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Graphical Abstract

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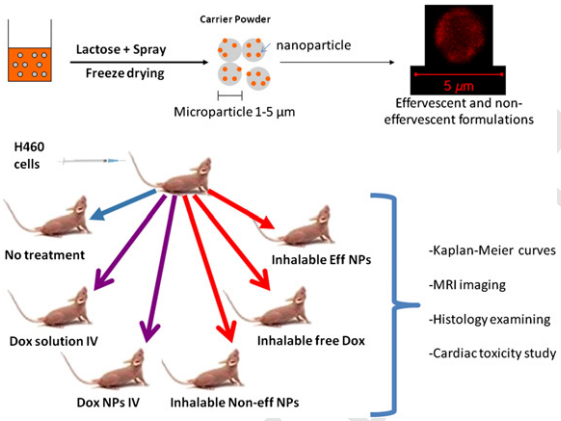

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Inhalable nanoparticles, a non-invasive approach to treat lung cancer in a mouse model

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ABSTRACT

Doxorubicin-loaded nanoparticles (NPs) were incorporated into inhalable effervescent and non-effervescent carrier particles using a spray–freeze drying technique. The prepared inhalable powders were tested in a tumor bearing Balb/c mouse model. Control treatments were treated with blank inhalable NPs, inhalable lactose powder containing free doxorubicin, and intravenous injections of a suspension of doxorubicin NPs, doxorubicin solution, or saline solution. The survival of treatment groups was plotted with Kaplan–Meier curves. Animals treated with inhalable effervescent nanoparticle powder containing 30 μg doxorubicin showed a highly significant improvement in survival compared to all other treatment groups. Mice in control groups treated with doxorubicin solution or doxorubicin NPs as intravenous injection, died in less than 50 days. Inhalable free doxorubicin showed high cardiac toxicity. Pathological samples showed large tumor masses in the lungs of animals not treated or treated with i.v. injections of doxorubicin NPs or doxorubicin solution. The lungs of animals treated with inhalable effervescent doxorubicin NPs showed fewer and much smaller tumors compared to the control groups, as visualized by MRI imaging which confirmed the observed pathology results. The present study demonstrates that inhalable effervescent doxorubicin NPs are an effective way to treat lung cancer. This non-invasive route of administration might change the way lung cancer is treated in the future.

1. Introduction

Lung cancer is one of the most lethal cancers and the second most common cancer in both men and women [1]. The long term survival rate of lung cancer patients treated by conventional modalities such as surgery, radiation, and chemotherapy remains far from satisfactory [2]. Anticancer drugs cause undesirable systemic side effects [3]. Improved drug delivery can play a key role in the fight against cancer by delivering anticancer drugs locally to the tumor site and decreasing systemic exposure to the drugs. Several studies are underway that investigate pulmonary delivery of chemotherapeutic solutions via inhalation [4–6]. However, the systemic side effects and dose limiting toxicity reported for pulmonary delivery are similar to those of intravenous administration [7].

Interest in the use of nanoparticles (NPs) as drug carriers in cancer therapy has increased over the last two decades, due to their ability to accumulate in cancer tissues by what is known as an enhanced permeability and retention (EPR) effect [8]. Moreover, NPs are able to modulate and overcome multidrug resistance *in vitro* and are targeted to special types of cells or tissues, passively and/or actively [9].

However, intravenous (i.v.) injection of nanosized carriers leads to carrier accumulation in the liver, which significantly reduces the dose that reaches the tumor site [10,11].

The recent developments of inhalable biodegradable nanoparticles [2,12–14], large porous particles [15] and liposomal dry powders [16,17] make inhalation a feasible alternative approach to deliver macromolecules such as insulin [18] and treat lung specific diseases like tuberculosis [19]. One issue with NP pulmonary delivery is that their size is not suitable for deep lung deposition. In fact, a carrier system such as lactose microparticles is required for deep lung delivery. The carrier particles should meet special requirements such as an appropriate mass median aerodynamic diameter (MMAD) and a suitable fine particle fraction (FPF) [13,14,20].

Direct delivery of drug loaded nanoparticles to the lungs combines the concepts of localized delivery with the advantages of using nanoparticles in lung cancer therapy.

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An *in vitro* study demonstrated the cytotoxicity of doxorubicin-loaded nanoparticles on H460 and A549 lung cancer cells [21]. We have also demonstrated that blank inhalable nanoparticles are well tolerated in a mouse model [14]. In the present study, effervescent and non-effervescent carrier particles optimized for local lung delivery of doxorubicin-loaded nanoparticles were tested in lung cancer bearing mice. The survival of these mice was compared with positive and negative control groups. Additionally, the acute cardiotoxicity of doxorubicin and doxorubicin-loaded nanoparticles was investigated.

