed in 5 mL sterile water. This suspension was labelled under vacuum with 370 MBq ^{99m}Tc in the presence of 200 µg stannous chloride. Quality of labelling was checked both *in vitro* and *in vivo* as detailed below.

Labelling control by *in vitro* high performance liquid chromatography (HPLC). Control of the radiochemical purity was performed by chromatography on 3 mm Chr Whatman paper using methanol/water (80/20 v/v) for elution; these conditions allowing sensitive quantitation of free ^{99m}TcO₄⁻.

To further define the labelled preparation, radio HPLC on a Lichrosorb Si 60-5 column was performed using an iso-octane/dichloromethane/isopropanol mixture (900/90/10, v/v). Radioactivity was detected using a LB506 C Berthold monitor equipped with a 150 µl BGo flow cell. Detection of nonradioactive molecules was performed using a Bischoff 8110 (Leonberg, Germany) refractive index device. The content in colloids labelled with ^{99m}Tc was determined using exclusion diffusion radio HPLC on a TSK 2000 SW column, using water as eluant.

Labelling control by *in vivo* ^{99m}Tc scanning in baboons. Because the nebulization process could alter the amphotericin B binding with ^{99m}Tc, *in vivo* control of stability of the labelling after nebulization was performed in three healthy Papio-papio baboons weighing 10-15 kg. Each of the baboons, unanaesthetized at the time of the experiments, inhaled the ^{99m}Tc-labelled amphotericin B aerosol three times over a 1 month period through a face-mask, using Fisoneb®, Respirgard II® and DP100®. A gamma-camera, Orbiter 75 (Siemens, Germany), equipped with a high resolution low energy collimator was used at the 140 keV photoelectric peak with a 15% spectral window. At the beginning of inhalation, a dynamic deposition study (20, 60 s images, posterior view) was instituted to measure deposition in real time, and position the animal to outline both lungs and neck. After completion of aerosol inhalation, serial 300 s images of the neck and chest were recorded over 180 min in order to assess stability of the labelling *in vivo*.

Aerosol characterization

Inhaled mass assessment. The inhaled mass is the quantity of drug actually reaching the mouth with a given nebulizer for a defined breathing pattern and period of time [3]. It is an important factor in terms of effectiveness of aerosol therapy, because it is related to the nebulizer efficiency [4]. Inhaled mass was assessed in duplicate with each of the nebulizers. For Respirgard II®, inhaled mass was measured from two different nebulizers. For DP100® and Fisoneb®, the same nebulizer was used for the duplicate experiments.

The nebulizer charge (N) was counted before nebulization in an activimeter for gamma-emitters Capintec CRC-15R (Aries, France) and expressed in µCi. The nebulizer was then connected to a piston ventilator (Harvard, USA) used at a frequency of 20 breaths·min⁻¹, 750 cc tidal volume and 0.5 inspiration/expiration time ratio, according to conditions described previously [5]. An absolute filter of low resistance, Pad (41.0522, Pari, Germany), was interposed between the nebulizer and the ventilator. This filter sampled all the aerosol particles inhaled by a patient breathing with the pattern described above. The Respirgard II® and DP100® were run continuously, and the Fisoneb® was activated during inspiration. Aerosolization was considered to be complete when a period of 2 min had elapsed without the nebulizer producing aerosol [6]. Radioactivity deposited on the filter was counted using the Capintec CRC-15 R activimeter (Aries, France). The inhaled mass was expressed as a percentage of the activity placed in the nebulizer, *i.e.* the nebulizer charge (N).

Particle size distribution

Five milligrams of amphotericin B in 5 mL sterile water was labelled, as described above. Particle size distribution was determined in duplicate using the cascade impaction method, with the same nebulizers used in the inhaled mass study. A 10 stage GS1 cascade impactor (Schaefer Techniques, Nozay, France) was connected to the Harvard ventilator (used in the same condition as described above) and to the nebulizer *via* a T-connector, just proximal to the filter. The aerosol was sampled isokinetically at a 1 L·min⁻¹ flow until nebulization was completed. Each of the slides of the cascade impactor was washed with 0.5 mL dimethylsulphoxide (DMSO) and collected into separate vials. Radioactivity was counted in a 5 inch NaI (TI) Packard-Canberra detector for 60 s. Data were expressed in counts per minute (cpm). Response of this system is linear until 600,000 cpm. Mass of amphotericin B present in each vial was then determined by HPLC, according to the method described below. Diagrams presenting cumulative percentages of radioactivity or mass of amphotericin B on the x-axis and aerodynamic diameter corresponding to each of the slides on the y-axis were drawn on log probability paper. Mass median aerosol diameter (MMAD) and geometric standard deviation (σ_g) were therefore determined from isotopic (MMAD_i and σ_{gi}) or HPLC (MMAD_c and σ_{gc}) curves as described previously [7, 8]. Isotopic and HPLC data on each stage were compared by linear regression analysis.

