RECEPTOR-TARGETED NANOCARRIERS FOR TUMOR SPECIFIC TREATMENT AND IMAGING

By

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Written under the direction of
Professor Tamara Minko, Ph.D.
And approved by

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Lung cancer is the leading cause of cancer death in the United States. The efficacy of chemotherapy in lung cancer is limited by the development of cancer cell resistance during the treatment, difficulties in the delivery of anticancer drugs specifically to lung tumors, and severe adverse side effects of high doses chemotherapy on healthy organs. The main objectives of the present research are: (1) to provide effective delivery of therapeutic and imaging agents to lungs, (2) to mitigate the above described resistance, (3) to enhance the efficacy of lung cancer chemotherapy and limit adverse side effects of the treatment. To achieve these goals, we developed, characterized, and evaluated in vitro and in vivo on orthotopic model of lung cancer using a novel multifunctional tumor-targeted Nanocarrier-based Drug Delivery System (NDDS) composed of (1) a carrier; (2) a synthetic analog of Luteinizing Hormone-Releasing Hormone (LHRH peptide) as a targeting moiety; (3) an anticancer drug or imaging agent; (4) suppressors of drug resistance. We tested and compared: linear polymer, dendrimers and liposomes as nanocarriers; antisense oligonucleotides or small interfering RNA targeted to MRP1 and BCL2 mRNA as suppressors of pump and nonpump cellular resistance respectively;
near-infrared cyanine dye Cy5.5, rhodamine, or fluorescein isothiocyanate as imaging agents.

The proposed multivalent NDDS exhibited preferential accumulation in the lungs, increased the sensitivity and specificity of tumor imaging, enhanced cancer treatment, and limited adverse side effects of the treatment on healthy organs. Targeting of nanocarriers to tumor specific receptors minimizes the influence of the architecture, composition, size, and molecular mass of nanocarriers on the efficacy of imaging and cancer treatment.

Local intratracheal administration sustained higher concentration of NDDS and its payload in the lungs and substantially limited their accumulation in other organs. Moreover, intratracheal local delivery of NDDS led to the more efficient treatment of lung cancer when compared with the intravenous administration of NDDS or free drugs. Simultaneous suppression of pump and nonpump resistance dramatically enhanced the cytotoxicity of the anticancer drug leading to a substantial increase in apoptosis induction.
DEDICATION

To My Mom
Who is the most loving, joyful, and positive person
I love you so much!

To my advisor: Professor Tamara Minko
Who is the definition of ‘PERFECTION’

To Dean John L. Colaizzi
Who gave me the support and chance to make my dream possible

To Dr. Shahied
Who has always been my friend, my mentor
His wisdom and fear of God have and will continue to inspire me

To My Family & Friends
Who always believed that I can achieve anything I want
ACKNOWLEDGEMENTS

I would love to thank my advisor, Professor Tamara Minko, for being an incredible leader. I joined her lab in a not so conventional way, after my former advisor decided to leave the school. Immediately, she made me feel ‘home’. She told me to focus on the future and really gave me the strength to start over.

Dr. Minko is very committed to her students. She is the perfect ‘boss’ because she is fair and treats everyone equally. She really loves science and if I needed assistance, she would come to the lab, get a pair of gloves, and teach me. She also likes to leave her office door open to answer any questions.

Dr. Minko did not just teach me great science and lab techniques, she also gave me the confidence that junior scientists need. She never belittled my questions. In fact, she has a very beautiful way of making her students feel significant. What I love most about Dr. Minko is her humble spirit. She is not arrogant at all, even though she is famous and very respected by everyone in the field, in addition to being the chair of the department, a quality that only truly noble people possess. I feel very fortunate I had the chance to work under such a dedicated and ethical scientist.

I would really like to thank my committee members: Dr. Michniak-Kohn, Dr. You, and Dr. Shahied. I am very happy you accepted to evaluate my work. I appreciate your time very much. I also admire that, each of you has a very balanced life, and yet, managed to build a very successful career.

I am also truly thankful to have known Dean John L. Colaizzi. When I expressed to him my strong desire to become Dr. Minko’s graduate student, he supported me and did everything in his power to make my transition as smooth as possible. He basically
gave me the chance to make my dream a reality. Then, I had the pleasure to work with
him as a teaching assistant and let me just say, he deserves that we, graduate and
undergraduate students, wear T-shirts that say ‘WE LOVE DEAN COLAIZZI’.

And of course, I want to thank all professors, as well as staff, researchers, and
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1. INTRODUCTION

Lung cancer is the number one cause of cancer deaths among men and women in the United States\(^1\). More Americans die each year from lung cancer than from breast, prostate, and colorectal cancers combined\(^2\).

Lung cancer is one of the most difficult cancers to treat because it metastasizes very early in its course. Therefore, its very early detection is a key factor for better prognosis. Table 1.1 lists the average 5-year survival rates of breast, ovarian, stomach, and lung cancers from stage of first diagnosis\(^3\).

Nanotechnology allows the manufacturing of drug delivery systems (DDS) at the nanometer scale which make them attractive diagnostic as well as therapeutic agents for many reasons, such as their multivalency (or multifunctionality). Figure 1.1 depicts a schematic structure of the assembly of the multifunctional pharmaceutical nanocarrier\(^1^7\) evolving from being traditional and simple to becoming more involved and quite complex.

For instance, due to the many binding sites on their surfaces, nanoscale carriers used in drug delivery such as liposomes, quantum dots (QD), and dendrimers can simultaneously attach targeting moieties, different protective groups--such as polyethylene glycol (PEG)--as well as contrast agents, such as fluorescent probes for imaging\(^3\).

In addition, these novel drug delivery systems (DDS) promise a greater stability and longer half-life in the bloodstream. They also minimize drug degradation, prevent
cytotoxic side effects of anticancer drugs, and increase the drug bioavailability at the
target site $^3, ^{17}$.

Unfortunately, novel targeted cancer therapy alone is not sufficient in most cases
because the targeted drug still has to overcome many types of resistance such as
multidrug resistance (MDR); as described by Minko et al. $^4, ^{18}$.
Multidrug resistance (MDR) can be further subdivided into 1) Pump (active drug efflux)
and 2) Non pump resistance. ATP-binding cassette (ABC) transporters, such as P-
glycoprotein (PgP) and Multidrug resistance associated protein transporters (MRP) are
examples of pump resistance, whereas, cellular defense mechanisms are considered a
type of non-pump resistance. Table 1.2. lists mechanisms of drug resistance for some
anticancer agents $^{13}$.

Today, antisense oligonucleotides therapy (ASO) and small interfering RNA
(siRNA) are being used to inhibit cellular resistance of various proteins by inhibiting
gene expression at the transcript level $^5, ^6$.

Hence, our lab proposed a multivalent drug delivery system (DDS), we refer to as
an ‘advanced proapoptotic anticancer DDS’ (APADDS), as described by Minko et al. $^4$
and illustrated in figure 1.2 $^4$. This APADDS is composed of 5 components:

1) **Drug Carrier:** In this research project, PEG, dendrimers, long circulating liposomes
will be employed.
2) **Targeting moiety** to cancer cells: To this aim, Luteinizing Hormone-Releasing Hormone (LHRH) peptide will be used. LHRH, also called gonadotropin-releasing hormone (GnRH), is a hormonal decapptide produced by the hypothalamus\(^7\)\(^-\)\(^11\).

LHRH receptors are overexpressed in various cancer cells. However, in healthy organs of the reproductive system such as the prostate, testes, and ovaries, these receptors are present in very low levels and they are virtually non-existent in most normal tissues\(^9\)\(^-\)\(^11\).

These findings make LHRH receptors a potential target for cancer therapy that limits the cytotoxic side effects associated with systemic administration of chemotherapeutic agents\(^7\),\(^8\),\(^11\).

3) **Anticancer drug**: We will employ both paclitaxel (Taxol\(^8\)) and doxorubicin in this project.

4) **A suppressor to pump resistance**: To further enhance the efficacy of cancer therapy, antisense oligonucleotides (ASO) will be used to suppress the overexpression of MDR-1, the gene encoding Pgp, hereby inhibiting pump resistance.

5) **A suppressor to non-pump resistance**: To this aim, we will use antisense oligonucleotides (ASO) to suppress the overexpression of BCL-2; a protein family that consists of proapoptotic and antiapoptotic members:
Antiapoptotic members inhibit apoptotic cell death. Proapoptotic members, on the other hand, stimulate apoptosis mainly by suppressing the activity of antiapoptotic BCL-2 family proteins\textsuperscript{6,12-14}.

BCL-2 homology 3 (BH3) domain of proapoptotic proteins from the BCL-2 family is responsible for the induction of apoptosis\textsuperscript{12}. Moreover, when short synthetic peptides bearing the minimal sequence of BH3 domain are conjugated to the antiapoptotic BCL-2 family proteins, the cellular antiapoptotic defense is suppressed\textsuperscript{9,15,16}, hereby decreasing the resistance of cancer cells to anticancer drugs.
1.1. References


Table 1.1. Average 5-year survival rates from stage of first diagnosis. 

