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ORIGINAL ARTICLE

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Gemcitabine aerosol: in vitro antitumor activity and deposition imaging for preclinical safety assessment in baboons

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Abstract *Aim*: To characterize gemcitabine aerosol, its in vitro activity against lung cancer cells, its deposition, and tolerance in a non-human primate model. *Methods*: In vitro cytotoxicity of nebulized gemcitabine against NCI-H460 and A549 lung cancer cells was tested using a growth inhibition assay and compared with non-nebulized gemcitabine. The ^{99m}Tc-DTPA-radiolabeled gemcitabine aerosol was characterized by cascade impaction and the gemcitabine mass/^{99m}Tc activity relationship was established for further quantitative nuclear imaging. Nine weekly inhalations at a target dose of 1 mg/kg body weight of gemcitabine were performed in three baboons using dynamic scintigraphic acquisitions for continuous monitoring of gemcitabine delivery during inhalation. Gemcitabine plasma concentrations were measured during the first inhalation. *Results*: Growth inhibition assays for both NCI-H460 and A549 cells did not differ between nebulized and non-nebulized gemcitabine. Aerosol characterization showed a particle mass median aerodynamic diameter of 3.7 ± 0.8 μm and a linear relationship between gemcitabine mass (y) and ^{99m}Tc activity (x) ($y = 0.82x - 10^{-5}$, $R^2 = 0.88$). No toxicity was observed after nine weekly inhalations of a mean dose of gemcitabine of 11.1 mg (88% of the target dose) as assessed from scintigraphic data. A dose-dependent peak plasma concentration of gemcitabine (20–74 ng/ml) was observed by the tenth minute of inhalation. *Conclusions*: We have characterized a gemcitabine aerosol suitable for intrathoracic airway deposition and demonstrated that jet nebulization does not alter the cytotoxic properties of the drug. In a primate model, we have developed a scintigraphic procedure for the monitoring of aerosol deposition, and we have demonstrated the safety of nine weekly aerosol administrations of gemcitabine.

Keywords Aerosol · Chemotherapy · Gemcitabine · Lung cancer · Scintigraphy · Safety

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Introduction

Regional chemotherapy has been proposed as a treatment modality in a number of situations in oncology in order to increase exposure of the tumor to the drug, while minimizing systemic side effects. Administration of drugs directly to the lungs via inhalation allows regional drug delivery to the lungs and airways with smaller doses and fewer systemic effects [13]. There is now increasing evidence to support the role of inhaled therapeutics in the treatment of various lung diseases. In lung cancer, regional chemotherapy could be useful in unresectable bronchioloalveolar carcinoma or main bronchus carcinoma with limited invasion, endobronchial tumor relapse after surgery, in situ carcinoma or synchronous or metachronous lesions in patients with an already detected lesion. Aerosol delivery of chemotherapy could be considered alone or in combination with other treatment modalities such as radiotherapy. However, few studies have documented the feasibility of inhalation delivery of anticancer agents [8, 18, 19, 22].

We recently demonstrated that gemcitabine can be administered via endotracheal spray in rats without any

marked toxicity and with a maximum tolerated dose of 4–6 mg/kg body weight once a week for 9 consecutive weeks [7]. Under these conditions, there were no chemotherapy-related deaths, and no clinical, histological, or hematological signs of toxicity apart from a slight decrease in platelet and red blood cell counts. The lung gemcitabine concentration after pulmonary administration of 4 mg/kg body weight was estimated to be 50-fold higher than that obtained after IV administration of 10 mg/kg body weight in rats [10]. At an equivalent dosage, we demonstrated an inhibition effect on tumor growth of pulmonary administration of gemcitabine in an orthotopic model of lung cancer [6]. In these previous studies, gemcitabine was delivered to anesthetized animals using an endotracheal sprayer facilitating pulmonary delivery of a coarse aerosol with droplet size [mass median aerodynamic diameter (MMAD): 18 ± 3 μm] that would be inappropriate for delivery into the bronchial tree using a conventional nebulizer. Before proceeding with clinical evaluation, we decided to focus on the effect of nebulizing gemcitabine in terms of in vitro activity of the nebulized drug against human lung cancer cells, aerosol characteristics, and its deposition in a non-human primate model.

Materials and methods

Nebulization

The area to be targeted for the delivery of chemotherapy via aerosol in lung cancer is the central airways. The study was performed using the Atomisor NL9M® jet nebulizer [La Diffusion Technique Française (DTF), St Etienne, France] with an Atomisor Abox + ® compressor (Atomisor, DTF, St Etienne, France). This nebulizer was previously validated for aerosol delivery of gene therapy in cystic fibrosis [12].