Amphotericin B assay. The assay was adapted from the method described by LECLERCQ *et al.* [9].

Reagents and standards. HPLC grade methanol and hydrochloric acid were purchased from Merck (Darmstadt, Germany) and DMSO from Aldrich (Saint Quentin-Fallavier, France). Ethylenediamine tetra-acetic acid (EDTA) was obtained from Aldrich and was analysis grade. A stock standard solution of amphotericin B ($1 \text{ g}\cdot\text{L}^{-1}$) was prepared in DMSO. Working standard solutions ($5,000, 1,000, 500, 100, 50$ and $25 \text{ ng}\cdot\text{mL}^{-1}$) were prepared by dilution of the stock solution with the mobile phase. The mobile phase was a mixture of methanol:5 mM EDTA (80:20), adjusted to pH 7.8 with dilute hydrochloric acid. It was vacuum-filtered before use with a solvent filtration system (Waters Assoc., Milford, MA, USA).

Chromatography and extraction procedure. The analyses were carried out using a Waters HPLC equipped with a 600 E Waters pump and a 712 wisp injector thermostated at 30°C . The detector was a Waters 991 photodiode assay model. The column was a reversed-phase Bondapak C 18 ($100\times 6.2 \text{ mm}$; $10 \mu\text{m}$ average particle size) provided by Interchim (Montluçon, France).

For each sample, 0.9 mL of mobile phase was added to 0.1 mL of DMSO containing amphotericin B. The mixture was spun for 30 s, and 100 μL were injected into the chromatograph.

The chromatography conditions were the following: flow of the mobile phase $1.2 \text{ mL}\cdot\text{min}^{-1}$, absorbance reading 405 nm.

Deposition study

Patients. Three patients (two males, aged 72 and 64 yrs, and one female, aged 72 yrs, hereafter designated as patients Nos. 1–3, respectively), suffering from post-tuberculosis lung aspergilloma, gave their informed consent to participate in this study approved by our local Ethics Committee. Aspergilloma had been diagnosed according to the usual criteria [10], and localized on computed tomographic (CT) scan. It was positioned in the left upper lobe in patient No. 1 and in the right upper lobe in patients Nos. 2 and 3. These patients could not be treated by surgery because of respiratory insufficiency. Their clinical status remained stable during the study.

Image acquisition. Each of the patients received amphotericin B aerosol through the mouth, at a daily dose of 5 mg for 4 weeks. It was mixed in 5 mL sterile water. In an attempt to optimize respiratory function before amphotericin B nebulization, patients inhaled two puffs of salbutamol as a pretreatment. Each nebulizer was used for a 1 week period. During the last, or fourth week, daily administration was completed *via* the Fisoneb® for all three patients. On the 5th day of each of the first three treatment weeks, patients inhaled $^{99\text{m}}\text{Tc}$ amphotericin B labelled as described above, with Respirgard II®, Fisoneb® or DP 100®. A new Respirgard II® was used each day. Each patient used the same Fisoneb®

and DP100® nebulizer. The nebulizer charge (N) was counted before nebulization, as described above, and expressed in μCi . Patients inhaled $^{99\text{m}}\text{Tc}$ amphotericin B aerosol until dryness of the nebulizer. As soon as this was achieved, a 300 s 128×128 pixel static image was obtained from the anterior view of the chest.

Image analysis. Images were processed using a Max Delta (Siemens, Germany) computer. For each patient, the lung images obtained after each nebulizer study were visually compared. Regions of interest (ROI) were drawn on the image which had the largest lung area and then superimposed onto the other two images. These ROIs were used to assess the lung deposition by the ratio of cpm to the nebulizer charge, *i.e.* deposition/N ($\text{cpm}/\mu\text{Ci}$). They were subdivided into a central zone (C) which is the region centered over the large central airways constituting 33% of the area of both lungs, and a peripheral zone (P) which is obtained after the deduction of the central zone from the whole lung zone [11]. ROIs corresponding to upper (U) and lower (L) halves were also defined in each of the lungs. In the three patients and with the three nebulizers, the lower half of the left lung was clearly separated from the gastric activity. A ROI corresponding to the upper half of the single lung where the aspergilloma was located was also drawn (A). The activity present in each ROI was expressed in counts per minute. Results were expressed by ratios either of the relative activity (cpm) in two different regions or of the activity in the region to the μCi in the nebulizer (N) at the beginning of inhalation. In each patient, the three nebulizers were, therefore, compared in terms of the following ratios: deposition/N ($\text{cpm}/\mu\text{Ci}$); C/P (cpm/cpm); U/L (cpm/cpm); C/N ($\text{cpm}/\mu\text{Ci}$); P/N ($\text{cpm}/\mu\text{Ci}$); U/N ($\text{cpm}/\mu\text{Ci}$); A/N ($\text{cpm}/\mu\text{Ci}$).