<table>
<thead>
<tr>
<th>Stage</th>
<th>Breast</th>
<th>Ovarian</th>
<th>Stomach</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>I(A)</td>
<td>100</td>
<td>93</td>
<td>78</td>
<td>47</td>
</tr>
<tr>
<td>II(A)</td>
<td>92</td>
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<td>III(A)</td>
<td>67</td>
<td>51</td>
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<td>8</td>
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<tr>
<td>IV(A)</td>
<td>20</td>
<td>17.5</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 1.2. Drug resistance mechanisms described for some anticancer agents.  

<table>
<thead>
<tr>
<th>Drug classes and examples</th>
<th>Mechanism of cytotoxicity</th>
<th>Molecules implicated in resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercalators</td>
<td>Topo II inhibitor, SO</td>
<td>Pgp, MRP, GST, Topo II</td>
</tr>
<tr>
<td>Alkylators</td>
<td>DNA alkylation</td>
<td>GSH, ALDH, AGT (?)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>DNA alkylation</td>
<td>GSH, MT, DNA repair enzymes, cMOAT (?)</td>
</tr>
<tr>
<td>Carboplatin</td>
<td></td>
<td>AGT</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>DNA alkylation</td>
<td>Amplification of DHFR, MRP (?)</td>
</tr>
<tr>
<td>BCNU</td>
<td>Folic acid antagonist</td>
<td>decreased RFC expression</td>
</tr>
<tr>
<td>Methotrexate</td>
<td></td>
<td>Amplification of TS</td>
</tr>
<tr>
<td>3-Fluorouracil</td>
<td>Uracil analog</td>
<td></td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>Tubulin</td>
<td>Pgp, MRP, tubulin</td>
</tr>
<tr>
<td>Vinblastine</td>
<td></td>
<td>kinase inhibition</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>Tubulin</td>
<td></td>
</tr>
<tr>
<td>Polyastringization inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topo II inhibitor</td>
<td>MRP, GSH, Pgp, Topo I</td>
</tr>
<tr>
<td>Tacrine</td>
<td>Inhibitor of microtubule assembly</td>
<td>Pgp, altered α/β tubulin</td>
</tr>
</tbody>
</table>

* AGT, O'-allylguanine-DNA alkytransferase; ALDH, aldehyde dehydrogenase; cMOAT, multispecific organic anion transporter (MRP2); DHFR, dihydrofolate reductase; GSH, glutathione; MT, metallothionein; Pgp, P-glycoprotein; RFC, reduced folate carrier (RFC); SO, superoxide and free radicals; TS, thymidylate synthase; Topo II, topoisomerase II.  
* Question mark means that a specific mechanism is suspected but not yet completely proven.
Figure 1.1. Schematic structure of the assembly of the multifunctional pharmaceutical nanocarrier\textsuperscript{17}.

1 – Traditional “plain” nanocarrier (a – drug loaded into the carrier)
2 – targeted nanocarrier or immunocarrier (b – specific targeting ligand, usually a monoclonal antibody, attached to the carrier surface)
3 – magnetic nanocarrier (c – magnetic particles loaded into the carrier together with the drug and allowing for the carrier sensitivity towards the external magnetic field and its use as a contrast agent for magnetic resonance imaging)
4 – long-circulating nanocarrier (d – surface-attached protecting polymer (usually PEG) allowing for prolonged circulation of the nanocarrier in the blood)
5 – contrast nanocarrier for imaging purposes (e – heavy metal atom – $^{111}$In, $^{99m}$Tc, Gd, Mn – loaded onto the nanocarrier via the carrier-incorporated chelating moiety for gamma- or MR imaging application)
6 – cell-penetrating nanocarrier (f – cell-penetrating peptide, CPP, attached to the carrier surface and allowing for the carrier enhanced uptake by the cells)
7 – DNA-carrying nanocarrier such as lipoplex or polyplex (g – DNA complexed by the carrier via the carrier surface positive charge)
8 – hypothetical multifunctional pharmaceutical nanocarrier combining the properties of the carriers # 1–7
Figure 1.2. Advanced Proapoptotic DDS (APADDs) ⁴.
2. BACKGROUND AND SIGNIFICANCE

2.1. Lung Cancer

2.1.1. Overview and Statistics

Lung cancer is the uncontrolled growth of abnormal cells in one or both lungs. It is life threatening and one of the most difficult cancers to treat because it tends to metastasize very early in its course. The most common sites for lung cancer metastasis are the adrenal glands, liver, brain, and bone\(^1,2\). Most of the time, lung cancer arises from the epithelial cells of the bronchi and bronchioles; for this reason lung cancer is sometimes called ‘bronchogenic carcinomas’\(^1\).

More Americans die each year from lung cancer than from breast, prostate, and colorectal cancers combined\(^4\). Figure 2.1.1. depicts the mortality of lung cancer estimated by the American Cancer Society in relation to other types of cancer such as colorectal, breast and prostate in 2005 (the most recent year for which statistics are currently available).

Annually, lung cancer kills more men than prostate cancer and more women than breast cancer\(^3,4\). While overall cancer incidence rates are declining, lung cancer incidence rates among women continue to rise\(^2,4\).

2.1.2. Major Causes and Types of Lung Cancer

2.1.2.1. Major Causes of Lung Cancer are:

1. **Smoking**: Cigarette smoking is the first leading cause of lung cancer in the United States. More than 87% of lung cancer is related to smoking\(^1,3\).
2. **Passive smoking**: 17% of cases of lung cancer in non-smokers are caused by second-hand smoke exposure in childhood and adolescence\(^9\).
3. Radon gas$^{1,3}$

4. Asbestos fibers$^{1-3,9,10}$. 

5. Familial predisposition$^1$. 

6. Lung diseases$^{1,2}$. 


8. Air pollution$^{1,2}$. 

2.1.2.2. Types of Lung Cancer

Lung cancer is broadly classified into 2 types: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC) $^{1-3,9,10}$. This classification is based upon the microscopic appearance of tumor cells. These two types of lung cancer grow and spread in different ways:

1) SCLC comprises about 20% of lung cancer and is the most aggressive of all lung cancer. SCLC is strongly related to cigarette smoking$^{1,2,9}$. 

2) NSCLC is the most common type of lung cancer, accounting for about 80% of all lung cancer. NSCLC has 3 main types, based upon the type of cells found in the tumor$^{1,2,9}$. They are:

1. **Adenocarcinomas** -- The most commonly seen type of NSCLC in the U.S. and comprise up to 50% of NSCLC$^{1,2}$. Most adenocarcinomas arise in the periphery of the lungs$^1$. 

2. **Squamous cell carcinomas** -- (or ‘epidermoid carcinomas’). They account for about 30% of NSCLC. Squamous cell cancers arise mainly in the central chest area in the bronchi$^{1,2}$. 
3. Large cell carcinomas -- (or ‘undifferentiated carcinomas’). These are the least common type of NSCLC\textsuperscript{1, 2}.

There are much less common types of lung cancer such as Bronchial carcinoids, which account for up to 5\% of lung cancers. These tumors occur mainly in people under 40 years old and are unrelated to cigarette smoking. They generally grow slowly and are treated by surgery\textsuperscript{1}.

### 2.1.3. Signs and Symptoms of Lung Cancer

1. **No symptoms** -- About 25\% of lung cancer is first discovered on a routine chest x-ray\textsuperscript{1}. These patients often report no symptoms.

2. **Symptoms related to the cancer** -- The growth of the cancer may lead to coughing, wheezing, chest pain, hoarseness, dysphagia, or infections\textsuperscript{1-3, 9, 10}.

3. **Symptoms related to metastasis** -- Lung cancer may spread to the bones causing extreme pain at bone sites. Lung Cancer can also spread to the brain causing neurologic symptoms in the form of seizures, blurred vision, or headaches\textsuperscript{1, 2, 9}.

4. **Paraneoplastic symptoms** -- Lung cancer cells usually produce hormone-like substances\textsuperscript{10} leading to oversecretion of cortisol by the adrenal glands\textsuperscript{1} or, elevated levels of calcium in the bloodstream\textsuperscript{1, 10}.

5. **Non specific symptoms** -- such as: Weight loss, weakness, and fatigue\textsuperscript{2, 9, 10}.

### 2.1.4. Current Treatments and Types of Chemotherapeutic Drugs

#### 2.1.4.1. Treatment(s) for Lung Cancer

Treatment for lung cancer can involve surgical removal of tumor, chemotherapy, or radiation therapy, as well as combinations of these methods, depending on the localization and extent of the tumor\textsuperscript{1-3, 9-11}.
2.1.4.2. Types of Chemotherapeutic Drugs

1. **Alkylating Agents** -- They directly damage DNA and they work in all phases of the cell cycle\(^5,11\). Examples include: Cisplatin, cyclophosphamide, and mechlorethamine (nitrogen mustard) \(^5-8,11\).

2. **Nitrosoureas** -- They interfere with enzymes that copy and repair DNA and they are not phase specific\(^11\). These agents are able to cross the blood brain barrier, so they are often used to treat brain tumors \(^5\). Examples include: Streptozocin, carmustine, and lomustine \(^5,11\).

3. **Antimetabolites** -- They interfere with DNA and RNA growth and they damage cells during the S phase\(^11\). Examples include: 5-fluorouracil, 6-mercaptopurine, and methotrexate\(^5\).

