Efficacy of a Magnetic Drug Delivery System and Development of an Orthotopic Lung Tumor Imaging Model

Amber McBride

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Efficacy of a Magnetic Drug Delivery System and Development of an Orthotopic Lung Tumor Imaging Model

by

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DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
Nanoscience and Microsystems Engineering

The University of New Mexico
Albuquerque, New Mexico

December 2014

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I dedicate this dissertation to my amazingly strong and supportive mother, Victoria Fisher. Growing up, she spent many a late night helping my brother and I with science fair projects, coaching me through oboe auditions, driving us to 4-H meetings, and striving to develop characteristics leading us to be hardworking, genuine people. She fostered our love of animals, gardening, and being outside. I often think about how all of this, unintentionally, molded my path towards science. She encouraged us to make the most of every opportunity that came our way, and impressed upon us the value of education. Most importantly, she has taught me that I am capable of doing nearly anything I put my energy toward.

Thanks, Mom, for always being my biggest supporter in life. If degrees could be conferred from one person to another, I would gladly give you this one. It is with deep gratitude that I dedicate this work to you.
ACKNOWLEDGEMENTS

Science is a collaborative effort for which I have many to thank.

First and foremost: Dr. Pavan Muttil, my Ph.D. advisor. Thanks, Pavan, for teaching me how to think more critically as a scientist, encouraging me to challenge assertions, for treating me as a valued colleague, and for the genuine care you have shown me as a graduate student. Socrates remarked that the bond between a teacher and a student lasts a lifetime, even once the two have parted company. I think this is especially true of a Ph.D. advisor and his first Ph.D. student. Here’s to collaborations in the future!

Thanks to my husband Ben who has supported this journey toward being a “perpetual” student.

Thanks to my dear lab mate, Dominique Price. The Ph.D. can be a long and lonely road, especially when writing the thesis. I am immensely thankful for your words of encouragement on days when the science didn’t come easily, and for your intellectual contribution to my project. I’m grateful to have gained a cherished friend and colleague.

To Dr. Pam Hall for setting an example of a woman in science who has been incredibly genuine with me. You are a rare gem among scientists, Pam. I also greatly appreciate Dr. Brad Elmore, my go-to guy for coffee, E.coli and random questions.

Thanks to Todd Thompson and Deb MacKenzie for all their intellectual and technical help and encouragement with the sodium iodide symporter project.

I would like to thank Dr. Deryl Troyer and Marla Pyle for mentoring me as an undergraduate student at Kansas State University. It can be very difficult to find an
investigator willing to incorporate an undergraduate student into a project. Undergraduate research made the conceptual biology come alive for me.

This study was funded in part by the Health Science Center Research Allocations Committee grant. Amber was generously supported by two pre-doctoral fellowships: the Integrating Nanotechnology and Cellular Biology with Neuroscience (INCBN) Integrative Graduate Education and Research Traineeship (IGERT) fellowship and the New Mexico Cancer Nanotechnology Training Center (CNTC) fellowship. Thanks to Linda Bugge, Dr. Janet Oliver, Ryan Tanner, and Dr. Abyaha Datye for their continual support and mentorship throughout my graduate career.

I was incredibly fortunate to be able to spend three months in 2013 conducting research in Munich, Germany at the Klinikum Rechts der Isar at the Technical University of Munich. Thank you to Dr. Christian Plank, Dr. Martina Anton, Dr. Carsten Rudolph, Dr. Olga Mykhaylyk, Dr. Yolanda Antiquia-Sanchez, and Dr. Ulrike (Riki) Schillinger.

A huge thanks to Monique Nysus in the Radiopharmacy imaging facility for helping me with the sodium iodide symporter *in vitro* and *in vivo* experiments. Also, to Dr. Jamie Hu of the UNM Animal Core Facility for helping me develop the orthotopically-induced xenograft tumor model.

I wish to thank the Graduate Resource Committee (GRC) on UNM main campus for their weeklong dissertation writing workshops (i.e. boot camps). All UNM grad students need to take advantage of this resource. These will lessen your misery, I promise!

Last, but certainly not least, a huge thank you and hug to Heather Armstrong. She is the woman that made the Nanoscience & Microsystems program successful. She has been
incredibly encouraging of the graduate student association, and pillar of strength for me professionally and personally. Many a graduate student owes her dearly. Thank you.

I write this dissertation to share with the graduate students that will follow in Pavan’s lab. May you stay focused, work hard, read constantly, publish early and often, and most of all – enjoy the opportunity that is graduation research education.
EFFICACY OF A MAGNETIC DRUG DELIVERY SYSTEM AND
DEVELOPMENT OF AN ORTHOTOPIC LUNG TUMOR IMAGING MODEL

by

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ABSTRACT

The aim of this work was the development of a novel drug delivery vehicle termed nano-in-microparticles (NIMs) to evaluate the magnetic-field dependent targeting of dry powder NIMs administered endotracheally using an ex vivo and in vivo rodent model. NIMs are a novel dry powder drug delivery vehicle containing 70% lactose (w/w), 20% SPIONs (w/w) and 10% fluorescent nanospheres (w/w). Mice were insufflated with NIMs delivery vehicle in the presence of magnetic field dependent targeting to the left ventral lung after thoracotomy; controls were insufflated with the NIMs in the absence of magnetic field dependent targeting. Quantification of deposition of the dye component (drug surrogate) of NIMS in left and right lung tissue showed an increase in fluorescence to the left lung, resulting in nearly a 3-fold increase in deposition of dye in NIMS when administered and targeted to the left lung. Quantification of iron in lung tissue showed significantly more in the left lung, corroborating deposition of the dye surrogate. The administration of an aerosol liquid suspension delivery vehicle in the presence and absence of magnetic field dependent targeting was also evaluated and did not show significance in magnetic field dependent targeting when compared to dry powder NIMs. Toxicity study results indicate that doxorubicin-containing NIMs showed more toxicity than the free doxorubicin control, and that a dose dependent increase in toxicity is observed over time in lung tumor cells exposed to doxorubicin-containing-NIMS. We conclude that 1) the NIMs vehicle can be targeted significantly better as a pulmonary drug delivery agent to specific regions of the lung than liquid suspension and 2) Doxorubicin containing NIMs are more toxic to A549 lung adenocarcinoma cells as free drug, making NIMs ideal for a targetable inhaled drug delivery vehicle.
Concomitantly, we developed a lung cancer animal-imaging model by subcutaneous xenograft and orthotopic lung implantation of NSCLC adenocarcinoma cells (A549) that were genetically modified with the human sodium iodide symporter gene (hNIS; SLC5A5) for the purpose of tumor imaging. Lung tumor growth was quantified using single-photon emission computer tomography (SPECT) and computed tomography (CT) imaging. hNIS is a membrane glycoprotein that normally transports iodide in the thyroid, and has the ability to symport the radiotracer $^{99m}$TcO$_4^-$. A549 cells were genetically modified with plasmid and lentiviral vector constructs to stably express hNIS. Modified tumor cells were then implanted into nude mice to develop two tumor models: the subcutaneous xenograft model and a xenograft orthotopic tumor model. Tumor progression was longitudinally imaged by SPECT/CT, and quantified by SPECT voxel analysis. hNIS expression in lung tumors was quantified using quantitative PCR (qPCR). In addition, hematoxylin and eosin staining and visual inspection of pulmonary tumors were also performed. Orthotopic tumors induced by the A549-hNIS-lentiviral vector cell line exhibited a logarithmic increase in SPECT scintillation over orthotopic tumors induced by the A549 hNIS-plasmid vector cell line and A549 unmodified control cells in vitro and in the tumor bearing mice models. Furthermore, orthotopic implantation of tumor cells, injected between the 5$^{th}$ and 6$^{th}$ intercostal rib space of the left lung achieved a 100% orthotopic tumor take rate showing the replicative precision of this model. We developed a highly sensitive spatial and temporal lung tumor-imaging model that could ultimately be used to test the therapeutic efficacy of novel inhaled chemotherapeutic agents.
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2014 Manuscript to be submitted to the Journal of Nuclear Medicine,
Impact Factor: 5.774, titled "A Longitudinal, Orthotopic Mouse Model Using
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Chapter 1: Introduction and Background

Lung cancer: a 20th and 21st century problem

Lung cancer is responsible for 27% of total cancer deaths in the United States and is the leading cause of death of all cancers diagnosed in 2014 (Siegel et al., 2014; American Cancer Society, 2014). Among men, lung and bronchus cancer is the second leading cancer site, accounting for 14% of total new cancer cases diagnosed and 28% of the total cancer deaths (American Cancer Society, 2014) (Fig. 1A). Similarly among women, lung and bronchus cancer is the second leading cancer site, accounting for 14% of total new cases diagnosed and 26% of the total cancer deaths (American Cancer Society, 2014) (Fig. 1A). The American Cancer Society estimates 224,210 new cases of lung cancer in the United States (2014; 116,000 in men and 108,210 in women) (Fig. 1 B, C). In January 2014, the Morbidity and Mortality Report from The Centers for Disease Control published that lung cancer incidence rates have dropped 2.6% per year among men, from 87 cases to 78 cases per 100,000 men from 2005 - 2009. In women, the rate decreased 1.1% per year, from 57 to 54 cases per 100,000 women. The fastest drop was among adults aged 35 – 44 years and is attributed to a decline in the number of young adults smoking in the United States.

While active smoking accounts for 85 - 90% of cases attributed to cause, 10 – 15% of all lung cancers arise in those who have never smoked (Samet et al. 2009; Thun et al. 2006; Siegel et al. 2014). Interestingly, lung cancer is currently rising among those non-smoking (Locher et al., 2013). The leading cause of lung cancer in nonsmokers according to the US Environmental Protection Agency (EPA) is exposure to radon gas.
Figure 1: Trends in incidence and mortality rates. Graphs depict trends in incidence and mortality from all cancers diagnosed from 1975 – 2010. Data from the American Cancer Association and the Surveillance, Epidemiology, and End Results (SEER) Program, National Cancer Institute 2013, accessed July 16, 2014.
Radon exposure accounts for approximately 20,000 deaths, or 10% of lung cancer cause (Krewski et al., 2005). Another 9 - 15% of lung cancer cases are caused by occupational carcinogen exposure (Field and Withers, 2012), including asbestos (Currie et al., uranium (Furlow 2014) and iron (Wild, Bourgkard, and Paris 2009) from smelters, blast furnaces and foundries. Air pollution causes another 1-2% of lung cancer (Cesaroni et al., 2013; Raaschou-Nielsen et al., 2011). Finally, a small portion (≤1%) of lung cancer occurs in people with no known risk factors for the disease. Lung cancers in non-smokers often have certain gene differences in tumors that are different from those from smokers, such as the TP53 (Mogi and Kuwano, 2011) or p16 (Andujar et al., 2010) tumor suppressor genes and the K-RAS oncogene (D'Arcangelo and Cappuzzo, 2012) in NSCLC.

The five-year survival rate for lung cancer is 53.5% for cases detected when the disease is localized within the lungs (Siegel et al., 2014). However, only 15% of lung cancer cases are diagnosed at an early stage. For lung tumors that have spread to other organs, the five-year survival rate is a mere 3.9%. Of all patients diagnosed with NSCLC, less than 10% of patients survive more than 5 years (Mazzone and Mekhail, 2012). Thus, efforts to detect and treat lung cancer have severe limitations and have failed to ameliorate lung cancer survival rates despite emerging therapeutics and delivery systems.

There are 2 major types of lung cancer: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 85-90% of lung cancers are NSCLC (Syrigos, Nutting, and Roussos, 2006). There are 3 main subtypes of NSCLC: 1) squamous cell carcinoma, 2) large cell (undifferentiated) carcinoma, and 3) adenocarcinoma. For this research, I have focused on lung adenocarcinoma because it is the most prevalent form of NSCLC, accounting for about 85% of all lung cancers.
(Society 2014). Adenocarcinoma is the most applicable cancer for our proposed mode of therapeutic drug delivery because it is confined to the lung, found in the outer pleural cavity, and chemotherapy can reach affected tumor areas when administered by the pulmonary route.

**Current therapies for NSCLC**

Treatment for NSCLC depends on the stage of the cancer. Surgery and resection of the tumor and affected areas is a common treatment for NSCLC that has not spread to nearby lymph nodes. Resection is invasive and often causes impaired lung function post-surgery. Radiation therapy can also be used if surgery is not possible, alone or in combination with chemotherapy. Platinum-based chemotherapy doublet therapy has showed modest improvement in patient outcome when compared with the best supportive care (Schiller et al., 2002). Doublet chemotherapy has become the standard treatment modality and is the cornerstone treatment for lung cancer. In metastatic disease, treatment is based on the combination of cisplatin or carboplatin with drugs such as paclitaxel, docetaxel, gemcitabine and vinorelbine (Azzoli et al., 2010; Goffin et al. 2010).

Over the last two decades, the development of oncogene-directed targeted therapies have significantly changed the treatment of NSCLC and quickly becoming a first-line therapy. The first available targeted therapies for advanced NSCLC were gefitinib (Kris et al. 2003; Cappuzzo et al., 2005) and erlotinib (Wang, Schmid-Bindert, and Zhou 2012), both of which are small-molecule tyrosine kinase inhibitors (TKI) against epidermal growth factors (EGFR) also known as HER 1 or ErbB-1. EGFR in lung cancer is commonly overexpressed in NSCLC (Jänne and Engelman 2005; Sridhar et al., 2003; Yang et al., 2014) and has been a motivational factor in the development of EGFR
TKIs. Phase III clinical trials have demonstrated their antitumor activity for both getfitinib and erlotinib (Shepherd and Pereira 2005; Thatcher et al. 2005). Both therapies have been incorporated into treatment for patients after standard first-line chemotherapy.

**Limitations to current therapies for NSCLC**

Although targeted and tailored therapies are currently being used for NSCLC, overall lung cancer survival rates have failed to improve for numerous reasons (Subramanian, 2011). First, conventional lung cancer therapies are systemically administered. They affect rapidly dividing cancerous cells as well as healthy cells. A major obstacle to lung cancer treatment is the inability to selectively target and deliver chemotherapeutics to cancerous cells. Second, effective drug concentrations in solid tumors is a key parameter for successful treatment. Conventional lung therapies fail to reach high concentrations at the target tumor site. Instead, drug is administered systemically instead of regionally and high drug concentrations becomes toxic to vital organs (Schiller et al. 2002; Minchinton and Tannock 2006). Third, multidrug resistance (MDR) is a well-characterized broad pattern of cross-resistance to unrelated drugs after exposure to a single drug. Interestingly, this pattern has been observed in *in vitro* culture models and in the clinic. Drug resistance is the main cause of the failure of chemotherapy in malignant tumors and is usually diagnosed during treatment after a long period of drug administration. The MDR phenotype can occur as a result of cellular adaptations including reduced drug uptake, increased drug efflux, alterations in intracellular drug distribution or inadequate induction of apoptosis (Wangari-Talbot, 2013).
The Pulmonary Drug Delivery Approach

The development of inhalational agents for oncological use has been limited (Sharma et al. 2001) in the clinical setting. There is a large amount of published data regarding aerosol delivery of chemotherapy in preclinical studies. Feasibility of aerosol delivery has been shown in cancer cell cultures (Azarmi et al. 2008; Tseng et al. 2009; Koshkina, Yang, and Kleinerman 2013), animal models (Roa et al. 2011; Hershey et al. 1999; Dames et al. 2007; Hasenpusch et al. 2012; Gagnadoux et al. 2008; Rodriguez and Crabbs 2010; Yi and Wiedmann 2010), and Phase I/II human trials (Otterson et al. 2007; Otterson et al. 2010; Tatsumura, Yamamoto, and Murakami 1983; Tatsumura et al. 1993; Zarogoulidis et al. 2013; Lemarie et al. 2011). Most of these publications focus on the use of nebulized drug delivery vehicles instead of dry powder delivery vehicles.

The chemotherapeutic agent, 5-fluorouracil (5-FU), was investigated in 1968 for inhalational therapy. Tatsumura et al. (Tatsumura, Yamamoto, and Murakami 1983) published research that treated patients with inhaled 5-FU preceded by surgery. They found higher drug concentrations in the tumors than in the surrounding tissue. In a later study, Tatsumura et al. (Tatsumura et al. 1993) found high 5-FU concentrations in the main bronchus and adjacent lymph nodes for nearly 4 hours post administration. Formulation of 5-FU with lipid-coated nanoparticles showed sustained drug release and enhanced anticancer properties (Hitzman and Elmquist 2006). Otterson et al (Otterson et al. 2007; Otterson et al. 2010) administered inhaled doxorubicin in a Phase I and later in a Phase I/II clinical study. They demonstrated the adverse effects of aerosol treatment such as metallic taste, mild bronchospasm, moderate reduction of pulmonary function, and pulmonary dose-limiting toxicity. However, none of these studies used a targeting
mechanism of any sort. Azarmi et al. formulated poly (butylcyanocrylate) nanoparticles with doxorubicin for inhaled, targeted drug delivery to the lung via encapsulation in lactose carrier particles made by spray-freeze drying. He showed successful *in vitro* uptake into H460 and A549 lung cancer lines and targeting of their drug delivery vehicle through endocytosis rather than passive diffusion.

**Advantages of the Pulmonary Drug Delivery Approach**

The direct drug administration of chemotherapeutics and adjuvants to the respiratory tract by inhalation offers many theoretical advantages over a traditional drug delivery system (Labiris and Dolovich 2003). 1) Drugs can be delivered regionally instead of systemically, directly to the disease site. 2) Regional delivery allows for a lower drug dose to be used resulting in 3) fewer systemic side effects 4) allowing for the bypass of barriers to therapeutic efficacy such as poor gastrointestinal absorption and first-pass metabolism of the drug in the liver. 4) The use of a non-invasive “needle-free” delivery system. 5) Inhalable carriers allow for a wide range of substances to be delivered, from small molecules to very large proteins (Wolff 1998). 6) The lung has an enormous absorptive surface area (100 m²) and a highly permeable membrane (0.2 – 0.7 µm thickness) in the alveolar region (Patton and Byron 2007). 7) Large molecules with low absorption rates can be absorbed in significant quantities due to the slow mucociliary clearance in the lung periphery resulting in prolonged residency in the lung (Agnew, Pavia, and Clarke 1981).

In addition, formulations of inhaled drug delivery vehicles as dry powders have many advantages as well. 1) Dry powders have greater chemical and physical stability compared to dispersions for nebulization thus increasing their shelf-life. A dry powder is
more stable than a nebulized suspension and can reach the tumor site without releasing its payload. The nebulized suspension can prematurely degrade or agglomerate, whereas we have shown that we can overcome this limitation with a dry powder. 2) Medication as an inhaled dry powder can be given as a single dose in one breath. This reduces treatment time and improves patient compliance. 2) Dry powder formulations may not require cold-chain storage.

**Limitations of the Pulmonary Drug Delivery Approach**

A number of acute and latent effects observed in a small number of human trial studies are still under investigation for inhaled chemotherapy administration. 1) Pulmonary dose limiting dose toxicity as shown by Otterson et al. (Otterson et al. 2007; Otterson et al. 2010) 2) Cases where studies have failed to demonstrate the long-term effects of the chemotherapeutic drug formulations in the lung parenchyma (Darwiche et al. 2013).

Transportation problems stemming from improperly functioning conducting airways may lead to impaired respiration and particle transport. 1) Patients with compromised lung function have impaired respiration through bronchoconstriction, which leads to inefficient drug delivery to the lung or even impaired pulmonary absorption ability. 2) Damage to the ciliated epithelium may also limit the mucociliary clearance of particles impacted in the upper airway, especially in the case of dry powders. 3) Respiratory flow rate can also affect the dose delivered to the patient, as well as dose reproducibility.
Physiological Factors Affecting Inhaled Therapeutic Effectiveness

The extent of particle deposition matters largely on the physiological factors of the patient. The method of inhalation by the patient influences the outcome of particle deposition in terms of location and degree. The airflow pattern and structure of the conducting airways will determine the particle deposition in the throat, how much is swallowed, and in turn, the dose available for lung deposition (Borgström, Olsson, and Thorsson 2006).

Diseases such as asthma or chronic pulmonary obstructive diseases can obstruct the airway. An obstructed throat causes recirculation of air in both directions, which greatly alters flow characteristics (Luo, Liu, and Yang 2007). Patients that suffer from asthma have constricted or narrowing of the pharyngeal airway. This results in increased oropharyngeal deposition of an aerosolized dose due to clearance impaction, reducing the total dose delivered to the lungs (Svartengren et al. 1996).

Use of Dry Powders to Generate a Pulmonary Immune Response

Pulmonary delivery of vaccines has gained major attention for achieving both mucosal and systemic immunity (Sou et al. 2011). Delivery of macromolecules in the form of dry powder aerosols to the pulmonary system has been widely suggested as a promising non-parenteral delivery route that provides improved stability when compared with conventional liquid formulations (Weers, Tarara, and Clark 2007) (Schüle et al. 2008). An optimum formulation containing NPs as dry powder nanoparticles can result in
greater stability, ambient storage conditions, and a better immune response compared to traditional liquid-based vaccines (Kunda et al. 2012).

Muttil et al. prepared PLGA NPs entrapping diphtheria CRM-197 antigen (size 200 ± 50 nm) by the emulsification solvent diffusion and double-emulsion methods. The NPs were then spray-dried with L-leucine and the resulting spray-dried powders were delivered in guinea pig lungs to evaluate the immune response following pulmonary and parenteral immunizations with the dry powders. The highest titer of serum IgG antibody was observed in guinea pigs immunized by the intramuscular route whereas high IgA titers were observed for dry powder formulations administered by the pulmonary route. This demonstrates that pulmonary immunization with dry powder vaccines leads to a high mucosal immune response in the respiratory tract and sufficient neutralizing antibodies in the systemic circulation to provide protection against diphtheria (Muttil et al. 2010). Particle size and surface charge of the material to be delivered plays an important role in determining the uptake by human dendritic cells (DCs) derived from blood (Foged et al. 2005). The excipients dissolve upon encountering the respiratory environment and thereby release the nanoparticles upon phagocytosis by antigen presenting cells such as dendritic cells, NPs release the antigen intracellularly and elicit CD8+ and CD4+ T cell responses (Doria-Rose and Haigwood, 2003). Furthermore, the optimal uptake by DCs occurs when the particle size is ~ 0.5 µm. Uptake of large particles (~ 1 µm) was greatly enhanced when they displayed a positive surface charge (Kunda et al. 2012) (Foged et al. 2005).