Pharmacokinetics and tolerance

In two patients (patients Nos. 1 and 3), amphotericin B serum levels were assayed using the HPLC technique described above on the days of the deposition studies, 30, 90, 150, 210, 270, and 330 min after the amphotericin B inhalation had been completed. To assess influence of the deposition pattern of the drug on its serum kinetics, amphotericin B serum peak values and peak times were compared to the different ratios characterizing regional deposition, using linear regression analysis.

Amphotericin B tolerance was checked by comparing dyspnoea and lung sounds before and after inhalation. At the end of the 4 week treatment, patients gave their subjective feeling regarding the effect of the treatment on their dyspnoea and well-being (improved, worsened, unchanged).

Results

Amphotericin B labelling control

In vitro control. HPLC analysis revealed less than 1.5% free $^{99\text{m}}\text{Tc}$. Labelled amphotericin B exhibited the same

chromatography pattern and retention volumes as the unlabelled molecule.

Baboon control. Absence of free circulating TcO_4^- was assessed in the baboons by imaging of the neck, which did not exhibit thyroid uptake over 200 min. The absence of circulating $^{99\text{m}}\text{Tc SnCl}_2$ was confirmed by the absence of bone fixation, both on neck and chest, over the same time period.

Aerosol characterization

Inhaled mass assessment. Inhaled masses, as assessed by duplicate runs were: 5.8 and 3.6% for Respirgard II®; 26.5 and 28.3%, for Fisoneb®; and 5.9 and 6.3% for DP100®.

Particle size distribution study. Particle size distributions are presented in table 1. Each nebulizer was studied twice, and for each run, particle size distribution was assessed both by isotopic and HPLC methods by linear regression. Results of both methods were similar ($r=0.981$ for Respirgard II®, $r=0.974$ for Fisoneb®, and $r=0.976$ for DP100®; $p<0.001$ for all 3 nebulizers). Mean \pm SD MMAD, expressed as the average of all data (HPLC and radioactivity) was higher with Fisoneb® ($4.82\pm0.78\text{ }\mu\text{m}$) than with DP100® ($2.27\pm1.14\text{ }\mu\text{m}$) and with Respirgard II® ($0.28\pm0.04\text{ }\mu\text{m}$). Mean $\sigma\text{g}\pm\text{SD}$ was also higher with Fisoneb® (3.67 ± 1.83) than with DP100® (1.73 ± 0.34) and with Respirgard II® (1.22 ± 0.10).

Deposition study

Because of differences in thoracic attenuation coefficient between patients (which were not measured), deposition data are best assessed by intrapatient comparisons for the different nebulizers (table 2). The greatest deposition to the whole lung was obtained with the ultrasonic nebulizers in all three patients. As expected (because of the larger MMAD), Fisoneb® also gave the greatest central deposition as illustrated by C/P ratios. However,

Table 1. – Particle size distributions determined by isotopic and HPLC techniques

	Respirgard II®	Fisoneb®	DP100®
MMAD _i * μm	0.27	4.15	2.00
MMAD _c * μm	0.30	5.5	2.55
σg_i	1.22	3.70	1.80
σg_c *	1.22	3.65	1.67
r-value	0.981	0.974	0.976
p-value	<0.001	<0.001	<0.001

HPLC: high performance liquid chromatography; MMAD: mass median aerodynamic diameter; σg : geometric standard deviation; i: results obtained by isotopic method; c: results obtained by assay method. *: mean of two runs. r and p-values by linear regression analysis of HPLC and isotopic results on each stage of the cascade impactor (n=20 samples from two runs).