4. ** Anthracyclines** -- They are anti-tumor antibiotics that interfere with enzymes involved in DNA replication\(^11\). They work in all phases of the cell cycle\(^5\). One major side effect of these agents is their cardiotoxicity \(^5-8,11\). Examples include: daunorubicin, and doxorubicin \(^8,11\).

Doxorubicin is one of the most widely used anthracyclines. It blocks RNA and DNA synthesis equally. The drug has two main mechanisms of action\(^11\):

- **Intercalation**: Doxorubicin intercalates between adjacent nucleotides forming a tight drug-DNA interaction. This interaction blocks DNA synthesis.

- **Enzyme inhibition**: Doxorubicin binds and inhibits topoisomerase II, a key enzyme involved in DNA synthesis. Also, metabolism of the drug produces oxygen free radicals which damage DNA and prevent DNA synthesis.
5. **Topoisomerase Inhibitors** -- These drugs interfere with topoisomerases, which are important in DNA replication\textsuperscript{11}. An example of topoisomerase I inhibitor is topotecan and an example of topoisomerase II inhibitor is etoposide\textsuperscript{5,8}.

6. **Mitotic Inhibitors** -- Mitotic inhibitors are plant alkaloids and other compounds derived from natural products\textsuperscript{11}. They can stop mitosis, thus, they work primarily during the M phase of the cell cycle but they are known for their potential to cause peripheral nerve damage\textsuperscript{5}. Examples include: paclitaxel (Taxol\textsuperscript{®}), docetaxel, and vinorelbine\textsuperscript{6}.

Mechanism of action of paclitaxel (Taxol\textsuperscript{®})\textsuperscript{11}:

Paclitaxel (Taxol\textsuperscript{®}) binds to microtubules and prevents their breakdown. As a result, the chromosomes cannot move to opposite sides of the dividing cell. Cell division is halted, and cell death is induced.

7. **Corticosteroid Hormones** -- They are useful in treating lymphoma, leukemia, and myeloma. An example is dexamethasone\textsuperscript{5}.

8. **Other Chemotherapy Drugs** -- Some chemotherapy drugs have different mechanisms and thus, do not fit in any other category. Examples include: L-asparaginase, hydroxyurea, and thalidomide\textsuperscript{5,11}.

There are also other types of cancer drug therapies that are not considered to be chemotherapy because these drugs do not kill cancer cells. Instead, they are targeted to work only on cancer cells\textsuperscript{46,56,57}. Thus, they tend to have fewer side effects than those caused by chemotherapy\textsuperscript{52,53}. An example of these drugs is ‘Sex hormones, or hormone-like drugs’ such as, anti-estrogens (tamoxifen), anti-androgens (flutamide), and LHRH agonists (leuprolide)\textsuperscript{5,11}. 
Another example is ‘immunotherapy’; which can be either active or passive \(^1-^3,^9\). Treatment with cancer vaccines is an example of an active immunotherapy\(^{11}\), while monoclonal antibody therapy is an example of a passive immunotherapy \(^5,^{11}\).

### 2.2. DELIVERY OF ANTICANCER DRUGS

**Potential Benefits of Nanotechnology and Drug Carriers**

New drug delivery systems (DDS) aim to minimize drug degradation and loss, prevent harmful side effects and increase the availability of the drug at the target site\(^{85}\).

Targeting mechanisms can also be either passive or active; an example of passive targeting is the preferential accumulation of chemotherapeutic drugs in solid tumors as a result of the differences in vascularization of tumor tissue compared with healthy tissue, a phenomenon known as the Enhanced Permeation and Retention (EPR) effect.

Active targeting involves the chemical modification of the surface of drug carriers with molecules enabling them to be selectively attached to cancer cells \(^{27,85}\).

Examples of drug carriers include polymers, nanoparticles, viral vectors, liposomes, and micelles, which can be designed to slowly degrade and be site-specific\(^{85}\).

Generally, a successful drug carrier system needs to have optimal drug loading and release properties, long shelf-life and low toxicity \(^{27,31}\).

#### 2.2.1. POLYETHYLENE GLYCOL (PEG)

Polyethylene glycol (PEG) is a linear polymer, available in a variety of molecular weights. It is made from the polymerization of ethylene oxide \(^{14,15}\). Figure 2.2.1.a.\(^{12}\) depicts the chemical structure of PEG.
PEG is soluble in water and most organic solvents\textsuperscript{16}. The two hydroxy groups located at each end of the polymer allow it to be coupled to various peptides and drugs. PEG used in drug delivery has generally a molecular weight that ranges from 1000 to 20,000 Da\textsuperscript{13-15,17}.

Although neutral PEGs are more commonly used, cationic PEGs are gaining popularity in gene delivery because their positive charge allows them not only to form better complexes with the negatively charged DNA but it also enhances their cellular uptake via endocytosis. DOTAP is the most widely used cationic lipid\textsuperscript{86}.

Figure 2.2.1.b\textsuperscript{16} is a diagram illustrating the main advantages of PEG-protein conjugates. PEG offers many advantages\textsuperscript{13-16}:

\begin{itemize}
  \item PEG increases the solubility of insoluble drugs and decreases the toxicity of others.
  \item PEG shows low antigen activity and does not harm the bioactivity of proteins or cells.
  \item PEG enhances the half-life of drugs by improving their pharmacodynamic (PD) and pharmacokinetic (PK) properties.
  \item PEG increases biocompatibility, prevents protein adsorption to surfaces, and aids in penetrating cell membranes.
  \item PEG coupled to low molecular weight drugs enhances the drug uptake by tumor cells through the Enhanced Permeation and Retention (EPR) effect.
  \item PEG has also been used as a stabilizer of nanoparticles.
\end{itemize}

These are just some of the reasons why PEG is useful in biotechnology and will be used in this research project.

\textbf{2.2.2. DENDRIMERS}
Dendrimers (Greek, ‘tree-like’) are quite new to the field of polymer chemistry. These structures are fully synthetic and cannot be found in nature. Dendrimers have promising applications in scientific disciplines, such as medicine and chemistry. Dendrimers are nanometre-sized, polymer macromolecules. They consist of:

- a central core,
- branching units, and
- terminal functional groups

Figure 2.2.2.a depicts the structure of a dendrimer. The core of a dendrimer determines the solubilizing properties of the cavity within the core, whereas its external chemical groups determine the solubility and chemical property of the dendrimer itself.

Targeting is achieved by attaching specific ligands to the external surface of the dendrimer which enable it to bind to a target site.

The preferential accumulation of drug-encapsulated dendrimers into tumors occurs also through the passive targeting mechanism known as the enhanced permeation and retention (EPR) effect.

Ideal dendrimers possess monodisperse properties, which means that all molecules are alike when it comes to structure and molecular weight. In most cases, however, dendrimers of higher generations contain some impurities.

At present, there are 2 defined methods of dendrimer synthesis:

1. **Divergent Synthesis** means that the synthesis starts at the core and continues towards the periphery. Tomalia introduced this method for synthesis of polyaminoamines, called PAMAM-dendrimers in 1985.
2. **Convergent synthesis** was developed by the Fréchet-group at the University of Cornell in the early 90s. Here, the synthesis begins at the periphery and proceeds to the core.

The convergent synthesis approach has several advantages: The desired product can be easily purified and can carry more than one type of functional groups on the outermost generation of the dendrimer. Problems with convergent synthesis of dendrimers include: the need for a much higher amount of raw material, sterical problems, and inadequate yield.

Figure 2.2.2.b. provides a schematic representation of a multifunctional dendrimer illustrating the binding sites for both targeting ligands and protective groups, and figure 2.2.2.c. is a close-up diagram that depicts the anatomy of a generation three dendrimer with its various key regions.

### 2.2.3. LIPOSOMES

Liposomes are microscopic vesicles that consist of one to several lipid bilayers. Figures 2.2.3.a. illustrates the components that make a liposome useful for drug delivery.

Liposomes can vary in particle size as well as lamellarity and are therefore, subdivided into multilamellar vesicles (MLV), consisting of several concentric bilayers, large unilamellar vesicles (LUV), in the size range 200–800 nm, and small unilamellar vesicles (SUV), in the size range 50–150 nm. Drug molecules can be encapsulated and solubilized within the bilayers.

Conventional liposomes have short half-lives because the reticuloendothelial system (RES) entrap them. The design of long circulating liposomes resolve the RES
trapping problem because their liposomal membrane is modified in a way that mimic cells circulating in the blood.\textsuperscript{27}

Liposomes are important nanocarriers, especially as drug delivery systems (DDS) of anticancer drugs. For instance, pegylated liposomal doxorubicin (Caelyx\textsuperscript{®}) is used in the treatment of relapsing ovarian cancer.\textsuperscript{29} Also, pegylated and non-pegylated liposomal doxorubicin are used in clinical trials for the treatment of metastatic breast cancer.\textsuperscript{30}

Liposomal preparations are also used for tumor imaging as agents for magnetic resonance imaging (MRI), computed tomography (CT) contrast agents, single photon emission computed tomography (SPECT), as well radionuclide therapies.\textsuperscript{26, 27}

Liposomes offer many advantages as drug delivery carriers:\textsuperscript{26-30}
\begin{itemize}
\item Liposomes are biocompatible, biodegradable, and nonimmunogenic.
\item Liposomes can be used for delivery of hydrophobic, amphipathic and hydrophilic drugs.
\item Liposomes protect their encapsulated drug from the external environment.
\item Liposomes can exist in various types of formulations and they can be administered through most routes of administration.
\item Liposomes reduce toxicity and increase stability of entrapped drug.
\item Liposomes increase efficacy and therapeutic index of encapsulated drug.
\item Liposomes limit the serious side effects associated with anti-cancer drugs because they reduce exposure of healthy tissues to toxic drugs.
\item Liposomes coupled to site-specific ligands achieve active targeting.
\end{itemize}

Figure 2.2.3.b.\textsuperscript{25} illustrates a liposome being accepted into a cell and emptying its contents.