2. Materials and methods

2.1. Preparation of blank polybutyl cyanoacrylate nanoparticles

Nanoparticles were prepared as described in previous work [21]. In brief, 100 μL of n-butylcyanoacrylate (Loctite, Ireland) monomer was added to 1% dextran 70 (Sigma, Canada) solution in 10 mL of 0.01 M HCl under constant stirring at 500 rpm. After 4 h the nanoparticle suspension was filtered through a 0.8-μm membrane filter (Nuclepore Track-Etch membrane, Whatman, USA) and stored at 2–8 °C.

2.2. Preparation of doxorubicin nanoparticles

Doxorubicin nanoparticles were synthesized as mentioned above with the following modifications. Doxorubicin powder (50 mg) (doxorubicin hydrochloride for injection, USP, Mayne Pharma (Canada) Inc., Montreal QC) was dissolved in 2 mL of distilled water. After 30 min the solution was added to the nanoparticle reaction flask under stirring at 500 rpm for 4 h. Final nanoparticle suspension was handled as described in Section 2.1.

2.3. Determination of drug loading efficiency and capacity

The loading efficiency was determined by calculating the difference between the added amount of doxrubicin and the unbound fraction of doxrubicin as previously reported [22]. The unwashed NP suspension was centrifuged at 100,000 *g* for 10 min using an Airfuge (Beckman, CA, USA). The supernatant was separated and analyzed for free doxrubicin by HPLC. The HPLC system consisted of a 851-AS_ auto sampler (Jasco Co., Tokyo, Japan), a LC-600_ isocratic pump (Shimadzu Co., Tokyo Japan), and a lichosphere_100 RP18e (5 lm) cartridge (Lichocart, Merck, Germany). Samples were eluted with a mixture of water, acetonitrile, methanol, and phosphoric acid (540:290:170:2) at a flow rate of 1 mL/min. DOX was detected with an FP-920_ fluorescence detector (Jasco Co., Tokyo, Japan) adjusted to 460-nm excitation wavelength and 550-nm emission wavelength. The centrifuged NP pellets were separated and dried, and the weight was used to calculate the loading capacity [22].

2.4. Particle size analysis of nanoparticles

Particle size and zeta potential were measured by photon correlation spectroscopy using a Zetasizer HAS 3000 from Malvern (Worecestershire, UK). 100 μL of the nanoparticle suspension was dispersed in 4 mL deionized water and sonicated for 1 min. Measurements were carried out at 25 °C. The *Z*-average value was used to express the mean hydrodynamic particle size in nm, and the polydispersity index was used to indicate the width of distribution.

2.5. Preparation of effervescent carrier particles

Effervescent carrier particles were prepared as described previously [14]. 250 mg sodium carbonate (Sigma, Canada) and 1000 mg spray dried lactose monohydrate (FlowLac 100, Meggle, Germany) were dissolved in 3 mL distilled water and 300 μL ammonium hydroxide 28–30% (Sigma, Canada) was added. The solution was kept in a tightly closed container. Before spray–freeze drying, 200 mg citric acid (Sigma, Canada) powder and 10 mL of nanoparticle suspension were added to the solution. The suspension was spray–freeze dried as previously reported [22]. In brief, a two-fluid nozzle (Spraying Systems Co., Wheaton, IL, USA) utilizing gaseous nitrogen at a flow rate of 0.6 scfm was used to atomize the nanoparticle suspension, which was supplied at a flow rate of 37 mL/min using a peristaltic pump (CTP-A, Chem-Tech, Punta Gorda, FL, USA). The nozzle was placed ~15 cm above a 600 mL flask containing 300–400 mL of liquid nitrogen. Following spraying, the flask contents were transferred to a Pyrex vacuum beaker, and the liquid nitrogen was allowed to evaporate. The vacuum container was attached to a freeze-dry system (Labconco Corp., Kansas City, USA) operating at 0.004 mbar with a collector at −52 °C. The powder in the flask was held at subzero temperature for 7 h, followed by 41 h at 23 °C. The powder was collected and stored in a sealed vial at 4 °C.

2.6. Preparation of non-effervescent carrier particles

For the preparation of nanoparticle loaded non-effervescent carrier particles, 1200 mg spray dried lactose monohydrate (FlowLac 100, Meggle, Germany) was added to the nanoparticle suspension. The suspension was spray–freeze dried as described in Section 2.5.

