In Vivo Assessment of Temozolomide Local Delivery for Lung Cancer Inhalation Therapy

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Abstract

The aim of this study was to compare the efficacy of local drug delivery by inhalation to intravenous delivery in a B16F10 melanoma metastatic lung model. Temozolomide was formulated as a suspension, which was elaborated and evaluated in terms of particle size, shape and agglomeration. An endotracheal administration device was used to aerosolize the suspension. This mode of delivery was evaluated at different temozolomide concentrations and was optimized for the uniformity of delivered dose, the droplet size distribution and the distribution of droplets *in vivo*. Of the particles in the stabilized suspension, 79% were compatible with the human respirable size range, and this formulation retained 100% *in vitro* anticancer activity as compared to temozolomide alone in three distinct cancer cell lines. The pulmonary delivery device provided good reproducibility in terms of both the dose delivered and the droplet size distribution. Most of the lung tissues that were exposed to aerosol droplets contained the particles, as revealed by fluorescent microscopy techniques. The global *in vivo* antitumor activity of the inhaled temozolomide provided a median survival period similar to that for intravenous temozolomide delivery, and three out of twenty-seven mice (11%) survived with almost complete eradication of the lung tumours. The present study thus shows that inhalation of a simple liquid formulation is well tolerated and active against a very biologically aggressive mouse melanoma pulmonary pseudo-metastatic model. This inhalation delivery could be used to deliver other types of anticancer drugs.

Keywords: NSCLC, temozolomide, aerosol therapy, mouse B16F10 melanoma, lung pseudo-metastases, fluorescent microparticles

1. Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of death from cancer in Western countries (Anabousi et al., 2006; Burris, 2009; Vincent, 2009), and the lungs are also a frequent site of metastatic processes (Carballo et al., 2009; Onaitis et al., 2009; Ricciardi et al., 2009; Yano et al., 2009).

The history of the management of NSCLC patients has recently been reviewed (Vincent 2009). NSCLC patients are typically treated with platinum-based chemotherapy, often in combination with radiation therapy and surgery, if feasible (Hildebrandt et al., 2009; Tiseo et al., 2009). This non-specific, non-selective cytotoxic chemotherapy causes significant toxicities to the patient and results in only a modest increase in survival time (Burris, 2009). To decrease systemic toxicities, a growing area of current NSCLC patient treatment is devoted to molecular-targeted therapeutic agents. These agents are initially effective in certain small subpopulations of patients, but eventually nearly all patients become resistant to further treatment (Burris, 2009; Vincent, 2009). The use of specific drug delivery systems represents an interesting strategy for concentrating the cytotoxic agent in a given organ (e.g., the lung), which would limit systemic toxicities (Anabousi et al., 2006; Gagnadoux et al., 2008).

Pulmonary administration is currently used to deliver drugs directly to the lungs for the local treatment of respiratory diseases such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, tuberculosis, pneumocystis carinii pneumonia and pulmonary hypertension (Smyth et al., 2008). New formulations based on the use of biocompatible lipids and surfactants have recently been proposed as acceptable

pharmaceutical carriers or fillers for inhalation deliveries; these formulations improve the drug deposition and reduce the inherent local irritation resulting from the administration of some drugs (Sebti and Amighi, 2006; Pilcer et al., 2006). Pulmonary delivery of aerosolized chemotherapy for the local treatment of lung tumours (primary lung cancers and lung metastases) could be useful for i) concentrating these toxic agents at the tumour site and ii) avoiding both systemic toxicities and invasive administration methods.

The goal of our work is to detail our methodology for delivering the alkylating agent, temozolomide (TMZ), directly to the lungs of mice bearing lung cancers. In this study, we evaluate the usefulness of a method for the local delivery of TMZ by inhalation for the treatment of lung cancer. Simple liquid formulations and an experimental administration device are employed, and this method is compared to conventional intravenous administration. We have chosen the B16F10 mouse melanoma model because it i) produces massive lung pseudo-metastases, ii) is very aggressive at the biological level and iii) is also more sensitive to TMZ than to platinum derivatives (Mathieu et al., 2007). TMZ is active against cancers associated with extremely poor prognoses, such as glioblastomas and melanomas (Lefranc et al., 2007). TMZ has recently been assayed in various clinical trials relating to lung cancer patients (Choong et al., 2006; Tamaskar et al., 2008; Kouroussis et al., 2009). Systemic long-term TMZ administration can lead to hematological toxicities in cancer patients (Dario and Tomei, 2006; Gerber et al., 2007), but the TMZ delivery system that we propose here could lower the TMZ-associated toxicity found with systemic TMZ delivery, while maintaining anticancer activity.

2. Materials and Methods

2.1. Materials

TMZ was purchased from Shilpa Medicare Limited (Raichur, India); 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-Dimirystoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from NOF Corporation (Hyogo, Japan). L-Histidine and potassium phosphate were purchased from Merck (Darmstadt, Germany), as were HPLC-grade acetonitrile and glacial acetic acid.

2.2. Preparation of TMZ formulations

2.2.1. Intravenous solution

TMZ is a water-insoluble compound (Sampson et al., 1999). We used the dissolution-enhancing agent L-histidine, which is an excipient approved by the Food and Drug Administration (FDA, USA), for intravenous injection (Sandeep et al., 2002). TMZ (40 mg) and L-histidine (40 mg) were solubilised in phosphate buffer (10 mL, pH 5.0) that had been sterilised by filtration using a Stericup-GV pre-sterilised vacuum-driven disposable filtration system with a 0.22 µm polyvinyl difluoride filter (Millipore, Massachusetts, USA). The resulting intravenous solution was 0.4% TMZ (m/v).

2.2.2. Suspension for inhalation

Because TMZ has limited solubility, suspensions were prepared with appropriate particle sizes and concentrations. Microcrystalline suspensions were used so that a large amount of TMZ (up to 2.4 mg in a total volume of 50 µl, used for inhalation at a concentration of 4.8% TMZ (m/v)) could be delivered into the lungs. However, only a handful of excipients are authorised by the FDA for pulmonary tract administrations. We thus chose biocompatible and biodegradable phospholipids as surfactants to

stabilise the aqueous TMZ suspensions. A mixture of phospholipids was used at a high concentration (56% in relation to TMZ) to coat and stabilise the microcrystals in the suspension. DLPC (180 mg) and DMPC (180 mg) in phosphate buffer solution (100 ml) were mixed using a high speed stirrer-homogeniser composed of an X620 motor coupled with a T10 dispersing tool (CAT M. Zipperer, Staufen, Germany). A phosphate buffer (pH 5.0) was used to ensure TMZ stability (Shen et al., 1995) and to guarantee good tolerance by the pulmonary tract (Atkins and Crowder, 2004). The procedure involved a homogenising time of 10 min at a stirring rate of 8,000 rpm, and then TMZ (6.4 g) was mixed with 100 ml of this phospholipid dispersion using the same homogenisation procedure. The size-reduction step and the formation of phospholipid-coated microcrystals were performed by means of an EmulsiFlex-C5 high-pressure homogeniser (Avestin Inc., Ottawa, Canada). Pre-milling low-pressure homogenising cycles (15 cycles at 7,000 PSI and 10 cycles at 12,000 PSI) were performed with the TMZ suspension (to avoid blocking the homogenising gap) before applying high-pressure homogenising (HPH) cycles (20 cycles at 24,000 PSI). This procedure allowed us to decrease the particle size from a range of 10-71 μm (initial TMZ particles) to 1-5 μm and, in addition, enabled the formation of phospholipid-coated microcrystals. The process was carried out using a “closed loop” approach, and stirring was maintained at 8,000 rpm in the sample reservoir to avoid sedimentation of the particles in the suspension. The HPH procedure causes a 30°C increase in the temperature of the suspensions after 20 cycles at 24,000 PSI. Thus, all experiments were performed using a heat exchanger downstream of the homogenising valve to maintain a relatively constant temperature of 10 ± 1°C, as detailed previously (Hecq et al., 2005).