\textbf{2.2.4. Micelles}
The core of a micelle entraps drugs while the hydrophilic shell protects its contents. A micelle can have various shapes and sizes and its outer shell can be chemically altered to escape recognition by the reticuloendothelial system (RES) or to target a specific site\textsuperscript{31}.

2.2.5. Liquid Crystals

Liquid Crystals have properties of both liquid and solid states. They can have different lamellar phases, within which aqueous drug solutions can be incorporated\textsuperscript{32, 33}.

2.2.6. Nanoparticles

Nanoparticles, including nanospheres and nanocapsules are able to adsorb or encapsulate a drug, thus protecting it against degradation.

In nanocapsules, the drug is confined to a cavity surrounded by a polymer, while nanospheres are matrix systems within which the drug is uniformly dispersed\textsuperscript{34}.

2.2.7. Hydrogels

A hydrogel is a network of hydrophilic polymers that can swell in water while maintaining its structure\textsuperscript{36}. Hydrogels protect the drug from enzymatic and chemical degradation\textsuperscript{35, 36}. Environment-sensitive hydrogels are ideal drug delivery systems as they can modulate drug release at the target site, in response to pH, temperature, or specific concentration differences\textsuperscript{36}.

2.2.8. Molecularly Imprinted Polymers

Molecular Imprinting is the design of a precise macromolecule that can recognize its target molecule from a group of closely related molecules\textsuperscript{37}. Molecularly imprinted polymers have an enormous potential as drug delivery systems. Examples include: rate-
programmed drug delivery, activation-modulated drug delivery and feedback-regulated drug delivery\textsuperscript{37-39}.

2.2.9. In-Situ Forming Implants

In-situ forming implants are liquid formulations that generate a semisolid depot after subcutaneous injection. They are important parenteral delivery systems because they are less invasive compared to implants, they enable drug delivery over prolonged periods of time, and they minimize side effects\textsuperscript{40}.

2.3. TARGETING OF ANTICANCER DRUGS TO LUNG TUMOR

The focus in this section is on Peptide Receptors overexpressed in cancer of the Respiratory system (Lung Cancer), which are broadly classified into 2 categories: 1) Receptors regulated by the G-Protein, and 2) Tyrosine-Kinase Coupled Receptors.

Figure 2.3.\textsuperscript{50} is a scheme of the principle of in vivo peptide receptor targeting of cancer.

2.3.1. G-Protein-Coupled Receptor Systems in Lung Cancer

2.3.1.1. Bombesin, Gastrin-Releasing Peptide (GRP)/ GRP Receptor

Gastrin-releasing peptide (GRP), a 27-amino acid peptide, is the main mammalian member of the bombesin family of peptides. Bombesin (BN), a 14 amino acid peptide, is an analogue of human gastrin-releasing-peptide (GRP)\textsuperscript{41,42,45}.

The bombesin receptor family includes at least four different subtypes: BBR1, BBR2, BBR3, and BBR4 subtypes\textsuperscript{41,43,45}. GRP receptors’ proteins are overexpressed in a variety of human tumors, including prostate cancer, breast carcinomas, small cell lung cancer (SCLC) and non-SCLC, as well as in renal cancer\textsuperscript{41-43}. Thus, several analogues of
BN have been radiolabeled with a variety of radionuclides for the early diagnosis of cancer\textsuperscript{41-43, 45}.

2.3.1.2. Cholecystokinin (CCK)/R-CCK2 Receptor

The cholecystokinin 2 receptor (R-CCK2) is also a member of the G-protein coupled receptor superfamily. R-CCK2 is overexpressed in small cell lung cancers\textsuperscript{42}.

Several groups have synthesized CCK peptide analogs that can be labeled with radionuclides\textsuperscript{42-44}.

2.3.1.3. Somatostatin (SST)/ Somatostatin Receptors (SSTR)

Somatostatin, a cyclic 14 amino acid peptide, is an endogenous inhibitor of cell proliferation and angiogenesis\textsuperscript{42, 48}. Five distinct receptor subtypes are known to date; sst1–5. The presence of somatostatin receptors permits the localization of primary tumors and their metastases by scintigraphy with radiolabeled somatostatin analogs\textsuperscript{42, 45-47}. Unfortunately, sensitivity is low but specificity is very high suggesting that SSTR may not be expressed on all tumor sections\textsuperscript{49}.

2.3.1.4. Luteinizing Hormone-Releasing Hormone (LHRH) / LHRH Receptors

LHRH, also called gonadotropin-releasing hormone (GnRH), is a hormonal decapeptide produced by the hypothalamus. LHRH plays a pivotal role in the regulation of the pituitary gonadotrophs, and thus, reproduction. Its effects are exerted through binding to high-affinity, G protein-coupled receptors on the plasma membrane of pituitary gonadotroph cells and subsequent release of follicle-stimulating hormone (FSH) and Luteinizing hormone (LH)\textsuperscript{45-47}.

LHRH receptors were found to be overexpressed in cancerous cells\textsuperscript{51-53}. LHRH receptors on human cancers appear to be similar to pituitary LHRH receptors and can mediate direct effects of LHRH agonists and antagonists\textsuperscript{45, 46}.
In addition to breast, ovarian, endometrial, and prostate cancers, which can be affected by the pituitary/gonadal axis\textsuperscript{45, 46, 51}, LHRH receptors were also expressed in cancer of nonreproductive organs such as renal cell carcinomas\textsuperscript{45, 55}, adenocarcinomas of the colon\textsuperscript{45, 56, 57}, and ductal pancreatic carcinomas\textsuperscript{58, 59}.

It had also been shown that the expression of LHRH receptors in tumors is associated with activation of the human epidermal growth factor receptor (EGFR)\textsuperscript{54}.

In contrast to tumor cells, LHRH receptors are present in very low levels in healthy organs of the reproductive system such as the prostate, testes, and ovaries, but are absent from most normal tissues\textsuperscript{51-53}. These findings make LHRH receptors a potential target for cancer therapy, hereby limiting the cytotoxic side effects of anticancer drugs\textsuperscript{45, 46}. Figure 2.3.1.4.\textsuperscript{45} is an illustration of LHRH receptor-mediated entry of cytotoxic LH-RH analog AN-152 into various human cancer cells.

2.3.1.5. Vasoactive Intestinal Peptide (VIP)/VIP Receptor

Vasoactive Intestinal Peptide (VIP), a 28-amino acid peptide, stimulates growth and proliferation of several lung cancer cell lines. VIP has 2 different types of receptors, V pac1 and Vpac2 receptors\textsuperscript{42, 50}. Lung cancers are poor candidates for \textsuperscript{125}I-VIP scintigraphy because VIP receptors are highly expressed in normal tissues of the lungs\textsuperscript{42, 50}.

2.3.2. Tyrosine-Kinase Receptor Systems in Lung Cancer

2.3.2.1. Epidermal Growth Factor (EGF)/ EGF Receptor

Increased levels of EGFR expression have been observed in lung, prostate, breast, gastric and ovarian cancers, among others\textsuperscript{61}. Several ligands, either radiolabeled peptides or monoclonal antibodies, have been developed for the purpose of in vivo EGFR
Monoclonal antibodies have also been developed for treatment; they are highly specific with few side effects and may be synergistic with chemotherapy and radiation\textsuperscript{60, 62}; an example is cetuximab (IMC-C225), a human–murine chimeric IgG monoclonal antibody that competitively binds to the extracellular domain of EGFR, preventing tyrosine kinase activation, inhibiting cell growth, and in some cases inducing apoptosis\textsuperscript{62}. EGFR targeted therapy may therefore, be useful in the early diagnosis and possible treatment of lung cancer\textsuperscript{60}.

### 2.3.2.2. Tyrosine-Kinase Receptor HER2

The orphan transmembrane tyrosine kinase receptor HER2 has been detected in adenocarcinomas and squamous cell carcinomas\textsuperscript{42}. Despite the absence of ligands for HER2, HER2 can be activated in response to EGF by forming heterodimers with EGF-R, resulting in cell proliferation\textsuperscript{62}.

The anti-HER2 monoclonal antibody ‘trastuzumab’ blocks tumor growth in patients with metastatic breast carcinoma\textsuperscript{42, 62}, and preliminary studies suggest that Trastuzumab may also hold a therapeutic potential for patients with NSCLC\textsuperscript{62}.