In vitro cytotoxicity study

In vitro cytotoxic activity of nebulized gemcitabine against NCI-H460 and A549 human lung cancer cells was tested using a growth inhibition assay and compared with non-nebulized gemcitabine. Gemcitabine (Gemzar, Eli Lilly and Co. Inc., IN, USA) was supplied in vials containing 200 mg of freeze-dried powder for injection. The nebulization solution was obtained by reconstituting the freeze-dried powder in 5 ml of 0.9% NaCl for a final concentration of 40 mg/ml. Gemcitabine was nebulized with the Atomisor NL9M® for 13 min. To check the integrity of the nebulized drug, we used the method previously described by Lerondel et al. [12] for verification of aerosolized virus bioactivity. According to this method, 100 μl samples were taken from the nebulizer reservoir before (*T0*) and after various nebulization times (7, 10, and 13 min). Gemcitabine concentrations of each sample were determined by UV spectrophotometry. NCI-H460 human large cell lung carcinoma cell line and A549 cell line initiated from a human alveolar cell carcinoma were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (Gibco, Invitrogen Ltd, Paisley, UK) containing 862 mg/l Glutamax I, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 10% fetal bovine serum (Gibco), 50 U/ml penicillin, and 50 mg/ml streptomycin, for NCI-H460 cell line, and in Ham's F12K medium (Gibco) with 2 mM L-glutamine and 10% fetal bovine serum for A549 cell line. They were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was replaced every 2 or 3 days and cells were subcultured weekly using 0.25% trypsin-1 mM EDTA (Gibco). Tumor cells were seeded in 24-well plates at 40,000 and 20,000 cells per well, respectively. All assays were performed with exponentially growing cultures. After 24 h, 1 ml of drug-containing medium was added. For each sample of nebulized gemcitabine, dose range from 10^{-10} to 10^{-5} M was tested. Cytotoxicity was assessed after 72 h of exposure using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay, which is dependent on the cellular reduction of MTT by mitochondrial dehydrogenase of viable cells to an insoluble colored formazan derivative, which is then solubilized in acidic isopropanol [1, 16]. Briefly, cells were incubated for 90 min in the presence of culture medium containing 500 μg/ml MTT (Sigma-Aldrich Co, St Louis, MO, USA). Then, the medium was removed and formazan was extracted with 400 μl per well of isopropanol containing 8% 1 N HCl. Absorbance was measured at 450 nm. Concentration of nebulized gemcitabine causing 50% growth inhibition (IC_{50}) was calculated using Table Curve 2D software. Four independent experiments were performed in triplicate.

Aerosol characteristics and radiolabeling process

The objective of this part of the study was to determine the aerosol particle size distribution and to validate the radiolabeling method to be used in the subsequent deposition study. The gemcitabine freeze-dried powder was reconstituted in 5 ml of ^{99m}Tc-diethylenetriaminopentaacetic acid (DTPA) (15 mCi) dissolved in 0.9% NaCl for a final concentration of 40 mg/ml. The nebulizer charge activity was measured, immediately before nebulization, in an activimeter (Capintec, CRC Ariès, France). Particle size distribution was measured using a 10-stage cascade impactor (IMPAQ GS-1, California Measurements, San Diego, CA, USA) operating at 1 l/min, connected to the jet nebulizer mouthpiece. Expelled aerosol was passed through an absolute filter (breathing filter, BB50TE, Pall, UK) connected to a 14-l/min vacuum pump. To start nebulization, the compressor and pumps were turned on. After 1, 3, and 7 min of nebulization, the compressor and pumps were stopped, and

the cascade impactor was dismantled. Each glass plate of the impactor stages was then introduced in a tube (T420, Simport, Canada) containing 20 ml of 0.9% NaCl solution. The activity in each tube was measured with the activimeter and the gemcitabine concentration was determined by UV spectrophotometry (204 nm). All experiments were performed in triplicate. For each measurement, the activity (expressed as the percentage of the activity charged in the nebulizer) and the mass of gemcitabine (expressed as the percentage of the mass charged in the nebulizer) were compared in order to determine the gemcitabine mass/^{99m}Tc activity relationship. Particle size distributions and MMAD according to gemcitabine mass measurement and ^{99m}Tc activity were compared. The respirable particle fraction i.e. the percent of particles with an aerodynamic diameter between 1 and 5 μm [15] was calculated.

Deposition study in baboons

Animal care

Three female baboons (*Papio papio*) weighing 11–14 kg, previously acclimatized to laboratory conditions, were included in this study. Baboons were lightly anesthetized with ketamine hydrochloride (10 mg/kg, intramuscular) and then placed on a contention chair. They were allowed to recover for 15–30 min to perform conscious inhalation. To obtain transient inhibition of lung mucociliary clearance, animals were given a subcutaneous injection of atropine sulfate (0.050 mg/kg) 30 min before inhalation. To prevent propagation of gemcitabine aerosol, inhalations were performed in a specially designed closed cabin equipped with an air extraction and filtering system (Fig. 1). Administration of the aerosol was performed with a conical mask directly connected to the reservoir of the Atomisor NL9M® nebulizer. In our previous study, the maximum tolerated dose of gemcitabine delivered via endotracheal spray in rats was estimated to be 3 mg/kg body weight effectively deposited in the lungs once a week for 9 weeks [6]. Taking into account the dosage conversion factors [9], we planned to deliver to the baboons a target dose of 1 mg/kg body weight once a week for 9 consecutive weeks. The clinical state of the animals was evaluated throughout the study to detect any signs of toxicity. Before each administration, the animal was weighed and a blood sample was collected from the saphenous vein for complete blood count. On day 64, the animals were sacrificed for histological examination. Nasal cavity, trachea, bronchi, lungs, esophagus, stomach, duodenum, jejunum, ileum, colon, sternum, and bone marrow, spleen, and liver were removed and analyzed by a veterinary pathologist to evaluate the adverse effects of chemotherapy. All procedures were performed according to the French and European regulations for animal experimentation and were approved by the institutional Animal Ethics Committee.