2.3. Characterisation of the suspension for inhalation

2.3.1. Particle size - Laser light-scattering technique

The particle size distribution of the pulmonary suspension was determined by means of a Malvern Mastersizer 2000 laser diffractometer using a wet sampling system (Hydro 2000, Malvern Instruments Ltd, Worcestershire, UK). To analyse the dispersed sample, we used a refractive index of 1.45 and an absorption index of 1.50. Malvern Mastersizer software Version 5.54 (Malvern Instruments Ltd, Worcestershire, UK) was used to characterise the volume median particle size (d(v; 0.5) in µm) and the two additional parameters d(v; 0.1) and d(v; 0.9) (the size, in microns, at which 10% and 90% of the particles are smaller than the remaining distribution, respectively).

2.3.2. Particle shape and agglomeration – Optical microscopy

The shape and the agglomeration of TMZ particles were evaluated by means of an Olympus BX60 optical microscope (Olympus Optical Co., Tokyo, Japan) coupled to a TK-C1381 digital camera (JVC, Yokohama, Japan). The data were analysed using analySIS 5.0 software (Olympus Optical Co., Tokyo, Japan).

2.4. Evaluation of the TMZ aerosol generated from the pulmonary delivery device

2.4.1. Device and *in vivo* administration procedure

An aerosolising system was used to deliver aerosol from liquid formulations (drug in suspension) to mouse lungs by the non-invasive endotracheal route; this system consisted of an aerosolising tip suitable for mice, i.e., a Microsprayer® IA-1C system

(Penn-Century, Philadelphia, USA) connected to an FMJ-250 high-pressure syringe (Penn-Century, Philadelphia, USA).

The *in vivo* administration procedure was nearly identical to that described by Bivas-Benita et al. (2005) but in our procedure, the mice were anaesthetised by an intraperitoneal injection of 112.5 mg/kg body weight (bw) of ketamine (Ketamine 1000, Ceva, Libourne, France) and 1.5 mg/kg bw of xylazine (Proxylaz, Prodivet pharmaceuticals, Eynatten, Belgium) to minimise the death by anaesthesia that occurs with 100 mg/kg bw of ketamine and 10 mg/kg bw of xylazine.

2.4.2. Uniformity of delivered dose - High Performance Liquid Chromatography (HPLC)

The uniformity of the dose of the TMZ suspensions delivered to the mouse lungs by the pulmonary delivery device was evaluated at different concentrations: 0.2%, 0.4%, 0.8%, 1.6%, 3.2% and 4.8% (m/v). Three measurements were performed at each concentration (50 µl volume). The aerosol was generated in hermetic vials containing 950 µl of the mobile phase, after the septum cap was pierced with the aerosolising tip of the device. Further dilution was then performed to achieve the range of linearity. The mean, standard deviation (SD) and coefficient of variation (CV) were evaluated for each concentration. A validated HPLC method was used to determine the amount of TMZ in the droplets that were generated by the pulmonary delivery device. The chromatographic system (HP 1200 series, Agilent Technologies, Brussels, Belgium) was equipped with a quaternary pump, an auto sampler and a diode array detector. The separations were performed on a reverse phase Hypersil Gold C-18 column, 5 µm, 250 × 4.6 mm (Thermo Fisher Scientific, Waltham, USA). The mobile phase consisted of 0.5% v/v aqueous acetic acid–acetonitrile (90:10 v/v), which was

delivered at a flow rate of 1.0 ml/min. The quantification was performed at 329 nm. The calibration curve was linear in the 25-300 µg/ml range. The TMZ samples and calibration standards were diluted in the mobile phase. The volume injected was 10 µl and the analysis time was 10 min.

2.4.3. Aerosol droplet size distribution – Laser light scattering

Aerosol droplet size distribution measurements were performed by means of a Spraytec® laser diffraction-based apparatus (Malvern, Worcestershire, UK). The size distribution of the aerosol droplets was characterised by the d(v; 0.1), d(v; 0.5) and d(v; 0.9) values (see section 2.3.1). The droplet size distributions were evaluated for aerosol droplets that were generated from the pulmonary delivery device; the droplets contained either water (50 µL) or suspensions (50 µL) composed of 0.2%, 0.4%, 0.8%, 1.6%, 3.2% and 4.8% (m/v) TMZ. Measurements were performed at room temperature (22 ± 2 °C), and mean ± SD values were determined for each sample (n=15).

The aerosol droplet size distribution was also determined for an aqueous 0.2% suspension (m/v) of polystyrene microparticles (monodisperse size of 1 µm) using the same procedure as that described above. This series of experiments was performed to reproduce the aerosol generated from the suspension of fluorescent particles (see section 2.4.4), which was used to evaluate the distribution of droplets in mouse lungs.

2.4.4. The distribution of aerosol droplets generated from the pulmonary delivery device in mouse lungs

A suspension of fluorescent particles was used to evaluate the distribution in mouse lungs of the droplets generated from the pulmonary delivery device. An aqueous suspension (0.2% (w/v), 50 µL) of yellow-green fluorescent polystyrene microparticles coated with biotin (monodisperse size of 1 µm, FluoSpheres, Invitrogen Molecular Probes, Oregon, USA) was administered to three healthy mice by the endotracheal procedure described in section 2.4.1. Mice were sacrificed by cervical dislocation 15 min after administration of the suspension, and the lungs were removed. The upper, median and lower right lobes, as well as the upper and lower left lobes, were separated, were cut lengthwise and were frozen with nitrogen liquid. For each lobe, 5 cryosections of 7 µm were cut and were collected on slides; 5 fields per slide were photographed at 200× magnification on the FITC and DAPI filters by means of a motorised inverted fluorescent IX81 microscope (Olympus Optical Co., Tokyo, Japan) that was equipped with a megapixel digital F-view camera (Olympus Optical Co., Tokyo, Japan). Image acquisition was carried out using the cell M 8.0 software package (Olympus Optical Co.) with the pulmonary tissue filling the acquired fields.

2.4.5. Description of image analysis for the fluorescent particle distribution in mouse lungs

The green channel was acquired as a grayscale 8-bit image (i.e., pixel value range of 0-255, see Figure 1A). The image analysis procedure provides the number of dots in the observed view field. The first step was to threshold (at a gray value of 30) the input grayscale images (Figure 1A) to obtain a binary image of the dots (Figure 1B). The binary image was labelled (i.e., separate objects were identified) to obtain the object count and the respective sizes. In this labelled image, the detected objects are

either single dots or dot clusters (i.e., tightly connected dots). To calculate the number of dots in an object, we determined a canonical dot surface by sampling a series of images that presented a large majority of separate dots and computed the mode of the surface distribution across all of the images. To determine the dot count, we divided each object surface by the canonical dot surface and rounded the result (the result is never rounded to 0, as each detected object includes at least one dot).

Some images were affected by noise and required an additional pre-processing step and a higher threshold value to generate binary images. In low-light situations, the camera automatically raises the gain at acquisition time, thereby introducing uniform noise across the entire image. The noise was detected automatically by applying a minimum filter (Dougherty and Lotufo, 2003) to the grayscale image and then subtracting the result from the original image. The sum of the pixel values in this difference provided a noise index, from which we identified the images that should be pre-processed. The pre-processing consisted of applying the minimum filter (Dougherty and Lotufo, 2003) with a radius of 6 pixels and then thresholding the result with a higher value (130) to obtain the binary image. The steps after thresholding remained the same as those described above.