### 2.3.3. Overcoming Cellular Resistance

Unfortunately, targeted cancer therapy alone is not always successful because the targeted drug still has to overcome many types of resistance; as described by Minko et. al.\textsuperscript{85}. One type is multidrug resistance (MDR), which can be further subdivided into 1) Pump (active drug efflux) and 2) Non pump resistance. ATP-binding cassette (ABC) transporters (P-glycoprotein, PgP and Multidrug resistance associated protein transporters, MRP) are examples of pump resistance, whereas, cellular defense mechanisms are a type of non-pump resistance.
Today, antisense oligonucleotides therapy (ASO) and small interfering RNA (siRNA) are being used to inhibit cellular resistance of various proteins by inhibiting gene expression at the transcript level\textsuperscript{90, 94}. ASO are designed to target the complementary sequence within a given RNA. Upon delivery into the cell, they hybridize to the RNA complement to interfere with gene expression and inhibit protein production\textsuperscript{90, 92, 94}, as illustrated in figure 2.3.3.a.\textsuperscript{90}.

Hence, to further enhance the efficacy of cancer therapy, our lab\textsuperscript{85, 91-94} employed antisense oligonucleotides (ASO) to suppress the overexpression of:

1) MRP-1, hereby inhibiting pump resistance and

2) BCL-2, a protein family that functions in the cellular protection against apoptosis. It consists of proapoptotic and antiapoptotic members. Antiapoptotic members limit activation of caspases and prevent leakage of cytochrome $c$ into the cytoplasm, hence, inhibiting apoptotic cell death. Proapoptotic members, on the other hand, stimulate apoptosis mainly by suppressing the activity of antiapoptotic BCL-2 family proteins\textsuperscript{91-94}.

The BCL-2 family is characterized by specific regions of homology termed BCL-2 homology (BH1, BH2, BH3, BH4) domains, which are critical for these proteins. BCL-2 homology 3 (BH3) domain of proapoptotic proteins from the BCL-2 family is responsible for the induction of apoptosis\textsuperscript{91}.

Hence, our lab proposed an advanced proapoptotic anticancer DDS (APADDS), as described by Minko et al.\textsuperscript{85} and illustrated in figure 2.3.3.b.\textsuperscript{85}. This APADDS is composed of at least 5 components: 1) drug carrier; 2) targeting moiety to cancer cells; 3) anticancer drug; 4) a suppressor to pump resistance; and 5) a suppressor to non-pump resistance.
2.4. In Vitro Imaging Using Microscopy

2.4.1. Optical (or light) microscopy\textsuperscript{63, 73}

Standard optical microscopy has many limitations, such as the lack of sufficient contrast and poor resolution.

2.4.2. Fluorescence Microscopy and Types of Fluorescent Probes

2.4.2.1. Fluorescence Microscopy

Fluorescence is when certain compounds are illuminated with high energy light (excitation light), they are able to emit a different light of a lower frequency (fluorescence emission)\textsuperscript{63, 73}. This method is important in biomedical research since a fluorescent image is extremely sensitive and highly specific\textsuperscript{68}. Another advantage of this technique is that it allows the simultaneous detection of many fluorescent dyes labeling different biological structures, while maintaining specificity\textsuperscript{63, 68, 73}.

Examples of commonly used fluorophores are fluorescein or rhodamine and green fluorescent protein (GFP)\textsuperscript{68}.

2.4.2.2. Types of Fluorescent Probes\textsuperscript{64, 68}

The development of stable and bright fluorescent probes allows the ability to visualize individual cells in vitro and in vivo. They include:

1. **Reactive and conjugated probes.** Examples: Fluorescein isothiocyanate (FITC, a reactive derivative of fluorescein), as well as rhodamine, and cyanine.

2. **Molecular Probes**, such as the Alexa Fluors and the DyLight Fluors. They are a newer generation of fluorophores more photostable, brighter, and less pH-sensitive than other standard dyes of comparable excitation and emission\textsuperscript{70}.
3. **Cyanine Dyes.** They belong to a synthetic dye family with the common molecular formula: $\text{ArN}^+=\text{CH}[\text{CH}=\text{CH}]_n=\text{NAr}$ (‘Ar’ is an aromatic moiety). Depending on the structure, they cover the spectrum from infra-red (IR) to ultra-violet (UV)\textsuperscript{75}.

4. **Nucleic acid probes.** Example: Molecular beacon probes, which are oligonucleotide probes that fluoresce when they bind to a target nucleic acid sequence. This is a novel nonradioactive method for detecting specific sequences of nucleic acids. Figure 2.4.2.2.a. depicts the operation of molecular beacons\textsuperscript{71}.

   A typical molecular beacon probe is 25 nucleotides long. The middle 15 nucleotides are complementary to the target DNA and do not base pair with one another, and the five nucleotides at each end are complementary to each other and not to the target DNA\textsuperscript{71, 72}.

5. **Fluorescent Proteins.** 2 examples are:

   1) **Green Fluorescent Protein (GFP).** GFP is a protein comprised of 238 amino acids, from the jellyfish *Aequorea victoria*. It fluoresces green when exposed to blue light\textsuperscript{74}. While most small fluorescent molecules such as FITC (fluorescein isothiocyanate) are strongly phototoxic when used in live cells, fluorescent proteins such as GFP are not toxic at all\textsuperscript{68, 74}. The GFP gene can be introduced into organisms and maintained in their genome. To date, many bacteria, yeast and other fungal cells, plant, and mammalian cells have been created using GFP as a marker\textsuperscript{68, 112-114}.

   2) **Fluorescence Resonance Energy Transfer (FRET).** FRET is an important technique used to study whether two proteins interact with each other. Examples: Cyan and Yellow fluorescent proteins\textsuperscript{68}. Recently, FRET was used to examine the activity
of calpain, a cysteine protease, 200 um deep in live mouse tissue using multiphoton microscopy\textsuperscript{69}.

7. Quantum Dots (QD). Example: Cadmium selenide (CdSe) quantum dots. They are inorganic nanocrystals, comprised of a crystalline core of semiconductor material, such as CdSe, coated with a shell that can be conjugated with proteins, lipids, or other moieties\textsuperscript{65-67}. Semiconductor quantum dots (QDs) offer several advantages over organic dyes in fluorescence imaging applications such as higher quantum yield, extreme photostability, and a high emission intensity\textsuperscript{65}. In addition, they are nanometers in diameter, their core size can vary, and they are ideal for multicolor applications\textsuperscript{66}.

Wang et al.\textsuperscript{67} labeled the ovarian carcinoma marker CA125 with QD in different types of specimens (fixed cells, tissues, and xenografts). They reported that the photostability of QD was much higher than that of the organic dye, FITC. Also, all labeling signals of QDs were more specific and brighter than those of FITC.

Figure 2.4.2.2.b.\textsuperscript{88} depicts a photostability comparison between quantum dots and FITC conjugated to Tca8113/BLM cells, their results show that in 30 minutes, FITC fluorescence almost disappeared meanwhile the fluorescent signal of QDs remained strong.

2.4.3. Confocal laser Scanning Microscopy vs. Multiphoton Microscopy

Both confocal laser scanning microscopy (CLSM) and multiphoton microscopy allow a much higher resolution and a significantly sharper 3D image than optical microscopy\textsuperscript{63}.
Both techniques use lasers as excitation sources to scan samples which fluoresce\textsuperscript{63, 73}. However, multiphoton microscopy offers several advantages over confocal LSM microscopy when imaging living tissue\textsuperscript{68, 76, 81}:

1. Multiphoton microscopy is much more efficient in imaging deep tissue than confocal microscopy.

2. Multiphoton microscopy uses the long wavelength, low energy (typically infra-red) excitation lasers. This is important in imaging live cells as they cause less damage than short wavelength lasers, so cells may be observed for longer periods with fewer toxic effects.

Figure 2.4.3.\textsuperscript{76} depicts a comparison of the same frontal sections of human skin in vivo images obtained using either a confocal (a-d) or multiphoton microscope (e-h). Their results show that the images acquired using the multiphoton microscope were of a much higher quality than those obtained with the confocal microscope, especially at deeper layers of the skin.

\textbf{2.4.4. Further Enhancements}

For fast motions, some techniques were developed:

\textbf{2.4.4.1. Multispot-Scanner Microscopy}

Multispot-scanner confocal microscopes (also called ‘slit-scanning confocals’) collect data from multiple spots at once. Some examples of commercially available multipoint scanners are the Perkin Elmer Ultraview spinning disk confocal microscope\textsuperscript{77}, or the Zeiss LSM 5 LIVE confocal microscope\textsuperscript{78}.

\textbf{2.4.4.2. Sophisticated Software}
Sophisticated software such as advanced spectrometers and analysis packages are very useful; for instance, they allow the simultaneous imaging of multiple fluorophores as well as the elimination of autofluorescence. Example of multispectral detectors include: The LSM 510 META detector (Zeiss)\textsuperscript{78} or the TCS SP system (Leica)\textsuperscript{80}.

2.4.5. Electron Microscopy

2.4.5.1. Transmission electron microscopy (TEM): The resolution limit of TEM nowadays is around 0.05 nanometer\textsuperscript{63}.

2.4.5.2. Scanning electron microscopy (SEM): Figure 2.4.5.2.\textsuperscript{89} illustrates SEM images of paclitaxel loaded PLGA nanospheres with (a) PVA and (b) DPPC as emulsifiers. The SEM images show that the DPPC emulsified nanospheres have a relatively smaller size.

2.4.6. X-ray microscopy

X-ray microscopy uses electromagnetic radiation to produce images. The resolution of X-ray microscopy lies between that of light (optical) microscopy and the electron microscopy\textsuperscript{63, 73}.