2.7. Measurement of mass median aerodynamic diameter

The mass median aerodynamic diameter (MMAD) of the powder was measured as described previously [14] using a Mark II Anderson Cascade Impactor (Graseby Anderson, Smyrna, GA, USA) with effective cut-off points recalibrated at 60 L/min. A passive dry powder inhaler that utilizes cyclonic action as well as mechanical impaction to disperse powder particles was used to de-agglomerate and deliver the powder [23]. The flow rate was monitored with a pneumotachometer (PT 4719, Hans Rudolph Inc., Kansas City, MO, USA).

2.8. Animal studies

Animal studies were approved by the animal ethics committee of the Alberta Cancer Board.

2.8.1. Implantation of lung cancer cells

Female 4–5 week old BALB/c nude mice were purchased from Charles River Laboratories (Senneville, Quebec, Canada). The human non-small cell lung carcinoma cell line NCl-H460 obtained from American Type Culture Collection (Rockville, MD, USA) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. On the day of inoculation, cultured cells were trypsinized, washed, and suspended in 0.9% saline. Each mouse received 100 μL of tumor cells with >95% viability at a concentration of 2×10^6 cells per 1 μL using a 27 gauge needle through the tail vein of anesthetized mice.

2.8.2. Treatment protocol

Tumor bearing mice with 5% weight loss were randomized into five different control and treatment groups (as listed below). For administration via inhalation, the animals were anesthetized with katemine/acepromazine, positioned against an angled restraining stand, and treated once a week for 4 weeks by either:

(i) 1 mg inhalable effervescent nanoparticle powder containing 30 μg doxorubicin ($n = 16$)
(ii) 1 mg inhalable non-effervescent nanoparticle powder containing 30 μg doxorubicin ($n = 16$).

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A DP-4M insufflator (Penn-Century Inc., Philadelphia, PA, USA) was used to administer the inhalable powders. The insufflator was weighed before and after powder filling as well as after administration of the powder to determine the delivered dose. The tip of the insufflator tube was positioned near the carina (first bifurcation of the pulmonary tracts) so that the measured dose of powder could penetrate deep into the lung. A fiber optic light (Nikon Inc., USA) was positioned on the animal's chest to visualize the location of the DP-4M device in the animal. For powder delivery, 0.25 mL of air was puffed through the DP-4M device.

For intravenous injection, animals were anesthetized by isoflurane and injected via tail vein by either:

(iii) 100 μL of 0.9% normal saline (negative untreated control) ($n=16$)
(iv) 30 μg doxorubicin in 100 μL of normal saline (positive control) ($n=8$)
(v) a nanoparticle suspension containing 30 μg doxorubicin in normal saline (positive control) ($n=8$).

Two other control groups were included in the study:

(vi) 1 mg inhalable lactose powder containing 30 μg doxorubicin powder ($n=8$)
(vii) 1 mg blank inhalable nanoparticle powder ($n=4$).

2.8.3. Animal monitoring

In order to assess a predetermined level of tumor burden in the lung, during the study period animals were monitored three times a week for weight loss and inactivity in addition to signs of morbidity such as scruffy appearance, listlessness, and compromised breathing. A morbidity scoring sheet was used as previously described [14]. Animals exhibiting higher morbidity scores (>10 out of 14) were euthanized according to the approved protocol. The study duration was 140 days, at which time the statistical endpoints of survival differences were reached according to the approved study protocol.

2.8.4. Histopathological study

During the gross postmortem examination, a set of organs was removed from each animal. Removed tissues were immediately immersed in 10% neutral buffered formalin and fixed for a minimum period of 24 h. Following fixation, tissues were trimmed with a scalpel to a thickness of 2–3 mm and a section of each organ was placed in a tissue cassette. Tissues in cassettes were processed into paraffin, embedded in a paraffin block, sectioned on a microtome to a thickness of 5 microns, placed on a microscope slide and stained with hematoxylin and eosin stain, following standard histology techniques. Slides were examined by a certified veterinary pathologist and each section of tissue was either recorded as normal, or a description was made of abnormalities or findings.

2.8.5. MRI imaging

Three mice from each treatment group were selected randomly and MRI imaging was performed to establish the location of the tumor burden. MRI images were acquired with a 9.4 T 21.5-cm horizontal bore magnet equipped with a 12.0-cm inner diameter gradient set (Magnex Scientific, Oxford, UK) interfaced to a TMX console. A 44-mm inner diameter birdcage coil was used for radiofrequency (rf) excitation and reception. The images are T1 weighted, and transverse spin echo scans were recorded with the following parameters: Echo time (TE)/repetition time (TR) was 13/1500 ms, the bandwidth (BW) was 50 KHz, the field of view (FOV) was 26*26 mm with 128×128 matrix, the number of averages was 4, and the thickness was 0.5 mm.