2.5. Determination of the *in vitro* anticancer activity

2.5.1. Cell culture conditions

The mouse B16F10 melanoma cell line (American Type Culture Collection (ATCC; Manassas, VA, USA) code CRL-6475), and the human A549 NSCLC (ATCC code CCL-185) and T98G glioblastoma (ATCC code CRL-1690) cell lines were maintained in a Roswell Park Memorial Institute (RPMI) medium supplemented with 10% foetal

bovine serum and a mixture of 0.6 mg/ml glutamine, 200 IU/ml penicillin, 200 IU/ml streptomycin, and 0.1 mg/ml gentamicin, as detailed elsewhere (Ingrassia et al., 2009). Trypsin-EDTA, foetal bovine serum, the cell culture media, and their supplements were obtained from GibcoBRL (Invitrogen SA, Merelbeke, Belgium). The foetal bovine serum was heat-inactivated for 1 hour at 56°C. The cell lines were incubated at 37°C in sealed (air-tight) plastic Falcon dishes (Nunc, VWR, Leuven, Belgium) in a 5% CO_2 atmosphere.

2.5.2. *In vitro* growth inhibition – MTT colorimetric assay

The *in vitro* growth inhibitions induced by naked TMZ, i.v. TMZ solution, and inhaled TMZ suspension were determined by means of the colorimetric 3-[4,5-dimethylthiazol-2yl]-dephenyltetrazolium bromide (MTT) (Sigma, Bornem, Belgium) assay, as detailed elsewhere (Van Quaquebeke et al., 2007; Ingrassia et al., 2009). The cells were incubated for either 72 or 144 hours in the presence or absence (using the vehicle as a control) of the drug with concentrations ranging between 0.5 µM and 1 mM, with semi-log concentration increases. The experiments were carried out in sextuplicate.

2.6. Determination of *in vivo* anticancer activity

2.6.1. Animals

All animal experiments (see sections 2.4.4, 2.6.2, 2.6.3, 2.6.4) used female B6D2F1 mice (18-22 g; Charles Rivers, Arbresle, France) and were performed on the basis of authorisation no. LA 1230355 from the Animal Ethics Committee of the Federal Department of Health, Nutritional Safety, and the Environment (Belgium).

2.6.2. Determination of the maximal tolerated dose

The maximal tolerated dose (MTD) of a drug is defined as the maximum dose that can be administered acutely (i.e., in a single endotracheal dose) to healthy animals (i.e., not grafted with tumours). The survival period and weight of the animals were recorded for up to 21 days post-injection. Seven different doses (5, 10, 20, 40, 80, 120 and 160 mg/kg bw) were used to determine the MTD index (with 3 mice per group). The MTD index is defined as the concentration causing the death of at least one mouse in a group of three. The pulmonary delivery device was used to administer the different doses. A volume of 50 µl was used for each dose, and the concentrations of the suspension for inhalation were 0.2, 0.4, 0.8, 1.6, 2.4, 3.2 and 4.8% of TMZ (m/v); for the dose at 160 mg/kg bw, 100 µl of the suspension at 3.2% TMZ (m/v) was aerosolised to avoid clogging of the aerosolising tip. When the MTD index is higher than 160 mg/kg, a drug is usually considered to be non-toxic, and the highest dose administered to tumour-bearing mice is MTD/2, i.e., 80 mg/kg (Darro et al., 2005).

2.6.3. The B16F10 melanoma pseudo-metastatic lung model

B16F10 melanoma pulmonary pseudo-metastases were obtained by the intravenous injection (lateral tail vein) of 2.5×10^5 B16F10 cells (200 µl), as we detailed previously (Mathieu et al., 2007). All mice were grafted with B16F10 tumour cells on the same day. Mice were randomised on the 6^{th} day post-tumour grafting, and treatments began on the 7^{th} day post-tumour grafting. Each experimental group included 11 to 27 mice, as detailed in the Results section and in the captions of the Figures.

2.6.4. Experimental protocols for determining the antitumour activity

TMZ (40 mg/kg bw) was administered either via i.v. (200 µl of an intravenous solution at 0.4% TMZ (m/v) in the tail vein) or directly to the lungs (50 µl of a suspension for inhalation at 1.6% TMZ (m/v) by the endotracheal route) either once a week (Wednesday) or three times a week (Monday, Wednesday, Friday) for three consecutive weeks. The experimental groups included 11 mice (control group and i.v.-treated), 17 mice (one endotracheal administration per week) or 27 mice (three endotracheal administrations per week). A larger number of mice were used in the endotracheally-treated groups to counteract the mouse deaths that can occur with repeated anaesthesia procedures. The mice that died during anaesthesia are labelled with a star, while those that died from their cancers are labelled with a black dot in the figure describing these data (Fig. 7B).

Mouse survival was checked at 9:00 am and 4:00 pm each day. Mouse weight was recorded three times per week (Monday, Wednesday, Friday). Each B16F10 melanoma-bearing mouse was sacrificed (by cervical dislocation) when it had lost 20% of its weight (compared to that determined at the time of the tumour graft) or if it was suffocating. The lungs were removed, fixed in buffered formalin, embedded in paraffin and then processed for conventional histopathological analyses.

2.7. Statistical analyses

Survival analyses were carried out by means of Kaplan-Meier curves and the log-rank test. Statistical comparisons of more than three independent groups of data were made using the Kruskal-Wallis test (a nonparametric one-way analysis of

variance). When this multi-group test was significant, post-hoc tests (Dunn procedure) were used to avoid multiple comparison effects when comparing the group pairs of interest. All statistical analyses were performed using Statistica (Statsoft, Tulsa, OK).

3. Results

3.1. *In vitro* evaluation of the TMZ formulations

3.1.1. The TMZ suspension for inhalation

The HPH process reduced TMZ particles from a d(v; 0.5) value of 27.6 µm to 3.2 µm, with 79% of the particles displaying a size spread of 1-5 µm, as compared to only 5% before the process (Figure 2). The shape of the particles became more spherical, and the suspension appeared to be stabilised by the high concentration of phospholipids (Figure 2). The suspension for inhalation presented significantly more individualised particles than suspensions that used surfactants other than phospholipids or that had a low concentration of phospholipids (data not shown).

3.1.2. *In vitro* anticancer activity

The *in vitro* growth inhibition assay was evaluated using murine B16F10 melanoma and human A549 NSCLC and T98G gliobastoma cells. No difference in antitumour activity was observed between the naked TMZ, the i.v. TMZ solution and the TMZ suspension for inhalation (Figure 3). The T98G GBM cells displayed higher sensitivity to TMZ when these cells were cultured for 144 hours instead of 72 hours. The same

observation was made for the A549 NSCLC cells but to a lesser extent. The length of time that B16F10 melanoma cells were cultured with TMZ had no effect on the TMZ sensibility of the B16F10 melanoma cells.

3.2. *In vitro* and *in vivo* evaluation of TMZ aerosols generated from the pulmonary delivery device

The three doses delivered by the pulmonary delivery device showed good uniformity, as demonstrated by the low CV (<5%) observed at different TMZ concentrations (Table 1). The droplet size distributions for the different suspensions, including the polystyrene suspension, were compared to the droplet size distribution in water. The means ± SD of d(v; 0.1), d(v; 0.5) and d(v; 0.9) in water were 10 ± 2 µm, 24 ± 4 µm and 44 ± 5 µm, respectively. The d(v; 0.1), d(v; 0.5) and d(v; 0.9) showed no statistically significant ($p > 0.05$) difference between the droplet size distributions in water and at different TMZ concentrations (up to 3.2%). The droplet size distribution for 4.8% was significantly different ($p < 0.05$) only for d(v; 0.9), which suggests that a high particle concentration promotes the agglomeration of droplets (Figure 4).

Figure 5 illustrates the distribution of fluorescent particles in the lungs of a healthy mouse (i.e., not bearing a B16F10 melanoma) when the particles are administered via the pulmonary delivery route. The fluorescent particle distribution was determined quantitatively in each part of the lungs of three distinct mice, as illustrated in Fig. 5B. Five fields per slide were analysed quantitatively, and five histological slides were analysed for each of the five parts of the lungs [URL (upper right lung), MRL (median right lung), LRL (lower right lung), ULL (upper left lung) and LLL (lower left lung)]. We

evaluated quantitatively the total number of fluorescent particles for each of the 25 fields analysed per lung part of a given mouse (labelled 1 to 25 in Fig. 5B), and this procedure was performed with three distinct mice. The data in Fig. 5B show that even though the distributions of fluorescent particles were heterogeneous in the various parts of the lungs, significant amounts of fluorescent particles were nevertheless present in each part of the lungs.