**Micro- and Nanoparticles as Drug Delivery Vehicles**

Spherical delivery vehicles are often called particles, capsules, or carriers and can
range in size from several nanometers to tens of micrometers. Microparticles for inhaled drug delivery are optimally in the size of 1 – 3 µm to achieve alveolar deposition (Gerrity et al. 1979).

Depending on the target delivery region, particles should exhibit desirable particle size distribution ideally with aerodynamic diameters \(d_a\) of 5-10 µm for airways and 1-5 µm for deep lung delivery with a standard density of 1 g cm\(^{-1}\). Particles less than 0.5 µm (NPs) are driven by diffusion and are most likely to be exhaled, hence they are therefore often encapsulated and delivered within dry powder microparticles.

**Brief History of Magnetic Particle Targeting**

Magnetic iron oxide has been used to impart a magnetic moment to particles in the field of biotechnology since the 1970s (Kakar, Batra, and Singh 2013). Kakar provides a very thorough review of the insights that magnetic particle systems have provided for biomedical application. Gilchrist et al. published a paper in 1956 on the selective inductive heating of lymph nodes after injection of 20 – 100 nm sized magnetite particles into the lymph nodes near surgically removed cancer (Gilchrist et al., 1957). This was a seminal paper using hyperthermia to thermally ablate tumor cells. Meyers et al. described how magnetic carriers were able to accumulate small iron particles when intravenously injected into the leg veins of dogs using a large, externally applied horse shoe magnet (Meyers, Cronic, and Nice, 1963).

Defined spherical magnetic microspheres containing albumin were made for the first time in the 1970’s for tumor therapy and as magnetic resonance contrast agents (Widder, Senyei, and Ranney 1979). Many attempts have been made to develop magnetic
drug targeting methods but none have progressed to clinical trials (A. K. Gupta and Gupta 2005; Häfeli 2004; Babincova and Babinec 2009). An example of a magnetic targeted carrier drug platform was the FDA-approved was FeRx, Inc (San Diego, CA) which contained up to 80% of metallic iron with the remaining comprised of activated carbon (Rudge et al. 2000). The systemic application of magnetic nanoparticles proved difficult because of their rapid clearance from the blood via the RES, but improvements in functionalization using polyethylene glycols (PEG) has overcome this limitation.

**Superparamagnetic Iron Oxide Nanoparticles (SPIIONs)**

Superparamagnetism is a phenomenon where magnetic materials exhibit a behavior similar to paramagnetism at temperatures below the Curie or the Neel temperature (Sadhukha, Wiedmann, and Panyam 2013). SPIONs are small synthetic Fe$_3$O$_4$ particles with core sizes ranging between 5 nm and 99 nm in diameter. SPIONs consist of a single (individual) magnetic domain of elements that can be aligned in the presence of an external magnetic field allowing for magnetic-field-dependent targeting, hyperthermia induced cancer-cell killing or MRI resonance imaging. SPIONs have unique physical and chemical features such as a large surface area to mass ratio, which make them ideal for ligand attachment binding or for the delivery of cargo payload.

**Formulation of Inhalable Dry Powders**

The process of spray drying is used to create fine dry powders for pulmonary delivery. The spray drying technique has been well recognized since the 1990s (Chan and Chew 2003). We use the B-290 mini-spray dryer in our lab, manufactured by the company Buchi. Spray drying is a pharmaceutical manufacturing process used to produce respirable particles in the solid state.
Figure 2A shows a schematic of the B-290 and Figure 2B details the mechanics of the spray drying process. First, the inlet air of the spray dryer is heated to average temperatures ranging between 50 °C to 185 °C, depending on the materials being spray dried, with a maximum of 220 °C (Figure 2B – circle 1). Next, using a two-fluid nozzle, droplets are formed. Conductive heat is exchanged between the drying nitrogen gas and the sample droplets in the drying chamber (Fig. 2B – circle 3). The dry powders are collected in the particle collection chamber attached to the cyclone (Fig. 2B – circle 4).

**Figure 2**: A) Schematic of the Buchi B-290 Spray dryer and B) step-wise mechanism of the spray drying process.

Compared to other methods of powder manufacturing like milling or lyophilization, spray drying yields dry powders with good aerodynamic properties (e.g. flow and
dispersability) and offers more control over their dry physicochemical properties because variables like atomization, drying dynamics and liquid properties can be fine tuned. Operation variables can be manipulated through interrelated parameters such as the inlet air temperature, the drying air flow rate, the liquid feeding rate, the pressure of atomizing air, as well as percent solids in the solution (Cal and Sollohub 2010).

Dry powders can be formulated with various excipients (co-polymers or sugars, e.g. manitol, trehalose, or maltodextrin) for controlled release (O'Hagan et al. 1991; O'Hagan et al., 2004). Spray drying excipients such as leucine or polymorphic forms of drugs results in corrugated particles that have highly and improved dispersion behavior (Hoppentocht, Hagedoorn, et al. 2014; Son et al. 2011; Son et al., 2012). Ester derivatives of trehalose can increase the lipophilicity, reducing the extent or rate of crystallization of spray dried powders, with a goal of sustained drug release (Davidson et al. 2003).

Factors Influencing Inhaled Microparticle Deposition

There are two primary modes of pulmonary aerosol administration: nasal and oral inhalation. Microparticle deposition in the respiratory tract is dependent on multiple factors:

1) **Particle size**: Particle size and its distribution are determined by the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD), respectively.

2) **Particle shape**: Particles that are long and fibrous particles, such as asbestos, are more likely to deposit and penetrate the alveoli because they orient parallel to air flow streamlines. A particle with a greater aspect ratio can potentially be
deposited deeper in the deep lung leading to a more efficacious therapeutic effect (Ally, Kappl, and Butt 2012).

3) **Particle density:** Particle density refers to the mass per unit volume of the particle itself. Liquid particles and earth-matter particles have a density equal to that of their parent material. Smoke and fume particles may have apparent densities significantly less than that of their chemical composition because of the large void spaces in their highly agglomerated structure. By inserting “pores” into particles by decreasing density (large porous particles), dispersion properties are improved by reducing van der Waals forces and is beneficial for pulmonary delivery (Crowder et al. 2002; Edwards et al., 1997).

4) **Particle airflow velocity:** The inhaled air follows through the nose or mouth – a torturous path of branching airways in the lung. Flow is cyclical and reverses many times a minute. At its peak, it may be turbulent in the trachea. As the Reynolds number decreases with increasing lung depth, airflow becomes increasingly laminar (Lippmann, Yeates, and Albert, 1980). Increasing air velocity increases impaction deposition, but decreases sedimentation and diffusion by decreasing residence time.

5) **Tidal volume:** The inhaled air at the start of each breath goes deeper into the lung and remains longer than air inhaled later in the breath. The deeper the air goes and the longer this air resides, the greater its depletion of inhaled particles.

6) **Inter-patient physiological variations:** Significant differences in respiratory tract anatomy can affect flow rate including the diameter of the airway. The diameter of the airway affects the particle as it contacts the airway surface. The surface of
the mucous layer defines the effective diameter of the conducting airways. A normal patient has a mucus layer that is ~5 µm thick; a patient with bronchitis has a much thicker mucus layer which causes increased small airway particle deposition by impaction and turbulent diffusion (Labiris et al., 2003).

7) **Inhaler design:** Differences in inhalers can lead to variable amounts of emitted dose to a patient, based on resistance from the inhaler.

**Mechanisms Governing Particle Deposition**

There are three generally accepted mechanisms by which particle deposition in the airway occurs:

1) *Inertial impaction* is the main deposition mechanism for particles > 3 µm, accumulated in the upper trachea bronchial regions. Inertial impaction occurs during passage through the oropharynx and large conducting airways if the particles possess a certain mass and velocity. Hyperventilation can also influence deposition by impaction.

2) *Sedimentation* by gravitational force occurs in the smaller bronchi, the bronchioles, and the alveolar spaces where the airways are small and have low air velocity. Sedimentation is influenced by breath holding, which increases residence time and therefore deposition (Li and Ahmadi, 1995). Sedimentation is less effective than diffusion when particles are < 0.5 µm (Lippmann, Yeates, and Albert 1980).

3) *Diffusion* impacts particles in the range of 0.5 – 1.0 µm and is based on Brownian motion (Lippmann, Yeates, and Albert 1980). Diffusion depends only on particle size and not on particle density or shape.
In most cases, the first three deposition mechanisms occur most frequently.

4) *Interception* occurs when the trajectory of a particle comes in contact with the bronchial bifurcation, hence is significant only for fibrous particles and must be a significant linear size and shape compared to the airway diameter because aerodynamic drag can affect orientation within the airway.

5) *Electrostatic mobility* can influence enhanced respiratory tract deposition.

   Particles carry a certain charge (e.g. freshly fractured mineral dust particles) and can induce charge on the surface of the airways. Most aerosols have small amounts of charge, however deposition due to electrostatic mobility is usually non-significant in comparison to the first three mechanisms discussed.

**Aerodynamics of Dry Powder Aerosols**

The distribution and the deposition sites of inhaled particles is strongly dependent on their aerodynamic diameters (Lippmann, Yeates, and Albert 1980). Airflow in the respiratory system is complex and has been understood mainly from experimental studies using physical models of lung airway. In the average adult, airflow is turbulent in the upper airways. Airflow becomes more laminar, as indicated by Reynold’s number, starting at generation 16 (as the generation number increases, airways become smaller).

**Administration of Dry Powders In Vivo**

Dry powders are delivered using a dry powder insufflator (DPI) (Penn-Century Inc.) attached to a commercially available syringe. Vaccine liquid droplets are administered using a Penn Century IA-1C Liquid Microsprayer Aerosolizer attached to a FMJ-250 High Pressure Syringe (Penn-Century Inc., Wyndmoor, USA) (Sakagami 2006;

Hoppentocht et al. (Hoppentocht, Hoste, et al. 2014) investigated the dispersion efficiency of dry powders with the DP-4M Insufflator™ by comparing particle sizing using RODOS dry powder sizing machine. He found many limitations of the DP-4M Insufflator™ such as variability in dispersion efficiency and in the dose delivered to the rodent. Two hundred µL of air, based on the Penn Century recommendation, was insufficient to adequately disperse dry powers in mouse lungs. We also observed this limitation and further discuss about the device in subsequent chapters.

A nose-only aerosol exposure chamber can be used to take advantage of a rodents’ natural breathing mechanism to inhale dry powders. A drawback of this aerosolization method is that aerosols can deposit in the nasopharynx cavity in the rodent, thereby becoming a subject of mucociliary clearance. This aerosolization device also requires the delivery of large volume of particles and a significant amount of powder can be wasted because of nasopharyngeal deposition.

**Forces Acting on Dry Powders**

All forces acting on dry powders were referenced from the book “Particulate Interactions in Dry Powder Formulations for Inhalation” (Zeng, Martin, and Marriott, 2000).

1. **Electrostatic Force (Coulombic)**: Electrostatic properties of formulation component materials play an important role in dry powder inhalation products. Fine powders exert charge on other particles and tend to be highly cohesive. Electrostatic charges can arise from the contact of an uncharged particle with
either a negatively or positively charged particle transferring electron and ions among particles. When two charged particles are brought together, they will experience attraction or repulsion depending on their electrical signs. The electrostatic charge distribution of the particles affects the efficiency of drug delivery by influencing both the transport and deposition of inhaled particles (Balachandran, Machowski, and Gaura 1997) (Beleca et al. 2010).

2. **Drag force**: Drag force is the predominant force acting on particles in the conducting airways. Drag force is due to static pressure gradients in the flow. (Dunber, Hickey, and Holzner 1998).

3. **Buoyancy force**: This force is caused by the Saffman lift force (a result of the velocity gradient of the air or fluid), the Magnus force (caused by particle contact and rebound from the conducting airway wall) and centrifugal force (affected by the mass and the diameter of the drug particle).

4. **Friction**: Forces of friction may play a role at the initiation of powder dispersion and to a lesser extent, during aerosol delivery (Podczeck 1997).

5. **Dispersion Forces (Van der Waals force)**: These forces are present in fine dry powders causing clumping behavior, an example of a cohesive force. The major force between uncharged particles is Van der Waals force.

**Particle Characterization**

1. **Size and size distribution**: The determination of aerodynamic diameter by measuring the mass median aerodynamic diameter (MMAD) is one of the most important characterization parameters in terms of particle deposition by impaction and sedimentation. MMAD and geometric standard deviation (GSD) are determined by
cascade impaction studies. Fine particle fraction (FPF) is also determined using the impactor, and is defined as the percentage of particles in the respirable size range of 1 – 5 µm (Telko and Hickey, 2005).

2. **Moisture content**: Hygroscopicity is the intrinsic tendency of a material to take on moisture from its surroundings (Telko and Hickey, 2005). Moisture uptake and loss can result in local dissolution and recrystallization, leading to irreversible aggregation through solid bridge formation, which can adversely affect aerosol generation and lung deposition (Braun, Oschmann, and Schmidt 1996). Moisture uptake can be quantified using a Karl Fischer (KF) colometric titration. Dynamic Vapor Sorption (DVS) can also be used to determine moisture uptake.

3. **Glass transition** ($T_g$): The crystalline or amorphous state of materials can be determined by differential scanning calorimetry (DSC).

4. **Drug release and dissolution properties**: Drug release profiles are critical for inhaled drugs in order to evaluate the pharmacokinetic profile and hence the bioavailability of the drug.

5. **Surface characteristics**: Electron microscopy (SEM/TEM) is used to determine corroborate particle size and determine physical surface characteristics morphology.

6. **X-ray diffraction**: XRD can be used to determine bond lengths and bond angles defined by intermolecular spacing, which is directly related to crystalline properties of the material (Brittain, 1995).

7. **Determination of metal content**: Inductively Coupled Plasma – Optical Emission Spectrometry can be used to determine amounts of metal in a drug delivery system, for example iron.
Brief History of the Subcutaneous Xenograft Animal Model

The most commonly used animal model is a tumor xenograft in immunodeficient mice. The term “xeno” has Greek origin meaning from a foreign place. In the case of lung cancer, a lung cancer cell or tissue is placed in a region other than the lung; usually in a flank or shoulder region. Xenografts initiated through the injection of tumor cells from cell culture or through transplantation of a small tumor mass.

Multiple types of tumor installation methodologies exist. Xenograft tumor models are most commonly used in immunodeficient mice (Olive et al, 2006). Xenograft models can be initiated through the subcutaneous or intravenous injection of tumor cells from cell culture or tissue transplantation. Xenograft transplants may be orthotopic, meaning that the tumor is placed in the site it would be expected to arise naturally in the host: human lung cancer cells placed in the mouse’s lung. Orthotopically implanted xenograft tumor models can be initiated through surgical transplantation of a small tumor mass or by injection of tumor cells from cell culture through intravenous injection or intratracheal injection.

An allograft mouse tumor system, also known as a syngeneic model, consists of cancerous cells or solid tumors that are transplanted into a host mouse, derived from the same genetic background as a given mouse strain (eMICE, NCI, nih.gov). Since the cancer tissue and the recipient mouse share ancestry, the transplant is not rejected by the host’s immune system. An advantage of a syngeneic model is that the host immune system is normal, which may more closely represent a translational tumor microenvironment. A disadvantage is that the transplanted mouse tissue will not fully represent a human tumor.
**Orthotopic Animal Model and Clinical Relevance**

In orthotopically-induced xenograft models human cells injected (or human tissue implanted) in the appropriate organ or tissue of origin. Orthotopic lung cancer models can be initiated multiple ways: using endobronchial, intrathoracic, or intravenous injection of tumor cell suspensions. McLemore developed the first orthotopic lung cancer model in 1987 by implanting either human lung cancer cells or fragments of tissue into the lung of nude mice by endobronchial injection (McLemore et al., 1987). An intrathoracic injection model was used and resulted in moderate tumor take rate – 35% of fresh primary human lung tumor specimens and 66% using cell inoculum.

Advantages of orthotopic tumor models include 1) Improved tumor take rate 2) Enhanced invasive and metastatic properties to the mediastinal lymph nodes. 3) Tumors arise in the organ-specific site providing a better representative host-tumor microenvironment. 4) Provides relevant pharmacokinetic and pharmacodynamics features more characteristic of human cancer than s.c. implanted tumors.

Disadvantages of orthotopic tumor models include 1) Animal lifetime is often shortened due to virulent tumor growth 2) Tumors imaging requires equipment that is expensive and may not be easily accessible 3) Tumors imaging is more time intensive than using digital calipers for s.c. xenografts 4) Orthotopic models are more costly to produce than s.c. models because imaging machinery is required. 5) The installation technique requires practice for tumor reproducibility.
Thesis Aims, Hypotheses and Expected Outcomes

The long-term goal of this research is two-fold: 1) to target a novel drug delivery vehicle containing a therapeutic agent to the respiratory tract of a healthy animal model 2) to develop an animal orthotopic imaging model using a modified symporter-A549 cell to longitudinally image resulting tumor burden in the same animal. The central hypothesis of this project is that magnetically responsive nano-in-microparticles (NIMs) in the presence of magnetic-field-dependent-targeting will be retained in specific regions of the respiratory tract and achieve high drug concentrations in lung regions.

Concomitantly, an orthotopic tumor model exploiting the sodium iodide (NaI) symporter (NIS) mechanism will allow for longitudinal SPECT/CT imaging of tumor burden. We tested our central hypothesis and accomplished the overall objective of this proposal by pursuing the following specific aims:

Specific Aim 1: Formulate, characterize and optimize an inhalable and magnetically responsive drug delivery dry powder containing doxorubicin (term nano-in-microparticles; NIMs) and develop a proof-of-concept in vitro tracheal mimic inhalation delivery system utilizing magnetic-field-dependent-targeting and retention of NIMs.

I hypothesized that by using the spray drying technique, a novel magnetically responsive dry powder drug delivery vehicle could be formulated for pulmonary administration. This formulation contains the active pharmaceutical ingredient doxorubicin, SPIONs, and the excipient lactose. Expected outcomes: These studies could demonstrate that by optimizing spray drying parameters and formulation component concentrations, the resulting dry powders will be of the correct mass median aerodynamic
diameter, geometry, morphology, charge and magnetic susceptibility for pulmonary administration. I also hypothesized that aerosolized NIMs will be targeted and retained in specific regions of respiratory tract influenced by magnetic-field-dependent-targeting. Expected outcomes: These studies could demonstrate, by the mechanism of magnetic targeting and inertial-impaction, that NIMs will be deposited and retained in a human tracheal mimic model and an ex vivo rat model.

**Specific Aim 2:** Target magnetically responsive NIMs to specific regions of the respiratory tract in healthy rats.

I hypothesized that pulmonary administration of NIMs could allow magnetic-field-dependent-targeting in a healthy mouse model. Expected outcomes: These studies could demonstrate that NIMs will be delivered and targeted to the left lung of the respiratory tract using a Dry Powder Insufflator™ in the presence of an externally placed permanent magnet. Dry powder NIMs could be compared to the administration of an aerosolized and targeted liquid suspension, again, in the presence and absence of magnetic-field dependent targeting.

**Specific Aim 3:** Develop a preclinical orthotopic tumor model for the purposes of longitudinal tumor imaging using SPECT/CT.

I hypothesized that an orthotopic tumor model exploiting the sodium iodide (NaI) symporter (NIS) mechanism could allow for longitudinal SPECT/CT imaging of tumor burden. Expected outcomes: These studies demonstrate that by developing an orthotopic tumor using A549-NIS modified cells, the tumor will sequester $^{99m}$Tc allowing for
SPECT/CT tumor burden imaging. Ultimately this will allow me to investigate the effects of inhaled therapeutic agents in the same animal over a longitudinal period of time.
Chapter 2:


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KEYWORDS: superparamagnetic iron oxide nanoparticles (SPIONs), doxorubicin, microparticles, spray drying, directed delivery, lung cancer therapy

INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide, with 1.4 million people dying from the disease each year, as of 2008 (1). Despite advances in surgery and treatment, the 5-year survival rate from 2001-2007 was 15% and has remained largely unchanged for decades (2). These statistics are due, in part, to a conventional drug delivery system that neither adequately delivers nor maintains sufficient drug concentration near solid lung tumors (3), leading to adverse effects in healthy tissues. Clearly, there is an unmet medical need in the field of cancer therapy to selectively deliver chemotherapeutics to lung tumors and minimize side effects observed in healthy tissues as well as to achieve effective therapy.

OBJECTIVE

We formulated and characterized microparticles containing Fe₃O₄ superparamagnetic iron oxide nanoparticles (SPIONs) and the chemotherapeutic agent doxorubicin in a lactose matrix. A proof-of-concept cylindrical glass tube was used to mimic the conducting airways of humans. In conjunction with an external permanent magnet, magnetically responsive nano-in-microparticles were directed and retained in a site-specific region inside the tube. Previously, Dames, et al. (4) targeted nebulized droplets containing SPIONs and pDNA, a therapeutic mimic, to specific lobe regions in a mouse lung. Although significant deposition was shown in the magnetized lobe, separation of pDNA from SPIONs during the delivery process was observed.
METHODS

Formulation of Inhalable Magnetic Microparticles

A suspension containing 77% lactose, 20% SPIONs, and 3% doxorubicin in ddH$_2$O (% w/w) were spray dried using a Büchi mini-spray dryer B-290 to achieve nano-in-microparticles dry powders (Büchi Corporation, Flawil, Switzerland). Spray drying parameters were as follows: inlet temperature 170 ± 2°C, outlet temperature 103 ± 2°C, aspirator rate 100%, and atomization air flow rate of 742 NL/h (equivalent to a spray rate of 60 mm). The yield was calculated as the ratio of the mass of solids collected after spray drying to the amount of solids in the feed suspension. Carrier control particles were manufactured containing 5% lactose only (% w/v) in ddH$_2$O, made into a solution and spray dried as previously stated.

Microparticle Characterization

The hydrodynamic and aerodynamic size and particle characteristics of the nano-in-microparticle dry powders were determined by laser diffraction, cascade impaction and electron microscopy. Energy-dispersive x-ray spectroscopy (EDS) via scanning electron microscopy (SEM) was used to elementally analyze Fe on the particle surface, whereas, inductively coupled plasma (ICP) quantified the amount of Fe loading in the microparticle.