2.8.6. Cardiac toxicity of inhaled doxorubicin

To evaluate the effect of local lung delivery of free doxorubicin inhalable powder, an inhalable doxorubicin powder was prepared using a spray–freeze drying technique. Cancer-free mice were treated in the same way as mentioned in Section 2.8.2. The animals were divided into 3 groups and treated with either:

1) 1 mg effervescent doxorubicin inhalable nanoparticle powder containing 30 μg doxorubicin ($n=3$)
2) 1 mg doxorubicin in lactose inhalable powder containing 30 μg doxorubicin ($n=3$)
3) Control group ($n=3$), untreated.

Effervescent doxorubicin inhalable nanoparticle powder was chosen for this study as it was proven to be the most effective treatment as it will be discussed later in the Result section.

To investigate cardiac toxicity, three enzymes that are indicators of cardiac toxicity—aspartate transaminase (AST), alanine transaminase (ALT) and creatine phosphokinase (CPK)—were measured in blood. Blood samples were collected 1 h after insufflation of designated treatment in heparinized glass tube and sent to IDEXX laboratories (Edmonton, Canada) to be analyzed.

2.9. Statistical calculation and analysis

The number of mice in each group was calculated to obtain a statistical power of at least 0.8. The Kaplan–Meier curves of the different groups were compared via log-rank tests (Mantel–Cox test). Another statistical analysis was performed using a single factor ANOVA test or Student *T* test, as appropriate, with a 0.05 level of significance using SSPS Statistics® program (SPSS Inc. Illinois, USA).

3. Results

The loading efficiency of doxorubicin in NPs was 85% and the loading capacity was 9.6 mg of doxorubicin/100 mg of NPs. These values are in agreement with those reported previously [24–26]. The prepared NPs had an average size of 137.22 ± 1.53 nm, the polydispersity index was 0.12, and the zeta potential was −23.5 ± 0.41 mV. The loaded amount of doxorubicin in the carrier particles after spray–freeze drying was calculated to be 30 μg/mg powder. The mean particle size of doxorubicin-loaded nanoparticles after redissolution was 145 ± 20 nm and 256 ± 29 of the effervescent and non-effervescent spray–freeze dried powder, respectively ($n=6$). The Student *T* test indicated that the increase in the size of nanoparticles after redissolution was only significant with the non-effervescent formula. The mass median aerodynamic diameters (MMAD) of carrier particles loaded with blank and doxorubicin NPs were 3.45 ± 0.11 and 3.41

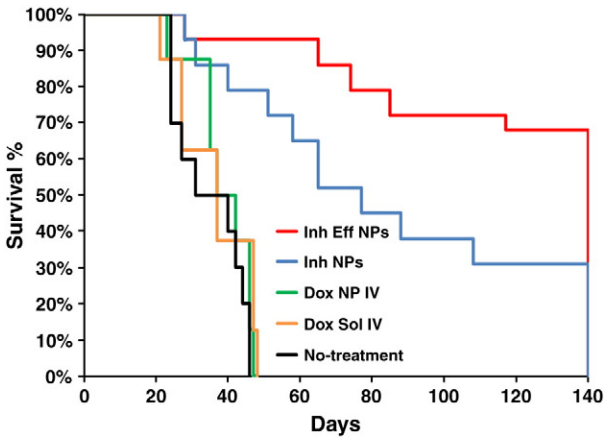

Fig. 1. Percent animal survival versus time. Animals were treated either with effervescent inhalable doxorubicin nanoparticle powder (Inh Eff NPs), inhalable doxorubicin nanoparticle powder (Inh NPs), doxorubicin-loaded nanoparticles IV (Dox NP IV), and doxorubicin solution (Dox Sol IV) or non-treated control group (No-treatment).

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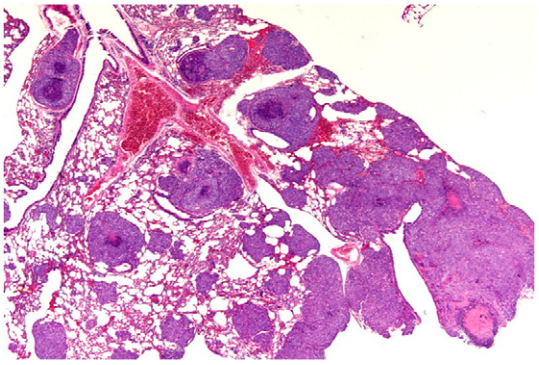

Fig. 2. Lung section of mouse from the non-treatment group. (20× magnification hematoxylin and eosin staining). Discrete tumor nodules are easily observed in the lung parenchyma.