Table 2. – Deposition results measured in specific lung regions as ratios

	Pt No.	Type of nebulizer		
		Respirgard II®	Fisoneb®	DP100®
Deposition/N	1	8.87	13.01	14.00
	2	2.05	18.49	3.46
	3	2.17	9.17	0.84
C/P	1	0.85	1.88	1.10
	2	1.10	1.36	1.23
	3	1.07	1.15	0.72
C/N	1	4.07	8.50	7.53
	2	1.08	11.04	1.91
	3	1.03	4.71	0.37
P/N	1	4.80	4.51	6.46
	2	0.97	8.11	1.55
	3	0.96	4.08	0.51
U/L	1	0.75	0.52	0.69
	2	0.97	1.35	0.55
	3	1.05	3.54	2.80
U/N	1	3.87	4.23	5.87
	2	0.96	9.62	1.26
	3	1.15	7.37	0.64
Aspergilloma/N	1	1.24	2.95	1.79
	2	0.59	5.28	0.57
	3	0.42	3.14	0.29

N: nebulizer charge (μCi); C: deposition in the central region (cpm); P: deposition in the peripheral region (cpm); U: deposition in the upper halves of the lungs (cpm); L: deposition in the lower halves of the lungs (cpm); Aspergilloma: mycetoma region; Pt: patient.

when regional counts were normalized for nebulizer activity, intrapatient comparisons revealed that the Fisoneb® also delivered significant particles to the lung periphery, exceeding peripheral delivery of drug from the other nebulizers in two of the three patients, and consistently depositing the greatest amount in the lung quadrant containing the aspergilloma.

Pharmacokinetics and tolerance

Table 3 presents results of amphotericin B serum concentrations measured in patients Nos. 1 and 3 at different times after nebulization had been completed. With Fisoneb®, the highest amphotericin B serum concentration (22 and $20\text{ ng}\cdot\text{mL}^{-1}$ in the two patients, respectively) was observed 30 min after the end of the inhalation. With DP100®, the peak value was $23.6\text{ ng}\cdot\text{mL}^{-1}$ at 150 min in patient No. 1 and $11.9\text{ ng}\cdot\text{mL}^{-1}$ at 210 min in patient No. 3. For technical reasons (haemolysis), amphotericin B serum levels obtained with Respirgard II® were available only in patient No. 3. The $5.7\text{ ng}\cdot\text{mL}^{-1}$ peak concentration was obtained 90 min after nebulization had been completed. With this nebulizer, amphotericin B serum concentrations remained stable during the 330 min monitoring period.

The peak serum concentrations correlated with the amount of drug deposited in the whole lung ($r=0.9$; $p=0.025$; $n=5$), the central regions ($r=0.9$; $p=0.037$) and

Table 3. — Amphotericin B serum concentrations (ng·mL⁻¹) in patients Nos. 1 and 3 between 30 and 330 min after nebulization had been completed

Time min	Patient No. 1		Patient No. 3		
	Fisoneb®	DP100®	Fisoneb®	DP100®	Respigard II®
30	22.0	10.90	20.00	7.06	4.10
90	7.50	22.00	17.80	10.10	5.70
150	8.40	23.60	14.40	9.30	4.90
210	8.40	21.70	14.30	11.90	3.30
270	6.80	15.40	13.90	2.50	4.10
330	5.20	9.60	3.80	2.30	4.10

Table 4. — Significance of the relationship between amphotericin B peak serum concentrations and time to peaks (n=5) *versus* deposition in the lungs

	Peak serum concentrations		Time to peak	
	r-value	p-value	r-value	p-value
Deposition/N	0.9	0.025	0.5	0.444
C/P	0.5	0.238	0.8	0.138
C/N	0.9	0.037	0.3	0.405
P/N	0.9	0.029	0.3	0.580
U/L	0.1	0.838	0.1	0.909
U/N	0.8	0.090	0.5	0.308
Aspergilloma/N	0.8	0.102	0.8	0.124

For abbreviations see legend to table 2.

the peripheral regions ($r=0.9$; $p=0.029$). There was a trend to a negative correlation between serum peak time and deposition in central and aspergilloma regions, but it was not significant ($r=-0.8$; $p=0.138$ and $r=-0.8$; $p=0.124$, respectively) (table 4).

No acute bronchial side-effect, such as dyspnoea or wheezing, was induced in any patient by the daily amphotericin B inhalation over the 28 day treatment period. At the end of that period, patients Nos. 1 and 3 felt a subjective improvement in their dyspnoea and well-being, and patient No. 2 did not report any change.

Discussion

This study, based both on bench and human experiments, demonstrates that amphotericin B suspension can be effectively aerosolized and deposited throughout the lungs without clinical evidence of acute toxicity.