Scintigraphic assessment of deposition

Scintigraphic imaging was performed using an Orbiter 75 gamma camera (Siemens, Munich, Germany) equipped with a high-resolution parallel collimator. Images were recorded using a 128×128 pixels and 16 bits matrix, on the 140 keV photopeak of the ^{99m}Tc with a 20 spectral width. In order to evaluate ^{99m}Tc γ-ray

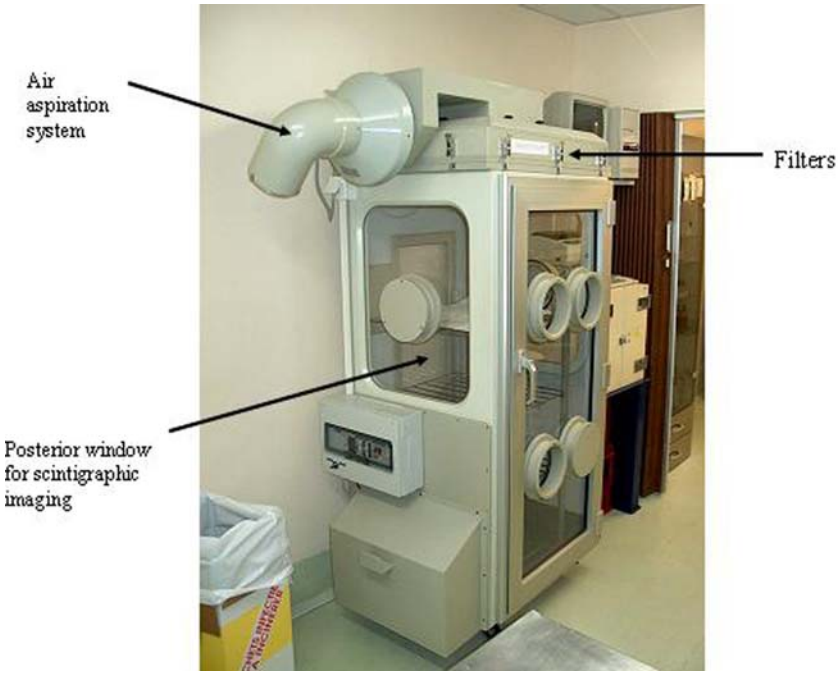

Fig. 1 Inhalation cabin equipped with an air extraction and filtering system for aerosol delivery of chemotherapy

absorption, perfusion scintigraphy was performed for each animal before starting gemcitabine inhalations. For this purpose, an I.V. injection of 1 ml of ^{99m}Tc serum albumin macroaggregates (^{99m}Tc-AMA) 20–90 μm in size (PULMOCIS Cis Bio International, France) was performed into a saphenous vein and followed by a single 120-s scintigraphic image. After ensuring that all ^{99m}Tc-labeled macroaggregates were trapped in the pulmonary capillaries, ^{99m}Tc γ-ray absorption was estimated by dividing the 120-s scintigraphy lung count by the total AMA activity. For scintigraphy of aerosol deposition ^{99m}Tc-DTPA was used as a tracer of the aqueous phase within the gemcitabine aerosol. Immediately before inhalation, 260 mg of gemcitabine freeze-dried powder were reconstituted in 3 ml of ^{99m}Tc-labeled DTPA (30 mCi) then made up with 3.5 ml of 0.9% saline to obtain a final volume of 6.5 ml and a final concentration of 40 mg/ml in the nebulizer reservoir. The activity deposited in the nebulizer was counted using a gamma-well counter (Capintec). During inhalation of the radiolabeled aerosol, animals were seated in the contention chair inside the inhalation cabin that was placed in front of the collimator of the γ camera. Dynamic 60-s posteroanterior scintigraphic acquisitions were performed during inhalation (Fig. 2) in order to estimate minute-by-minute the equivalent dose of gemcitabine delivered to the animal, taking into account the activity deposited in the nebulizer, the isotope decay, the ^{99m}Tc γ-ray absorption, and the gemcitabine mass/^{99m}Tc activity relationship previously established. Inhalation was continued until the 1 mg/kg body weight target dose was obtained without exceeding a maximum duration of 30 min. At the end of inhalation, a 60-s posteroanterior static scintigraphic acquisition was performed to calculate the activity of regions of interest (ROIs) corresponding to the whole body, the total lung surface, and the stomach. The dose of gemcitabine deposited in the whole body, total lungs, and stomach were estimated and expressed in mg equivalent.

Plasma gemcitabine concentrations

Plasma gemcitabine concentrations were measured after the first aerosol administration to determine preliminary pharmacokinetics of the aerosolized drug. Heparinized 5 ml blood samples were collected from the saphenous vein before aerosol administration, after 10 min of