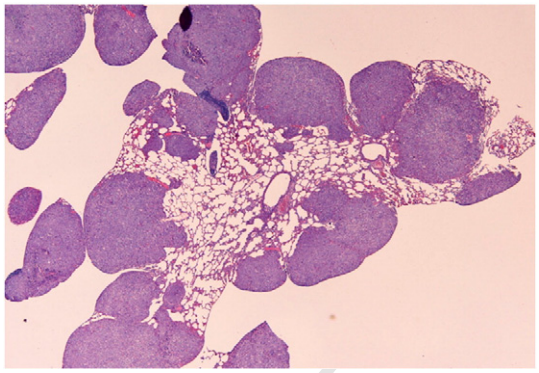

Fig. 4. Lung section of a mouse treated with doxorubicin solution intravenously. Bulky tumor nodules are easily observed. (20× magnification, hematoxylin and eosin staining).

±0.22 μm, respectively ($n=6$). The geometric standard deviations (GSD) were 1.99 and 1.91, respectively.

The animal survival data are shown in Fig. 1. All mice in the no-treatment control group (group iii) died or were euthanized during the first 45 days due to reaching the predetermined morbidity scores for euthanizing. The no-treatment control group showed significant weight loss after 3 to 4 weeks. The sacrificed or deceased animals had massive tumors in their lungs which showed metastasis to the liver, heart, and bone. For evaluating the effect of blank NPs (NPs without doxorubicin), a group (vii) of animals was treated with 1 mg blank nanoparticle inhalable powder. In this group nearly 90% of the mice died or were sacrificed within 50 days. The animals in this group also showed significant weight loss after 3 to 4 weeks. Similar to the no-treatment group, massive tumor burden was found in lungs, liver, heart, and bone.

Fifty percent of the mice treated with 30 μg doxorubicin solution as intravenous injection (group iv) died or were sacrificed within 40 days and all died or were sacrificed in less than 50 days. This group showed body weight loss after 28 days.

The positive control group injected intravenously with a suspension of NPs containing 30 μg doxorubicin (group v) survived comparably to animals injected intravenously with doxorubicin solution (group iv); all the animals died or were sacrificed in less than 50 days.

Groups of mice treated via inhalation with 1 mg nanoparticle effervescent powder containing 30 μg doxorubicin (group i) or 1 mg non-effervescent powder containing 30 μg doxorubicin (group ii) showed different results. The animals treated with inhalable non-effervescent nanoparticle powder containing 30 μg doxorubicin showed longer survival times compared to the no-treatment groups and the groups injected intravenously with doxorubicin as solution or NPs suspension (groups iv and v) (p values compared to either group were smaller than 0.0001). Fifty percent of the animals treated with inhalable non-effervescent NPs containing 30 μg doxorubicin (group ii) survived over 70 days and 30% of the mice survived until the termination of the study at day 140. The animals treated with inhalable effervescent nanoparticle powder containing 30 μg doxorubicin (group i) showed a significantly longer survival time compared to all other groups ($p<0.05$). Seventy percent of the animals in this group survived 140 days.

During the postmortem examination, three animals were randomly selected from each group and lungs, liver, and heart were removed for a pathology study. One representative picture is shown from each treatment group. Fig. 2 shows a section of a mouse lung representative of the no-treatment group (group 3). The sections of lung revealed multiple large and often confluent tumor masses in the parenchyma. There were also numerous smaller metastases throughout the alveolar septa. The central core of some of the larger masses was necrotic. The tumor cells were undifferentiated and mitoses were common.

Fig. 3 shows the heart of the animal of Fig. 2, showing tumors in the ventricular lumen. In the heart, there were large masses of tumor cells growing within and occupying most of the lumen of the right atrium and half of the lumen of the right ventricle. In addition to the primary metastases seen grossly in the lung, there were numerous secondary metastases also present.

Fig. 4 shows a representative lung section of an animal injected intravenously with doxorubicin. This animal showed multiple pulmonary tumor metastases. The tumor masses in the lungs were of the same size as those in non-treated mice but they were not as numerous. Microscopic examination confirmed the presence of multiple pulmonary tumor metastases.