3.3. *In vivo* anticancer activity of the TMZ inhalation method on the pseudo-metastatic lung model

Figure 6 illustrates the pseudo-metastatic lung model, which displays very aggressive biological behaviour, as observed both macroscopically (Fig. 6A) and microscopically (Fig. 6B). Macroscopically, it appears that the mouse B16F10 melanoma model destroyed more than 80% of the healthy pulmonary tissue within 23 days after grafting (Fig. 6B). This feature is also apparent microscopically, where >80% of the pulmonary parenchyma was replaced by the melanoma (Fig. 6B).

The MTD index of TMZ administered by inhalation was greater than 160 mg/kg; no death and no significant changes in weight were observed between the different experimental groups. The TMZ doses that were delivered either via i.v. (the IV groups in Fig. 7A) or endotracheally (the ET groups in Fig. 7A) did not provoke toxic side effects, as compared to the controls (the wV and V groups in Fig. 7A), in terms of the body weight changes over time.

Fig. 7B shows that the method we developed to deliver TMZ endotracheally (directly into the lungs) to mice bearing B16F10 melanoma pseudo-metastases displayed

antitumour activity that is quite similar to the results obtained when delivering TMZ by means of an i.v. route. Indeed, no statistically significant ($p > 0.05$) difference was apparent when one-weekly i.v. administration was compared with one-weekly endotracheal TMZ delivery (Fig. 7B); similar results were observed when comparing three-weekly i.v. versus endotracheal TMZ delivery (Fig. 7B). We obtained three "long-survivors" (3/27 = 11%), which are mice that survived >70 days post tumour-grafting. These mice were given the three-weekly endotracheal TMZ delivery protocol; long-survivors were not observed with the three-weekly i.v. TMZ delivery (Fig. 7B). The three long-survivors were sacrificed on the 72nd day post tumour-grafting, and their lungs were removed and processed for histological analyses, which revealed an almost complete eradication of the tumour from the lungs (Fig.7C).

Moreover, there are no statistically significant ($p > 0.05$) differences between the non-treated group (the wV group in Fig.7B) and the vehicle-treated group (the V group in Fig.7B), and in both cases, the body weight evolution was similar (Fig.7A).

4. Discussion

There are several advantages associated with inhaled chemotherapy for the direct local treatment of lung tumours and lung metastases. In general, chemotherapy is characterised by a dose-dependent response in the targeted cancer (usually through pro-apoptotic effects), but is associated with a large amount of non-specific, toxic side-effects. Directing the chemotherapy of interest directly to a cancer-damaged organ could increase its efficacy and could reduce systemic side-effects (Gagnadoux et al., 2008). Administration of chemotherapy in multiple doses (through parenteral or oral routes) is often interrupted to allow the normal tissues to recover, such as the

bone marrow and the gastrointestinal tract. Pulmonary delivery could reduce the intervals between doses and could thus prevent tumour cell repopulation (Smyth et al., 2008). Moreover, both parenteral and oral administrations suffer from drug dilution during systemic circulation, which results in a significant drop in the concentration of the drug that reaches the target tumour site (in our case the lung cancer or pulmonary metastases). In addition, these parenterally-delivered chemotherapy agents are only present at the tumour site for a very brief period of time (Smyth et al., 2008). Furthermore, cancers must be treated chronically with various types of cytotoxic and/or cytostatic drugs, and the repeated parenteral administration of these types of drugs leads to severe and limiting toxic side-effects. Additional pain is also an issue because chronic parenteral administrations damage veins. Finally, trauma to veins can lead to thrombophlebitis, and extravasations of the drug solution into the extra vascular spaces may, in turn, lead to irritation and tissue necrosis; infection can occur at the site of catheter introduction, and air embolisms can occur because of air drawn in via the intravenous line (Jain, 2008).

Pulmonary drug delivery is a non-invasive delivery method, in contrast to parenteral delivery, and inhalation has the advantage of potentially minimising the risk of systemic side-effects by delivering a high drug concentration directly to the disease site. Moreover, the oral route presents some barriers to effective treatment, such as poor gastrointestinal absorption and first-pass metabolism in the liver, and these issues are avoided with the inhalation method. Inhalation exhibits a similar or superior therapeutic effect as that observed with oral or parenteral administration (Labiris and Dolovich, 2003).

Inhalation has been used as a method of drug delivery for hundreds of years, but this historical use has been focused on vapour inhalation or the smoking of herbal

preparations. Beginning in the middle of the twentieth century, current methods of pulmonary drug delivery were developed and employed to treat respiratory disorders. This method was enabled by the availability of effective drugs and portable delivery systems (e.g., pressurised metered-dose inhalers). Since then, remarkable advances have been made in the technology of devices and formulations for inhaled drugs (Anderson, 2005).

Nowadays, more than 65 different inhaled products containing one of more than 20 active ingredients are marketed to treat respiratory diseases (Labiris and Dolovich, 2003). However, few studies have focused on aerosolised chemotherapy for the treatment of lung cancer and pulmonary metastases (Smyth et al., 2008). Several reports have been published, but these reports mainly relate to pre-clinical models (Knight et al., 1999; Koshkina et al., 2000; Koshkina et al., 2001; Gautam et al., 2002; Zou et al., 2004; Gagnadoux et al., 2005).

The animal tumour models used in these studies are human xenograft (Knight et al., 1999), metastatic (Koshkina et al., 2000; Koshkina et al. 2001; Gautam et al., 2002) and orthotopic models (Zou et al., 2004; Gagnadoux et al., 2005). Metastatic and orthotopic mouse models tend to be more appropriate than the xenograft model in the prediction of a clinical response because the former models mimic the morphology and growth characteristics of clinical diseases at the tumour site (Suggitt and Bibby, 2005). In addition, the mouse seems to be the leading preclinical model for pulmonary delivery chemotherapy. In our study, we chose the B16F10 melanoma lung pseudo-metastatic model to reproduce these metastases in the target organ. Cheng et al. (2005) described this mouse model and report that the development of the metastases in the lung seems to be a function of time. In addition, no significant metastatic lesions are observed in the liver, spleen or brain. The lung metastatic

lesions not only extravasate and grow near blood vessels, but they also grow near and at the lung surface. In addition, this model is highly invasive, as shown by our results, and more than 80% of the pulmonary parenchyma is replaced by metastatic tissue in the final stages of the disease.

Pulmonary drug delivery is highly problematic in small animal models such as mice because the efficiency of delivery is dramatically reduced in these species. The techniques for delivering drugs to mouse lungs include the inhalation chamber, nose-only aerosol exposure and intratracheal instillation. The inhalation chamber and nose-only aerosol exposure are the main techniques used in the previous studies of aerosolised chemotherapy (Knight et al., 1999; Koshkina et al., 2000; Koshkina et al., 2001). These techniques are usually used in inhalation toxicology but are used only rarely in pulmonary biopharmaceutics research because the lung-deposited dose is difficult to determine. Moreover, only a small amount of drug is inhaled by the animal during the process, and a long period of time is required to administer the dose. There is drug loss on the skin, in the nose and in the throat of the animal (Driscoll et al., 2000; Bivas-Benita et al., 2005; Sakagami, 2006). Intratracheal instillation was used in recent aerosolised chemotherapy studies (Zou et al., 2004), and this method has many advantages: it is quick, simple and the administered dose is easily quantifiable. Moreover, only small amounts of drug are required, and there is a minimal drug loss in the upper airway area (Driscoll et al., 2000; Sakagami, 2006; Bivas-Benita et al., 2005). Initially, intratracheal instillation was an invasive method: after anaesthetising the animal, an incision was made between the tracheal cartilaginous rings, and an endotracheal tube was inserted through the incision to position the tip just before the tracheal bifurcation (carina). Typically, 10-200 µl of an aqueous solution or a suspension of drug was placed into the airway through the