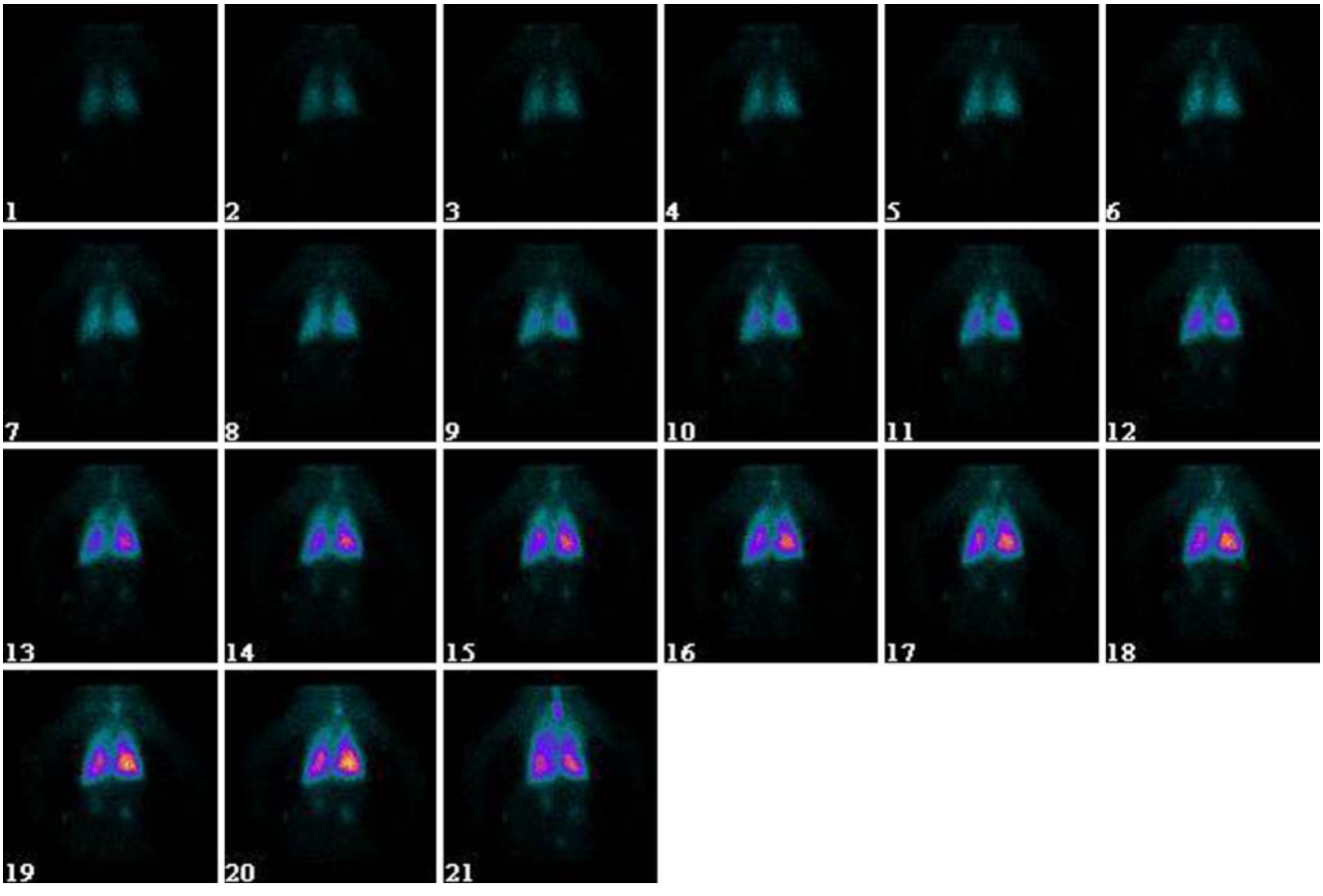

Fig. 2 Dynamic 60-s scintigraphic acquisitions (posteroanterior view) during inhalation in baboon PP9513. The dose of gemcitabine delivered to the animal was estimated minute-by-minute from the whole body activity by taking into account the activity deposited in the nebulizer, the isotope decay, the ^{99m}Tc γ-ray absorption and the gemcitabine mass/^{99m}Tc activity relationship. In this case, the 1 mg/kg body weight target dose was obtained by the 21st minute of inhalation

inhalation, at the end of inhalation (30 min), and 60, 120, 240, and 360 min after starting the inhalation. All blood samples were collected in tubes containing 0.5 mg of tetrahydrouridine to prevent ex vivo metabolism of gemcitabine. Plasma was prepared by centrifuging these samples at 2,000×*g* for 10 min. All plasma samples were stored at −20°C until gemcitabine assay. The plasma gemcitabine concentration was quantified by high performance liquid chromatography (HPLC). First, 25 µl of internal standard (doxifluoridine) were added to 500 µl plasma samples and vortex-mixed. Blank human plasma for calibration was prepared in pooled normal human plasma from heparinized whole blood centrifuged at 2,000×*g* for 10 min. Plasma proteins were then precipitated with 1 ml of acetonitrile. After vortex mixing for 2 min and centrifuging for 10 min at 12,000×*g*, the supernatant was transferred to a glass tube and evaporated at 56°C for 30 min under a stream of nitrogen. The dry extract was reconstituted with 200 µl of mobile phase before injection into the column. The volume of injection was 40 µl. HPLC separation of analytes was performed on a Waters ODS2 (4.6×250 mm^2; 5 µm) reversed-phase analytical column coupled with an ODS2 (4.6×10 mm^2; 5 µm) pre-column (Waters, St Quentin en Yvelines, France). The eluant was a potassium dihydrogen phosphate buffer pH 3 delivered at 1.3 ml/min. The detector was set at 280 nm and the limit of quantification was 12 ng/ml.