Proof-of-Concept Tracheal Mimic Tube Study

A cylindrical borosilicate glass tube (two-centimeter diameter by 25-centimeter length) was designed to mimic the conducting airways of the human respiratory tract. Two different formulations were administered: the aforementioned nano-in-microparticle dry powder formulation, and a nebulized liquid suspension. Both contained a fluorescent dye as a surrogate for doxorubicin. For our in vitro investigation, the highest magnetic grade commercially available neodymium permanent magnet (grade 52, ΔB < 0.58 T) was used. This was placed on the external vertical axis of the tube to achieve targeting of the magnetically responsive nano-in-microparticle dry powders to a specific region of the tube. When conducting in vivo experiments, we plan to use a higher magnetic flux gradient as reported in the literature (4). An insufflator and microsprayer (Penn Century) were used to aerosolize the dry powders (n = 3) and liquid suspension, respectively. Relative fluorescence intensity was quantified using the Carestream Molecular Image Station 4000 MM Pro.

RESULTS AND DISCUSSION

TEM and SEM images of the nano-in-microparticle dry powder containing SPIONs and doxorubicin indicated the microparticle to be spherical in shape (Figures 1A and 1B). The geometric diameter of the microparticle was 1.6 µm as determined by SEM imaging. TEM images suggested that SPIONs are uniformly distributed throughout the microparticle delivery vehicle. SEM reveals the rough outer surface of individual microparticles due to the protrusion of the SPIONs from the microparticle surface (Figure 1C). An average geometric diameter of 1.6 µm was measured
with laser diffraction, and corroborated with SEM. The aerodynamic particle size characterization was determined using a next generation cascade impactor (NGI) and showed a mass median aerodynamic diameter (MMAD) of 3.27 µm, administered using the Penn Century insufflator at a flow rate of 15 L/min, simulating compromised lung function. The average fine particle fraction of the microparticles was 96% with a geometric standard deviation (GSD) of ±1.69 µm. The theoretical powder yield was 60.9%. EDS and ICP showed the microparticle surface composition to be 16.08% ± 0.5 Fe and 7.44% ± 0.3 Fe, respectively, compared to the theoretical 20% (w/w) Fe loading in the microparticle. Based on this data, we suggest differential SPION partitioning to the outer microparticle surface versus the inner microparticle matrix. Proof-of-concept tracheal tube studies showed significant spatial deposition of the nano-in-microparticle dry powders in regions experiencing a strong magnetic flux (Figure 2A). This correlates the amount of drug delivery vehicle that could be deposited to a specified location using our delivery method and aerosolized dry powders. Quantitative fluorescence analysis showed increased deposition of aerosolized dry powders (mean = 430, SE = 101) near the magnet based on greater fluorescence intensity, compared to the aerosolized liquid formulation (mean = 211.0) (Figure 2B). Therefore, more therapeutic could be directed and deposited in specific lung tumor regions with a dry powder formulation than a liquid suspension.

Figure 1.  A) SEM micrograph of blank lactose dry powder with a smooth external morphology. B) TEM micrograph of the outer edge of a particle incorporating electron dense SPIONs in a lactose matrix. C) SEM micrograph of the rough external morphology of magnetically responsive dry powder.
CONCLUSIONS

The formulation and characterization of a novel nano-in-microparticle dry powder was performed. This work is novel because a magnetic dry powder containing a chemotherapeutic agent can be directed to specific regions of the lung more efficiently compared to aerosolized liquid suspensions containing SPIONs. The solid matrix of a dry powder will allow a better association between the chemotherapeutic agent and SPIONS, thus preventing the separation of the drug from the SPIONs during the inhalation process. This was demonstrated in a proof-of-concept tracheal tube study, assessed by relative fluorescence. Magnetic dry powder microparticles have nearly twice the fluorescent intensity and will therefore better accumulate in a specific region compared to the magnetic aerosolized liquid suspension. Accordingly, the aerosolized liquid suspension showed decreased fluorescence, due to the premature separation of the drug surrogate dye from the SPIONs before reaching the region of interest. Therefore, targeting magnetically responsive powders containing a chemotherapeutic agent could lead to efficient targeting to lung tumor regions. This novel delivery system could minimize the side effects observed in healthy tissues due to the chemotherapeutic agent, and has great potential for inhalation therapy in the future.

ACKNOWLEDGEMENTS

This research has been supported in part by the NSF-IGERT Integrating Nanotechnology with Cell Biology and Neuroscience Fellowship (DGE-0549500) and the NCI Alliance for Nanotechnology in Cancer New Mexico CNTC Training Center. We would like to thank Chemicell GmbH and DMV-Fonterra, respectively, for their gifts of magnetic nanoparticles and lactose.
Preparation and Characterization of Novel Magnetic Nano-in-Microparticles for Site-Specific Pulmonary Drug Delivery

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ABSTRACT: We propose the use of novel inhalable nano-in-microparticles (NIMs) for site-specific pulmonary drug delivery. Conventional lung cancer therapy has failed to achieve therapeutic drug concentrations at tumor sites without causing adverse effects in healthy tissue. To increase targeted drug delivery near lung tumors, we have prepared and characterized a magnetically responsive dry powder vehicle containing doxorubicin. A suspension of lactose, doxorubicin and Fe3O4 superparamagnetic iron oxide nanoparticles (SPIIONS) were spray dried. NIMs were characterized for their size and morphological properties by various techniques: dynamic light scattering (DLS) and laser diffraction (LS) to determine hydrodynamic size of the SPIIONS and the NIMs, respectively; next generation cascade impactor (NGI) to determine the aerodynamic diameter and fine particle fraction (FPF); scanning (SEM) and transmission (TEM) electron microscopy to analyze particle surface morphology; electron dispersive X-ray spectroscopy (EDS) to determine iron loading in NIMs; inductively coupled plasma atomic emission spectroscopy (ICP-AES) and superconducting quantum interference device (SQUID) to determine Fe content in the microparticles; and high performance liquid chromatography (HPLC) to determine doxorubicin loading in the vehicle. NIMs deposition and retention near a magnetic field was performed using a proof-of-concept cylindrical tube to mimic the conducting airway deposition. The hydrodynamic size and zeta potential of SPIIONS were 56 nm and −49 mV, respectively. The hydrodynamic and aerodynamic NIM diameters were 1.6 μm and 3.27 ± 1.69 μm, respectively. SEM micrographs reveal spherical particles with rough surface morphology. TEM and focused ion beam–SEM micrographs corroborate the porous nature of NIMs, and surface localization of SPIIONS. An in vitro tracheal mimic study demonstrates more than twice the spatial deposition and retention of NIMs compared to a liquid suspension, in regions under the influence of a strong magnetic gradient. We report the novel formulation of an inhaled and magnetically responsive NIM drug delivery vehicle. This vehicle is capable of being loaded with one or more chemotherapeutic agents, with future translational ability to be targeted to lung tumors using an external magnetic field.

KEYWORDS: pulmonary delivery, magnetic microparticles, inhalable dry powders, SPIIONS, lung cancer therapy, spray drying

1. INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide, with 1.4 million people dying from the disease each year, as of 2008.† Lung cancer accounts for more deaths than breast, prostate, colon, liver and kidney cancers combined.‡ In the United States alone, 160,000 people died of lung cancer in 2010.§ Despite the use of new chemotherapeutic agents for lung cancer, the average patient five-year survival rate is 5–15% and has remained largely unchanged for decades.†

These statistics are due, in part, to conventional drug delivery systems that neither deliver nor maintain sufficient drug concentration near solid lung tumors, leading to adverse effects in healthy tissues. The lung offers a unique and challenging route for drug delivery with high absorption and surface area, ca. 100 m2.¶ Inhaled drug delivery is widely used for diseases such as asthma, COPD and cystic fibrosis and has shown promise as an alternate delivery method for lung cancer chemotherapeutics. However, it has been associated with side effects.¶

A major unmet medical need in the field of cancer therapy is to selectively deliver chemotherapeutic agents to lung tumors. The objective is to minimize side effects observed in healthy lung tissues as well as to achieve effective therapy. A phase I/II study of inhaled doxorubicin combined with oral cisplatin and docetaxel-based therapy for advanced non-small cell lung cancer showed the efficacy of inhaled therapy. Although seven evaluable patients responded to the combined inhaled and oral therapy, dose-limiting pulmonary toxicity was observed in two patients due to a lack of drug-tumor targeting.¶ This

Received: December 22, 2012
Revised: August 19, 2013
Accepted: August 21, 2013
Published: August 21, 2013
study showed the benefit of delivering agents directly to the lung with diminished systemic side effects.

Researchers have used Fe₃O₄ superparamagnetic iron oxide nanoparticles (SPIONs) as a means to target drugs to specific regions of the lung in animal models to mitigate the toxicity observed in healthy lung tissues. If successful, this approach will lead to improved tumor targeting, minimize the side effects observed in healthy tissues due to the chemotherapeutic agent, and maximize the therapeutic outcome. Targeted pulmonary drug delivery using SPIONs will also significantly lower the total drug dose required to achieve therapeutic response at the tumor site, thus further alleviating the side effects observed in healthy tissues.

Previously, Dames et al. and Rudolph et al. targeted nebulized suspension containing SPIONs and pDNA, a therapeutic mimic, to specific regions in a mouse lung. Although significant deposition of SPIONs was shown in the magnetized lobe, separation of pDNA from SPIONs during the delivery process was observed. Nebulized formulations used in these studies lead to separation of the drug surrogate from the SPIONs before reaching the intended target site. We propose formulating and characterizing an inhalable dry powder vehicle containing dye to target drugs to specific regions in the lung in animal models to mitigate the toxicity observed in healthy lung tissues.

The objective of this study was to formulate and characterize nano-in-microparticles (NIMs) containing SPIONs and a chemotherapeutic agent doxorubicin in a lactose matrix. NIMs, the first of their kind formulated in a dry powder form, will prevent premature separation of the chemotherapeutic agent and SPIONs.

**2. EXPERIMENTAL SECTION**

**2.1. Materials.** Alpha-α-(+) -lactose monohydrate Respitose ML-001 was a gracious gift from DMV-Fonterra Excipients GmbH & Co. KG (Goeh, Germany). FluidMAG-UC SPIONs with a hydrodynamic diameter of 50 nm were gifted and purchased from Chemicell GmbH (Berlin, Germany). FluoroMAX Green Fluorescent Polymer nanospheres (0.025 μm diameter) were purchased from Thermo Fisher Scientific. Adriamycin (doxorubicin) was purchased from Selleck Chemicals, LLC (Houston, TX). Adriamycin (doxorubicin) was purchased from Selleck Chemicals, LLC (Houston, TX). Adriamycin (doxorubicin) was purchased from Selleck Chemicals, LLC (Houston, TX).

**2.3. Microparticle Characterization.**

2.3.1. Yield and Encapsulation Efficiency of Doxorubicin. NIM yield was calculated as the ratio of the mass of solids collected after spray drying to the amount of solids in the feed suspension. The percentage encapsulation efficiency (EE) and percentage doxorubicin loading in NIMs were determined using eqs 1 and 2, respectively:

\[
\% \text{ EE} = \frac{\text{actual weight of doxorubicin in NIMs} \times 100}{\text{theoretical weight of doxorubicin in NIMs}}
\]

\[
\% \text{ doxorubicin loading} = \frac{\text{actual weight of doxorubicin in NIMs} \times 100}{\text{NIMs weight}}
\]

2.3.2. SPION Characterization: Hydrodynamic Size and Zeta Potential. Hydrodynamic size (Dₜ) was determined using dynamic light scattering (Zetasizer Nano ZSP, Malvern Instruments Ltd.). The samples were prepared by dispersing 1 μL [50 mg/mL] of SPIONs in 1 mL of ddH₂O (n = 9). The zeta potential of SPIONs was characterized by dispersing 0.5 μL [50 mg/mL] of SPIONs in 1 mL of ddH₂O (n = 9), expressed as size distribution by average intensity.

2.3.3. NIM Hydrodynamic Size. The volume median diameter (D₀) of the NIMs was determined by laser diffraction using the cuvette disperser (Helos/KF-OM, Symptec GmbH, Germany). Briefly, 5.0 mg of NIMs was suspended in 1 mL of acetonitrile and gently vortex-mixed. A 200 μL aliquot of this suspension was added dropwise to the 6 mL cuvette containing 5 mL of acetonitrile. Measurements were taken for 10 s using the R3 lens in triplicate.

2.3.4. In Vitro Aerosolization Studies (Aerodynamic Diameter). The aerodynamic size of the spray dried NIMs containing dye was determined using a Next Generation Impactor (model 170 NGR, MSP Corporation, Shoreview, MN). NIM samples (6 mg) were aerosolized using a model DP4 dry powder insufflator for rat (Penn Century, Inc., USA). A pump (Copley Scientific, Nottingham, UK,) was operated at a flow rate of 30.0 L/min for 10 min. Following aerosolization, particle deposition was measured by gravimetric method from collection cups. The percent cumulative mass fractions were plotted versus log aerodynamic diameters. The mass median aerodynamic diameter (MMAD) was estimated by linear interpolation that links the curve points at 50% deposition. The fine particle fraction (FPF; stage 3 to stage 7, i.e. <6.4 μm) was calculated as a percentage of total emitted dose (n = 3).

2.3.5. NIM Morphology and Cross Sectional Analysis. To determine particle size, surface morphology and elemental analysis, NIMs were visualized using a high-resolution scanning electron microscope.
We adapted an HPLC method reported by Mikan et al. to our system. To prepare the solutions, doxorubicin standards with concentrations of 0.078, 0.156, 0.31, 0.63, 1.25, 2.5, 5, 10, and 20 μg/mL were prepared by dispersing NIMs into a suspension, separating the SPION component using a magnetic gradient, and transferring supernatant to HPLC vials. The HPLC system consisted of a 1260 Infinity Agilent LC (Agilent Technologies, Santa Clara, CA). Integrations, calculations, and plotting of chromatograms were performed with a Chemstation computing integrator (Agilent Technologies, Houston, TX). A C18 HPLC column was used, and the mobile phase was prepared by mixing acetonitrile and water, adjusted to pH 3.0 with phosphoric acid, at 72/28 (v/v) proportions. Doxorubicin eluted at 7.2 min with a flow rate of 1 mL/min. The UV detector was set at a wavelength of 254 nm. The HPLC apparatus was operated at room temperature.

2.3.8. SPION Magnetization Measurement. Magnetic properties were measured with an MPMS-7XL SQUID Susceptometer (Quantum Design, San Diego, CA) integrated to a Physical Properties Measurement system (PPMS, Quantum Design, San Diego, CA). The ac susceptibility measurements were conducted in the temperature range from 4 to 300 K with nominal magnetic field of 20 kOe. These measurements allowed us to calculate the magnetic moment of the SPIONs as well as the concentration of SPIONs in the NIMs.

2.3.9. Permanent Magnet Characterization. The cylindrical permanent magnet (2.5 cm length × 2 cm diameter) was characterized using a Bell-5180 series Hall effect gaussmeter (Sypris Test and Measurement, F.W. Bell, Orlando, FL). The Hall effect gaussmeter was used to measure the flux density (B) at increasing distances from the surface of the magnet. A ruler was taped parallel to the magnet, and the flux density was measured with the axial gaussmeter probe in 1 mm increments from a distance of zero to 30 mm from the magnet. Because magnetic force is proportional to the magnetic field gradient (ΔB/Δx), the gradient was determined by dividing ΔB, the change in the flux density between each successive measurement, by Δx, the change in distance between each successive measurement. The magnetic field gradient measurements were used to indicate the relative strength of the magnetic attractive force on the NIMs at different positions for the subsequent tracheal mimic tube study. The dimensions of the tube were similar to those of a human male adult (20 mm diameter × 200 mm length × 1 mm length)
thickness). Two different formulations were examined: NIMs and a nebulized liquid suspension. Both formulations contained the green fluorescent nanospheres as a surrogate for doxorubicin. Briefly, 5.0 mg of NIMs was weighed for each run; for the preparation of the liquid suspension 5.0 mg of the NIMs were suspended in 3 mL of water to have equal proportions of the nanospheres and SPIONs. The permanent magnet was placed on the external vertical axis of the tube to achieve retention of the NIMs near the magnet (Figure 1). The magnetic retention of the NIMs was examined by placing the magnet at 0 and 4 mm from the tube based on the magnetic strength characterized earlier. These two distances were measured from the outer surface of the glass tube (thickness 1 mm). Aerosol generators (model DP-4 dry powder insufflator and model IA-1C liquid suspension microsprayer (Penn Century, Inc., Philadelphia, PA, USA)) were used to aerosolize the NIMs and the liquid suspension, respectively (n = 3) (Figure 1). The glass tube was connected to a vacuum source that was adjusted to a flow rate of 15 L/min. Relative fluorescence intensity was quantified using the Carestream Molecular Image Station 4000 MM Pro using excitation and emission wavelengths of 488 and 508 nm, respectively. Student t tests were used to quantify statistical significance (Graphpad prism, La Jolla, CA).

3. RESULTS

3.1. NIM Formulation. NIMs were formulated by spray drying doxorubicin and SPIONs in a lactose matrix. For the purpose of drug delivery and bioavailability to tumors, NIMs were characterized on the basis of size, surface morphology and magnetic properties. SEM and TEM micrographs indicate NIMs to be spherical in shape with a diameter of 1.6 μm (Figure 3C and Figure 3E, respectively). TEM micrographs revealed increased electron dense (black) areas indicating the presence of iron atoms. Cross-sectional analysis by TEM shows that SPIONs are preferentially distributed on the outer surface of the NIMs (Figure 3E). SEM micrographs revealed a rough outer surface due to SPIONs protruding from the lactose matrix (Figure 3C). The surface area of NIMs may be influenced by the extent of the surface roughness. This is an important characteristic of dry powders when mitigating drug–drug particle agglomeration formation.16

3.2. Characterization of SPIONs: Hydrodynamic Size, Zeta Potential, and TEM. SPIONs had an average radius of 56 ± 6 nm (Figure 2B) and a density of 2.5 g/cm³. SPIONs had an average zeta potential of −49 mV (Figure 2C) and confer colloidal stability against aggregation. TEM micrographs of SPIONs showed magnetite crystals on the order of 6 nm that are combined to form a single-domain 50 nm core (Figure 2A).

3.3. Yield, Encapsulation Efficiency and Doxorubicin Loading. The theoretical powder yield was 60.9% based on a
mass balance performed on the solids (SPIONs, doxorubicin, lactose) and NIMs before and after spray drying, respectively. Using HPLC, % EE was quantified in NIMs to be 57%. Doxorubicin eluted with a retention time of 7.2 min. Theoretically, NIMs contained 2.8% (w/w solids) doxorubicin, and actual doxorubicin loading was quantified to be 1.6%. The doxorubicin loss is attributed to the spray drying process; small particles of doxorubicin (which have a characteristic red color) were visually observed trapped in the filter of the machine.

3.4. NIM Characterization. The average hydrodynamic NIM diameter was 1.6 μm using laser diffraction. This size correlates with the size assessed using SEM (Figure 3B). A MMAD of 3.27 μm was obtained using a NGI. The aerodynamic diameter takes the density of the NIMs into account and is fundamental to particle deposition in the lung and hence for inhaled drug delivery. The average fine particle fraction (FPF) (≤6.4 μm) of the NIMs was >90%, and the geometric standard deviation (GSD) was ±1.69.

3.5. Permanent Magnet Characterization. Single and two combined permanent magnets were characterized for their magnetic field strength. The magnetic field strength on the surface of the single magnet was 0.58 T. Magnetic field strength did not increase when two permanent magnets were combined (data not shown). Magnetic field lines are parallel to the surface of the magnet and diverge with increasing distance, resulting in a weaker flux density at larger distances; the magnetic force acts on the NIMs when they enter this region of diverging magnetic field. The direction of the force moves magnetic objects from...
regions of lower flux density to regions of greatest flux density (i.e., the surface of the magnet). This study also shows that the geometry and orientation of the magnet is critical for significant deposition of the NIMs near the magnet. Figure 5A shows that the magnet was characterized over a distance of 30 mm with decreasing magnetic gradient strength (G/mm).

3.6. Magnetization Measurement. A SQUID is a sensitive magnetometer that is used to measure extremely small magnetic fields. With this device we measured the magnetic moment of NIMs. Susceptibility curves were obtained to provide the specific magnetization (s) in emu/g at a particular magnetic field strength. This is related to the susceptibility through the equation $\chi = (s\rho)/\mu_0$ where $\rho$ is the particle density. The zero coercivity and the reversible hysteresis behavior indicate the superparamagnetic nature of the SPIONs (Figure 4E). The specific saturation magnetization for the NIMs was calculated to be 16 emu/g. This value is below the saturation magnetization known for bulk $\text{Fe}_3\text{O}_4$ ($M_s = 92$ emu/g) as expected given that lactose does not contribute to the magnetic moment of NIMs. SUSCEPTIBILITY curves were obtained converting this to magnetite, $\text{Fe}_3\text{O}_4$. The magnetic moment was corrected by the diamagnetic response of the SPIONs (Figure 4E). The specific saturation magnetization for the NIMs was calculated to be 10.6% by weight, in good agreement with the $\text{Fe}_3\text{O}_4$ fraction obtained from the magnetization measurements (9%). EDS-SEM and EDS-TEM quantified Fe on the NIM surface, and $\text{Fe}_3\text{O}_4$ was calculated to be 16 ± 0.5% and 19 ± 0.5%, respectively (Figure 4C,D). These numbers are further understood knowing that EDS-SEM has a depth resolution of 50 nm whereas EDS-TEM penetrates an average sample depth of ~1 μm. TEM micrographs (Figure 3E) qualitatively supported this observation that the SPIONs are less densely distributed/localized in the center of the NIMs. Thus EDS-SEM quantitates a lower amount of $\text{Fe}_3\text{O}_4$ given this analytical tool penetrates the sample more deeply than EDS-TEM.

3.7. Proof of Concept Tracheal Tube Study. Magnetic retention of NIMs containing fluorescent nanospheres was quantified in relation to its fluorescence intensity (Figure 5B,C). Quantitative fluorescence analysis showed a 2-fold increased deposition of aerosolized NIMs at 0 mm (mean = 534.97, SD = 131.17) near the magnet based on greater fluorescence intensity, compared to the aerosolized liquid formulation at 0 mm (mean = 221.67, SD = 26.70) ($t = 4.054$, $p = 0.0154$, df = 4, two-tailed Student t-test) (Figure 5B,C,D). Control studies of untargeted NIMs (absence of magnetic targeting) qualitatively show no mean fluorescence over background in the region of interest (Figure 5B,C). Quantitative fluorescence analysis showed increased deposition of NIMs at 4 mm (mean = 385.65, SD = 30.15), compared to the aerosolized liquid formulation (mean = 277.30, SD = 88.16) (Figure 5D). A 28% decrease in the fluorescence intensity of NIMs was observed when the magnet was placed 4 mm away from the tube compared to 0 mm.