Amphotericin B dissolved into saline is unstable at body temperature [2]. It was used by RODENHUIS *et al.* [1] as a solution in 5% dextrose. We believed that bacteriological safety was not insured by such a preparation. Therefore, we used a suspension of amphotericin B in sterile water. Whilst use of ^{99m}Tc radiolabelled tracers, such as human serum albumin or colloids, have been shown to be relevant to assess *in vivo* kinetics of pentamidine [12] or gentamicin [13] aerosols, for example, such an "indirect isotopic method" has not been validated, to our knowledge, with suspensions. Therefore, we decided to directly label amphotericin B itself after reduction by stannous chloride, as previously described for albumin [14]. We recently applied this method to

label a new scintigraphic tracer (J001) and pentamidine [15, 16]. Quality of amphotericin B radiolabelling was demonstrated by reduced content of labelled colloids, similar chromatography pattern and retention volumes of labelled and unlabelled amphotericin B. Stability was assessed *in vitro* by detection of less than 1.5% free ^{99m}Tc and *in vivo* lung retention in baboons. Occurrence of free circulating ^{99m}Tc or ^{99m}Tc SnCl₂ in these animal experiments would have led to thyroid or bone marrow imaging, which were not observed during the 200 min continuous period of follow-up in animals. Finally, comparison of the gamma-counting method and HPLC assessment of the particle size distribution assessment did not exhibit significant differences. We concluded, therefore, that the direct isotopic method was accurate for *in vivo* study of aerosolized amphotericin B in patients.

The major factor responsible for differences in lung deposition was the type of nebulizer. For example, the highest deposition was recorded with the Fisoneb® and the lowest with Respigard II®. These observations were predictable from the bench measurements of inhaled mass. Similar measurements of inhaled mass were reported with Fisoneb® for nebulization of pentamidine [17]. To analyse the regional deposition, regions of interest were drawn from the image with the largest lung area. Although less accurate than outlines obtained after radioactive gas inhalation, this method allows an intrapatient comparison of the three nebulizers. We did not attempt to perform inter-subject comparisons in this study. As expected, central deposition (as assessed by C/P and C/N ratios) was strongly related to the MMAD and was higher with Fisoneb®. Nevertheless, the amount deposited in the peripheral region was also greater with Fisoneb® in two patients because of the greater inhaled mass. Peripheral deposition (P/N ratio) and upper lobe deposition (important for most aspergillomas) as measured by U/N ratio, were higher with the ultrasonic nebulizers. Whilst there was considerable variability in the quantity and distribution of deposited drug in the patients, the consistently higher deposition in the mycetoma lung region measured with the Fisoneb® emphasizes the importance of the inhaled mass, and illustrates the ability of bench studies to predict drug delivery *in vivo*.

Ultrasonic nebulizers are usually considered to be less efficient than jet nebulizers for aerosolizing drug suspensions [18] (budesonide, for example [19]). The present study, however, demonstrates that ultrasonic nebulizers can nebulize an amphotericin B suspension and deposit it peripherally in the lungs. For this formulation, the

measurement of inhaled mass suggested that the ultrasonic nebulizer would deliver significant quantities of drug to the patient. Whilst *in vitro* testing is useful, the *in vivo* patient studies were required to assess the influence of particle size on drug delivery, especially to the peripheral lung regions. For example, the MMAD data predicted more central deposition and the σ_g was greater for Fisoneb® indicating a very polydisperse aerosol. Both of these parameters could reduce peripheral deposition. However, because of the high inhaled mass of the Fisoneb®, it effectively delivered drug throughout the lungs.

The amphotericin B peak serum levels, which correlated with the amount deposited in the lungs, were less than 25 ng·mL⁻¹, even with Fisoneb®. This concentration is 20 times less than the 0.5 µg·mL⁻¹ steady-state concentration reported after intravenous treatment at usual doses [20].

Clinically, the three drug delivery systems were well-tolerated by the patients, who did not exhibit any sign of airway irritation.

In conclusion, our findings based on consistent *in vitro* bench testing and direct patient studies demonstrate that amphotericin B suspension can be successfully nebulized and delivered to the lungs of patients colonized with *Aspergillus* without acute toxicity. The techniques applied in this study to measure pulmonary deposition of amphotericin B aerosols cannot be applied to a large number of patients. However, demonstration of a consistent deposition of the aerosol in the lungs, even in a small number of patients and, moreover, the correlation of deposition with peak serum concentration of the drug justifies further clinical evaluation of this treatment in patients with clinically invasive fungal disease.

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