Statistical analysis

Statistical analysis was performed using StatXact statistical software (Cytel Software Corp., Cambridge, MA, USA). All values were summarized by descriptive statistics and expressed as mean ± SEM (standard error of the mean). Gemcitabine mass measurements and ^{99m}Tc activities were correlated by linear regression and this correlation was characterized using Pearson's correlation exact test. The exact permutation test was used for comparison of the MMAD obtained by gemcitabine mass measurement and ^{99m}Tc activity. Pearson's correlation exact test was used to determine the correlation between the particle size distributions determined with gemcitabine mass and ^{99m}Tc activity. A P value < 0.05 was considered significant.

Results

In vitro cytotoxicity study

As demonstrated by Fig. 3, growth inhibition assays of the various samples of nebulized gemcitabine and the non-nebulized drug were similar for either NCI-H460 or A549 cell lines. The IC_{50} values were 5.72 nM for H460 cells and 29.9 nM for A549 cells after 72 h of exposure.

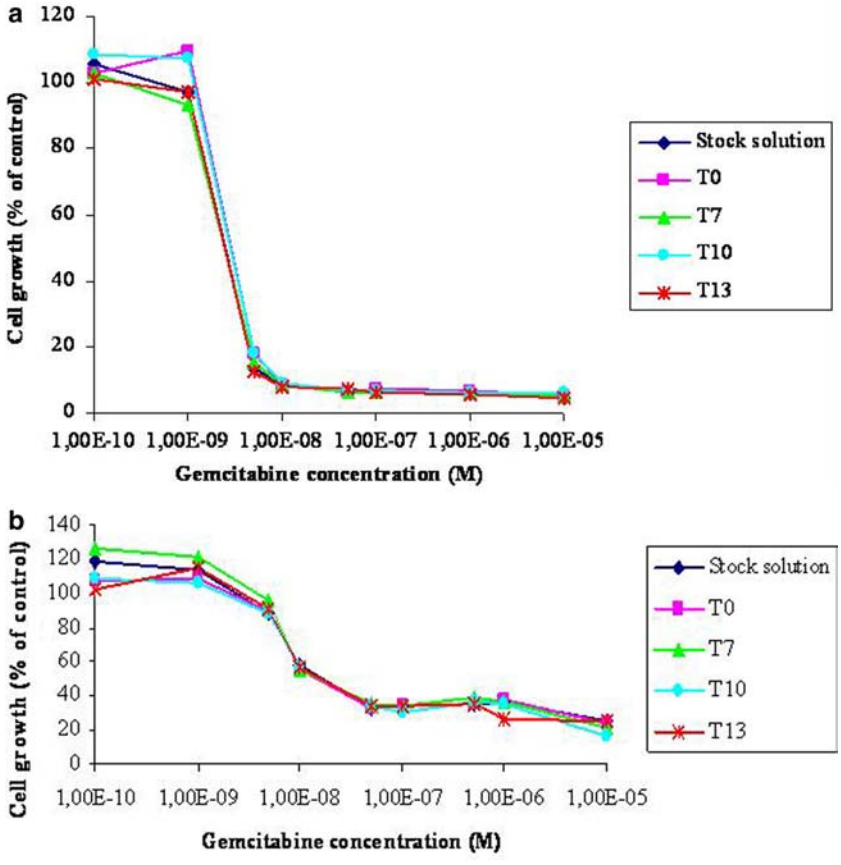

Fig. 3 Growth inhibition assay of non-nebulized and nebulized gemcitabine on NCI-H460 (**a**) and A549 (**b**) tumor cells. *T0* Gemcitabine before nebulization; *T7, T10*, and *T13* gemcitabine after 7, 10, and 13 min of nebulization, respectively

Aerosol characteristics and radiolabeling process

Comparison of gemcitabine mass and ^{99m}Tc activity measurements showed a significant correlation of 94% ($P < 0.0001$). Gemcitabine mass (y)/^{99m}Tc activity (x) relationship ($y = 0.82x - 10^{-5}$, $R^2 = 0.88$, $n = 80$) indicated that ^{99m}Tc activity overestimated the actual mass of gemcitabine by 18%. Particle size distributions according to gemcitabine mass measurement and ^{99m}Tc activity showed a strong correlation of 99.8% ($P < 0.0001$) with a MMAD of 3.7 ± 0.8 µm according to gemcitabine mass and 3.8 ± 0.5 µm according to ^{99m}Tc activity ($P = 0.41$, $N = 8$). The respirable particle fraction was 55%.

Deposition study in baboons

Nine weekly inhalations were performed in the three animals without any clinical signs of toxicity during aerosol administration. The duration of inhalation ranged 18–30 min. Scintigraphic imaging of the thorax at the end of inhalation revealed intense and homogeneous deposition in the trachea and central lungs. A lower peripheral activity was observed, indicating moderate but significant deposition in the peripheral compartment (Fig. 4). The doses of gemcitabine deposited in the whole body, total lungs, and stomach, as assessed by scintigraphic imaging, are shown in Table 1. On average, the total dose of gemcitabine delivered to the animal was 11.1 mg, i.e. 88% of the target dose and 4.5% of the dose deposited in the nebulizer. Marked variations of the delivered dose were observed from one administration to another in two-third of the animals (PP9611 and PP863). Eighty-two percent of the delivered dose was deposited in the lungs and 18% was deposited in the stomach.