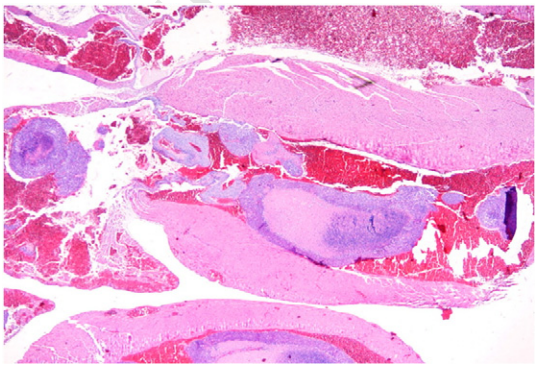

Fig. 3. Heart section of the animal in Fig. 2 showing growth of tumor in the ventricular lumen. (20× magnification hematoxylin and eosin staining).

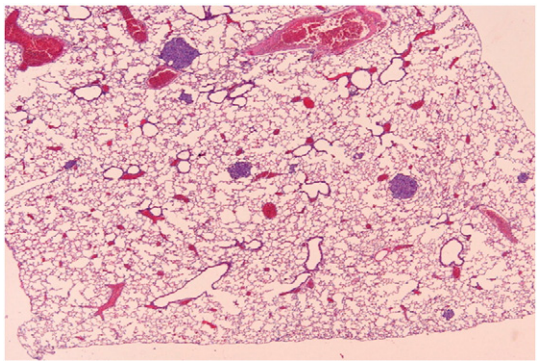

Fig. 5. Lung section of a mouse treated with non-effervescent doxorubicin nanoparticle powder. Small tumor nodules are observed. (20× magnification, hematoxylin and eosin staining).

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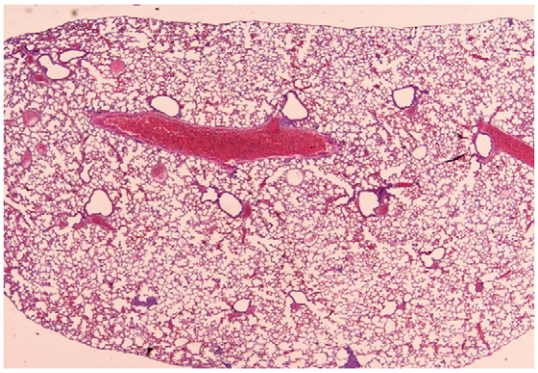

Fig. 6. Lung section of mouse treated with effervescent doxorubicin nanoparticle powder. (20× magnification, hematoxylin and eosin staining).

Fig. 5 shows a representative histopathology of a lung belonging to a mouse treated via inhalation with non-effervescent doxorubicin nanoparticle powder (group ii). This mouse showed multiple pulmonary tumor metastases. The metastases in the lungs of this animal were fewer in number compared to non-treated animals and mice injected intravenously with a doxorubicin solution (group iv). A small number of fairly uniform individual metastases were randomly located in the lungs.

Fig. 6 shows a representative histopathology of the lung of a mouse treated via inhalation with effervescent doxorubicin nanoparticle powder (group i). Metastases in the lungs were few and small. The lungs of this animal had only a few small metastases present.

The MRI images demonstrated an excellent soft tissue contrast which enabled the localization of intra-thoracic lung cancer and any extra cancerous manifestations. Fig. 7a is a T1 weighted, transverse spin echo scan of an untreated mouse 4 weeks after cancer inoculation. Tumor masses showed intense signals relative to adjacent tissues such as normal lung, and tumor boundaries were well delineated. Fig. 7a shows big tumor masses (arrow 1) with the involvement of the visceral and mediastinal pleura (arrow 2), chest wall (arrow 3), parietal pericardium, and the heart (arrow 4). The sections of lung isolated from the same animal revealed multiple large and often confluent tumor masses in the parenchyma with numerous metastases throughout the alveolar septa; the tumor cells are undifferentiated and mitoses are common. In the heart, there were large masses of tumor cells growing within and occupying most of the lumen of the right atrium and half of the lumen of the right ventricle (Figs. 2 and 3). Fig. 7b and c shows MRI scans of mice treated via inhalation with 1 mg of effervescent doxorubicin nanoparticle powder (group i) and a control mouse (not injected with cancer cells), respectively. The MRI images of the mouse treated with inhalable effervescent doxorubicin nanoparticle powder (group i) showed no signs of intra-thoracic lung cancer. The MRI results were consistent in each group and one representative image was chosen.

The animal study results showed a good correlation between MRI and histology data, as all tumor manifestations detected in MRI were confirmed later by histology.