4. DISCUSSION

This research proposes the use of regional chemotherapy by the pulmonary route with the intent of increasing drug exposure near a solid tumor. Despite inhaled drug delivery being used for respiratory diseases for over 30 years, targeted drug delivery to specific regions of the lung has not been adequately explored. We show here, for the first time, the formulation of magnetic NIMs containing SPIONs and doxorubicin, by the process of spray drying. NIMs can be guided to a region of interest in a tracheal tube with strategic placement of an external magnet. Inhalable dry powders will allow higher doses of drug to be delivered to cancerous lung regions without increasing side effects observed in surrounding healthy tissues, compared to a liquid suspension. Using this novel delivery method, we do not expect to overcome oropharyngeal deposition. This delivery method will only localize drug near the target region that does not impact the upper regions of the respiratory tract.

4.1. Formulation of a Novel Inhalable NIMs Delivery Vehicle. NIMs were formulated using pulmonary-compatible lactose rather than a biodegradable polymer. This is to allow for the immediate release of drug from the vehicle after deposition and to minimize the patient contact time with the magnetic field. After NIM deposition at the target site, the NIM will disintegrate quickly in the lung parenchyma to release drug that willdiffuse into the target tumor mass. Neither doxorubicin nor SPIONs are conjugated to the delivery vehicle thus eliminating any limitations on the release of bound drug near the target site.

4.2. Characterization of NIMs. NIMs exhibit a rough outer surface, as seen from the SEM images (Figure 3B,C). This facilitates a beneficial disaggregation of particles when administered as a dry powder. Currently, dry powder flow and dispersion are improved by incorporating larger particles (50–100 μm) as carriers to facilitate disaggregation of dry powders during inhalation. Should NIM flow or dispersion be a problem, the addition of carrier particles would be considered. The flow dynamics of the NIMs after inhalation can also affect the drug deposition and would require further studies. A logical next step, and our next pursuit, will involve the administration of NIMs in animal models to investigate the targeting capabilities in the upper and lower respiratory tract.

NIMs can be designed to modulate drug release after the particles have been guided to the targeted lung regions in the future. If this concept is successful, NIMs can also be used to deliver doublet chemotherapeutic agents since doublet therapy is the cornerstone of treatment for lung cancer. Future studies also need to investigate the effect of SPIONs on cellular uptake and pulmonary toxicity in animal models, since surface properties of SPIONs are known to affect the cellular uptake and cytotoxicity. However, the biodegradability and biocompatibility of SPIONs have been proven for many years in the clinical setting as a contrast agent in magnetic resonance imaging.

4.3. Magnetic Properties of Aerosolized NIMs. The small magnetic moment of individual SPION in an aerosolized liquid droplet cannot be guided easily in the presence of an external magnet. However, when SPIONs are assembled together in NIMs, the net magnetic moment of aggregated SPIONs is large enough to be manipulated with a medically compatible external magnet. In addition, droplets containing nanosized SPIONs for inhaled drug delivery are easily exhaled due to their small size; therefore encapsulating the SPIONs into micrometer sized NIMs will help to resolve this problem. The combined magnetic moment of the SPIONs present in the NIMs may allow the particles to be retained at the target site until the NIMs disintegrate therefore releasing the drug. In addition, NIMs could also be retained at the target site by entrapment from lung mucus and cilia.
tube compared to 0 mm. This decrease in fluorescence, and therefore magnetic force, is a challenge that needs to be addressed since the magnetic field has to penetrate a larger distance if this targeting mechanism is to be applied in humans. Since the magnetic gradient decreases with the distance to the target tissue, the main limitation of this delivery mechanism could relate to the strength of the external field that can be applied to the patient to obtain the necessary magnetic gradient; however, high field gradient electromagnets are being used in animals for magnetic targeting and substantial field gradients are also used in MRI imaging. Further studies are needed to validate the safety of the strength of an external magnet as it relates to magnetic nanoparticles.

The magnetization s-curve of NIMs loaded with 9% w/w Fe₃O₄ is shown (Figure 4E) and has negligible coercivity, and consequently no remanence was observed once the magnetic field was removed, indicating the superparamagnetic behavior of the SPIONs. Localization of SPIONs on the NIM surface was qualitatively and quantitatively observed. Surface analytical techniques were used to understand the elemental depth profiles of SPIONs in NIMs. EDS analysis is based on the beam voltage which is applied to the sample; a lower voltage will excite electrons to a lesser depth in the sample allowing for the quantification of elemental Fe composition. Both EDS-TEM and EDS-SEM analysis were used to determine the distribution of SPIONs in the surface from the interior to the surface of the particles (Figure 4C,D). Uncoated SPIONs were chosen for this formulation to exploit the superparamagnetic properties of the core iron oxide nanoparticles, since coating leads to loss of magnetic strength per volume. However, coating SPIONs with a polymer is known to prevent aggregation and to provide colloidal stability to the suspension. In our study, the suspension containing SPIONs, drug and lactose was adequately stirred prior to and during the spray drying process, to prevent aggregation of SPIONs.

4.4. Proof-of-Concept Tracheal Mimic and NIMs Targeting. Dames et al.¹¹ and Hasenpusch et al.²⁴ have proven the feasibility of magnetic aerosol delivery using nebulized SPIONs and a drug surrogate to target specific lung regions in mice using an external permanent magnet. A drawback of this delivery system was that the drug surrogate separated from the magnetic carrier during pulmonary delivery resulting in premature release of the surrogate in the upper respiratory tract. This separation would result in a lower amount of the drug surrogate reaching the target site in a liquid suspension. We demonstrated a similar result in the tracheal mimic experiment as seen by a uniform nanosphere distribution suspension. We demonstrated a similar result in the tracheal mimic experiment. This study brings about new perspectives for specific disease treatment by increasing delivery of chemotherapeutic agents to the tumor mass and reducing unwanted drug-related side effects in nondiseased tissue. In conclusion, SPIONs with very high saturation magnetization were successfully encapsulated in NIMs by spray drying. These novel formulations have the potential to be targeted to specific regions of the lung using an external magnet.

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We thank Dr. Mehdi Ali for his assistance analyzing Fe quantification, and Ms. Erin Weeda for her help with the tracheal mimic tube studies. We also thank the New Mexico Cancer Nanotechnology Training Center and fellowship program for their support.

ABBREVIATIONS USED
MMAD, mass median aerodynamic diameter; NIMs, nano-in-microparticles; SPIONs, superparamagnetic iron oxide nanoparticles; ICP-AES, inductively coupled plasma atomic emission spectroscopy; EDS, energy dispersive spectroscopy; SQUID, super quantum conducting interference device; PPE, personal protective equipment; GSD, geometric standard deviation

REFERENCES


A Pilot Study Targeting Nano-in-Microparticles (NIMs) in Rat Lungs Ex Vivo

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KEYWORDS: magnetic drug targeting, lung cancer therapy, superparamagnetic iron oxide nanoparticles (SPIONs), spray drying, microparticles

INTRODUCTION

Pharmaceutical aerosols are of growing interest in the field of therapeutic drug delivery. Recently, inhaled pharmaceuticals have gained preclinical trial attention for the treatment of lung cancer [1, 2]. A major obstacle in lung cancer treatment is the ability to selectively deliver chemotherapeutics to lung tumors, therefore minimizing side effects observed in healthy tissues. There have been several different approaches to site-specific targeted drug delivery [3-5] using magnetic-field-dependent-targeting. This research attempts to improve upon previous drug delivery studies using a targeted and inhaled dry powder approach.

METHODS

Nano-in-microparticles (NIMs) were formulated and characterized containing Fe₃O₄ super-paramagnetic iron oxide nanoparticles (SPIONs) and a far-red fluorescent probe (Invitrogen Molecular Probes, FluoroSpheres®) in a lactose matrix. NIMs were characterized for size and magnetic strength. An ex vivo pilot study was conducted to target NIMs to the right lung lobe in healthy rats, in the presence or absence of a magnetic field. Total radiance (fluorescence intensity) was qualitatively and quantitatively analyzed to assess NIMs deposition and retention.
Formulation of Inhalable Magnetic Microparticles

A suspension containing 70% lactose (DMV-Fonterra Excipients, Respitone, ML001), 20% SPIONs (Chemicell, fluidMAG-UC/A, 0.050 mm diameter) and 10% Fluorospheres (Invitrogen Molecular Probes, 660 nm excitation and 680 nm emission, 0.024 mm diameter) (% w/w) was spray dried using a Büchi mini-spray dryer B-290 to achieve NIMs dry powders (Büchi Corporation, Flawil, Switzerland). Spray drying parameters were as follows: outlet temperature 100 ± 4°C, inlet temperature 170 ± 2°C, aspirator rate 90%, and atomization air flow rate of 742 NL/h (equivalent to a spray rate of 60 mm). Powder yield was calculated as the ratio of the mass of solids collected after spray drying to the amount of solids in the feed suspension. Carrier control particles were formulated containing 5% lactose only (% w/v) in ddH$_2$O, made into a solution and spray dried as previously stated.

Microparticle Characterization

The hydrodynamic and aerodynamic size and particle characteristics of NIMs were determined by laser diffraction, cascade impaction and electron microscopy. Internal particle characteristics were determined by focused ion beam ablation (FIB) and visualized by Transmission electron microscopy (TEM). The magnetic strength of NIMs was assessed by inductively coupled plasma atomic emission spectroscopy (ICP-AES), energy-dispersive x-ray spectroscopy (EDS) was used for elemental analysis, and surface morphology via scanning electron microscopy (SEM).

Ex Vivo NIM Magnetic-Field-Dependent Targeting in Rat Lungs

The overall pulmonary deposition of fluorescent, magnetic NIMs was targeted to rat lungs by insufflation (Penn Century, Dry Powder Insufflator, DP-4) in the presence of a neodymium permanent magnet (Applied Magnets, grade 52, D $B < 0.58$ T). Rats ($n=3$) were sacrificed, and lungs lobes with trachea (attached) were excised. Five puffs of air were administered to the lungs by insufflation to counteract lung collapse. A permanent magnet was placed at a distance of 1 μm from the right lung tissue. A total of ten milligrams of fluorescent NIMs powder was insufflated intratracheally. An IVIS Lumina II imager following intratracheal delivery imaged NIMs powder deposition. Fluorescent regions of interest (trachea, right lung lobe and left lung lobe) were quantified.

RESULTS AND DISCUSSION

SEM micrographs indicate NIMs to be spherical in shape (Figure 1A and 1C) with a rough external surface (Figure 1B, NIM rendering). An average geometric diameter of 1.6 μm was measured with laser diffraction, and corroborated with SEM. The aerodynamic particle size characterization was determined using a next generation cascade impactor (NGI) and showed a mass median aerodynamic diameter (MMAD) of 3.2 μm, administered using the Penn Century insufflator at a flow rate of 15 L/min simulating compromised lung function, as this targeted delivery vehicle is for lung cancer patients. The average fine particle fraction (FPF) was > 90% with a geometric standard deviation (GSD) of ± 1.7 μm. The theoretical powder yield was 58%.
Using ICP-AES, Fe₃O₄ was calculated to be 10.6% by weight. EDS-TEM and EDS-SEM quantified Fe on the NIM surface, and Fe₃O₄ was calculated to be 19 ± 0.5% and 16 ± 0.5%, respectively. EDS-TEM has a spatial resolution of 50 nm, whereas EDS-SEM penetrates an average sample depth of ~ 1 µm. EDS-TEM quantitates a higher amount of Fe₃O₄ given this analytical tool penetrates the sample at a shallower depth than EDS-SEM.

Table 1. Magnetic-field-dependent accumulation of NIMs in rat lungs.

<table>
<thead>
<tr>
<th>Tissue region</th>
<th>Rat #1</th>
<th>Rat #2</th>
<th>Rat #3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>R lung lobe (treatment, magnetized)</td>
<td>7.633 x 10⁸</td>
<td>2.110 x 10⁸</td>
<td>2.246 x 10⁸</td>
<td>1.706 x 10⁸</td>
</tr>
<tr>
<td>L lung lobe (treatment, unmagnetized)</td>
<td>3.765 x 10⁸</td>
<td>5.865 x 10⁸</td>
<td>3.438 x 10⁸</td>
<td>4.356 x 10⁸</td>
</tr>
<tr>
<td>Trachea (treatment)</td>
<td>1.470 x 10¹⁰</td>
<td>7.404 x 10⁹</td>
<td>4.108 x 10⁹</td>
<td>8.737 x 10⁹</td>
</tr>
</tbody>
</table>

Non-magnetic-field accumulation of NIMs in rat lungs (n = 1)

| +Control (treatment, unmagnetized, average in both lobes) | 2.435 x 10¹⁰ |
| -Control (no treatment, unmagnetized, average in both lobes) | 1.532 x 10⁹ |

a NIMs were insufflated into excised lungs (n = 3) with the application of a magnetic field
b Deposition is given in total radiant efficiency [pJ/(µW·cm²)]

A four-fold increase in magnetic-field-dependent accumulation of NIMs was seen in the magnetized lung (right lobe) over the unmagnetized lung (left lobe) (Table 1 and Figure 2A). NIM deposition in the trachea remained high which could be attributed to the method of delivery (insufflation) (n = 3, Table 1, Figure 2). The insufflated NIM dry powders were widely distributed in the pulmonary tract of the rat when magnetic targeting was not applied (Figure 2B). No fluorescence background was seen in control lungs (no treatment, unmagnetized) (Figure 2C). Lactose has an intrinsic tendency to take up moisture (a hygroscopic property). When passing through the humid environment of the airways, this results in increased particle size [6] and therefore more proximal NIM deposition (increased radiance intensity) and non-magnetic-field accumulation in the trachea (Figure 2A and 2B).
CONCLUSION

This pilot study showed approximately a four-fold magnetic-field-dependent accumulation of NIMs containing SPIONs and FluoroSpheres in the right lung lobe of rats ex vivo. By aligning NIMs in one direction using a magnetic field, microparticles would become randomly oriented with airway flow, leading to a higher probability of deposition in the targeted lung lobe [7]. Investigational method development for dry powder pulmonary insufflation in animals is particularly relevant for aerosol delivery as it closely mimics clinical use in humans.

ACKNOWLEDGEMENTS

This study was supported by the University of New Mexico Health Science Center Research and Allocations Committee (RAC) grant and graduate student support provided by the New Mexico Cancer Nanotech Training Center (CNTC). The authors thank Paul Durfee for his assistance with the IVIS Lumina II small animal imager, and Dr. Mehdi Ali for analysis of ICP-AES.

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NIMs Deposition Analysis - A Rat lung Pilot Study

An *ex vivo* pilot study was conducted to target NIMs to the right lung in healthy rats, in the presence or absence of a magnetic field. Deposition was quantified by fluorescence intensity (excitation 660 nm; emission 680 nm) of the rat lungs left lung, right lung and trachea for an exposure time of 20 seconds. A region of interest (ROI) was drawn around the left lung, right lung, trachea and background with area remaining constant (2.5 cm$^2$). Fluorescence was measured and expressed as units of radiance efficiency:

$$\text{Radiance efficiency} = \frac{(p/sec/cm^2/sr)}{uW/cm}$$

Targeting Efficiency Ratios As A Means to Analyze Fluorescence

In addition to comparing fluorescence intensity, data were further analyzed by calculating and comparing targeting efficiency ratios (Chen et al. 2002). Targeting efficiency was calculated as follows: in the numerator, fluorescence intensity of the left lung, right lung or trachea ($I_{\text{individual}}$) was divided by the sum of the fluorescence intensities of the three tissues ($I_{\text{sum}}$) in the denominator (Equation 1).

$$\text{Targeting efficiency ratio} = \frac{I_{\text{individual}} \ (e.g. \ left\ lung)}{I_{\text{sum}} \ (left\ lung+right\ lung+trachea)}$$

The key issue in this analysis was to determine whether a ratio was high or low in order to conclude whether magnetic field dependent targeting was significantly increased or decreased to the right lung, in comparison to the untargeted left lung.

In the following chapter, Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) will be used to quantify the deposition of iron in the lung from
either the dry powder NIMs vehicle or a liquid suspension vehicle (liquid suspension vehicle was used by Dames, et al. 2007 and Hasenpusch et al. 2012). An analysis strategy was needed to empirically corroborate the results of ICP-OES (iron deposition in the targeted lung) and the IVIS Lumina II Imager (dye deposition in the targeted lung). We believe that the ratio analysis method, in addition to reporting fluorescence (radiance efficiency) allows for a more objective comparison of raw fluorescence values of the \textit{ex vivo} targeting pilot study data and subsequent \textit{in vivo} targeting study data. Targeting efficiency ratio comparisons help normalize and compare values with minimal data manipulation. In the future \textit{in vivo} targeting study, expressing fluorescence and iron deposition in terms of targeting efficiency ratios allowed us to best corroborate these analytical methods, ultimately allowing for more meaningful data comparison.

\textbf{Statistical Analysis}

Statistical analyses were performed using Windows SPSS version 16 (SPSS Inc, Chicago, IL, USA). Paired t-tests with bonferroni correction were used to compare two or more groups of independent data. The significance level was set at $\alpha = 0.05$. Descriptive statistics were also employed if data were not statistically significant.

\textbf{Results}

Fluorescence intensity was quantified in the presence and absence of magnetic field dependent targeting, reported as mean intensity ± SEM. In the absence of magnetic field dependent targeting (no magnet placed on either lung), fluorescence intensity was similar in both the right and left lungs ($8.34 \times 10^7 \pm 8.89 \times 10^6$ right lung; $9.89 \times 10^7 \pm 2.02 \times 10^7$ left lung) (Fig. 3C). In terms of targeting efficiency ratios, in the absence of magnetic field dependent targeting, 20% NIMs deposition was observed in the right lung
versus 23% NIMs deposition in the left lung. This resulted in an absolute difference of 3% deposition to the left lung. Therefore, no preferential deposition of NIMs was observed in the right or left lungs.

A 3.9 fold increase in fluorescence (drug surrogate) was observed in the right lung compared to the left lung from the administration of dry powder NIMs (right lung mean: $1.71 \times 10^9 \pm 4.73 \times 10^8$; left lung mean: $4.36 \times 10^8 \pm 7.60 \times 10^7$, $P = 0.057$, $n = 3$) (Fig. 3A). Using the targeting efficiency ratio, a 20% increase in fluorescence from NIMs was observed in the right lung versus 4% NIMs deposited in the left lung (Table 3.1). Significantly more fluorescence was quantified in the right lung of rats that had been exposed to an external magnetic gradient ($1.71 \times 10^9 \pm 4.73 \times 10^8$) during insufflation than compared to the control mice in the absence of magnetic targeting ($8.34 \times 10^7 \pm 8.89 \times 10^6$ right lung; $P = 0.0035$) (Fig. 3A, B).
Figure 3. Deposition of the Model Drug Surrogate in the Lungs of Rats upon NIMs

**Dry Powder Administration** A) Comparison of magnetic dependent targeting in the right lung, left lung and trachea in the presence and absence of targeting, quantified by average fluorescence intensity. Differences in fluorescence intensity are significant comparing the right lung in the presence of magnetic targeting and the right lung in the absence of magnetic targeting (\( **P = 0.0035, n = 3 \)). B) Comparison of fluorescence targeting and deposition between the right (magnetized) and left lung (unmagnetized; \( *P = 0.057 \)).
Comparison of fluorescence deposition in the absence of targeting of right and left lung (not significant). D) Comparison of between right lungs (P = 0.076, n = 3). E) Comparison between left lungs (neither lung magnetized) (*P = 0.055, n = 3).

Discussion

Rats were chosen for this pilot ex vivo targeting study for a number of reasons:

1) Although mortality or safety data is scantily published using dry powder inhalers in rodent models, previous literature reported that rats observed a 3% mortality rate versus a 9% mortality rate in mice (Guillon et al., 2012) when dry powders were administered using the Insufflator™ or Microsprayer™ instrument (Morella et al., 2009). We previously observed that if mice were over stimulated when endotracheally intubated with the Dry Power Insufflator™ or Microsprayer™, laryngeal spasms and death could occur, a result of reflex adduction of the vocal cord.

2) We felt rats would serve as a better translational model than mice in determining targeting efficiency of magnetically responsive dry powders because of their larger scale trachea and lungs.

3) Intubation of a rat trachea is easier in a bigger rodent model. Our goal was not to achieve maximum NIMs dose delivery, but to target the NIMs to a specific lung as efficiently as possible.

Hasenpusch demonstrated a two-three fold higher accumulation of their model drug, fluorescein, in the lung region of mice that had been exposed to an external magnetic gradient during nebulization compared to the untargeted mice without any exposure to a magnetic gradient (Hausenpusch et al., 2012). In Hasenpusch’s study, the deposition rate of iron was found to increase 2.1-fold in the right lung exposed to the
magnetic field compared with the left lung (control) independent of the nebulization device (nose-only (NOIS) inhalation system and a whole body delivery chamber (WBIC)). We demonstrated a 5-fold higher accumulation of our model drug in the lung region of rats that had been exposed to an external permanent gradient during insufflation compared to the control rat without any exposure to a magnetic gradient.

Hasenpusch observed that there was no significant difference in fluorescein deposition, nor luciferase activity in the analysis of transgene expression, when comparing the left and right lobes in the presence of magnetic field dependent targeting of mice. In comparison, we saw 4-fold higher accumulation of our model drug in the lung region of rats that had been exposed to an external magnetic gradient during insufflation.

Fluorescein deposition increased not only in the magnetized lung lobe but also in the non-magnetized control lung lobe; they concluded that the influence of the magnetic gradient was strong enough to increase deposition of aerosol droplets in the right lungs exposed to the magnetic field but weak enough to avoid rapid clearance of the SPIONs by mucociliary clearance or phagocytosis. There are two proposed implications of increased deposition to the untargeted lung: beneficial deposition to the untargeted lung and unbeneficial uncontrolled deposition to the untargeted lung. Many diseases are restricted to segregated areas of the lung so it could be advantageous to attain high deposition concentrations in the diseased parts of the lung, while leaving the healthy tissue unaffected making uncontrolled deposition to the untargeted lung unbeneficial.