tube using a microsyringe (Sakagami, 2006). More recently, to avoid a tracheotomy, some studies have employed either direct injection or spraying of the drug through an orotracheal intubation, as reviewed by Driscoll et al. (2000). Sophisticated endotracheal devices have recently become available for use in mice. They are now able to deliver multiple administrations of both liquid and powder formulations into the lungs in a non-invasive, relatively accurate and reproducible manner (Sakagami, 2006; Bivas-Benita et al., 2005; Amorij et al., 2007). One of these endotracheal devices was used to deliver our pulmonary suspension at different TMZ concentrations. Up to 79% of the particles in TMZ suspensions for inhalation were in the human respirable size range (1-5 µm). The particle size reduction was required both to avoid clogging the aerosolising tip of the pulmonary delivery device and to limit the sedimentation of particles to maintain the homogeneity of the suspension during the pulmonary administration. The aerosol droplet size distribution was not affected by the TMZ concentration and was in the same particle size ranges as both the aerosol droplets generated with water and with the suspension of polystyrene. The results demonstrated that this device delivers a reproducible dose and a reproducible aerosol, and that both of these variables are independent of the particle concentrations, with the exception of the d(v; 0.9) value with a 4.8% TMZ concentration. The d(v; 0.5) range obtained with the aerosols from the water and the suspensions was 21-25 µm, and this result corresponds to the mass median diameter of 16-22 µm and 20 µm provided by the supplier and by Dennett et al. (2006), respectively. In spite of the high droplet size generated with this pulmonary delivery device, the aerosol seems to reach most of the lung tissue in the mouse.

The *in vivo* evaluation of the aerosol droplets in mouse lungs reveals that the deposition is heterogeneous between the different lobes and between the three mice.

The heterogeneity observed with this unique mode of administration in mouse lungs could be decreased with multiple administrations, as performed with a chronic treatment, suggesting that the morphology and variability of the pulmonary tract play important roles in the deposition of the aerosol droplets. However, a significant number of fluorescent particles reached each lobe and most of the analysed fields. The broad exposure of the lung tissue to the aerosol droplets is confirmed by the results obtained in other studies where pulmonary deposition was evaluated by scintigraphic imaging (Gagnadoux t al., 2005; Delepine et al., 2002). In addition, the efficacy in reaching the mouse lungs was confirmed by the gene transfection that was observed in the lung epithelium after gene delivery by the pulmonary delivery device (Delepine et al., 2002; Schughart et al., 1999).

The repeated endotracheal procedure (including anaesthesia) affected neither the body weight evolution nor the median survival period, as shown by the non-statistically-significant ($p > 0.05$) difference between the non-treated and vehicle-treated mice in the evaluation of the *in vivo* antitumor activity.

The TMZ suspension for inhalation was as tolerable as an intravenous solution. A MTD index of the TMZ suspension that is greater than 160 mg/kg bw is considered to be non-toxic, as defined in Darro et al. (2005). In addition, the excipients used in the suspension for inhalation (e.g., DLPC) are known to be non-toxic and well-tolerated in volunteers (Gilbert t al., 1997; Waldrep et al., 1997), and DMPC is currently being used in the development of pulmonary formulations (Darwis and Kellaway, 2001; Desai et al., 2002).

The antitumor activity of the TMZ suspension delivered by the endotreachal method was similar to that delivered by intravenous administration, in terms of median

survival period. These results demonstrate that this method efficiently delivers the drug to numerous tumours that are localised at different sites of the lungs. In addition, three “long-survivors” (3/27) were observed with the pulmonary delivery but not with the intravenous route. The pulmonary chemotherapy delivery seems able to deliver the drug at an adequate concentration and for a sufficiently long time period to eradicate the tumour sites.

However, pulmonary delivery does not reduce the frequency of administration, which suggests that TMZ is eliminated rapidly from the pulmonary tract.

The efficient and safe TMZ suspension for inhalation that was developed in this study could be administered easily to humans via a nebuliser, and this method would be particularly useful for NSCLC patients and patients with pulmonary metastases. However, the occupational exposure of healthcare workers to the nebulised chemotherapy presents a safety issue, but this potential problem could be addressed by using a well-ventilated room with an air filtering system. A recent study demonstrated the effectiveness of a mobile HEPA filter air-cleaning system combined with a demistifier tent, and this system prevented aerosol propagation during the inhalation of nebulised chemotherapy (Wittgen et al., 2006). Another approach would be to develop a dry powder form of the drug to deliver chemotherapy safely and easily. In addition, the effectiveness of the treatment could be improved by increasing the residence time of particles in the lungs, thereby decreasing the necessary delivered dose.

In conclusion, the B16F10 melanoma lung pseudo-metastatic model is an interesting preclinical model for studying aerosolised chemotherapy. Both the pulmonary delivery device and the endotracheal administration are safe and efficient for

administering cytotoxic/cytostatic drugs for inhalation in pre-clinical models (e.g., mice); these drugs are formulated as a solution or suspension of particles that are of a human respirable size. The TMZ suspension for inhalation that was developed in this study was well-tolerated, and the efficacy was comparable to that of intravenous treatment at the same dosage. This TMZ suspension could be easily administered via a nebuliser in NSCLC patients and in patients with pulmonary metastases. TMZ is currently undergoing a large number of clinical trials (Lefranc et al., 2007), including trials with NSCLC patients (Choong et al., 2006; Tamaskar et al., 2008; Kouroussis et al., 2009), and its therapeutic benefits have been demonstrated in cancers associated with extremely poor prognoses, such as glioblastomas (Lefranc et al., 2009).

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Tables

Table 1

The uniformity of the dose delivered by the pulmonary delivery device for inhalation.

Doses are delivered as suspensions at different TMZ concentration (m/v).

	0.2%	**0.4%**	**0.8%**	**1.6%**	**3.2%**	**4.8%**
Mean	0.195	0.359	0.71	1.35	3.03	4.48
SD	0.001	0.003	0.02	0.05	0.04	0.04
CV %	0.7	0.9	3.2	3.5	1.4	0.8

Figure Legends and Figures

Figure 1: (A) A grayscale original image and (B) a binary image obtained after thresholding.

Figure 2: The particle size distribution, shape and agglomeration of TMZ in the pulmonary suspension. The images were observed using an optical microscope at 200× magnification (bar = 50 µm), and the volume particle size distribution was characterised by a size range (d(v; 0.1)-d(v; 0.9)) that was evaluated using a laser diffractometer (**A**) after and (**B**) before the size reduction step.

Figure 3: The *in vitro* anticancer effects of TMZ. The global growth inhibition was evaluated on the A549, B16F10 and T98G cell lines. Naked TMZ, i.v. TMZ solution and TMZ pulmonary suspensions were administered over 72 and 144 hours.

Figure 4: The size distribution analysis of the aerosol droplets. The droplet size distributions were characterised by d(v; 0.1), d(v; 0.5) and d(v; 0.9), and were evaluated for both water and pulmonary suspensions with 0.2%, 0.4%, 0.8%, 1.6%, 3.2% and 4.8% (m/v) TMZ. The suspensions were aerosolised by the pulmonary delivery device. Fifteen measurements were performed to obtain mean ± SD values.

Figure 5: The distribution of fluorescent particles in the lungs of healthy mice.

(**A**) An example of a 7-µm longitudinal lung cryosection that was observed using a fluorescent microscope at 200× magnification. This image shows the distribution of yellow-green fluorescent microparticles (on a FITC filter) in the blue fluorescent lung tissue (on a DAPI filter) of a healthy mouse after administration of a 0.2% suspension of these particles (50 µl) using the pulmonary delivery device. (**B**) A total of 25 fields per lobe were photographed at 200× magnification on the FITC and DAPI filters for the upper, median and lower right lobe, as well as for the upper and lower left lobe (URL, MRL, LRL, ULL and LLL, respectively). For each picture, the counting of fluorescent microparticles was performed by the software. The 25 numbers of fluorescent microparticles counted in each picture are detailed for each lobe and for each mouse.