To investigate the cardiac toxicity, three enzymes, AST, ALT and CPK were measured in blood samples collected 1 h after insufflation of inhalable powder. Table 1 shows the concentration of AST, ALT and CPK in treatment and control groups. The results show a significant increase in all enzyme concentrations where animals were treated with inhalable doxorubicin powder compared to inhalable effervescent doxorubicin-loaded NPs or non-treated. No significance difference in the enzyme levels were observed between animals treated with inhalable doxorubicin NPs and non-treated mice.

Table 1 t1.1
Concentration of AST, ALT and CPK in control mice and mice treated with either 1 mg nanoparticle powder containing 30 μg doxorubicin or 1 mg doxorubicin powder containing 30 μg doxorubicin, all values are expressed in IU/L.

	Control	1 mg Dox inhalable effervescent NPs	1 mg Dox inhalable powder
AST	141 ± 22	160 ± 27	1356 ± 249
ALT	67 ± 3.9	70 ± 12	767 ± 156
CPK	524 ± 56	600 ± 71	4359 ± 1663

4. Discussion

The present study investigated a new concept for the treatment of lung cancer using doxorubicin-loaded NPs incorporated into micrometer-sized carrier particles for local delivery to the lungs. Aerosol

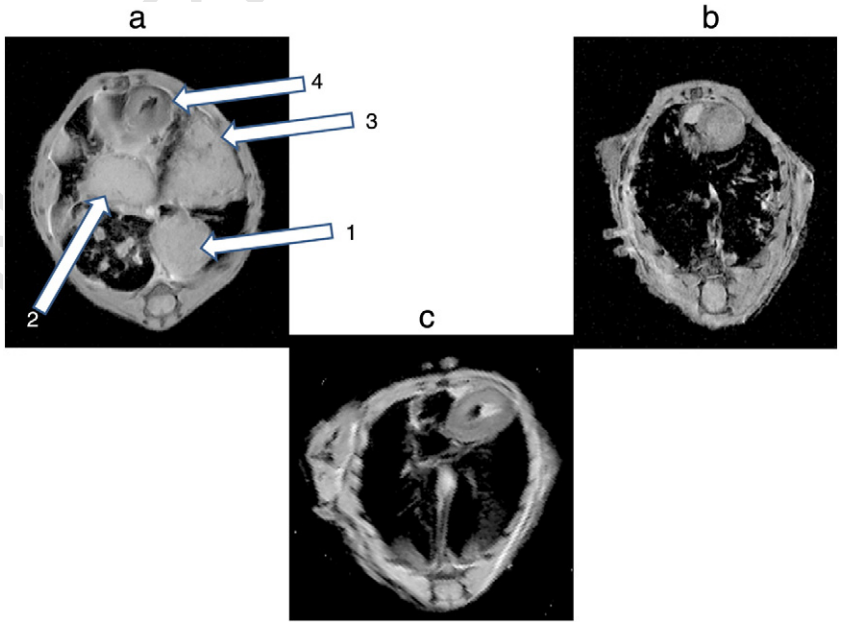

Fig. 7. T1 weighted, transverse spin echo scans (TE/TR = 13/1500 ms, BW = 50 KHz, the FOV = 26*26 mm with 128 × 128 matrix, thickness = 0.5 mm) of; (a) control non-treated mouse, (b) mouse treated with effervescent doxorubicin nanoparticle powder, and (c) control mouse (not injected with cancer cells).

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powders with particle diameters ranging from approximately 1 to 5 μm are considered the optimum size for deposition in the lung [27]. In our previous studies we showed that NPs can be incorporated into carrier particles to make inhalable particles for alveolar deposition [21]. Spray–freeze drying rather than conventional spray drying or freeze drying was used to prepare the carrier particles with a desired MMAD. Using spray–freeze drying, the drug is not exposed to high temperatures as it may be with spray drying, and the NPs are naturally prepared with a uniform distribution throughout the carrier particle rather than accumulating at the evaporation front as may occur in spray drying [15]. The active release mechanism used in the effervescent formulations disperses the NPs and prevents agglomeration when the carrier matrix dissolves and releases the NPs as previously reported [13].

An *in vivo* pilot study showed that Balb/c nude mice tolerate inhalable blank NPs powders [14]. These animals did not show any significant body weight loss or increased morbidity score over eight weeks. To test different formulations *in vivo*, animals were implanted with H460 lung cancer cells through the tail vein. This method was been successfully used in the past to evaluate drug treatments in mice [28].