I propose a different theory: Fluorescein was not bound to the SPIONs but free in solution allowing for premature dissociation from the delivery vehicle before it reached the intended target site, causing rapid tissue biodistribution of fluorescein after reaching
the lung surface. A dry powder delivery system would overcome the limitation of a weakly interacting delivery vehicle with its drug. Encapsulation of the candidate drug into a liposome was suggested, but this option likely will result in a similar problem because liposomes inherently leak cargo (Torchilin et. al., 2005). Hasenpusch’s experiments were conducted using either a NOIS or WBIC inhalation delivery system and it is known that a large amount of delivery vehicle is lost in the nose and mouth of the animal, further observed by Hasenpusch et al.

With this study, we have shown that dry powder NIMs can be reliably administered and targeted to a specific lung in the presence of magnetic field dependent targeting in an animal model ex vivo. In addition, we have shown that NIMs, unlike other aerosol delivery systems, do not prematurely separate during the delivery process and can be targeted and retained in the lung of a rat ex vivo.
Chapter 4: *In vitro* Pulmonary Delivery and Targeting of a Magnetically Responsive Dry Powder Nano-in-Microparticles

**Abstract**

The aim of this study was to evaluate the magnetic field-dependent targeting of dry powder nano-in-microparticles (NIMs) administered endotracheally using an *in vivo* healthy rodent model. NIMs are a novel drug delivery vehicle containing 70% lactose, 20% SPIONs and either 10% fluorescent nanospheres or 1.6% doxorubicin (percentages expressed as w/w solids) (McBride et al., 2013; Tewes et al., 2014). Mice were insufflated with 2.0 mg NIMs delivery vehicle in the presence of magnetic field dependent targeting to the left lung after thoracotomy; controls were insufflated with NIMs in the absence of magnetic field dependent targeting. Quantification of deposition of the fluorescent dye component (drug surrogate) of NIMS in left and right lung tissue showed an increase in fluorescence intensity to the left lung \((1.4 \times 0.26) \times 10^8\) radiance efficiency (RE), \(n = 3\); right lung: \((0.52 \pm 0.23) \times 10^8\) RE, \(n = 3\), \(P < 0.01\), expressed in units of average relative efficiency (RE) ± SEM). Differences in mean intensity were analyzed by ratios, resulting in a significant increase in deposition of NIMS when administered endotracheally and targeted to the left lung compared to the right lung. Quantification of iron showed significantly more deposition in the left lung compared to the right lung (left lung: \(1520 \pm 579\) mg/L iron, \(n = 3\); right lung: \(186 \pm 112\) mg/L iron, \(n = 3\), \(P < 0.005\)). In contrast to dry powder NIMs, the administration of an aerosol liquid suspension of NIMs in the presence and absence of magnetic-field dependent targeting was also evaluated and did not show increased deposition in response to magnetic...
targeting to the left lung. *In vitro* cytotoxicity study results indicate that doxorubicin-containing NIMs were more toxic to A549 lung adenocarcinoma cells than the free doxorubicin control. In addition, a dose dependent increase in cytotoxicity was observed over time in lung tumor cells exposed to doxorubicin-containing-NIMs. We conclude that 1) Doxorubicin-containing NIMs are more toxic to A549 lung adenocarcinoma cells compared to free drug and 2) dry powder NIMs vehicle can be magnetically targeted significantly better as a pulmonary drug delivery strategy to specific regions of the lung compared to a NIMs liquid suspension, thus making dry powder NIMs an ideal tool for targeted inhaled drug delivery.

**Introduction**

Lung cancer is the second most commonly diagnosed cancer in both men and women, and has the highest mortality rate compared to all other cancers (World Health Organization, 2007; Siegel et al., 2014; Society, 2014). Pharmaceutical aerosols have become a field of growing interest to the field of therapeutics drug delivery (Patton and Byron, 2007; Hickey, 2013) gaining preclinical trial attention for the treatment of lung cancer (Otterson et al., 2007; 2010; Albert Einstein College of Medicine of Yeshiva University, 2014; Memorial Sloan-Kettering Cancer Center, 2014; Eleison Pharmaceuticals LLC, 2014). For example, a phase I/II study of inhaled doxorubicin combined with platinum-based therapy was administered to twenty-eight patients with advanced non-small cell lung cancer (Otterson et al., 2010). Pulmonary delivery has been used to treat local pulmonary diseases such as asthma, microbial infections, as well as systemic diseases, but its application to the treatment of cancer is also giving promising results, specifically, with the use of inhaled micro- and nanocarrier-mediated drug
targeting for cancer therapy (Tomoda et al., 2009; Roa et al., 2011). Most recently, inhaled dry powder insulin was approved by the FDA for the treatment of type 1 or 2 diabetes (Santos Cavaiola and Edelman, 2014; Traynor, 2014).

Conventional chemotherapy administered intravenously for lung cancer damages both healthy and tumor cells, and accumulates in the liver, kidney and spleen, with a lower drug endpoint concentration available in the lung milieu. Inhalable chemotherapy can reduce systemic insult to healthy cells and organs by delivering drugs directly to the lungs thus achieving adequate concentration near the tumor mass (Shim and Williams, 1980; Lewinski et al., 2008).

The lung is a unique organ for drug delivery. Lungs are well suited for administration of inhaled chemotherapeutic agents due to their large surface area, thin alveolar epithelium, easily permeable membrane and extensive vasculature, which allow substantial and fast drug absorption for local and systemic efficacy (Johnson et al., 2002; Sakagami, 2013).

Inhalational chemotherapy was first described by Shevchenko and Resnik in 1968 and tested in dogs and subsequently in 58 patients, of which antitumor efficacy was observed in 24 patients (Shevchenko and Resnik, 1968). This trial established the feasibility of administering chemotherapy by inhalation, but failed because it was as toxic to healthy cells as cancer cells. A variety of chemotherapeutic agents have been evaluated in preclinical and clinical trials including cisplatin (Hao et al., 2014), mitomycin, 5-fluorouracil (Tatsumura et al., 1993), and adjuvants (Huland et al., 2003; Yi and Wiedmann, 2010).
Although inhaled drugs have existed for over 50 years, drawbacks such as difficulties with formulation, administration, uniform dosing, compliance and stability of inhaled drugs have limited their clinical success. Delivery vehicle formulation plays an important role in producing an effective inhalable medication. Limited clinical efficacy and significant dose-limiting pulmonary toxicity have represented two critical barriers restricting progress in the therapy of advanced lung cancer (Hershey et al., 1999; Otterson et al., 2007; 2010).

Magnetic-field dependent targeted delivery of drug-containing vehicles to specific regions of the model airways in vitro has been demonstrated previously by us and others (Dames et al., 2007; Xie et al., 2010; McBride et al., 2013; Tewes et al., 2014). The aim of this study was to evaluate the magnetic-field dependent targeting of dry powder nano-in-microparticles (NIMs) administered endotracheally into the lung of healthy mice. Using a novel inhaled dry powder drug delivery, this research attempts to improve the targeting of chemotherapeutic agents relative to previous magnetic-field based delivery strategies.

**Materials and Methods**

**Materials**

Alpha-D-(+)-lactose monohydrate Respitose ML-001 was a gift from DMV-Fonterra Excipients GmbH & Co. KG (Goch, Germany). FluidMAG-UC SPIONs with a hydrodynamic diameter of 50 nm was purchased from Chemicell GmbH (Berlin, Germany). Fluorescent dye-containing NIMs were formulated with Molecular Probes® FluoSpheres® Carboxylate-Modified Nanospheres (F8783, 0.02 µm, dark red fluorescent, excitation 660 nm and emission 680 nm, Molecular Probes®, Life

**Formulation and Characterization of the Dry Powder Nano-In-Microparticles (NIMs) Delivery Vehicle**

Spray drying was used to prepare the NIMs delivery vehicle as previously described (McBride et al., 2013). Briefly, a suspension containing approximately 78.4% lactose, 20% SPIONs and 1.6% doxorubicin (Selleck Chemicals, LLC, Houston, TX, USA) (w/w) for in vitro viability and toxicity studies and 70% lactose, 20% SPIONs and 20% fluorescent nanosphere (drug surrogate) for animals studies was spray dried in double distilled water (dd H$_2$O; 2.5% solution w/v feed concentration) using a B-290 mini-spray with a standard two-fluid nozzle (0.7 mm diameter; Büchi Corporation, Flawil, Switzerland), as previously described (McBride et al, 2013). Spray drying parameters were as follows: inlet temperature 170 ± 2°C, outlet temperature 103 ± 2°C, aspirator rate 100%, and an atomization air flow rate of 742 NL/h (equivalent to a spray rate of 60 mm). Carrier control particles were formulated containing 5% lactose only (% w/v) dd H$_2$O, made into a solution and spray dried, as previously stated.

**Dry Powder NIMs Particle Sizing**

Dry powder particle size was measured by laser diffraction using a Mastersizer 3000 analyzer (Malvern Instruments Ltd, Worcestershire, UK) attached to a dry-dispersion accessory device (Aero S, Malvern Instruments). A pressure of 4 bars was used for the Venturi dispenser and a feed rate vibration of 90% was used during the measurements. The refractive index of the sample was taken to be the average of the NIMs components multiplied by the percent (w/w) contained in the formulation: 70% w/w lactose (1.35), 20% SPIONs (2.42) w/w and 10% fluorescent nanospheres (1.52) for
an average of 1.58. The results were expressed in terms of \(D_{4,3}\) (the volumetric mean diameter) and \(D_{v50}\) (volumetric mean diameter). All samples were analyzed in triplicate and are expressed as mean ± standard deviation.

**In Vitro Cytotoxicity Studies**

Human lung adenocarcinoma cancer cells (ATCC® CCL-185™) (American Type Tissue Culture, Manassas, Virginia, USA) were exposed to NIMs dry powder and its components (doxorubicin; lactose; SPIONs). These novel formulations were tested for viability using the CytoTox96® Non-Radioactive MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, Wisconsin, USA) and the CytoTox96® Non-Radioactive lactate dehydrogenase (LDH) cytotoxicity assay (Promega, Madison, Wisconsin, USA) were cultured with Ham’s F12K (Kaighn’s) medium and supplemented with fetal bovine serum (FBS), 5% L-glutamine, 3% antimycotic, and 3% antibiotic (Life Technologies, Grand Island, NY, USA).

Briefly, NIMs were dispersed in F12K non-supplemented media and FBS yielding concentration of 1000 µg/mL, 100 µg/mL, and 10 µg/mL. The same protocol was used to generate doxorubicin solutions with concentrations of 30 µg/mL, 3 µg/mL, and 0.3 µg/mL; lactose solutions concentrations of 770 µg/mL, 77 µg/mL, 7.7 µg/mL; and SPIONs solution concentrations of 200 µg/mL, 20 µg/mL, 2 µg/mL. Prior to exposure, A549 cells were washed with fresh media and 100µL of the aforementioned suspensions/solutions (NIMs; doxorubicin; lactose; SPIONs) were pipetted in triplicate for exposure periods of 1, 8, 24, 48 hours.
MTS Viability Assay

CellTiter 96® Aqueous One Solution Reagent was mixed according to the manufacturer’s instructions. In brief, at time periods of 1, 8, 24, 48 hours post-exposure, 120 µL of MTS reagent mixed with media at a ratio of 1:6 and aliquoted into each well of a black optical bottom 96-well plate and incubated at 37°C and 5% CO₂ for 3 hours to maximize the development of formazan salt. Absorbance was measured at a wavelength of 490 nm. Results were plotted as absorbance over time with respect to increasing amounts of doxorubicin contained in the NIMs (0.03 µg, 0.3 µg and 3.0 µg).

LDH Toxicity Assay

The CytoTox96® Non-Radioactive Assay was mixed according to the manufacturer’s instructions. At time periods of 1, 8, 24, 48 hours post-exposure, 80 µL of media was aspirated from each well and spun at 10000 rpm for 10 minutes to settle any SPIONs or cells. 50uL of each sample supernatant was plated into wells of a black optical bottom 96-well plate, followed by 50uL of reagent substrate. The plate was incubated in the dark at room temperature for 30 minutes. Fifty microliters of stop solution was added to each well. Absorbance was measured at 490 nm and results were plotted as absorbance over time with respect to increasing amounts of doxorubicin contained in the NIMs (0.03 µg, 0.3 µg and 3.0 µg).
Magnetic Field Dependent Targeting *In Vivo* Animal Studies

**Animals**

Male balb/c mice (6-8 weeks old; Jackson Laboratory, Sacramento, CA, USA) were used for the *in vivo* magnetic-dependent targeting studies. All rodents were housed in a temperature and light cycle controlled facility, and their care was under the guidelines of the National Institutes of Health and an approved institutional animal care and use committee protocol (IACUC protocol number 11-100747-HSC) at the University of New Mexico.

**Targeting of Dry Powder NIMs Compared to NIMs liquid suspension in Murine Lungs**

Mice were anesthetized with xylazine/ketamine by intraperitoneal injection. Dry Powder Insufflators™ are specifically developed for pulmonary drug administration to mice and variants suitable for rats, guinea pigs and larger animals exist.

A dry powder Insufflator™ for mouse (DP-4-M; Penn-Century Inc., Wyndmoor, PA, USA) was attached to a 1 ml disposable plastic syringe that was used to endotracheally administer the dry powder NIMs. The insufflator delivery tube (cannula) was inserted gently into the trachea of the animal, proximal to the carina, until the curve of the cannula was positioned approximately 5 mm from the incisors. The mouse left lung was surgically exposed by thoracotomy and a commercially available neodymium–iron–boron (NdFeB) permanent cylindrical magnet (grade N52, 22 mm long × 20 mm in diameter; Applied Magnets, Plano, TX) was centered above, with edge perpendicular to the upper section of the left lung (Fig.1A). This permanent magnet was previously characterized to have a magnetic field of 0.58 Tesla (McBride et al., 2013).
The dry powder vehicle was administered using the syringe with an air volume of 500 µl for total of 10 puffs (10 actuations). A liquid suspension delivery vehicle was administered using the MicroSprayer® aerosolizer (1A-1C; Penn-Century) for mouse and was attached to a hand-operated, high-pressure syringe (FMJ-250; Penn-Century). Mice were endotracheally intubated with the MicroSprayer® and 50 µL saline containing 2.0 mg of NIMs (w/v solids) was administered directly into the airways in the presence of the magnetic field. Control mice were treated using the same dry powder NIMs and liquid suspension regime in the absence of magnetic-targeting. To prepare the liquid suspension delivery vehicle, NIMs were dissolved in saline to prepare a liquid suspension that contained equivalent amounts of the fluorescent dye (or drug) and SPIONs. Two thousand micrograms (2.0 mg) of dry powder NIMs were mixed in 50 µl of saline to prepare a single dose suspension for in vivo administration. Mice were euthanized humanely; lungs and trachea were removed en bloc, separated (Fig. 1B), and fluorescence and iron deposition were quantified in the respective tissue.
**Figure 1: Magnetic-field-dependent lung targeting study**

A) Position of the neodymium-iron-boron magnet during magnetic-field-dependent targeting. The magnet was positioned in the upper quadrant of the left lung. B) Orientation of imaged trachea, right lung, and left lung. Accumulated SPIONs are markedly visible in trachea and left lung (black arrows).

**Fluorescence Quantification**

Dry powder NIMs and liquid suspension NIMs in the presence of magnetic field-dependent targeting were administered endotracheally with or without magnetic targeting to the left lung. Individual lung lobes and trachea were excised and imaged immediately for fluorescence measurement (excitation: 660 nm; emission: 680 nm) with an exposure time of 10 seconds using a Caliper IVIS Lumina II (Caliper Life Sciences). A region of interest (ROI) was drawn around the trachea, left lung, right lung, and background. The
ROI area was kept constant at 2.5 cm$^2$ and fluorescence expressed as units of average radiance efficiency (RE) (p/sec/cm$^2$/sr)/(µW/cm$^2$).

Targeting efficiency ratio was calculated by determining ratios of the fluorescence intensity ($F_{I \text{individual}}$) of ROIs from an individual tissue (e.g. left lung) in the numerator, divided by the sum of fluorescence intensity ($F_{I \text{sum}}$) of the three ROI areas in the denominator (Equation 1).

$$Targeting \ Efficiency \ Ratio = \frac{F_{I \text{individual}}}{F_{I \text{sum}}}$$

**Iron Quantification In Murine Lungs and Trachea**

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to measure iron (ferrous) content in mouse lungs. Briefly, tissue was digested with 0.5 ml nitric acid (HNO$_3$) at 105 °C for 1 hour and vortexed until completely dissolved (protocol modified from Niazi et al. 1993)(Niazi et al., 1993). After cooling, the samples were brought up to a known volume with 18.1 megaohm MilliQ water and vortexed. Samples were analyzed with a PerkinElmer Optima 4300 DV ICP-OES. The recommended wavelength for iron was used (Fe(II)-λ-259.939 nm). A blank and range of calibration standards were used to establish a five-point calibration curve (0.1 ppm to 25 ppm (mg/L). Endogenous iron was analyzed in untreated lungs (n = 3). Calibration and instrument verification samples (ICV – integrity check values) were incorporated before and after analyzing the samples, as well as periodically throughout the measurements.
Statistical Analysis

Statistical analyses were performed using Windows SPSS version 16 (SPSS Inc, Chicago, IL, USA). Paired t-tests with bonferroni correction were used to compare two or more groups of independent data, respectively. The significance level was set at $\alpha = 0.05$. Descriptive statistics were also employed if data were not statistically significant. A power calculation post hoc was run using the free program G*power (Dusseldorf, Germany) (Faul et al., 2007; 2009). For in vivo animal experiments with equivalent sample sizes with an effect size of $n = 3$, the power was calculated to be $> 0.95$.

Results and Discussion

Microparticles for pulmonary delivery have been previously prepared by spray drying lactose as the main bulking agent (Sham et al., 2004, McBride et al., 2013). Spray drying nanoparticles (SPIONs in our case) with a lactose matrix would decrease the residence time of NIMS in the lung tissue after inhalation due to its solubility in the lung fluid. This would allow the immediate release of the drug and potentially decrease the chance of NIMs being phagocytosed by alveolar macrophages thus enhancing the bioavailability of the drug payload (Bailey and Berkland, 2009)

Dry Powder NIMs Formulation and Characterization

NIMs were previously characterized for particle size, zeta potential, morphology, iron and drug loading (McBride et al., 2013). The particle size was measured by laser diffraction using a Mastersizer 3000 Aero S dry powder disperser and in triplicate. The average $D_{4,3}$ was 3.86 $\mu$m $\pm$ 0.24 $\mu$m and the average $D_{50}$ (volumetric median diameter) was 2.71 $\pm$ 0.04 $\mu$m, with a span of 2.25, which expresses the distribution width. These
results are fairly typical in that the D_{4,3} is greater than the D_{50} (the volume-basis median value) (Horiba Scientific, 2012). NIMs were sized for optimal pulmonary delivery in the size range 1 – 5 µm (Gerrity et al., 1979). The advantage of encapsulating SPIONs into larger micron-sized NIMs will also overcome the disadvantage of the nanoparticle being exhaled when administered by the pulmonary route due to their small size (Hickey 2014).

The doxorubicin loading was previously quantified to be 1.6% (w/w) in the NIMs using HPLC. In addition, Fe_{3}O_{4} was calculated to be 10.6% (w/w) in the NIMs using ICP-AES (McBride et al., 2013).

**Viability of A549 cells exposed to NIMs quantified by the MTS assay**

The MTS assay was used to analyze the viability of A549 lung adenocarcinoma cells when exposed to NIMs. We hypothesized that doxorubicin-containing NIMs would be as toxic to A549 cells compared to free doxorubicin alone.

Figure 2 compares tumor cells treated with free doxorubicin solution versus free doxorubicin solution, lactose alone, and SPIONs alone. The viability of A549 control cells (n = 3) was 2.468 ± 0.007 at 1 hour, 2.380 ± 0.133 at 8 hours, 2.651 ± 0.025 at 24 hours, and 2.735 ± 0.004 at 48 hours. Three doxorubicin dose levels were compared.

When A549 tumor cells exposed to 0.03 µg free doxorubicin solution were compared to cells exposed to NIMs containing 0.016 µg doxorubicin, cells exposed to NIMs (2.706 ± 0.108, n = 3) were as viable as untreated A549 cells (2.735 ± 0.004, n = 3) after 48 hours of exposure (Fig. 2A), showing similar rates of killing. A549 cells treated with free doxorubicin (2.524 ± 0.011, n = 3) were 7% less viable after 48 hours of exposure than cells exposed to NIMs or untreated A549 cells (Fig. 2A).
Figure 2. MTS viability assay of A549 cells exposed to doxorubicin-loaded-NIMs delivery vehicle MTS assay measuring viability of A549 lung adenocarcinoma cells exposed to a) 1 µg NIMs containing 0.016 µg doxorubicin compared to 0.03 µg free doxorubicin and b) 10 µg NIMs containing 0.16 µg doxorubicin compared to 0.3 µg free doxorubicin and c) 100 µg NIMs containing 1.6 µg doxorubicin compared to 3.0 µg free doxorubicin.

Concomitantly, A549 cells exposed to 0.3 µg free doxorubicin solution were compared to cells exposed to NIMs containing 0.16 µg doxorubicin. Interestingly, cell viability was reduced 17% when exposed to NIMs (0.16 µg doxorubicin; 1.743 ± 0.157, n = 3) versus free doxorubicin (0.3 µg doxorubicin; 1.439 ± 0.046, n = 3) after 48 hours of exposure, even though the NIMs vehicle contained 46.67% less doxorubicin than the doxorubicin solution (Fig 2B). With a 10-fold increase in NIMs and free doxorubicin concentration exposure at 48 hours exposure (1.6 µg doxorubicin in NIMS; 3.0 µg free doxorubicin), a slight loss in cell viability was observed for free doxorubicin over NIMs. Cell viability was significantly reduced when exposed to NIMs (1.6 µg doxorubicin;
1.155 ± 0.014, n = 3) versus free doxorubicin (0.3 µg doxorubicin; 0.908 ± 0.046, n = 3) after 48 hours of exposure (Fig. 2C). However, there was an overall reduction in cell viability in both doxorubicin alone and NIMs, compared to controls. A549 cells exposed to solutions of controls (lactose alone and SPIONs alone) were viable over the entire 48 hour experimental time course. We conclude that neither lactose alone nor SPIONs alone decreased cell viability.

At 1 and 8 hours post-exposure, the tumor cell death rate was essentially equal with both the NIMs and free doxorubicin treatments. At time points beyond 8 hours, an increase in cell death was observed for the NIMs treated cells (0.16 µg doxorubicin) compared to free doxorubicin (0.3 µg) treatment.