Figure 6: **The macroscopic and microscopic examples of mouse lung with metastases.**

(**A**) An example of mouse lungs that developed black pulmonary tumour nodules 23 days after 2.5 × 10^5 B16F10 melanoma cells were injected into the tail vein. (**B**) The typical histology of such a tumour, shown at an optical magnification of 40×.

Figure 7: The toxicity and activity of pulmonary administration of TMZ in comparison with intravenous administration on a B16F10 lung metastases model.

(**A**) The weight curves and (**B**) the Kaplan-Meier survival curves of female B6D2F1 mice grafted with 2.5 × 10^5 B16F10 melanoma cells (injected into their tail veins) on

day 0. The mice were untreated (continuous lines) or treated (dashed lines) with 40 mg/kg bw of TMZ beginning on day 7. The treatments were administered intravenously via the tail vein (IV; circles) or by inhalation via the endotracheal route (ET; squares). Treatments were performed once (open) or three (filled) times per week for three consecutive weeks. Each experimental group was composed of a minimum of 11 animals (for intravenous administration) and a maximum of 27 animals (for pulmonary administration) to compensate for death during anaesthesia. The survival time and body weight of each mouse were evaluated each day and three times per week, respectively. (**C**) The macroscopic and microscopic examples of “long-survivor” lungs, which were dissected 72 days post-tumour grafting. These lungs presented an almost complete eradication of the tumours. The hematoxilin-eosin stained example was obtained by optical microscope at 100× magnification.

Figure 1

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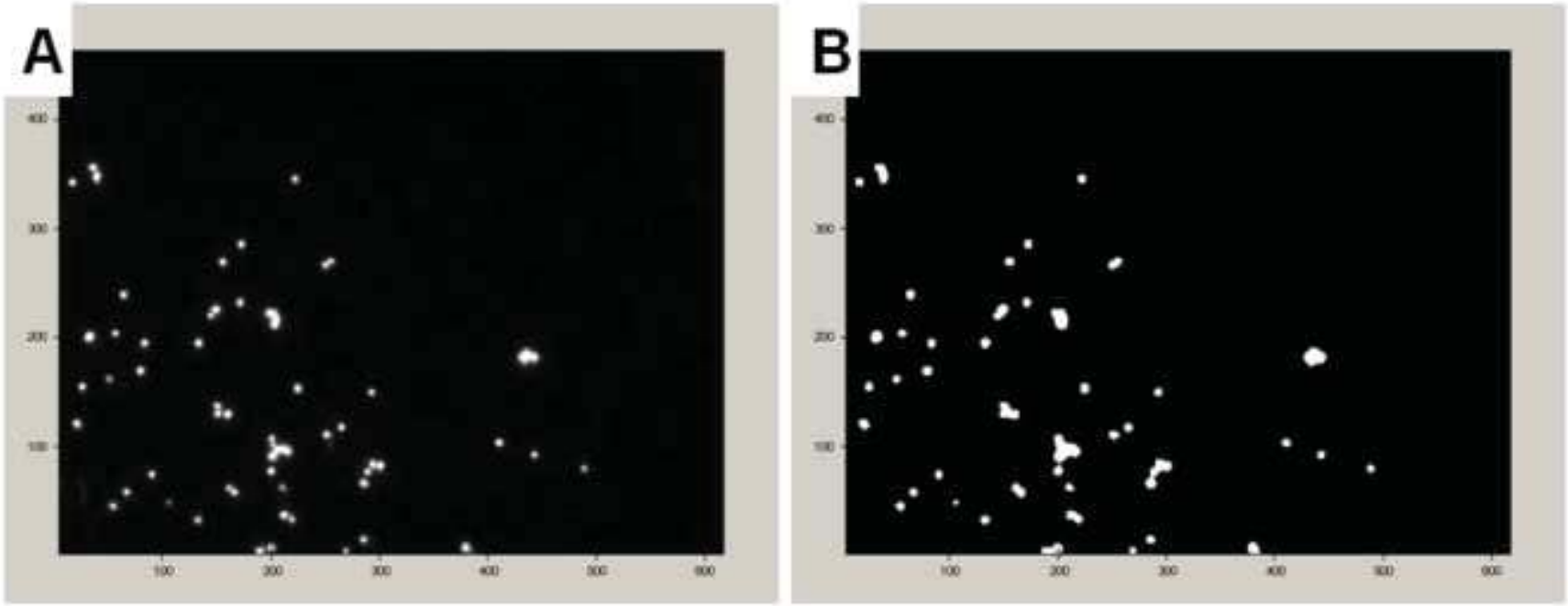

Figure 2

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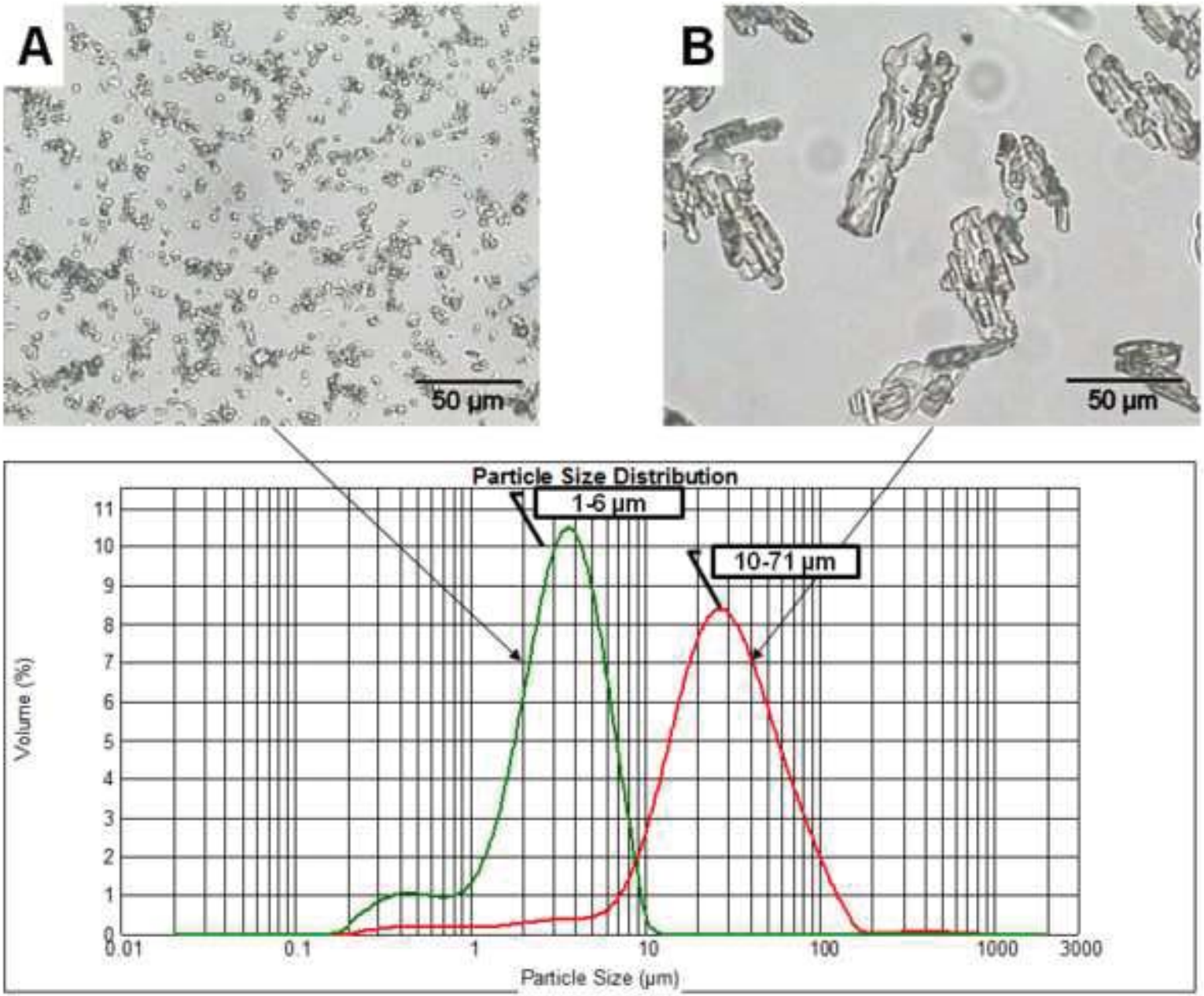