In this study tumor bearing mice were treated with two different types of inhalable drug loaded nanoparticle powders, effervescent and non-effervescent. The animals treated with effervescent nanoparticle carrier showed longer survival times than animals treated with non-effervescent nanoparticle carrier. The active release mechanism resulted from the effervescent reaction of the carrier powder proved to prevent nanoparticle agglomeration and enhance the dispersion of NPs over the non-effervescent powder as shown *in vitro* [13].

To compare the effects of the route of administration, an equivalent drug dose of 30 μg doxorubicin was injected intravenously. The animals in this group did not show any improved survival time compared to the no-treatment control group. The same dose of doxorubicin was injected in the form of a nanoparticle suspension, however this group of animals also did not show any better survival time compared to the control group. This result might be due to a high accumulation of the NPs in the liver as shown by other studies after intravenous injection [10,29–31]. The same dose of inhalable free doxorubicin in lactose powder was associated with high cardiac toxicity indicated by the huge increase in the AST, ALT and CPK.

The present study showed that the route of administration along with the delivery system have a crucial impact on the treatment outcome. All animals were treated with the same amount of doxorubicin but using different formulations and different routes of administration. There were significant differences in the survival times among groups. Local delivery of the drug via inhalation is expected to place the doxorubicin NPs in the vicinity of lung cancer cells with less systemic exposure resulting in less toxic side effects. The loading of doxorubicin into NPs significantly reduced the cardiotoxicity due to the lower concentration of free drug. The enhanced tolerability of doxorubicin-loaded inhalable NPs over the inhalable free doxorubicin made the pulmonary delivery of this anticancer agent a viable option and significantly increased the therapeutic effect of the treatment.

It was expected that the inhalable nanoparticle powder would decrease the tumor mass and tumor number in the lungs due to localized delivery. It was not necessarily expected that the mice would have an increased survival time. Animals that died in the inhalable nanoparticle treatment groups died of huge tumor burdens in other parts of their body but not because of high tumor burden in the lungs. This study showed that inhalable nanoparticle powders significantly increased the survival time of the mice compared to all control groups. After deposition and dispersion in the lungs, doxorubicin NPs may migrate to other organs through the lymphatic system or by translocation from the alveolar space to the blood. NPs could then passively accumulate in tumors located outside the lungs. Further studies are ongoing to establish this mechanism. The lungs, as a part of the MPS, are extensively rich in alveolar macrophages [32]. Therefore, macrophages are expected to have a substantial effect on the fate of inhaled NPs and the efficacy of any treatment using inhalable NPs as a delivery system [22,33]. In general, a portion of the inhaled NPs evades macrophages and translocates out of the alveolar spaces to other lung tissues and to the general circulation [34]. Another fraction of the inhaled NPs will be cleared by the alveolar macrophages, similar to other foreign objects brought into the lungs during breathing [35]. Consequently, the effectiveness of any anticancer therapy using inhalable NPs will be affected by the extent to which inhaled NPs are cleared by macrophages. The presence of alveolar macrophages limits the chance of NPs to reach cancer cells and decreases their efficacy as anticancer agents.

Evading macrophages by using hydrophilic surfactants, such as polysorbate 80 to coat the NPs surface is not a viable option in the pulmonary delivery of NPs as polysorbate-80-coated inhalable NPs were associated the pulmonary toxicity and decrease the tolerance *in vitro* and *in vivo* [36].

Another strategy to evade macrophage was using smaller NPs as they are associated with less macrophage uptake and enhanced accumulation at tumor sites [37–40].

The fact that effervescent carrier powder has an active release mechanism that prevents NPs agglomeration and enhances NPs dispersion after inhalation might contribute to the difference observed between effervescent and non-effervescent carrier powders. NPs released from effervescent carrier powder after pulmonary delivery are smaller in size than the ones released from the non-effervescent carrier powders [13].

The present study shows that inhalable drug loaded NPs are effective in treating lung cancer and may have potential to be used in cancer treatment of organs other than the lung.

5. Conclusion

The present study demonstrated a therapeutic effect on the lungs of tumor bearing mice of inhaled doxorubicin-loaded NPs. Inhalable doxorubicin nanoparticle powders increased the survival time of cancer bearing mice compared to intravenous administration of the same drug dose. The inhalable nanoparticle preparation showed less cardiotoxicity than free doxorubicin. This might be used clinically to increase the total doxorubicin dose. In addition, an effervescent carrier was more effective compared to a non-effervescent formulation. This study opens a new frontier in the treatment of lung cancer using a non-invasive pulmonary route of administration. Effective drug delivery is essential in achieving improved therapeutic outcomes for lung cancer.

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