**Cytotoxicity of A549 Cells Exposed to NIMs Quantified by the LDH Assay**

The LDH assay was used to analyze NIMs toxicity when exposed to tumor cells (Fig. 3). LDH released from damaged cells oxidizes lactate to pyruvate, which promotes conversion of tetrazolium salt (INT) reagent to a purple formazan product, a water-soluble molecule with absorbance at 490. The amount of LDH released is proportional to the number of cells damaged or lysed (Haslam et al., 2000; Lewinski et al., 2008). We hypothesized that the doxorubicin-containing-NIMs would be as toxic as free doxorubicin alone.

Figure 3 compares tumor cells treated with free doxorubicin solution versus the doxorubicin loaded-NIMs, lactose alone, and SPIONs alone. Increased absorbance signifies low cell viability or lactate dehydrogenase leakage from the cell membrane. When A549 tumor cells exposed to 0.03 µg free doxorubicin solution were compared to cells exposed to NIMs containing 0.016 µg doxorubicin, cells exposed to NIMs (0.552 ±
0.080, n = 3) were as toxic as tumor cells treated with free doxorubicin (0.425 ± 0.002, n = 3) after 48 hours of exposure (Fig. 3A).

Likewise, when A549 tumor cells exposed higher dose of free doxorubicin solution (3.0 µg) were compared to cells exposed to NIMs containing (1.6 µg doxorubicin) (1 µg NIMs containing 1.6% doxorubicin), cells exposed to NIMs (1.146 ± 0.045, n = 3) were as toxic as tumor cells treated with free doxorubicin (1.046 ± 0.068, n = 3) after 48 hours of exposure (Fig. 3C). Having shown that NIMs express more toxicity than the free doxorubicin control, despite containing 46.7% less doxorubicin, we attribute the increased toxicity of NIMs to be a synergistic effect of increased cellular uptake of
SPIONs and ROS production, coupled with greater cytotoxic effects of doxorubicin (Jones et al., 2011). SPIONs alone were found to be non-toxic to A549 cells. While bare iron oxide nanoparticles exert some toxic effects, coated SPIONs have been found to be nontoxic (Shim and Williams, 1980; Lewinski et al., 2008). SPIONs are composed of simple iron oxides that are biodegradable and their safety has been previously demonstrated by being used as diagnostic agents in humans in imaging of the lung (Stahlhofen and Möller 1993, Polyakt and Friedman 2009).

**Magnetic-Field-Dependent Targeting for Particle Deposition Visualization**

Dry powder NIMs containing fluorescent nanospheres were endotracheally insufflated in the presence of magnetic field dependent targeting to the left lungs of mice. Fluorescence associated with NIMs was detected in the mouse trachea, left lung and right lung.

A proof of concept magnetic-field dependent targeting study was conducted to optimize dry powder pulmonary delivery, and to show the feasibility of NIMs targeting in a healthy *ex vivo* rat model. Although the data was non-significant (P = 0.057), this study showed feasibility of magnetic field dependent targeting of the NIMs delivery vehicle to one lung over the other. Dry powder NIMs were targeted 3.9 fold more to the right lung than the left, and provided insight into dry powder NIMs formulation optimization, as well as optimization of the use and dispersion and delivery of dry powders from the DP-4R insufflator device.

In control animals that were not administered NIMs, both lungs exhibited little to no background fluorescence intensity (1.69 x 10^6 ± 2.77 x 10^5 RE). In the presence of
magnetic field dependent targeting with NIMs, significantly higher fluorescence units of radiance efficiency was detected in the left lung \((1.42 \pm 0.26) \times 10^8 \) RE; \((5.2 \pm 2.3) \times 10^7 \) RE in the right lung, \(P < 0.01\) as shown in Figure 4D, or 2.7-fold more deposition to the left lung than the right. We achieved slightly better targeting than Dames et al., 2007 who observed a two-fold higher dose of pDNA in the magnetized right lung than the unmagnetized left lobe. When NIMs were administered to the lungs in the absence of magnetic field dependent targeting, intensity averages were similar in both the left and right lung \((6.12 \pm 3.0) \times 10^7 \) RE; right lung: \((5.79 \pm 2.4) \times 10^7 \) RE.

Significantly more fluorescence was quantified in the trachea for dry powder NIMs than liquid suspension. We attribute this pitfall to the DP-4M insufflator delivery tool. Hoppentocht et al. recently published data stating that the recommended 200 µL air volume did not provide adequate dispersion or emitted dose to administer dry powders in mice lungs (Johnson et al., 2002; Huland et al., 2003; Yi and Wiedmann, 2010; Sakagami, 2013; Hoppentocht et al., 2014). We confirmed this observation and showed that a larger pulsed air volume of 500 µL was required for adequate NIMs dispersion in the lung. However, a higher air volume could have led to significant powder deposition in the trachea (Fig 4 B, D). The insufflator deposits the dry powder in the respiratory tract based on firm plunging of the syringe, which is necessary for the proper aerosolization and delivery of NIMs into the lung (Morello et al. 2009). The high velocity air stream generated by the syringe (attached to the insufflator) would lead to powder deposition in the upper conducting airways and the main tracheal bifurcation due to inertial impaction. Dry powder NIMs exiting the insufflator delivery tube had enough momentum to follow its trajectory until it collided with the tracheal wall (Mortensen et al. 2014). However,
NIMs that did not impact on the upper respiratory tract were available for magnetic targeting deeper into the lung lobes as shown in Figure 4D. We believe that using an existing passive dry powder inhaler that will allow slow and deep inspiration would mitigate the upper respiratory tract deposition of our NIMs.

We relied on information from the Penn Century manufacturer with respect to the emission velocity of the liquid suspension and the dry powder, but did not test this. The syringe volume (1 ml) and force used to dispense the liquid or powder from the instrument were maintained during the experiment.

Neither an increase nor decrease in the mass of the dose administered had a profound effect on the dispersion efficiency. An average of 2.0 mg NIMs (w/w solids) was loaded into the insufflator; based on gravimetric analysis, an average of 0.4 – 0.6 mg dry powder NIMs was administered. This suggests that limitations of the dose should not be based entirely on weight, but rather on the basis of powder properties and air volume for optimal powder dispersion and deposition. Therefore, it is necessary to optimize dispersion efficiency of the dry powders before using the insufflator.
Figure 4: Effect of magnetic-field-dependent targeting by fluorescence quantification of in murine lungs

A) Control, no treatment lungs show no fluorescence background (n = 1). The trachea is oriented in upper half of image; right lung is oriented on left side of image; left lung is oriented on right side of image. B) Dry powder NIMs vehicle and the liquid suspension vehicle in the absence of magnetic-field-dependent
targeting (n = 3). C) Dry powder NIMs vehicle in the presence of magnetic-field-dependent targeting (n = 3). D) Liquid suspension vehicle in the presence of magnetic-field-dependent targeting (n = 3).

Figure 5: Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension

A) Comparison of fluorescent dye in dry powder NIMs and liquid suspension in left and right lungs (targeted to left lung) in the presence of magnetic-field-dependence targeting and B) in the absence of magnetic-field-dependent targeting. C) Fluorescence differential between vehicles in the presence and absence of targeting. D) Fluorescence quantified in
left lung of NIMs (presence and absence of targeting) and liquid suspension (presence and absence of targeting).

**Effect of Magnetic-Dependent Targeting As Iron Quantification Using ICP-OES**

Inductively coupled plasma optical emission spectroscopy (ICP-OES) is an analytical tool used to detect trace metals and is also commonly used for iron oxide nanoparticle quantification (Taylor et al, 2014; Chertok et al, 2010; Cole et al, 2011; Sun et al, 2006; Moroz et al 2002; Huang et al, 2009; Wuang et al, 2006, Xie et al, 2008; Simbert et al, 2007; Graczyk et al, 2014). Our goal was to use this technique to accurately quantify nanoparticle iron in the lung and tracheal tissue after magnetic-dependent targeting of the NIMs or liquid suspension vehicle.

One drawback of ICP-OES is its inability to distinguish between endogenous and exogenous sources of iron. We compensated for this shortcoming by quantifying and subtracting endogenous iron in untreated left and right lung tissues, as well as tracheal tissue (n = 3). All data are presented as mean ± SEM unless otherwise noted. The standard curve had an $R^2$ value of 0.99975. The left lung was found to have an average iron concentration of $156.0 \pm 3.1 \text{ mg/kg}$, the right lung $135.0 \pm 16.3 \text{ mg/kg}$ iron, and the trachea $88.2 \pm 11.4 \text{ mg/kg}$ iron.
Figure 6: Iron accumulation and effect of magnetic-field-dependent targeting:
corroboration of iron and dye components from the delivery vehicle A) Accumulation of iron measured by ICP-OES to the left/right lung and trachea, targeted with NIMs vehicle. B) Accumulation of iron measured by ICP-OES to the left/right lung and trachea, targeted with the liquid suspension vehicle. C) Comparison of differences: fluorescence and iron to the left lung in the presence and absence of magnetic targeting and administration of NIMs dry powder vehicle. D) Comparison of differences: fluorescence and iron to the left lung in the presence and absence of magnetic targeting and administration of the liquid suspension.
In the case of targeted NIMs to the left lung, more iron was quantified in the left lung \((1520 \pm 579 \text{ mg/kg})\) than the right lung \((186 \pm 112 \text{ mg/kg})\) \((P < 0.005, n = 3)\), an 8.2 fold more difference, showing corroborative evidence that dry powder NIMs were targeted to the left lung over the right lung. In contrast, iron quantified in the left and right lungs with liquid suspension targeting did not show significant targeting \((\text{left lung } 394 \pm 141 \text{ mg/kg}, \text{right lung } 232 \pm 53 \text{ mg/kg})\). In the absence of magnetic targeting, variable amounts of iron were found in the left and right lungs with both the dry powder NIMs and liquid suspension vehicles.

**Corroboration of Targeting By Comparing Targeting Efficiency Ratios**

Furthermore, targeting efficiency was calculated by taking the ratios of intensity quantified by fluorescence and by ICP-OES in the left lung, right lung, and trachea, respectively, and divided by either the sum of the total fluorescence intensity or intensity given by ICP-OES of the three ROI areas. Quantification of fluorescence or iron was analyzed by comparing fluorescence or iron targeting efficiency ratios (e.g. iron/fluorescence in left lung divided by the sum of fluorescence/iron in left lung, right lung and trachea). The key issue was to determine whether a ratio was significantly high or low in order to conclude whether magnetic field dependent targeting was increased or decreased to the left lung, significant to the untargeted right lung.

Corroborating fluorescence and iron data, similar outcomes are seen when comparing the differences between left and right lung of NIMs in the presence and absence of magnetic targeting (fluorescence accumulation of NIMs, average differential of fluorescence in left and right lung in the presence of targeting \(30\% \pm 6\%\); iron accumulation of NIMs, average differential of iron in left and right lung in the presence
of targeting 30% ± 5%) (Figure 6C). In the absence of magnetic targeting, iron and fluorescence data corroborate each other (fluorescence accumulation of NIMs, average differential of fluorescence in left and right lung in the absence of targeting 1% ± 9% to left lung; iron accumulation of NIMs, average differential of iron in left and right lung in the absence of targeting 1% ± 2% to right lung) (Figure 6C).

Significantly more NIMs (61% ± 7%) than liquid suspension (28% ± 3%) were deposited in the trachea. Stopping or inertial force (F_s) is constantly acting on all particles (NIMs and liquid suspension) in a field of gravity, which results in a stationary particle settling velocity (U_{TS}) leading to particle contact with airway walls by sedimentation (Hershey et al., 1999; Frijlink and de Boer, 2004; Faul et al., 2007; Otterson et al., 2007; Faul et al., 2009; Otterson et al., 2010). We propose that both dry powder NIMs and liquid suspension impinge on the tracheal walls, but the liquid suspension continues to move down the trachea, therefore less iron accumulation was observed.

Although magnetic targeting of drugs using NIMs could be used for many diseases impacting the lung, localized tumors stands to benefit most significantly from this targeting mechanism and will be the focus of our future studies. We will be targeting our dry powder NIMs in an orthotropic lung cancer mice model developed in our laboratory to demonstrate NIMs ability to cause tumor regression when delivered by the pulmonary route.
Conclusion

Combination chemotherapy for lung cancer by the pulmonary route has shown promise in a Phase I/II study, however, its effectiveness was limited due to potential pulmonary dose-limiting toxicity to healthy tissues. Therefore, the ultimate goal of this targeted drug delivery vehicle is to carry high drug payloads of one or more chemotherapeutic agents to specific regions of the lung and minimize toxicity to healthy tissues that are not affected by cancer. Delivery of magnetically responsive NIMs to specific regions of the lung will be advantageous for the treatment of non-metastasized solid lung tumors, and this work provides the foundation for targeted drug delivery in rodent models of lung cancer.

Acknowledgements

We acknowledge Dr. Mehdi Ali of the Earth and Planetary Sciences Department at UNM for his technical assistance with ICP-OES, and Dr. Denis Wafula for his careful eye with revisions.
Chapter 5: A longitudinal, orthotopic mouse model using modified A549-hNIS human adenocarcinoma cells and SPECT/CT imaging

Abstract

We have developed a murine lung cancer imaging model by subcutaneous xenograft and orthotopic lung implantation of human lung adenocarcinoma cells (A549) that were genetically modified with the human sodium iodide symporter gene (hNIS; SLC5A5) for the purpose of tumor imaging. Lung tumor growth was quantified using single-photon emission computer tomography (SPECT) and computed tomography (CT) imaging. The radiotracer Tc-99m pertechnetate ($^{99m}$TcO$_4^-$) was selected for its short half-life (6 hours) and rapid clearance from the body. hNIS is a membrane glycoprotein that normally transports iodide (I$^-$) in the thyroid, and has the ability to symport the I$^-$ analog $^{99m}$TcO$_4^-$ Here, we have exploited the hNIS mechanism through genetic modification of lung cancer cells to sequester the radiotracer $^{99m}$TcO$_4^-$ in a lung tumor mouse model for the purpose of longitudinal SPECT/CT imaging.

Methods: A549 cells were genetically modified with plasmid and lentiviral vector constructs to stably express hNIS. Modified tumor cells were then implanted into nude mice to develop two tumor models: the subcutaneous xenograft model and the orthotopic xenograft tumor model. Tumor progression was longitudinally imaged by SPECT/CT, and quantified by SPECT voxel analysis. hNIS expression in lung tumors was quantified using quantitative PCR (qPCR). In addition, hematoxylin and eosin staining and visual inspection of pulmonary tumors were also performed.

Results: Orthotopic tumors (n = 6) induced by the A549-hNIS-lentiviral vector cell line exhibited a logarithmic increase in SPECT scintillation over orthotopic tumors induced
by the A549 hNIS-plasmid vector cell line (n = 6) and A549 unmodified control cells in vitro and in the tumor bearing mice models. No significant uptake was observed in cells infected with the plasmid DNA vector expressing hNIS. Orthotopic implantation of tumor cells, injected between the 5th and 6th intercostal rib space of the left lung achieved a 100% orthotopic tumor take rate showing the replicative precision of this model.

**Conclusion:** We developed a highly sensitive spatial and temporal lung tumor-imaging model. Through stable incorporation of the hNIS gene in A549 cells, a tumor model was developed and imaged longitudinally allowing for SPECT/CT imaging and precise tumor burden quantification at multiple time points across the study. This tumor model could significantly improve the quality of imaging data by avoiding inter-animal variation, reducing imaging invasiveness, and allowing long-term longitudinal monitoring of the same animal treated with new anti-cancer drugs. Ultimately, this model allows each animal to be used as its own control leading to improved study significance and a reduction in study animals.
Introduction

Animal tumor models are the preclinical standard for the evaluation of potentially useful cancer chemotherapeutic agents; however, drawbacks exist in animal models (Curt, 1994). Some of these drawbacks include the use of large cohorts of animals, variability in tumor size between animals, and the inability to precisely follow tumor growth and metastasis (Olive and Tuveson, 2006; Phalen et al., 2008).

Genes encoding for the transport of membrane proteins have emerged as an innovative strategy for non-invasive visualization of longitudinal tumor growth in preclinical animal models. Reporter gene imaging is based on vector-mediated overexpression of a transgene in host tissue which encodes for a protein that is normally not present in the target area (Miyagawa et al., 2005; Riesco-Eizaguirre and Santisteban, 2006). The feasibility of noninvasive imaging using PET or SPECT/CT and radiolabeled reporter probes has been demonstrated in small animals (Inubushi et al., 2003) and in large animals (Bengel et al., 2003; Dobrucki and Sinusas, 2009).

Xenograft tumor models are most commonly used in immunodeficient mice (Olive and Tuveson, 2006) and multiple types of tumor instillation methodologies exist. Xenograft models can be initiated through the subcutaneous or intravenous injection of tumor cells from cell culture or tissue transplantation. Xenograft transplants may also be orthotopic, meaning that the tumor is placed in the site where it would be expected to arise naturally in the host: human lung cancer cells placed in the mouse lung. Orthotopically implanted lung xenograft tumor models can be initiated through surgical transplantation of a small tumor mass or by injection of tumor cells from cell culture through intravenous injection, intratracheal injection or by surgical implantation. We
have established an orthotopic tumor model which simulates the clinical and pathological features of human lung cancer, similar to other published literature (Yamaura et al., 2000; March et al., 2001; Kang et al., 2010; Li et al., 2011).

The hNIS is a plasma membrane glycoprotein that mediates active iodine uptake in tissues such as thyroid, salivary glands, gastric mucosa, and lactating mammary glands (Tazebay et al., 2000). hNIS-mediated iodide uptake is an active transport process that occurs against the electrochemical gradient in negatively charged iodine anions (Fig. 1) (Chung, 2002; Dohán et al., 2003). Here, we propose the implantation of hNIS modified A549 human lung cancer cells into immune compromised mice (athymic nude nu/nu mice) to develop a longitudinal lung cancer animal-imaging model (Fig. 1). We show the development of an innovative preclinical tumor animal model using A549 lung adenocarcinoma cancer cells that were modified to express the human sodium iodide (Na⁺I⁻) symporter (hNIS) for reporter gene imaging. These cells enabled the development of subcutaneous and orthotopic xenograft lung cancer animal models and their tumor burden radioimaging, for the ultimate purpose of novel chemotherapeutic evaluation and assessment of tumor response.
Figure 1: Schematic of the vector-mediated overexpression of a transgene in a host cell encoding for a protein that is normally not present in the host cell. Plasmid DNA and lentiviral vectors expressing the hNIS gene were transfected or transduced to genetically modify A549 cells.

We have constructed plasmid DNA and lentiviral vectors expressing the hNIS gene. These vectors were transfected or transduced to genetically modified A549 cells. Cells were characterized for hNIS expression and implanted as a either a subcutaneous xenograft or orthotopic xenograft model to the lung. Resulting tumors were imaged using SPECT/CT and algorithmically quantified. We hypothesize that lung cancer cells expressing the hNIS gene will allow selective radioimaging of vector-modified cells in an orthotopic lung cancer animal model, thus providing a preclinical in vivo model to evaluate the efficacy of novel chemotherapeutic formulations.
Materials and Methods

Cell lines

The human epithelial carcinoma cell line CCL-185™ A549 was purchased from American Type Culture Collection (ATCC, Manassas, VA). A549 cells and genetically modified cells were cultured in F-12K nutrient mixture (kaighn’s modification) 1x media from Invitrogen (F-12K) (Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin from Invitrogen (Grand Island, NY). Cells were maintained at 37°C under an atmosphere of 5% CO₂.

Creation of the plasmid DNA vector and cell transfection

The A549-pDNA cell line was generated encoding the hNIS gene. Primers were designed based on the cDNA sequence SLC5A5 (Entrez Nucleotide, National Center for Biotechnology Information, Bethesda, MD) to form a 1932 basepair insert into the multiple cloning site of the pIRES2DsRedExpress vector. This modified vector was then transfected into CCL-185™ A549 cells using the FuGene 6® reagent (Promega, Madison, WI) according to the manufacturer’s protocol. Modified cells were plated in serial dilution resulting in a cell density of 10⁶ to 10 in 10 mm plates. Cells were treated for selection with 500 µg/ml of G418 Sulfate antibiotic (VWR, Radnor, PA, USA) for 2 weeks. Clones were screened for ⁹⁹mTcO₄⁻ activity. Those cells with highest activity were chosen for subsequent characterization studies.
Creation of the lentiviral vector and cell transduction

The A549-hNIS lentiviral cell line was generated using a lentiviral vector that encodes the sodium iodide gene driven by a household gene (PGK) promoter or strong viral (SF) promoter, both being eGFP-positive. The lentivirus was produced in 293T cells; A549 cells were then infected with the virus-containing medium. After infection, fresh F-12K medium was added to allow cells to recover for 24 to 48 h. Clones were screened for eGFP activity by flow cytometry and those with the highest activity were chosen for follow up in vivo studies.

In vitro hNIS –mediated Radioactive $^{99m}$TcO$_4^-$ uptake assay

A549 lung adenocarcinoma cells stably expressing the plasmid hNIS vector were plated 24 hours before the start of the assay at a cell density of 1 x $10^6$/well in a 12 well plate. One hour prior to the start of the uptake assay, cells were counted by hemocytometer using 0.4% solution of trypan blue (HyClone™ Trypan Blue Stain, GE Healthcare Life Sciences, Logan, Utah, USA) and cell counts were recorded. Cells were treated with 1.0 microcurie of $^{99m}$TcO$_4^-$ (Cardinal Health, Albuquerque, NM) for 1 hour at 37°C with 5% CO$_2$. Cells were washed twice with cold PBS (Invitrogen, Grand Island, NY). One mL of 20mM sodium acetate (Sigma-Aldrich Corporation, St. Louis, MO) in PBS was added to each well, incubated at room temperature for ten minutes and supernatant removed. Then 1 mL of 0.1 M NaOH (Sigma-Aldrich Corporation, St. Louis, MO) was added to each well to de-adhere and lyse cells. The cell lysate was collected and radioactivity was counted by a gamma counter (Wallac Wizard 1470-005, Perkin Elmer). For each sample, $^{99m}$TcO$_4^-$ activity (counts) emitted was divided by the number of cells in each well to normalize data. Experiments were performed in triplicate. Cells
were screen for $^{99m}$TcO$_4^-$ activity. Those cells with highest activity were chosen for subsequent *in vivo* studies.