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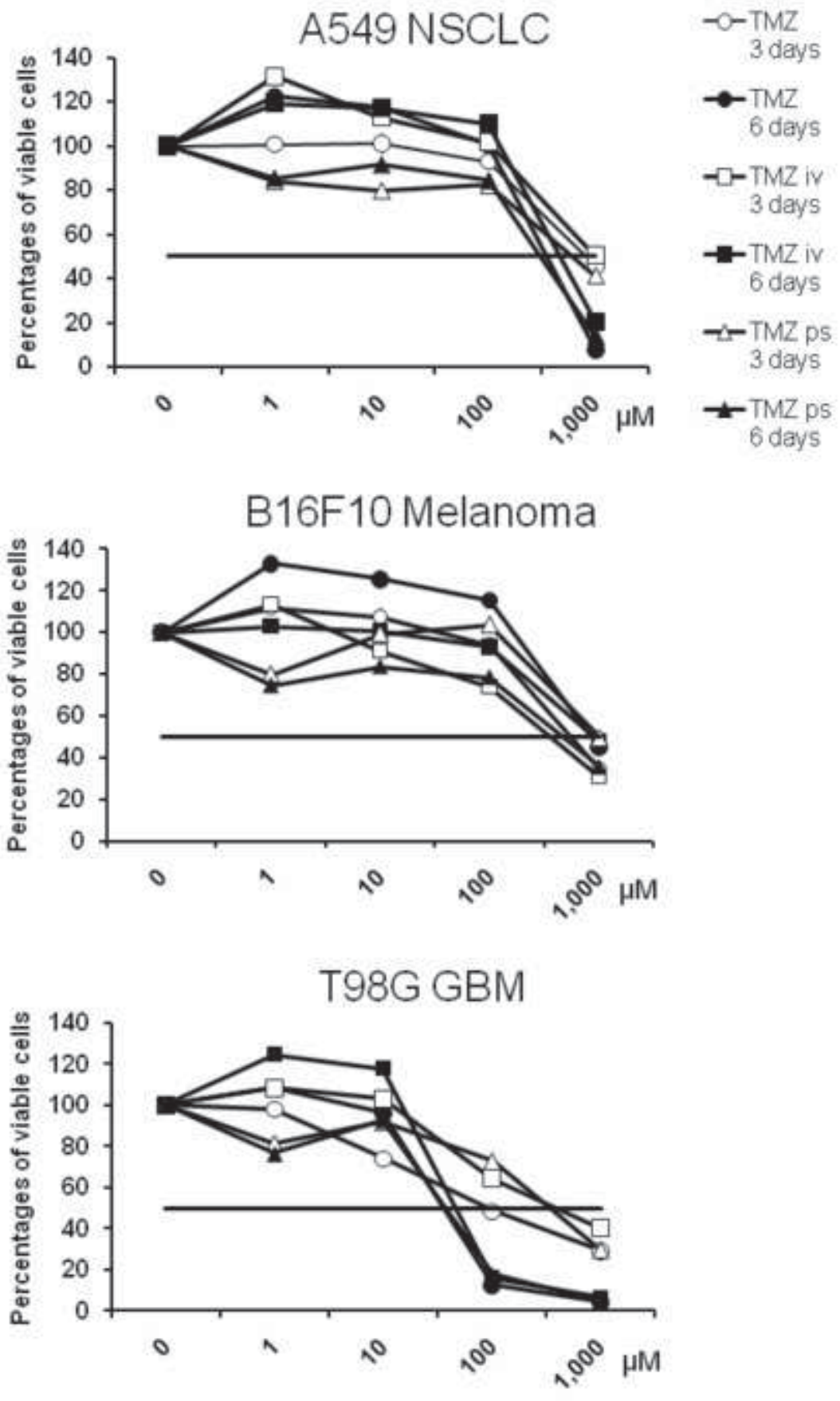

Figure 4

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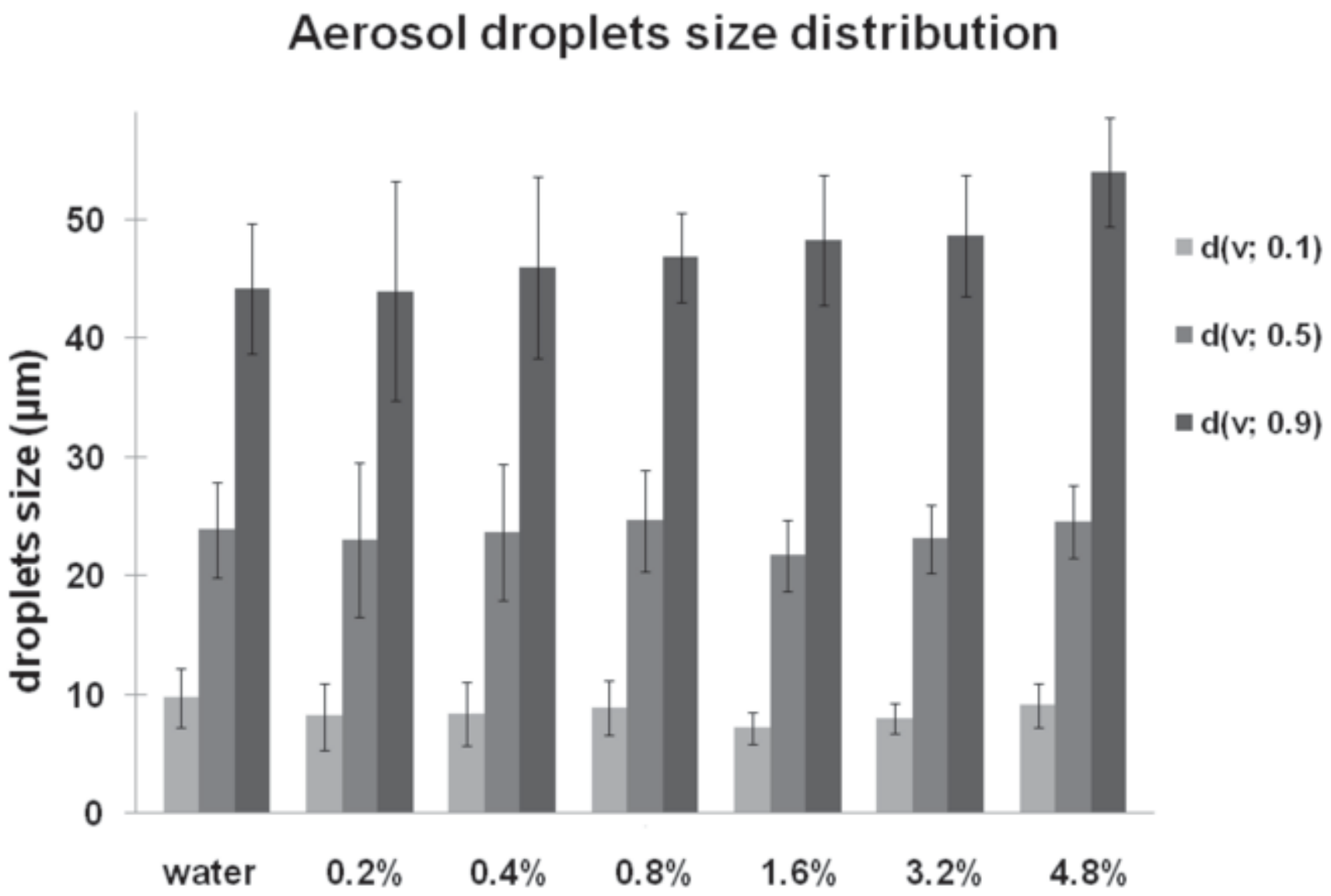