A pharmacokinetic study was performed for the first aerosol administration corresponding to a total delivered dose of gemcitabine of 9, 5, and 12 mg for PP9611, PP863, and PP9513, respectively. The corresponding peak plasma concentrations of gemcitabine were 62, 20, and 74 ng/ml, respectively, and were observed at 10 min i.e. in the middle of aerosol administration. Gemcitabine was undetectable in the three animals after 30 min (Fig. 5).

No clinical sign of toxicity was observed throughout the study. Mean weight on day 64 (13.05 kg) did not differ from that before the first aerosol administration (12.93 kg). Blood cell count throughout the study did not reveal any signs of hematologic toxicity. Histological examination did not reveal any signs of toxicity of nebulized gemcitabine on nasal, tracheal, and bronchial epithelium and in the lung parenchyma.

Discussion

Few studies have documented the feasibility of delivering chemotherapy by inhalation. Hershey et al. [8] treated dogs with advanced stages of primary lung cancer or lung metastases with paclitaxel or doxorubicin aerosols administered twice weekly. Tumor regression

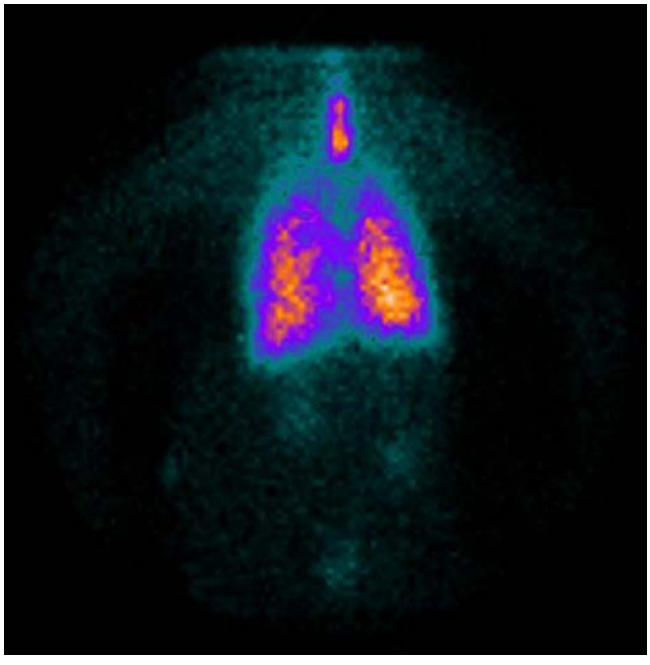

Fig. 4 Lung scintigraphy (posteroanterior view) in baboon PP9513 after inhalation of gemcitabine with ^{99m}Tc-labeled DTPA using an Atomisor NL9M® breath-activated jet nebulizer

Table 1 Equivalent doses of gemcitabine delivered to the baboons via aerosol as estimated from scintigraphic data

Baboons	Target dose (mg)	Total delivered dose (mg equivalent)	Dose deposited in the lungs (mg equivalent)	Dose deposited in the stomach (mg equivalent)
PP9611	11	10 ± 1.8 (2–12)	8.7 ± 1.9 (2–12)	1.3 ± 0.6 (0–4)
PP863	14	11.1 ± 1.2 (5–14)	8.5 ± 1.2 (2–11)	2.6 ± 0.4 (1–4)
PP9513	13	12.1 ± 0.5 (10–14)	10.1 ± 0.9 (7–14)	2 ± 0.6 (0–4)

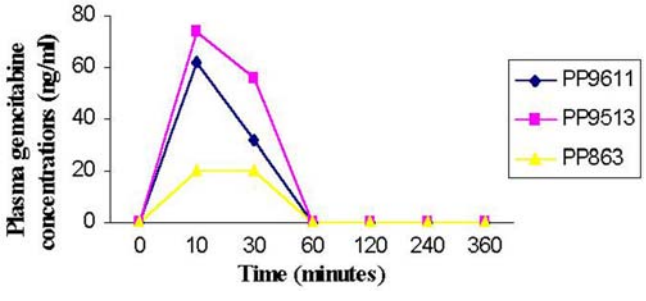

Fig. 5 Plasma concentrations before, during and after aerosol delivery of 9, 5, and 12 mg of gemcitabine to PP9611, PP863, and PP9513 baboon, respectively

was achieved in 25% of dogs with measurable tumors without the side effects normally associated with systemic administration of these drugs and without pulmonary toxicity in the dogs treated with paclitaxel. Tatsumura et al. [19] demonstrated that 5-fluorouracil (5-FU) administered by inhalation accumulated in the trachea, bronchi, and, interestingly, in regional lymph nodes of patients treated 2 h before thoracic surgery for lung cancer. They also investigated the antitumor effect of 5-FU administered by inhalation in ten selected patients with unresectable lung cancer and obtained four partial responses and two complete responses without stomatitis or any other notable side effects. Recently, a phase I study demonstrated the feasibility and safety of aerosol administration of 9-nitrocamptothecin in a liposomal formulation in 25 patients with primary or metastatic lung cancer [22]. Partial remissions and stabilizations were observed in two and three patients, respectively.