**Animals**

Male, 6-week old, athymic nude mice (Nu/Nu) (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were housed and maintained in specific pathogen-free conditions in a facility approved by the American Association for Accreditation of Laboratory Animal Care under National Institutes of Health Guidelines. Food and water were provided *ad libitum* to the animals in standard cages. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee, at the University of New Mexico under IACUC protocol number HSC100853.

**Implantation and development of subcutaneous xenograft lung tumors**

Modified A549-hNIS cells (3 x 10$^6$ cells in 100 µl) were suspended in 1:1 F 12K media and matrigel (BD Biosciences, San Jose, CA, USA) per injection site. Only cell suspensions with >90% viability determined by trypan blue exclusion was used to inject the cell inoculum. Mice were anesthetized by continuous flow of 3% isofluorane and cells were injected subcutaneously (s.c; 25 gauge, 5/8’’ needle). Each animal received 4 tumor injections; one each in the a) left anterior lateral thoracic wall b) right anterior lateral thoracic wall c) left posterior lateral aspect of the flank d) right posterior lateral aspect of the flank (Kyriazis and Kyriazis, 1980). Tumor growth was monitored with a digital caliper, and their volume was estimated according to the following formula: $V = (S^2 \times L)/2$, in which S and L are the smaller and larger measures of the tumor, respectively (Tomayko and Reynolds, 1989)(data not shown, for monitoring purposes). Animals were observed for 45 to 60 minutes after tumor inoculation, until fully
recovered.

**Implantation and development of orthotopic xenograft lung tumors**

Modified A549-hNIS cells (3 x 10^6 cells in 100 µl) were suspended in 1:1 F 12K media and matrigel per injection site. Mice were anesthetized by continuous flow of 3% isofluorane (2.5% LPM oxygen), shaved, and a 5 mm skin incision was made to the left chest, ~ 5 mm below the scapula. A needle (25 gauge) containing cells was quickly, at an angle of approximately 45°, advanced to a depth of 5 mm in the mediastinum of the left lung between the 5th and 6th intercostal rib space (protocol modified from Ichite et al. (Ichite et al., 2009) and Saha, et al. (Saha et al., 2010)). Animals were observed for 45 to 60 minutes after tumor inoculation until fully recovered.

**SPECT/CT imaging**

Tumors were imaged using single-photon emission computed tomography (SPECT) and computed tomography (CT) (NanoSPECT/CT, Mediso USA, LLC, Boston, MA). Mice were anesthetized with 3% isofluorane (2.5% LPM oxygen) in an anesthesia induction chamber and injected by tail vein with 1 millicurie of $^{99mTc}$O$_4^-$. Mice were placed on a heated bed (37°C) for 30 minutes of SPECT imaging followed by 15 minutes of CT imaging. Mice remained anesthetized at 2% isofluorane (2.5% LPM oxygen) during the entire imaging process and were monitored to ensure proper recovery from the anesthesia.

**Tumor histology**

Lungs and trachea bearing tumors were removed en bloc. During lung fixation, it was important to prevent lung collapse, deflation, and disruption of lung structures and to
avoid fixation artifacts, which could lead to alveolar wall thickening, hypercellularity, change in shape or volume, and blood in the lungs (van Kuppevelt et al., 2000). Therefore, a canula was inserted into the trachea and fixed with a ligature. The lungs were fixed by gentle infusion of the fixative (10% formalin) through the cannula by continuous release of fixative under pressure for 20 minutes (Braber and Verheijden, 2010). The final fixative pressure was defined as the height of the fixative reservoir above the surface of the fixative in which the lungs were immersed. Tissues were stored in 10% formalin until processing, and then embedded in paraffin. Seven-micron sections were sliced in a sagittal plane and stained with hematoxylin and eosin. Tumor samples were analyzed for histological changes including the presence of tumor necrosis, apoptosis, mitotic activity and cytologic atypia.

**Analysis of SPECT/CT and data processing**

An automated library-based segmentation routine robust to high-density lung tissue was used to segment the lungs from the CT image data, with manual QC and intervention as necessary. For the SPECT and CT data, separately, the Otsu method (Otsu, 1979) was applied within segmented lung regions of interest to calculate a tumor threshold. Lung voxels where intensity exceeded the threshold were classified as tumor voxels. The final tumor region was defined as the intersection of the modality-specific tumor regions.

**Statistical Analysis**

Data were presented as the mean and 95% CI, and the statistical significance of differences in mean values was assessed by a one-way ANOVA and Dunnett’s multiple comparisons post hoc test. The differences in radioactivity uptake (expressed in units of
percent injected does of per gram of tissue (%ID/g)) were considered significant at values of $P < 0.05$.

**Results**

**In vitro expression of hNIS in modified cell lines**

Plasmid and lentiviral-vector mediated hNIS expression was characterized for stable integration in A549 cells. An *in vitro* hNIS–mediated radioactive $^{99m}$TcO$_4^-$ uptake assay was used to characterize $^{99m}$TcO$_4^-$ uptake of A549-pDNA vector cells lines. $^{99m}$Tc uptake analysis of transiently transfected A549 cells (unstable incorporation of hNIS into the A549 cell genome) compared to A549 cells (untransfected cells) showed 124-fold higher $^{99m}$TcO$_4^-$ uptake in transiently transfected A549 cells, and was used as positive control of hNIS expression. Untransfected A549 cells showed no hNIS activity. Thirty-four cell lines were characterized for stable hNIS incorporation. Of those, two cell lines were chosen (named A549-pDNA clone 1 and A549-pDNA clone 2; abbreviations used going forth) for increased hNIS expression and $^{99m}$TcO$_4^-$ uptake activity (Fig 1C), and further used for *in vivo* studies.

Lentiviral vectors using two different promoters were also used to infect A549 cells. Flow cytometric analysis showed that for the housekeeping gene (PGK) as well as for the strong viral (SF) promoter, all cells were positive for eGFP. The SF promoter results in a 4-fold stronger mean fluorescence intensity signal compared to the housekeeping gene promoter (Figure 1A). Flow cytometric analysis showed 30.6% hNIS positive cells for household gene (PGK) promoter and 73.0% hNIS positive cells for the SF promoter. The SF promoter further resulted in a 2.9-fold stronger MFI signal than the household gene promoter (Fig. 1B) therefore the lentiviral vector cells containing the SF
promoter (named A549-LV clone, and abbreviation used going forth) were used for in vivo studies.

Quantitative PCR (qPCR) was used to compare hNIS expression of A549-pDNA clone 1, A549-pDNA clone 2, and A549-LV clone relative to A549 vehicle absent of the hNIS gene. A549-LV vector-mediated clone expressed 2067-fold more gene expression than vehicle. A549-pDNA vector-mediated clone 1 and A549-pDNA clone 2 expressed 14-fold and 21-fold more hNIS than A549 vehicle (Fig 1D). Based on qPCR results, we hypothesized that the A549-lentiviral vector using the SF promoter would best sequester $^{99m}$TcO$_4^-$ in lung cancer cells in an in vivo tumor model.

Figure 2: Characterization of lentiviral and pDNA vector-mediated hNIS expression in A549 lung cancer cells. A) PGK and SF promoter lentiviral clones characterized by
eGFP percent positive and mean fluorescence intensity B) hNIS expression characterization of PGK and SF promoter lentiviral clones C) pDNA vector clones characterized by *in vitro* uptake of $^{99m}$TcO$_4^-$ D) Comparison of hNIS expression of mRNA in lentiviral and pDNA vector-mediated cells.

**SPECT/CT imaging of subcutaneous xenograft tumors and hNIS expression**

Tumors were allowed to grow for a total of 47 days and imaged by SPECT and CT at two time points: 18 days (time point 1) and 47 days (time point 2). Phantom images show orientation of s.c. xenograft tumor placement and overall tumor growth, qualitatively determined by CT imaging (Fig. 3A). At time point 1, only the A549-LV tumor was observed (Fig. 3B). Again, at time point 2, only the A549-LV tumor was observed (Fig. 3C) even though A549 control, A549-pDNA clone 1, A549-pDNA clone 2 and A549-LV tumors were quantifiably present both visually and by CT (Fig. 4A). After sacrifice at time point 2, radioactivity was determined in the tumor tissue by SPECT voxel analysis (Fig. 3B) and showed significantly more activity in the A549-LV tumor compared to the A549 control tumor, and A549-pDNA clone 1 and 2 tumors ($p < 0.01, n = 6$) even though the tumor size was an average of 200 mm$^3$ for all tumors (Fig. 4A).
Figure 3. Subcutaneous xenograft tumor quantification A) Orientation of xenograft tumors as shown by phantoms images on a CT image: upper L: A549 control, lower L: A549-lentiviral clone, upper R: A549-plasmid clone 1 and upper L: A549-plasmid DNA clone 2 B) SPECT/CT first imaging time point (day 18 tumor growth) (n = 6) C) SPECT/CT second imaging time point (day 47 tumor growth) (n = 7; representative image)
Figure 4: SPECT analysis of subcutaneous injected xenograft tumors at the end of the study (day 47) A) Tumor volume quantification using CT analysis. B) Percent injected dose $^{99m}$TcO$_4^-$ per gram of tumor tissue. C) mRNA hNIS expression of tumors. One-way ANOVA and Dunnett’s multiple comparisons post hoc test ($p < 0.01$, $n = 6$), error bars graphed as mean with 95% CI.
Quantification of hNIS expression by qPCR

We further corroborated hNIS expression by qPCR of mRNA from excised tumors (Fig. 4C). Interestingly, A549-pDNA tumors had very low levels of hNIS expression. A549-LV tumors expressed an average of 4.8 fold more hNIS than A549-pDNA tumor 1, and 27 fold more hNIS expression than A549-pDNA tumor 2.

SPECT/CT imaging of orthotopically-induced xenograft tumors expressing hNIS

Like the s.c. xenograft tumor model, orthotopically-induced xenograft tumors were imaged by SPECT/CT at 2 time points (day 18 and 36). SPECT images show minimal radioactivity for A549-LV tumors at imaging time point 1 (Fig. 5 A, upper right panel). A significant increase in radioactivity is seen at time point 2 (Fig. 5 B upper right panel). No radioactivity is observed for A549-pDNA tumors 1 or 2 at either imaging time point even though CT detected similar tumor volume among all groups (Fig. 5C). We observed a 25 fold increase in $^{99m}$TcO$_4^-$ radioactivity (% ID/g) in the A549-LV tumor compared to other tumor groups (Fig. 5D). When quantifying hNIS expression of mRNA from excised tumor tissue, the A549-LV tumor group exhibited a 13.8 fold higher hNIS expression over the A549-pDNA tumor group (data not shown).
Figure 5: Tumor scintillation of orthotopically-injected xenograft tumors compared to A) Tumor volume quantification on day 18: A549 control, lentiviral-A549, plasmid DNA-A549 clone 1 and plasmid DNA-A549 clone 2. B) Orthotopic xenograft tumor volume quantification on day 36. C) Comparison of tumor growth between saline control, A549 control, A49-pDNA clones and lentiviral-A549 clone groups, as measured by CT. D) Percent injected dose $^{99m}$TcO$_4^-$ per gram tumor tissue for all animal groups day 36.

**** p < 0.0001, error bars graphed as mean 95% CI.
Primary tumors in mice confirmed by histological analysis

To confirm SPECT/CT detection of tumor burden and the presence of tumor tissue in mice, we performed histological analysis of H&E stained tissues. Tumor tissue induced by s.c. xenograft transplantation of A549-pDNA and A549-LV modified cells lines showed areas of invasive adenocarcinoma tissue growing nests of cells of solid pattern (Fig. 6 A-D). No metastasis was found in mice with s.c. xenograft transplantation.

Tumor tissue induced by orthotopic xenograft transplantation to the lung using A549-pDNA vector and A549-LV vector-modified cell lines showed primary tumors in the left lung (Fig. 7 A – C). We saw widespread nodules and tumors preferentially invading and extensively disseminated along the pleura. Metastases were observed in the pleural cavity as well as the right lung. No metastases were observed in any other organs of the mouse. Purulent neutrophilic exudate fluid was also observed in the pleura due to inflammation and was creamy and yellow in appearance compared to normal thin serous fluid.
Figure 6: Representative H&E histological images of xenograft tumors of A) A549 controls; no genetic modification. B) A549 – pDNA clone 1  C) A549 – pDNA clone 2 D) A549 – Lentivirus clone. Montage images are 4x magnification. Inset images are 40x magnification (n = 3).
Figure 7: Histopathological analysis of orthotopically-induced tumor tissue

A) Representative H&E montage images depicting primary tumors (4x magnification) of saline control, A549 control, A549-pDNA clone 1, A549-pDNA clone 2, and A549-LV clone. B) Inset images comparing tumor and normal tissue (40x magnification) C) Dissected specimen showing solitary nodule (as shown by arrow) n = 6 per group.
Discussion

Animal models are the preclinical standard for the evaluation of potentially useful cancer chemotherapeutic agents. These models are essential for understanding the fates, effects, pharmacokinetic and pharmacodynamic interactions of novel drugs. Although drugs may be metabolized differently in animal models and humans, these data provide a baseline to assess drug efficacy in a preclinical animal model. Our hypothesis was that an innovative preclinical model could be developed using modified A549 human lung adenocarcinoma cells expressing the hNIS gene to enable non-invasive, longitudinal imaging of tumor burden. For this purpose, we constructed and characterized genetically modified lung cancer cell lines expressing the human hNIS gene and developed subcutaneous and orthotopic implanted xenograft tumors that could be longitudinally imaged by SPECT/CT to evaluate tumor growth. We took advantage of the hNIS mechanism to concentrate a radiotracer to tumor-specific regions and showed that hNIS-A549 modified cells sequestered \(^{99m}\text{TcO}_4^-\) in the orthotopically-induced xenograft lung cancer animal model.

The major finding of this work was that the significantly more hNIS expression and \(^{99m}\text{TcO}_4^-\) uptake was observed in A549-lentiviral-vector-mediated tumors in subcutaneous xenograft and orthotopically-implanted xenograft tumor models. To track the uptake of the radiiodine tracer \(^{99m}\text{TcO}_4^-\) by the human hNIS-modified tumor, SPECT/CT was used to longitudinally image tumors over a 47-day period. Tumors were imaged at two time points, at 18 days and 42 days growth, and showed significant growth in tumor volume based on injected dose of \(^{99m}\text{TcO}_4^-\) per gram of tissue. Orthotopically-
induced xenograft A549-LV tumors expressed $^{99m}$TcO$_4^-$ activity by 200 fold more than A549 control tumors. These findings are significant because they illustrate a new methodology that allows for precise tumor growth monitoring in a single mouse over multiple imaging time points without sacrificing animals throughout the study.

The second major finding of this study is that using algorithmic modeling of SPECT scintillation, tumor volume was empirically quantified to determine precise tumor burden compared to bio-distribution of $^{99m}$TcO$_4^-$ and H&E analysis by traditional animal sacrificing. The longitudinal imaging of tumor burden in this animal model will overcome drawbacks of having more animals to be sacrificed at multiple time points to evaluate tumor growth. In addition, this animal model will allow for precise tumor burden quantification over multiple imaging time points.

S.c. xenograft tumors in nude mice are the most common method of transplanting human tumor material. Recently some authors have questioned the accuracy of xenograft tumor data when applied to human drug trials (Syrigos et al., 2006). Orthotopically-induced xenograft models have advantages over s.c. xenograft models such as improved tumor take rates and enhanced invasive and metastatic properties, which are especially important for lung cancer because the organ-specific site provides tumor cells with the most appropriate milieu to evaluate the efficacy of novel therapeutics.

The SPECT/CT imaging method is advantageous over bioluminescence imaging; specifically, its signal is not limited by depth of tumor in the animal and it is better suited for identifying 3D localization of tumors (Deroose et al., 2007). Also, voxel number and intensity can be precisely quantified allowing for extremely accurate tumor burden
quantification, which is important when using an orthotopically-induced xenograft tumor model, as tumors cannot be manually measured by caliper (Ayers et al., 2010).

Limitations of this research are that SPECT/CT imaging and voxel quantification are costly and not widely available. Development and execution of the orthotopic tumor model is time-consuming and expensive, but more clinically relevant to human lung cancer. Also, the plasmid DNA vector-mediated expression of the hNIS gene was very low compared to lentiviral-vector medicated expression. Decreased hNIS gene expression in tumor progression has been related to dysregulation or epigenetic mechanisms. Transcriptional downregulation of the hNIS gene is caused by methylation of the hNIS DNA sequence in critical regulatory regions and maybe reversed by the application of chemical demethylation through the inhibitor of deacetylase (Nakamoto et al., 2000; Guo et al., 2010). Improved efficiency of baculovirus-mediated hNIS reporter gene imaging in lung adenocarcinoma cells was shown with sodium butyrate treatment (Guo et al., 2010). Stable incorporation of the hNIS gene, as shown by the lentiviral transduction method, is crucial for $^{99m}$TcO$_4^-$ uptake in vivo.

The hNIS gene has many advantages as an imaging reporter gene due to the wide availability of its substrate radioisotopes like $^{99m}$TcO$_4^-$, as well as their well-understood metabolisms and clearance mechanisms. In addition, hNIS is unlikely to interact with the underlying cell biochemistry, and iodide is not metabolized in most tissue, therefore ameliorating background signal. We have demonstrated significant increase of $^{99m}$TcO$_4^-$ uptake in mice injected with modified cells orthotopically, over a 200-fold increase, compared to mice injected with A549 cells alone.

**Conclusion**
We have constructed plasmid DNA and lentiviral vectors expressing the hNIS gene. These vectors were transfected or transduced to genetically modify A549 cells. Cells were characterized for hNIS expression and implanted as a subcutaneous xenograft or orthotopic model to the lung. Resulting tumors were imaged using SPECT/CT and algorithmically quantified. This tumor model could significantly improve the quality of imaging data by avoiding inter-animal variation, reducing imaging invasiveness, and allowing long-term longitudinal monitoring of the same animal treated with novel anti-cancer drugs.

**Acknowledgements:**

This research was supported in part by funding from University of New Mexico Research Allocation Committee Grant and the NCI New Mexico Cancer Nanotechnology Training Center (NIH R25CA153825 supporting Amber McBride). SPECT/CT images in this article were generated at the Keck-UNM Small Animal Imaging Resource established with funding from the W.M. Keck Foundation and the UNM Cancer Center Imaging Core. The technical assistance of Tamara Anderson and Monique Nysus is greatly appreciated. We thank Harmony Bowles and Erin Weeda for their valuable contributions to this project.
Future Directions and Outstanding Questions

Dissolution Studies

The efficacy and systemic exposure of inhaled drugs depends on the site of deposition, the rate and extent of absorption of the inhaled particle, and the physicochemical properties of the drug formulation. Developing an appropriate dissolution method for the *in vitro* assessment of inhaled NIMs is a key step in establishing any meaningful *in vitro* / *in vivo* correlations. The *in vitro* dissolution method must be optimized to be sufficiently sensitive to detect differences in critical compositional or processing variables (Sood et al, 2009; McConville, J.T., 2000). The purpose of this dissolution study would be to identify a relevant *in vitro* dissolution screening method to predict the *in vivo* drug release properties of spray dried NIMs. A number of issues remain in order to understand the deposition of drugs and dissolution rates of novel inhaled delivery vehicles in the lungs. For *in vitro* testing, considerations for a number of test parameters include:

- The dissolution rate of the excipient lactose
- Water, the medium composition
- Flow rates
- Dissolution pump type of a Type IV dissolution bath system

*In Vivo Delivery and Targeting of NIMs to an Orthotopic Tumor Model*

To bring full circle the testing of inhaled and targeted NIMs therapy with the sodium iodide symporter tumor imaging model, a cohort of mice could be induced with orthotopic lung tumors using hNIS lentiviral vector modified-A549 cells, as previously
shown. Based on the development of the orthotopic tumor model, we expect A549-LV orthotopic tumors to grow to a significant size within 15 – 20 days, accurately quantified by CT/SPECT.

Three treatments would be given: 1) Intravenous (IV) chemotherapeutic (doxorubicin or cisplatin) to understand tumor regression from a systemic drug delivery perspective. 2) Insufflated NIMs treatment containing the same intravenous chemotherapeutic agent given IV, in the absence of magnetic targeting. 3) Insufflated chemotherapeutic-containing NIMs treatment with magnetic field dependent targeting. We expect to understand tumor growth rate kinetics, and pharmacokinetic parameters of inhaled chemotherapeutics including liberation, absorption, distribution, metabolism and excretion of the three treatments.

- Do NIMs appear to cause more inflammation in the lung than the equivalent systemically administered dose?