Figure 5
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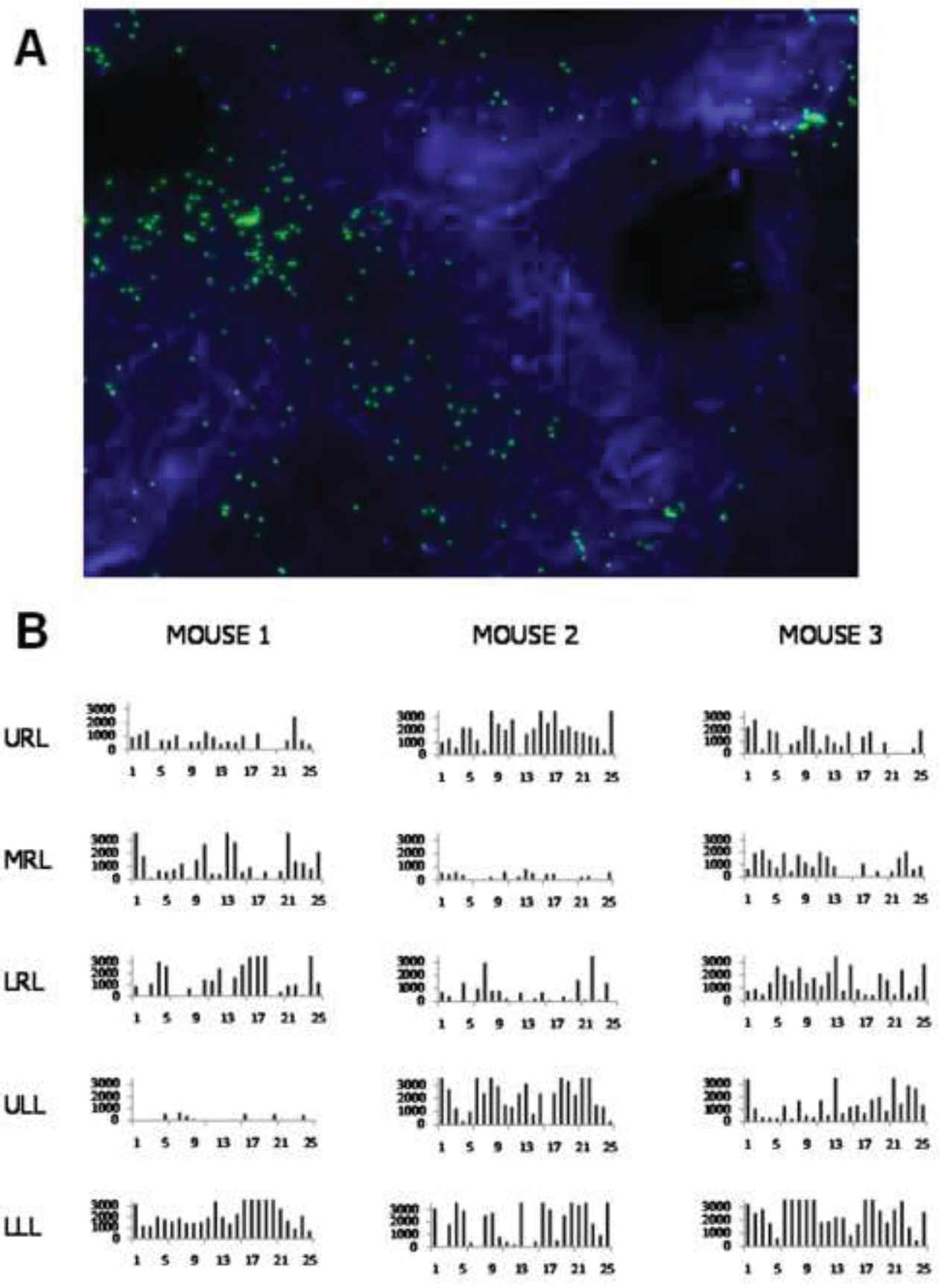

Figure 6

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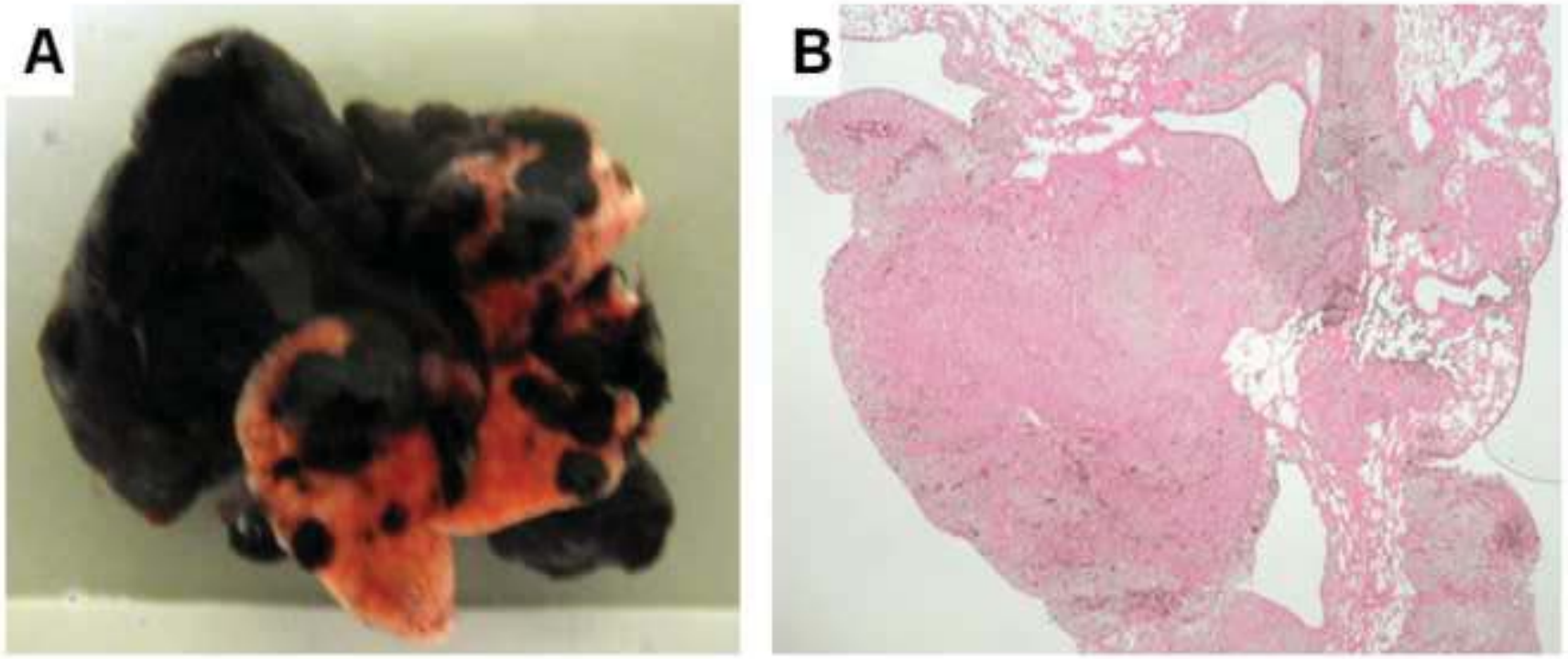

Figure 7
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