2.4.7. Scanning probe microscopy

Examples of scanning probe microscopy include: The Atomic Force Microscope (AFM), the Scanning Tunneling Microscope (STM) and the Photonic Force Microscope (PFM)\textsuperscript{63, 73}.

2.4.7.1. The atomic force microscope (AFM)

AFM is one of the most popular microscopes in nanoscale imaging.
The AFM has several advantages over the Scanning Electron Microscope (SEM)\textsuperscript{63, 73, 82, 83}. For instance, AFM provide a 3D images, samples viewed by AFM do not need any treatment that would damage them, and AFM provide higher resolution than SEM. Disadvantages of AFM compared with the scanning electron microscope (SEM) include the following: The AFM can only image a maximum height on the order of micrometers and a maximum scanning area of 150x150 micrometers\textsuperscript{63}, while the SEM can image an area on the order of millimetres by millimetres with a depth of field on the order of millimetres. Also, AFM cannot scan images as fast as an SEM\textsuperscript{63, 73}.

Figure 2.4.7.1\textsuperscript{89} shows topography (amplitude analysis) of poly(lactic-co-glycolic) (PLGA)/paclitaxel nanospheres characterized by atomic force microscopy using (a) PVA, (b) DPPC as emulsifiers. These AFM images show that the surface of the nanospheres fabricated with DPPC was much smoother than that of the nanospheres fabricated with PVA.

2.4.7.2. The scanning tunneling Microscope (STM) scans an electrical probe over a surface to be imaged\textsuperscript{63}.

2.4.7.3. The Photonic Force Microscopy (PFM) permits 3D image detection with very high accuracy and at a very high rate. Hence, images obtained have excellent resolutions\textsuperscript{84}.

2.5. Non-invasive In Vivo Imaging

In vivo molecular imaging is much more challenging than in vitro detection for many reasons: For instance, probes must be biocompatible, delivery barriers must be
overcome, amplification techniques (such as, suitable ligands and targeting moieties), and highly sensitive imaging systems must be available.

Figure 2.5.95 depicts a diagram comparing key elements for in vitro and in vivo molecular imaging. This section 2.5. is divided by type of imaging into six main categories: 1. Nuclear imaging, which includes PET and SPECT, 2. Magnetic Resonance Imaging (MRI), 3. Computed Tomography (CT) and Ultrasound Imaging, 4. Fluorescence Imaging, 5. Near-infrared Fluorescence (NIRF) Imaging, and 6. Bioluminescence Imaging. Table 2.6.96 provides a summary of currently available imaging techniques and their characteristics.

2.5.1. Nuclear (Radionuclide) imaging

Radionuclide imaging, which includes positron emission tomography (PET) and single photon emission tomography (SPECT), provides high sensitivity and moderate spatial resolution in evaluating gene expression via the protein product96-99, which can be an enzyme for the radiolabeled substrate101 or a receptor that binds to the radiolabeled receptor ligands102.

2.5.1.1. Positron Emission Tomography (PET)

PET is an important technique used for the in vivo visualization of various molecular processes related to cell proliferation within tumors100. PET is used to detect decaying nuclides such as $^{11}$C, $^{18}$F, $^{15}$O, $^{124}$I and images the distribution in vivo of trace quantities of positron emitting molecular probe administered. Examples of PET probes include [$^{18}$F]FHBG (9-[4-[$^{18}$F]fluoro-3-(hydroxymethyl) butyl]guanine) and [$^{124}$I]FIAU (5-iodo-20-fluoro-20-deoxy-1-b-D-arabino-furanosyl-uracil) and thymidine kinase (TK) is used as a proliferation marker.
As part of their triple retroviral vector reporter system, Doubrovin et al. used the herpes simplex virus type 1 thymidine kinase (HSV1-tk) as a reporter gene and 5-iodo-2’-fluoro-2’deoxy-1-ß-D-arabino-furanosyl-uracil (FIAU) as a reporter probe for PET imaging. Figure 2.5.1.103 illustrates the PET image acquired (image C, right panel). The other 2 components of this system are enhanced green fluorescent protein, eGFP; firefly luciferase, FLuc which will be described under fluorescence imaging and Bioluminescence, respectively.

MicroPET is a noninvasive system that allows serial and longitudinal imaging in small animals, which is great for monitoring the effects of therapy96, 103.

2.5.1.2. Single photon computed tomography (SPECT)

SPECT relies on gamma emitting radionuclides, such as 111In, 123I, 201TI or 99mTc introduced into an organism. These radionuclides emit a single photon97, 104.

The gene encoding the human somatostatin receptor type-2 has been used as a marker gene along with the marker, receptor binding compound [99mTc]-P2045 in SPECT imaging105. 99mTc-methoxyisobutylisonitrile(MIBI) SPECT has also imaged P-glycoprotein overexpression in vivo106.

2.5.2. Magnetic Resonance Imaging (MRI)

Normal and abnormal tissues respond differently to the slight magnetic field alteration produced by MRI giving different signals, which are then transferred into images108. Supramagnetic nanoparticles can be used as probes in MRI107, 108. Imaging of gene expression using MRI may be done by imaging of introduced reporters or enzymes, which bind paramagnetic substrates. For instance, the human transferrin
receptor (TfR) is expressed at higher levels on a variety of tumor cell types and can be imaged using transferin (Tf) bound supramagnetic particles\textsuperscript{109}.

2.5.3. Computed tomography (CT) and Ultrasound Imaging

Computed tomography (CT), originally known as computed axial tomography (CAT or CT scan) uses X-rays to acquire images. CT scans are ideal for examining bone and calcifications within the body\textsuperscript{108,110}. Ultrasound imaging, on the other hand, is effective in imaging soft tissues of the body\textsuperscript{108,111}.

2.5.4. Fluorescence imaging

Fluorescence imaging is used in our studies because it is a noninvasive, rapid, and inexpensive technique used to visualize cells. For instance, GFP fluorescence imaging has many cancer applications: For example, human ovarian tumor cells were infected with a replication-deficient adenovirus vector encoding GFP, and the GFP-positive cells were imaged both in culture and in vivo by fluorescence stereomicroscopy in mice\textsuperscript{115}.

As part of their triple-reporter retroviral vector system, Doubrovin et al. used enhanced Green Fluorescence Protein \textit{eGFP}. Figure 2.5.1\textsuperscript{103} illustrates the acquired green fluorescent image (image B, right panel) of mice bearing subcutaneous xenografts infected with the triple reporter retroviral vector.

GFP is very limited in imaging within organs in living animals because GFP excitation and emission wavelengths, which fall in the range of 500 nm (i.e., green light), have a very low tissue penetration (1–2 mm)\textsuperscript{117,118}.

2.5.5. Intravital microscopy (IVM)

IVM allows noninvasive monitoring of cellular activities in living tissue\textsuperscript{119,120}. IVM has improved cancer detection and treatment\textsuperscript{120,121}; for instance, when subcutaneous
pancreatic tumors in SCID mice were injected with adenovirus vectors encoding the VEGF flt-1 receptor (Adsflt) or control vectors (AdLacZ), IVM showed that cells infected with AdLacZ underwent strong tumor angiogenesis, whereas Adsflt-infected cells failed to exert such an effect\textsuperscript{122}.

2.5.6. Near-infrared Fluorescence (NIRF) imaging

Imaging in the near-infrared (NIR) spectrum (700–900 nm) maximizes tissue penetration and minimizes background auto fluorescence. NIR fluorescence imaging has light as a source of photons that encounters a fluorescent molecule, which, in turn, emits a signal with specific spectral characteristics that can be captured with an emission filter and a CCD camera\textsuperscript{123, 124}.

There are several commercially available devices for NIR imaging in vivo, such as the IVIS system (Xenogen Corporation, Alameda, CA)\textsuperscript{125}, which we use in our studies.

Figure 2.5.6\textsuperscript{124} depicts a diagram that shows the general experimental set up for the near-infrared fluorescence imaging system, which to some extent, resembles fluorescence microscopy systems.

NIRF probes have been used in detecting very small tumors\textsuperscript{126, 127}. For instance, today, using NIRF imaging, it becomes possible to determine directly and noninvasively the efficacy of Matrix metalloproteinase (MMP) inhibitors, in clinical testing\textsuperscript{128}.

2.5.7. Fluorescence-mediated tomography (FMT)

FMT can be used in clinics for early detection of a variety of cancers; for instance, using FMT and NIRF probes, a relationship has been established between protease levels and invasiveness in breast cancer\textsuperscript{129}.

2.5.8. Bioluminescence imaging
Bioluminescence is the emission of visible photons at specific wavelengths based on ATP-dependent reactions catalyzed by luciferases. Luciferases are photoproteins that emit detectable photons in the presence of oxygen and ATP during metabolism of substrates such as luciferin into oxyluciferin. Examples of luciferase systems include the eukaryotic luciferase luc and ruc genes from firefly species and Renilla reniformis, respectively. The light from these enzyme reactions can extend beyond 600 nm, with the red components of the emission spectra being the most useful in imaging due to easy tissue penetration.

Bioluminescence imaging is a sensitive and rapid technique used to study tumor cell growth and regression, and to track gene expression in living animal models. Sensitive imaging systems have been invented to detect small numbers of cells or organisms expressing luciferase; the IVIS system (Xenogen Corporation, Alameda, CA), which we use in our studies, is a good example.