The current best device to administer dry powders to rodents is the Penn Century Insufflator™. Compared to inhalation towers, tracheal insufflation delivers a more reproducible and predictable emitted dose. From our experience and others (Duret et al, 2012; Hoppentocht et al. 2014), the insufflator is an unreliable delivery strategy and even the best-planned and executed experiment has a high probability of failure. As better tools are developed to administer dry powders to animal models, we hope to reconcile some of the following issues:

- Reproducibility and precision of dose emitted
- Flow properties of the dry powders emitted
- Anti-aggregation and anti-clogging properties
APPENDICES

Spray drying SOP for nano-in-microparticles (NIMs) containing dye

Aim: To spray dry NIMs (10.0% dye, 20.0% SPIONs, 70.0% lactose) (% w/volume) for *in vivo* magnetic-field-dependent targeting (left mouse lung)

Spray drying plan:
1. 20 ml batch of 3% lactose (3% total solids) → for control purposes
   a. To verify that spray dryer works correctly, to characterize and optimize particle size
2. 20 ml batch of NIMs containing dye (2.5% total solids) → yields approximately 250 mg dry powder

Materials:
- FluoroSpheres® fluorescent microsphere far red dye, 10.0% (w/volume) of total microparticle
  - FluoroSpheres®-Carboxylate Modified Microspheres
  - Nominal bead size: 0.02 µm
  - Dark red fluorescent (633 emission)
  - 2% solids (concentration: 0.02 g/ml)
  - 4 ml dye purchased from Life Technologies rec’d 2/10/14
  - **Product Number: F-8783**
  - Life Technologies (Molecular Probes®) from Carlsbad, CA
- SPIONs, 20.0% (w/volume) of total microparticle
  - 50 nm diameter
  - [50 mg/ml] stock concentration
  - FluidMAG- UC/A, catalog # 4114-5
  - Synthesized 11/25/11 from Chemicell GmbH, Berlin, Germany
- Lactose, 70.0% (w/volume) of total microparticle
  - Gift from DMV Fonterra Excipients
  - Product name: Respitose, ML 002
  - Product code: 42510-7365

Consumable materials:
- 500 ml autoclaved ddH2O
- Autoclaved beakers:
  - (2) 200 ml beakers
  - (1) 50 ml beaker
- (10) Autoclaved 20 ml scintillation vials – plastic cap insert removed
- (3) Sterilized spatula (ethanol)
- (3) Sterilized magnetic stirrers (ethanol)
- Sterilized weigh boats large & small (ethanol)
- Autoclaved pipet tips
1. **20 ml batch of 3% lactose → for control purposes**

   - 3 grams lactose in 100 ml ddH$_2$O = 3% lactose solution (w/v)
   - Solubility of lactose = 210 mg/ml or 21% w/v

   Actual
   
   3.0131 g lactose
   
   100.0 ml milliQ H$_2$O

1. Weigh 3 g (3000 mg) lactose and add to a 100 ml volumetric flask.
2. Add ddH$_2$O to the fill line of the 100 ml volumetric flask.
3. Heat solution and stir with bar at 60 °C (lowest setting) for 5 minutes.
4. Aliquot 20 ml of lactose solution into new 50 ml beaker. Cover with parafilm.
   a. 20 ml yields [30 mg/ml] lactose solution = 600 mg lactose solids

a. **Spray-dry protocol:**

4. Spray drying conditions: (based off of previous lactose-only run)

<table>
<thead>
<tr>
<th>Theoretical</th>
<th>Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Inlet: 190°C ± 2 °C</td>
<td>190 ± 2 °C</td>
</tr>
<tr>
<td>ii. Outlet: 50°C ±4°C</td>
<td>50 ± 2 °C</td>
</tr>
<tr>
<td>iii. Aspirator: 90%</td>
<td>0%</td>
</tr>
<tr>
<td>iv. Pump: 10%</td>
<td>10%</td>
</tr>
<tr>
<td>v. Filter gauge*: - 50 mbar</td>
<td>- 57 mbar initial</td>
</tr>
<tr>
<td>vi. Nitrogen Q-flow: 63 mmbar</td>
<td>63 mmbar</td>
</tr>
<tr>
<td>vii. Filter used: PTFE (Teflon)</td>
<td>Teflon</td>
</tr>
</tbody>
</table>

   * Exhaust hose connected to HEPA filter

4. Calculate powder yield percentage: 

   a. Weight of vial + cap before spray drying: 161611 mg
   b. Weight of vial + cap + powder after spray drying: 161998 mg
   c. Weight of powder: 387 mg
   d. Percent yield: x/600 mg (100) = 64.5 %

4. Characterize particle size (Malvern Mastersizer 3000 Aero S):

Aero S particle SOP notes:

i. Assume non-spheroid particle geometry
   a. Lactose density: 1.52 g/cm$^3$
   b. Lactose absorptivity: 0.010
   c. Lactose refractive index: 1.347
   d. SPION density: 1.55 g/cm$^3$
   e. Dye density: 1.05 g/cm$^3$
2. **20 ml batch NIMs containing dye** (500 mg solids or 2.5% total solids)
   - Contains 20% SPIONs theoretically (50 nm diameter)
   - Contains 10% FluoroSpheres™ far red dye theoretically (stock concentration: 0.02 g/ml)
   - Contains 70% Lactose theoretically (6.33% lactose solution in water)

Calculation:

$$\begin{align*}
\text{Lactose:} & \quad 350.0 \text{ mg w/w} \quad 350/500 = 70.0\% \text{ total solids} \\
\text{SPIONs:} & \quad 100.0 \text{ mg w/v per 2 ml} \quad (2 \text{ ml @ [50 mg/ml]}) \rightarrow 100/500 = 20.0\% \text{ total solids} \\
\text{Dye:} & \quad 50.0 \text{ mg w/v per 2.5 ml} \quad (2.5 \text{ ml @ [20 mg/ml]}) \rightarrow 50/500 = 10.0\% \text{ total solids}
\end{align*}$$

$\Rightarrow$ 350 mg powder solids + 4.5 ml liquid solids + 15.5 ml H$_2$O for a 20 ml final volume

- Lactose solubility in H$_2$O: 21.9 gm in 100 ml

1. Weigh **350 mg lactose** and add to 5.0 ml ddH$_2$O in 50 ml beaker
2. Add **2.5 ml dye** (vortexed and sonicated)
3. You have 5.0 ml total volume solution at this point
4. Add **2 ml SPIONs**
   a. You have 7 ml total volume at this point
5. QS with another 13 ml water to flask line for 20 ml final volume. Parafilm and label.

**Spray drying**

1. Spray dry conditions:
   i. Inlet: 190°C
   ii. Outlet: 50°C ±4°C
   iii. Aspirator: 90%
   iv. Pump: 10%
   v. Filter gauge: - 52 mbar
   vi. Nitrogen Q-flow: 63 mmbar
   vii. Filter used: Teflon

2. Note powder characteristics: Particles that collect on the sides of the cyclone are nearly 100x bigger (average: 466 µm) than particles that fall into collection vial. No longer will I scrap sides of cyclone to retrieve any powders.

3. Calculate powder yield percentage:
   a. Weight of vial + cap before spray drying: 162519 mg
   b. Weight of vial + cap + powder after spray drying: 162722.1 mg
   c. Weight of powder: 203.1 mg
   d. Percent yield: x/500 mg (100) = 40.6%

4. Characterize particle size (Malvern Mastersizer 3000 Aero S):
### Table 1 Effect of Magnetic Field Dependent Targeting in Rat Lungs, Ex vivo

#### Ex Vivo Magnetic-Field-Dependent Targeting of NIMs

<table>
<thead>
<tr>
<th>Tissue region</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Ratio Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Right lung&lt;sup&gt;a&lt;/sup&gt; targeted</td>
<td>7.63E+08 5%</td>
<td>2.11E+09 21%</td>
<td>2.25E+09 34%</td>
<td>20%</td>
</tr>
<tr>
<td>Left lung</td>
<td>3.77E+08 2%</td>
<td>5.87E+08 6%</td>
<td>3.44E+08 5%</td>
<td>4%</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.47E+10 93%</td>
<td>7.40E+09 73%</td>
<td>4.11E+09 61%</td>
<td>76%</td>
</tr>
<tr>
<td>Total</td>
<td>1.58E+10</td>
<td>1.01E+10</td>
<td>6.70E+09</td>
<td></td>
</tr>
<tr>
<td>Differential (R-L lung)</td>
<td>3.87E+08 2%</td>
<td>1.52E+09 15%</td>
<td>1.90E+09 28%</td>
<td>16% to R lung</td>
</tr>
</tbody>
</table>

#### Ex Vivo Absence of Magnetic-Field Dependent Targeting of NIMs

<table>
<thead>
<tr>
<th>Tissue region</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Ratio Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Intensity (RE)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Right lung</td>
<td>8.78E+07 24%</td>
<td>6.63E+07 14%</td>
<td>9.62E+07 22%</td>
<td>20%</td>
</tr>
<tr>
<td>Left lung</td>
<td>6.58E+07 18%</td>
<td>9.54E+07 20%</td>
<td>1.35E+08 30%</td>
<td>23%</td>
</tr>
<tr>
<td>Trachea</td>
<td>2.14E+08 58%</td>
<td>3.10E+08 66%</td>
<td>2.15E+08 48%</td>
<td>57%</td>
</tr>
<tr>
<td>Total</td>
<td>3.67E+08</td>
<td>4.72E+08</td>
<td>4.46E+08</td>
<td></td>
</tr>
<tr>
<td>Differential (R-L lung)</td>
<td>2.20E+07 6%</td>
<td>-2.91E+07 -6%</td>
<td>-3.92E+07 -9%</td>
<td>-3% to R lung</td>
</tr>
</tbody>
</table>

<sup>a</sup> NIMs were administered to excised lungs and targeted to the right lung in the presence of a magnetic field

<sup>b</sup> Fluorescence expressed as radiant efficiency (RE) in units of [p/s]/[µW/cm²]
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Air volume</th>
<th>NIMs (mg) loaded</th>
<th>NIMs (mg) insufflated</th>
<th>Tissue region</th>
<th>Fluorescence $^c$</th>
<th>Percent fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500 µl $^a$</td>
<td>2.2</td>
<td>0.6</td>
<td>Right lung</td>
<td>1.66E+08</td>
<td>13.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left lung</td>
<td>2.63E+07</td>
<td>21.2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trachea</td>
<td>8.10E+07</td>
<td>65.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sum</td>
<td>2.73E+08</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>200 µl $^b$</td>
<td>2.3</td>
<td>0.3</td>
<td>Right lung</td>
<td>2.04E+07</td>
<td>11.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left lung</td>
<td>2.26E+07</td>
<td>12.7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trachea</td>
<td>1.35E+08</td>
<td>75.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sum</td>
<td>1.78E+08</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Air volume</th>
<th>NIMs (mg) loaded</th>
<th>NIMs (mg) insufflated</th>
<th>Tissue region</th>
<th>Fluorescence $^c$</th>
<th>Percent fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>500 µl $^a$</td>
<td>2.3</td>
<td>0.6</td>
<td>Right lung</td>
<td>1.33E+07</td>
<td>16.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left lung</td>
<td>2.63E+07</td>
<td>18.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trachea</td>
<td>4.80E+07</td>
<td>65.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sum</td>
<td>8.76E+07</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>200 µl $^b$</td>
<td>2.1</td>
<td>0.2</td>
<td>Right lung</td>
<td>9.13E+07</td>
<td>17.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left lung</td>
<td>7.79E+07</td>
<td>14.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trachea</td>
<td>3.56E+07</td>
<td>67.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sum</td>
<td>2.05E+08</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 500 µl air volume from 3 ml syringe
$^b$ 200 µl air volume using Penn-Century Air Pump
$^c$ Deposition units of average Relative Efficiency (RE) in $[\text{p/s/cm}^2\text{sr}]$/[$\mu\text{W/cm}^2$]. The IVIS Imager analyzed fluorescence.
$^d$ When a magnetic field was applied, the magnet was placed on the ventral surface above the left lung after surgically opening the animal, to expose the chest cavity.
Table 3 Pilot Study to Determine Reproducibility of Powder Administration From the Penn-Century Dry Powder Insufflator™

<table>
<thead>
<tr>
<th></th>
<th>Mass balance of dry powders before and after insufflation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactose dry powder (mg)</td>
</tr>
<tr>
<td></td>
<td>n = 1</td>
</tr>
<tr>
<td>Mass of clean mouse insufflator&lt;sup&gt;a&lt;/sup&gt;:</td>
<td>3492.87</td>
</tr>
<tr>
<td>Mass of dry powder:</td>
<td>1.59</td>
</tr>
<tr>
<td>Mass of insufflator with dry powder:</td>
<td>3494.27</td>
</tr>
<tr>
<td>Mass of dry powder after loaded into insufflator:</td>
<td>1.40</td>
</tr>
<tr>
<td>Mass of loaded insufflator after 10 actuations:</td>
<td>3493.20</td>
</tr>
<tr>
<td>Mass of insufflated dry powder:</td>
<td>1.07</td>
</tr>
<tr>
<td>Mass of insufflator after additional 10 actuations:</td>
<td>3493.26</td>
</tr>
<tr>
<td>Mass of powder/NIMs left in device:</td>
<td>0.39</td>
</tr>
<tr>
<td>Percent mass powder/NIMs not insufflated:</td>
<td>27.9%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dry Powder Insufflator™ Model DP-4M for mouse
### Table 4 Effect of Magnetic-Field-Dependent Targeting Quantified by Fluorescence – Intensity Values

**IVIS Fluorescence Study - Intensity Values**

#### Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension

<table>
<thead>
<tr>
<th>Dry powder NIMS (^a)</th>
<th>Liquid suspension (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 1)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>R lung</td>
<td>8.34E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>1.70E+08</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.05E+08</td>
</tr>
<tr>
<td>Total</td>
<td>3.58E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.50</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.79E+08</td>
</tr>
<tr>
<td>(n = 2)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>R lung</td>
<td>6.50E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>1.66E+08</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.84E+08</td>
</tr>
<tr>
<td>Total</td>
<td>4.15E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.40</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.66E+08</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>R lung</td>
<td>7.69E+06</td>
</tr>
<tr>
<td>L lung</td>
<td>8.94E+07</td>
</tr>
<tr>
<td>Trachea</td>
<td>9.66E+07</td>
</tr>
<tr>
<td>Total</td>
<td>1.94E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.60</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.16E+08</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>R lung</td>
<td>5.20E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>1.42E+08</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.29E+08</td>
</tr>
<tr>
<td>Total</td>
<td>3.22E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.50</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.54E+08</td>
</tr>
<tr>
<td>SEM</td>
<td>2.28E+07</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>R lung</td>
<td>8.34E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>1.70E+08</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.05E+08</td>
</tr>
<tr>
<td>Total</td>
<td>3.58E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.50</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.79E+08</td>
</tr>
</tbody>
</table>

#### Absence of Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension

<table>
<thead>
<tr>
<th>Dry powder NIMS (^a)</th>
<th>Liquid suspension (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 1)</td>
<td>(n = 1)</td>
</tr>
<tr>
<td>R lung</td>
<td>9.91E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>7.78E+07</td>
</tr>
<tr>
<td>Trachea</td>
<td>3.20E+08</td>
</tr>
<tr>
<td>Total</td>
<td>7.09E+07</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.60</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.29E+08</td>
</tr>
<tr>
<td>(n = 2)</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>R lung</td>
<td>1.46E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>2.92E+06</td>
</tr>
<tr>
<td>Trachea</td>
<td>7.09E+07</td>
</tr>
<tr>
<td>Total</td>
<td>8.84E+07</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.60</td>
</tr>
<tr>
<td>Total*dose</td>
<td>5.30E+07</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>R lung</td>
<td>6.02E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>1.03E+08</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.05E+08</td>
</tr>
<tr>
<td>Total</td>
<td>2.68E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.50</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.34E+08</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>R lung</td>
<td>5.79E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>6.12E+07</td>
</tr>
<tr>
<td>Trachea</td>
<td>1.65E+08</td>
</tr>
<tr>
<td>Total</td>
<td>2.84E+08</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.50</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.29E+08</td>
</tr>
<tr>
<td>SEM</td>
<td>2.44E+07</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>R lung</td>
<td>9.91E+07</td>
</tr>
<tr>
<td>L lung</td>
<td>7.78E+07</td>
</tr>
<tr>
<td>Trachea</td>
<td>3.20E+08</td>
</tr>
<tr>
<td>Total</td>
<td>7.09E+07</td>
</tr>
<tr>
<td>Dose (mg)</td>
<td>0.60</td>
</tr>
<tr>
<td>Total*dose</td>
<td>1.29E+08</td>
</tr>
</tbody>
</table>

\(^a\) Deposition units of average Relative Efficiency (RE) in \([\text{p} / \text{s} / \text{cm}^2 / \text{sr}] / [\mu \text{W/cm}^2]\). Fluorescence was analyzed by the IVIS Imager II

\(^b\) When a magnetic field was applied, the magnet was placed on the ventral surface above the left lung, after surgically opening the animal, to expose the chest cavity.

\(^c\) NIMs or liquid suspension were administered to the lungs of mice (n = 3) with or without application of the magnetic field.
Table 5 Effect of magnetic-field-dependent targeting quantified by fluorescence – ratio values

<table>
<thead>
<tr>
<th>IVIS Fluorescence Study - Ratio Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dry powder NIMS $^a$</th>
<th>Liquid suspension $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 1</td>
<td>23%</td>
</tr>
<tr>
<td>n = 2</td>
<td>16%</td>
</tr>
<tr>
<td>n = 3</td>
<td>4%</td>
</tr>
<tr>
<td>Average</td>
<td><strong>14%</strong></td>
</tr>
<tr>
<td>SEM</td>
<td>6%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Absence of Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Dry powder NIMS $^a$</th>
<th>Liquid suspension $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>R lung ($^c$)</td>
<td>L lung $^b$ (LL)</td>
</tr>
<tr>
<td>n = 1</td>
<td>20%</td>
</tr>
<tr>
<td>n = 2</td>
<td>17%</td>
</tr>
<tr>
<td>n = 3</td>
<td>22%</td>
</tr>
<tr>
<td>Average</td>
<td><strong>20%</strong></td>
</tr>
<tr>
<td>SEM</td>
<td>1%</td>
</tr>
</tbody>
</table>

$^a$ Deposition units of average Relative Efficiency (RE) in [p/s/cm²/sr]/[µW/cm²]. The IVIS Imager analyzed fluorescence.

$^b$ When a magnetic field was applied, the magnet was placed on the ventral surface above the left lung after surgically opening the animal, to expose the chest cavity.

$^c$ NIMs or liquid suspension were administered to the lungs of mice (n = 3) with or without application of the magnetic field.
Table 6 Effect of magnetic-field-dependent targeting quantified by iron – intensity values

Inductively Coupled Plasma - Optical Emission Spectroscopy - Intensity Values of Iron

<table>
<thead>
<tr>
<th></th>
<th>Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension</th>
<th>Absence of Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry powder NIMS  a, b</td>
<td>Liquid suspension  a, b</td>
</tr>
<tr>
<td></td>
<td>R lung L lung (^b) Trachea Total Fe</td>
<td>R lung L lung (^b) Trachea Total</td>
</tr>
<tr>
<td>n = 1</td>
<td>7.28E+04 3.34E+05 4.14E+05 8.22E+05</td>
<td>5.51E+04 2.12E+04 3.72E+04 1.13E+05</td>
</tr>
<tr>
<td>n = 2</td>
<td>2.42E+04 2.34E+05 6.73E+05 9.31E+05</td>
<td>1.56E+05 1.64E+05 1.01E+05 4.21E+05</td>
</tr>
<tr>
<td>n = 3</td>
<td>-2.13E+03 1.38E+05 2.32E+05 3.68E+05</td>
<td>7.09E+04 5.72E+04 4.48E+04 1.73E+05</td>
</tr>
<tr>
<td>Average</td>
<td>3.16E+04 2.36E+05 4.40E+05 7.07E+05</td>
<td>9.41E+04 8.08E+04 6.08E+04 2.36E+05</td>
</tr>
<tr>
<td>SEM</td>
<td>2.19E+04 5.66E+04 1.28E+05 1.72E+05</td>
<td>3.15E+04 4.28E+04 2.00E+04 9.41E+04</td>
</tr>
</tbody>
</table>

|                  |                                                                      |                                                                      |
|                  | Dry powder NIMS  a                                                  | Liquid suspension  a                                                  |
|                  | R lung L lung Trachea Total                                        | R lung L lung Trachea Total                                          |
| n = 1            | 2.77E+04 3.37E+04 3.12E+05 3.73E+05                              | 3.01E+05 2.30E+05 6.93E+04 6.00E+05                                 |
| n = 2            | 4.74E+04 2.22E+04 6.91E+05 7.61E+05                              | 1.36E+04 2.29E+04 3.03E+04 6.68E+04                                 |
| n = 3            | 9.76E+04 8.64E+04 4.71E+05 6.55E+05                              | 1.00E+05 4.32E+04 1.24E+05 2.67E+05                                 |
| Average          | 5.75E+04 4.74E+04 4.92E+05 5.96E+05                              | 1.38E+05 9.86E+04 7.44E+04 3.11E+05                                 |
| SEM              | 2.08E+04 1.98E+04 1.10E+05 1.16E+05                              | 8.50E+04 6.58E+04 2.71E+04 1.55E+05                                 |
Table 6 (continuation) Effect of magnetic-field-dependent targeting quantified by iron – intensity values

<table>
<thead>
<tr>
<th>Control: Endogenous Iron (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R lung Intensity</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>n = 1</td>
</tr>
<tr>
<td>n = 2</td>
</tr>
<tr>
<td>n = 3</td>
</tr>
<tr>
<td>Average</td>
</tr>
<tr>
<td>SEM</td>
</tr>
</tbody>
</table>

*a* Values normalized for endogenous iron  
*b* When a magnetic field was applied, the magnet was placed on the ventral surface above the left lung, after surgically opening the animal, to expose the chest cavity.
Table 7 Effect of magnetic-field-dependent targeting quantified by iron – ratio values

<table>
<thead>
<tr>
<th></th>
<th>Inductively Coupled Plasma - Optical Emission Spectroscopy – Iron Ratio Values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dry powder NIMS (^a)</td>
<td>Liquid suspension (^a)</td>
</tr>
<tr>
<td></td>
<td>R lung</td>
<td>L lung</td>
</tr>
<tr>
<td>n = 1</td>
<td>9%</td>
<td>42%</td>
</tr>
<tr>
<td>n = 2</td>
<td>3%</td>
<td>25%</td>
</tr>
<tr>
<td>n = 3</td>
<td>0%</td>
<td>38%</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>4%</td>
<td>35%</td>
</tr>
<tr>
<td>SEM</td>
<td>3%</td>
<td>5%</td>
</tr>
</tbody>
</table>

|                      | Absence of Magnetic-Field-Dependent Targeting: NIMs vs. Liquid Suspension |  |
|                      | Dry powder NIMS \(^a\)                                                                 | Liquid suspension \(^a\) |
|                      | R lung | L lung | Trachea | Outcome (L lung - R lung) | R lung | L lung | Trachea | Outcome (L lung - R lung) |
| n = 1                 | 7%     | 9%     | 82%     | 2%  | Fe to L lung | 50%     | 38%    | 21% | -12% | Fe to L lung |
| n = 2                 | 6%     | 3%     | 87%     | -3% | Fe to L lung | 31%     | 34%    | 32% | 3%  | Fe to L lung |
| n = 3                 | 15%    | 13%    | 70%     | -2% | Fe to L lung | 38%     | 16%    | 31% | -22% | Fe to L lung |
| **Average**           | 9%     | 8%     | 80%     | -1% | Fe to L lung | **40%** | 29%    | 28% | -10% | Fe to L lung |
| SEM                  | 3%     | 3%     | 5%      | 2%  |              | 6%      | 7%     | 4%  | 7%  |              |

\(^a\) Deposition units of average Relative Efficiency (RE) in \([\text{p/s/cm}^2/\text{sr}] / [\text{µW/cm}^2]\). The IVIS Imager analyzed fluorescence.

\(^b\) When a magnetic field was applied, the magnet was placed on the ventral surface above the left lung after surgically opening the animal, to expose the chest cavity.

\(^c\) NIMs or liquid suspension were administered to the lungs of mice (n = 3) with or without application of the magnetic field.
REFERENCES


Für Pharmazeutische Verfahrenstechnik E.V, June. doi:10.1016/j.ejpb.2014.06.014.