Gemcitabine (difluorodeoxycytidine, dFdC) is a chemotherapy molecule belonging to the nucleoside analog family. It has been demonstrated to be effective in the treatment of non-small cell lung cancer [17] both as monotherapy and in combination with other drugs. It is a prodrug, which is inactive in the extracellular compartment. It only becomes cytotoxic after reaching a nucleated cell, in which it undergoes several phosphorylations [14]. The gemcitabine formulation does not contain any chemical ingredients incompatible with aerosol delivery. These advantages, in addition to solubility in saline and absence of irritant properties, make gemcitabine an attractive candidate for local administration. We recently demonstrated that gemcitabine could be administered via endotracheal spray in rats without any marked toxicity at a maximum tolerated dose of 4–6 mg/kg body weight once a week for 9 consecutive weeks [7]. At an equivalent dosage, we demonstrated an inhibitory effect of pulmonary administration of gemcitabine on tumor growth in an orthotopic model of lung cancer [6]. Koshkina et al. [11] demonstrated that gemcitabine administered via aerosol inhibits the growth of lung metastasis in two osteosarcoma lung metastasis animal models. Interestingly, aerosolized gemcitabine was also effective against primary tumor in this study, although intraperitoneal administration of the drug at similar dosage had no effect on lung metastasis.

Before administering a drug by aerosol, it has been recommended to verify the absence of any harmful effects of nebulization on the therapeutic properties of this drug [4]. We therefore performed growth inhibition assays with nebulized and non-nebulized gemcitabine on NCI-H460 and A549 lung cancer cell lines. Our results confirmed that nebulized gemcitabine retains its activity against both NCI-H460 and A549 lung cancer cells. IC50 obtained with nebulized gemcitabine from the 13th minute of nebulization was similar to those previously observed with non-nebulized gemcitabine [5, 20]. Our results are in agreement with those recently published by Wang et al. [23] with nebulized farnesol.

A second part of the present study was designed to characterize a gemcitabine aerosol and to develop a non-human primate model of gemcitabine aerosol delivery for preclinical safety studies. We used a disposable breath-activated jet nebulizer to nebulize gemcitabine, a potentially toxic substance. Aerosol characterization was performed as previously described [2] using a 10-stage cascade impactor connected to the nebulizer. According to the guidelines for aerosol therapy [3], particle size distribution of our gemcitabine aerosol is suitable for deposition in the intrathoracic airways with an MMAD of 3.7 ± 0.8 μm. Under these conditions, inhaled gemcitabine may directly target primary lung tumors located in or near the central airways, or may be absorbed from the bronchial airways and reach the tumor via the bronchial circulation [19]. A previous study with the Atomisor NL9M® showed an inhaled fraction of 33% of the drug charge from the 12th minute of nebulization [21]. Considering the respirable particle fraction of 55% determined in the present study, we can extrapolate that 36 mg (200 mg×33%×55%) of gemcitabine, i.e. 0.5 mg/kg body weight for a 70 kg adult could be delivered to healthy human lungs in 12 min. This dosage corresponds approximately to the maximum tolerated dose determined in rats by weekly pulmonary administrations of gemcitabine [7, 9]. However, impaired respiratory function in patients with lung carcinoma with possibly associated obstructive lung disease is likely to alter the parameters of this extrapolation. In further clinical studies, aerosol delivery will have to be adjusted to potentially impaired and diseased lungs and airways.

Baboons, that were previously found to be relevant for extrapolation of aerosol deposition in humans [12], were used as an animal model of aerosol delivery of gemcitabine. Prevention of occupational exposure of healthcare workers to ambient air levels of the drug administered is a major safety consideration in order to treat patients with nebulized chemotherapy. In our study, inhaled treatments were given in a specially designed cabin which aspirated and filtered any free aerosol through HEPA filters. Administration of chemotherapy by inhalation also requires precise determination of the dose delivered in order to be safe and consistent. Scintigraphic imaging is able to assess the actual dose delivered via aerosol after correction for isotope decay and attenuation factors. To avoid direct labeling of the drug, nuclear imaging of aerosol deposition was performed with a routinely used radiopharmaceutical agent ^{99m}Tc-DTPA. Dynamic scintigraphic acquisitions during inhalation allowed continuous monitoring of gemcitabine delivery. The gemcitabine mass/^{99m}Tc activity relationship, indicating that ^{99m}Tc activity overestimated the actual mass of gemcitabine by 18%, was taken into account in the estimation of the dose of gemcitabine delivered to the animal. Previous studies of alveolar epithelial permeability of aerosolized

^{99m}Tc-DPTA in baboons showed a mean half-time clearance of about 70 min [24]. We did not integrate this factor in our study. Considering the duration of inhalation (18–30 min), we probably underestimated the actual gemcitabine deposition. At a target dose of 1 mg/kg body weight once a week for 9 consecutive weeks corresponding to the maximum tolerated dose previously determined in rats [7, 9], aerosolized gemcitabine was well tolerated with no clinical, hematological, or histological signs of toxicity. Our pharmacokinetic data demonstrated a dose-dependent increase of the peak plasma concentration of gemcitabine that was observed from the tenth minute of inhalation. Interestingly, gemcitabine concentrations decreased from the tenth minute until the end of inhalation. This may have been due to a saturation effect of the lungs' capacity to absorb the nebulized drug. Peak plasma gemcitabine concentrations were about 25 times lower than those observed 5 min after IV administration of 1 mg/kg body weight of the drug to dogs [10]. Further pharmacokinetic studies are required to document gemcitabine metabolites after aerosol administration. It is possible that gemcitabine is metabolized via different pathways than after other routes of administration.