In one study by Rehemtulla et al., a recombinant adenovirus coexpressing cytosine deaminase (CD) and firefly luciferase (Fluc) injected directly into the gliomas in mice showed high expression of luciferase. Further treatment of animals with 5-fluorocytosine (5-FC) has lead to a decrease in the luciferase imaging signal. The group used MRI, in parallel to confirm their results.

The luciferases from Renilla and firefly have different substrates, coelenterazine and D-luciferin, respectively, and can be imaged in tumors in the same living mouse because time of light production can be different by giving separate injections of these two substrates, thus, luciferases can be used to image two or more biological events in a single animal. Recently, dual bioluminescence imaging has been used to monitor gene
delivery via a vector bearing the therapeutic protein TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in gliomas\textsuperscript{135}; Shah et al. injected subcutaneously glioma cells stably expressing Firefly luciferase (Fluc) into nude mice and monitored tumor growth in vivo over time by luciferin administration and bioluminescence. They also injected HSV amplicon vectors bearing the genes for TRAIL and Renilla lucirefase (Rluc) into these Fluc-positive gliomas allowing superimposition of gene delivery to the tumor by coelentrazine administration and bioluminescence. Figure 2.5.8.\textsuperscript{135} is an illustration of their results that shows bioluminescence images with D-luciferin for Fluc activity to monitor tumor growth (Left side, image a) and bioluminescence images with coelenterazine for Rluc activity to track gene delivery to the tumor (Right side, image b). This dual imaging approach is useful in studying gene delivery vectors and simultaneously tracks their therapeutic effects in vivo.

As part of their triple-reporter retroviral vector system, Doubrovin et al. used bioluminescence with Firefly luciferase (FLuc). Figure 2.5.1.\textsuperscript{103} illustrates the acquired bioluminescence image with D-luciferin for Fluc activity of mice bearing subcutaneous xenografts infected with the triple reporter retroviral vector (image A, right panel).

Bioluminescence in experimental animals is fast, relatively inexpensive, and very useful in cancer research, and can be used in parallel with other molecular imaging techniques.

2.6. Animal Models of Lung Cancer

Model systems of lung cancer are important to understand and treat the disease:
1. **Spontaneous or chemical induced lung cancer models:**

These models are well representatives of human lung cancer\textsuperscript{136-141}. Strain A mice are used extensively to assess carcinogenic activity of chemicals agents, including benzopyrene, metals, aflatoxin, and polyaromatic hydrocarbons and nitrosamines\textsuperscript{142-144}. Unfortunately, these tumors are measurable late in their course, their metastatic pattern is not uniform, and their response to therapy is poor. Because of their limitations, these model systems are mainly used to study the early stages of carcinogenesis\textsuperscript{145}.

2. **Transgenic lung cancer models:**

Transgenic technology has produced a variety of mouse lung cancer models. Gene transfection can be achieved with microinjection\textsuperscript{146-148}, retroviral infection, or embryonic stem cell transfer\textsuperscript{149-152}.

Unfortunately, since malignant cells are directly inoculated into the host animal, early events, such as initiation and carcinogenesis, are not well suited for study but testing of new therapies can be done with these models.

3. **Human lung tumor xenografts:**

Since human neoplasms are rejected when implanted into another species, the host animal must be immunosuppressed. Examples of immunosuppressed laboratory animals available for transplantation of human tumors are: Hairless nude mouse mutants (nu/nu homozygotes), severe combined immunodeficient (SCID) mice and Rowett nude rats. Subcutaneous implantation in nude mice is the most common method of transplanting human tumors. The site, usually the dorsal lateral flank, is easily
accessible. These models have some disadvantages; for instance, tumor growth in an unusual site (the subcutis), which might affect study results\textsuperscript{153-155}.

4. Orthotopic lung cancer models:

In orthotopic models, human tumors are implanted directly into the appropriate organ or tissue of origin in the laboratory animal. Advantages include improved tumor uptake and enhanced metastatic properties\textsuperscript{154, 156, 157}. Examples: Colon carcinoma cells grown in the cecal wall, bladder carcinoma in the bladder, renal carcinoma cells under the renal capsule, and melanomas implanted subdermally (all yield metastases at much higher frequency than when grown subcutaneously)\textsuperscript{158, 159}, thereby supporting Paget's hypothesis that malignant cells have special affinity for growth in the environment of their origin (The seed and soil theory)\textsuperscript{160}. The models, however, are much more complex and more costly than subcutaneous models.

Orthotopic lung cancer models are developed using endobronchial, intrathoracic or intravenous injection of tumor cell suspensions\textsuperscript{161-165} and by surgical implantation of fresh tumor tissue\textsuperscript{166, 167} all using immunocompromised mice.

McLemore et al. developed the first orthotopic lung cancer model by implanting lung cancer cell lines into the lung of nude mice by endobronchial injection\textsuperscript{164}. A second model was developed by McLemore by injecting lung tumor cells into the pleural space\textsuperscript{168}. There are several other intrathoracic human lung cancer models; for instance, the traditional intravenous model via tail vein injection\textsuperscript{169, 170}, or tumor injection into lungs\textsuperscript{171, 172}. Recently, Miyoshi et al.\textsuperscript{173} developed a SCID lung cancer model by
percutaneous injection of cancer cells into the mouse lung. It is also possible to implant human cancer cells into mouse lung\textsuperscript{174}. In 1992, Wang et al. implanted human small cell lung cancer tissue into mouse lung\textsuperscript{172}. Table 2.6\textsuperscript{145} provides an overview of orthotopic lung cancer models.
2.6. References

5. http://www.cancer.org/docroot/ETO/content/ETO_1_4X_What_Are_The_Different_Types_Of_Chemotherapy_Drugs.asp?sitearea=ETO
10. http://www.plwc.org/portal/site/PLWC/menuitem.6067beb2271039bcfd748f68ee37a01d/?vgnextoid=1d27ea7105daa010VgnVCM100000ed730ad1RCRD&vgnextfmt=cancer

18. http://www.nasa.gov/images/content/81688main_Synthesis_Structure_med.jpg


35. http://www.dekker.com/sdek/abstract~db=enc~content=a713491611


64. http://pingu.salk.edu/flow/fluo.html


http://en.wikipedia.org/wiki/Molecular_beacon
http://www.biologie.uni-hamburg.de/b-online/e03/03.htm
http://en.wikipedia.org/wiki/Green_fluorescent_protein
http://en.wikipedia.org/wiki/Cyanine
http://www.zeiss.de/lsm
http://www.fz-borstel.de/flowcytes/leica/spbroch.pdf
http://www.embl-heidelberg.de/ExternalInfo/stelzer/photonicfm.html


Table 2.3. Peptide Receptors in Cancer\(^5\).
<table>
<thead>
<tr>
<th>Imaging methods</th>
<th>Isotopes</th>
<th>Molecular marker</th>
<th>Tissue depth</th>
<th>Potential for human imaging</th>
<th>Advantages</th>
<th>Reporter/jmocess</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>$^{18}$F, $^{14}$C, $^{18}$F, $^{32}$P, $^{82}$Rb, $^{123}$I</td>
<td>HSV-1-7K, NGF receptor, D2 receptor, NIS</td>
<td>No limit</td>
<td>Yes</td>
<td>Gene expression, reporter enzyme targeting, high sensitivity and use in quantitative translational research</td>
<td>$^{[18]}$F FHBG, $^{[82]}$Rb FIAU, $^{[123]}$I NGF, $^{[32]}$P FESP</td>
</tr>
<tr>
<td>SPECT</td>
<td>$^{111}$In, $^{123}$I, $^{99m}$Tc</td>
<td>Somatostatin receptor subtype 1, EHHDR2, MDRI</td>
<td>No limit</td>
<td>Yes</td>
<td>Gene expression, imaging multiple probes simultaneously</td>
<td>$^{[99mTc]}$-P2045</td>
</tr>
<tr>
<td>MRI</td>
<td>Vascular permeability, endocytosis, transferrin receptor, tyrosinase, beta-galactosidase</td>
<td>No limit</td>
<td>Yes</td>
<td>Gene expression, high spatial resolution and has the ability to combine both functional and morphological imaging</td>
<td>Gadolinium, MION transferrin, iron-oxide particles, galactopyranose</td>
<td></td>
</tr>
<tr>
<td>MRS</td>
<td>CD, CK</td>
<td>No limit</td>
<td>Yes</td>
<td>Gene expression</td>
<td>5-FC dFlucyl</td>
<td></td>
</tr>
<tr>
<td>FRI-visible</td>
<td>GFP, BFP, CFP, YFP, RFP</td>
<td>1 cm</td>
<td>Yes, under certain limits</td>
<td>Sensitive, detects fluorochroms in live and dead cells</td>
<td>Fluorescent proteins</td>
<td></td>
</tr>
<tr>
<td>FRI-NIR</td>
<td>Proteases: Cathepsin-D and -H, MMP-2, Caspase-3</td>
<td>1 cm</td>
<td>Yes, under certain limits</td>
<td>Activatable, detects fluorochroms in live and dead cells</td>
<td>Peptide-caged activatable NIR fluorochroms</td>
<td></td>
</tr>
<tr>
<td>FMT</td>
<td></td>
<td>No limit</td>
<td>Yes</td>
<td>Morphological, tumor imaging</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLI</td>
<td>Fluc, Bluc</td>
<td>1-2 cm</td>
<td>Yes, under certain limits</td>
<td>Gene expression, cell tracking, quick and easy</td>
<td>Luciferin, coelenterazine</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. Characteristics of currently available in vivo imaging techniques$^{96}$. 
Table 2.5. Orthotopic Lung Cancer Models