In this study, we characterized a gemcitabine aerosol suitable for intrathoracic airway deposition and demonstrated that jet nebulization does not alter the cytotoxic properties of the drug. In a primate model, we have developed a scintigraphic procedure for the monitoring of aerosol deposition and we have demonstrated the safety of nine weekly aerosol administrations of gemcitabine. Based on these preclinical results, a phase I study is planned using this scintigraphic procedure during the first inhalation to adjust aerosolization parameters to human and potentially damaged lungs and airways.

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References

1. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:936–942
2. Dequin PF, Delatour F, Faurisson F, Valat C, Lemarié E, Le Pape A, Diot P (1997) Characterization on the bench of an amikacin aerosol before clinical studies. J Aerosol Med 10:221–230
3. Diot P, Bonfils P, Faurisson F, Fauroux B, Dautzenberg B (2000) Proposed guidelines for aerosol therapy by means of nebulizers. Eur Respir Rev 10:206–209
4. Diot P, Dequin PF, Rivoire B, Gagnadoux F, Faurisson F, Diot E, Boissinot E, Le Pape A, Palmer L, Lemarié E (2001) Aerosol and anti-infectious agents. J Aerosol Med 14:55–64
5. Edelman M, Quam H, Mullins B (2001) Interactions of gemcitabine, carboplatin and paclitaxel in molecularly defined non-small-cell lung cancer cell lines. Cancer Chemother Pharmacol 48:141–144
6. Gagnadoux F, Le Pape A, Lemarié E, Lerondel S, Valo I, Leblond B, Racineux JL, Urban T (2005) Aerosol delivery of chemotherapy in an orthotopic model of lung cancer. Eur Respir J (in press)
7. Gagnadoux F, Le Pape A, Urban T, Montharu J, Vecellio L, Dubus JC, Leblond V, Diot P, Grimbert D, Racineux JL, Lemarié E (2005) Safety of pulmonary administration of gemcitabine in rats. J Aerosol Med 18:198–206
8. Hershey AE, Kurman ID, Forrest LJ, Bohling CA, Stonerook M, Placke ME, Imondi AR, Vail DM (1999) Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: proof of principle using dogs with spontaneously occurring tumors as a model. Clin Cancer Res 5:2653–2659
9. Hollinger MA (1995) Tables. In: MJ Derelanko, Hollinger MA (eds) Handbook of toxicology. CRC Press, New York
10. Kaway M, Esumi Y, Ishizaki S, Gunji S, Seki H (1995) Metabolism of gemcitabine in rat and dogs. Xenobiotica 25:405–416
11. Koshkina NV, Kleinerman ES (2005) Aerosol gemcitabine inhibits the growth of primary osteosarcoma and osteosarcoma lung metastases. Int J Cancer 116:458–463
12. Lerondel S, Le Pape A, Sené C, Faure L, Bernard S, Diot P, Nicolis E, Mehtali M, Lusky M, Cabrini G, Pavirani A (2001) Radioisotopic imaging allows optimization of adenovirus lung deposition for cystic fibrosis gene therapy. Hum Gene Ther 12:1–11
13. Lipworth BJ (1996) Pharmacokinetics of inhaled drugs. Br J Clin Pharmacol 42:697–706
14. Mackey JR, Mani RS, Selner M, Mowles D Young JD, Belt JA, Crawford CR, Cass CE (1998) Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 58:4349–4357
15. Morrow PE (1974) Aerosol characterization and deposition. Am Rev Respir Dis 110:88–89
16. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
17. Perng RP, Cheng YM, Ming-Lui J, Tsai CM, Lin WC, Yang KY, Whang-Peng J (1997) Gemcitabine versus the combination of cisplatin and etoposide in patients with inoperable non-small-cell lung cancer in a phase II randomized study. J Clin Oncol 15:2097–2102
18. Sharma S, White D, Imondy AR, Placke ME, Vail DM, Kris MG (2001) Development of inhalational agents for oncologic use. J Clin Oncol 19:1839–1847
19. Tatsumura T, Koyama S, Tsujimoto M, Kitagawa M, Kagamimori S (1993) Further study of nebulisation chemotherapy, a new chemotherapeutic method in the treatment of lung carcinomas: fundamental and clinical. Br J Cancer 68:1146–1149
20. Tolis C, Peters GJ, Ferreira GC, Pinedo HM, Giaccone G (1999) Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur J Cancer 35:796–807
21. Vecellio L, Chantrel G, Grimbert D, De Monte M, Diot P (2003) Influence of drug physico chemical characteristics on the performances of a new single use jet nebulizer. The Aerosol Society, DDL 14 (Drug Deliv Lungs XIV)
22. Verschraegen CF, gilbert BE, Loyer E, Huaringa A, Walsh G, Newman RA, Knight V (2004) Clinical evaluation of the delivery and safety of aerosolized liposomal 9-nitro-20(s)-camptothecin in patients with advanced pulmonary malignancies. Clin Cancer Res 10:2319–2326
23. Wang Z, Chen HT, Roa W, Finlay WH (2003) Farnesol for aerosol inhalation: nebulization and activity against human lung cancer cells. J Pharm Pharmaceut Sci 6:95–100
24. Yeates D, Hameister WM (1992) Alveolar epithelial permeability in baboons: histamine and capsaicin. J Physiol 450:363–74