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal</th>
<th>Tumor material</th>
<th>Inoculation method</th>
<th>Take rate</th>
<th>Regional metastatic sites</th>
<th>Distant metastatic sites</th>
<th>Average growth time</th>
</tr>
</thead>
<tbody>
<tr>
<td>McLemore [80]</td>
<td>Nude mice</td>
<td>H125, H358, H460, A549</td>
<td>Endobronchial</td>
<td>90%</td>
<td>Trachea 2%, peritracheal 6%, Lymph node 90%</td>
<td>Left lung, liver, spleen 3%</td>
<td>9–61 days</td>
</tr>
<tr>
<td>Howard [78]</td>
<td>Nude rats</td>
<td>H125, H460, A549, H345</td>
<td>Endobronchial</td>
<td>100%, 83%, 90%</td>
<td>Regional lymph nodes</td>
<td>H-125, A549 to contralateral lung</td>
<td>H460 (3 wks)</td>
</tr>
<tr>
<td>Howard [9]</td>
<td>Nude rats</td>
<td>Tumor fragment derived from H460 lung tumors</td>
<td>Endobronchial</td>
<td>100%</td>
<td>Lymph node 100%</td>
<td>Bone, brain, kidney, left lung, soft tissue</td>
<td>32–35 days</td>
</tr>
<tr>
<td>Wang [89]</td>
<td>SCID mice</td>
<td>Tumor fragment derived from A549 subcutaneous tumor</td>
<td>Thoracotomy</td>
<td>3/5</td>
<td>Chest wall</td>
<td>Contralateral lung</td>
<td>N/A</td>
</tr>
<tr>
<td>Wang [79]</td>
<td>SCID mice</td>
<td>Human SCLC tumor fragment</td>
<td>Thoracotomy</td>
<td>100%</td>
<td>Mediastinum, chest wall lymph nodes</td>
<td>Contralateral lung</td>
<td>18.5–62 days</td>
</tr>
<tr>
<td>Nagamachi [88]</td>
<td>Nude mice</td>
<td>A549, H23, H441, H157, Lu65, Lu69/A PC9, PC14</td>
<td>Intraperitoneal</td>
<td>100% except H23</td>
<td>Mediastinum, lymph nodes</td>
<td>Contralateral lung</td>
<td>Depends on specific cell line, PC14 within 30 days</td>
</tr>
<tr>
<td>Miyoshi [91]</td>
<td>SCID mice</td>
<td>Muc-44</td>
<td>Percutaneous intrapulmonary</td>
<td>N/A</td>
<td>Lymph node 52%</td>
<td>Contralateral lung</td>
<td>17.5 ± 6.0 days</td>
</tr>
<tr>
<td>Cuencas [92]</td>
<td>SCID mice</td>
<td>Human NSCLC biopsy specimens</td>
<td>Anterior thoracotomy</td>
<td>31%</td>
<td>N/A</td>
<td>Metastasis rate 50%; Contralateral lung 37.5%</td>
<td>4–6 months</td>
</tr>
</tbody>
</table>
Figure 2.1. Estimated Mortality of Lung Cancer in 2005\textsuperscript{4} (The most recent year for which statistics are currently available).
Figure 2.2.1.a. Chemical Structure of polyethylene glycol (PEG)\textsuperscript{12}.

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Figure 2.2.3.b. Acceptance of Liposome into Cell\textsuperscript{25}.
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Within minutes, aggregation of the fluorescent signal of AN-152 on the cell membrane was observed, indicating binding of the ligand to the receptors. This was followed by the strengthening of signals in the cytoplasm, demonstrating internalization of AN-152. Within 60 mins after exposure, the fluorescent signal was accumulated in the nucleus.
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Most ASO are single-stranded sequences which induce degradation of the target mRNA by activating the endonuclease RNase H in the nucleus (shown on the left).

RNA interference using double-stranded siRNA molecules occurs in the cytoplasm. It is initiated by formation of the RISC followed by siRNA unwinding, binding of the antisense strand to the target mRNA and its subsequent degradation by an endonuclease (shown on the right).
Figure 2.3.3.b. Advanced Proapoptotic DDS (APADDS)\textsuperscript{85}.
Figure 2.4.2.2.a. Operation of molecular beacons.\textsuperscript{21}
Figure 2.4.2.2.b. Photostability comparison between quantum dots and FITC conjugated to Tca8113/BLM cells\textsuperscript{88}.

Top row is the fluorescence of quantum dots and bottom row is that of FITC. The images from left to right were obtained at A = 0; B = 5; C = 10; D = 15; E = 20; F = 25; G = 30min.
Figure 2.4.3. A comparison of the same frontal sections of human skin in vivo images obtained using either a confocal (a-d) or multiphoton microscope (e-h)\textsuperscript{76}.

Images were acquired at the regions: 10 microns below surface in stratum corneum (a and e), cells of stratum spinosum (b and f), cells of basal layer (c and g), and within the dermis (d and h). Scale bars show 50 microns.
Figure 2.4.5.2. SEM images of paclitaxel loaded PLGA nanospheres with (a) PVA and (b) DPPC as emulsifiers.\textsuperscript{89}
**Figure 2.4.7.1.** Topography (amplitude analysis) of poly(lactic-co-glycolic) (PLGA)/paclitaxel nanospheres characterized by atomic force microscopy using (a) PVA, (b) DPPC as emulsifiers\textsuperscript{89}. 
Figure 2.5. Comparison of key elements for in vitro and in vivo molecular imaging\textsuperscript{95}. 
Figure 2.5.1. Noninvasive in vivo multimodality imaging of mice bearing subcutaneous xenografts produced from HSV1-tk/eGFP-cmvFluc transduced U87 cells (right shoulder) and wild-type (nontransduced) U87 cells (left shoulder). The xenografts were 4-5 mm in diameter.

Right Panel: (A) Whole-body bioluminescence imaging, (B) whole-body fluorescence imaging, and (C) axial and coronal microPET images of $[^{18}F]$FEAU accumulation are shown for the same mouse.

Left Panel: The multimodality triple-reporter system, which is a retroviral vector (TGL) for mammalian expression of a triple-fusion reporter gene (HSV1-thymidine kinase, TK; enhanced green fluorescent protein, eGFP; firefly luciferase, FLuc) driven by an LTR promoter.
Figure 2.5.6. Diagram depicts the experimental setup for the near-infrared fluorescence imaging system\textsuperscript{124}.

Fluorescent photons are selected with a 700-nm long pass filter. The emission signal is focused with a zoom lens and is recorded with a cooled CCD camera.
Figure 2.5.8. Dual imaging of amplicon vector delivery and glioma volumes\textsuperscript{135}.

(a) Mice bearing subcutaneous Gli36fluc\textsuperscript{+} gliomas were injected i.p. with D-luciferin and imaged for Fluc activity. (b) TRAIL-Rluc amplicon vector injected into the same tumor and 36 h later, coelenterazine (substrate for Renilla Luciferase) was injected into the tail vein and the mice were imaged for Rluc activity. Each image in (a) and (b) represents a scan time of 1 min. The dashed circle around the tumor indicates the tumor periphery.
3. SPECIFIC AIMS

Since multivalent tumor targeted nanocarrier drug delivery systems (DDS) are attractive diagnostic as well as therapeutic agents, hence three types of tumor-targeted delivery systems using linear polymer, polymeric dendrimer, and liposomes as carriers were developed, characterized, and compared to similar non-targeted systems. Tumor targeting was achieved by using Luteinizing Hormone-Releasing Hormone (LHRH) as a targeting moiety. We then evaluated in vivo treatment efficacy of targeted and non-targeted delivery systems loaded with anticancer drug paclitaxel (Taxol®).

Moreover, model systems of lung cancer are important for understanding the disease as well as development of new therapies. To this aim, we investigated 2 different orthotopic lung cancer models using (1) intrathoracic and (2) intravenous (Tail Vein) injection of luciferase positive human lung tumor cells. We compared tumor uptake rate, body and organ distribution, degree of invasiveness, among others.

Fourth, a body and organ distribution comparison of our nanocarriers-based delivery systems was done in orthotopic mice models of lung cancer.

Fifth, evaluation of the efficacy of intratracheal vs. intravenous treatment of lung tumor was performed in vivo.

Lastly, we will evaluate the efficacy of small interference RNA (siRNA) in overcoming cellular drug resistance. To this aim, small interfering (SiRNA) targeted to MRP1 mRNA (to suppress pump resistance) and BCL2 mRNA (to suppress non-pump resistance) were investigated.
Hence, we summarize our objectives in the following specific aims:

1. To study receptor-targeted nanocarriers specific for human lung cancer treatment and imaging.

2. *In vivo* evaluation of treatment efficacy of targeted and non-targeted delivery systems loaded with anticancer drug paclitaxel (Taxol®).

3. To develop a reproducible orthotopic mice model of human lung cancer.

4. *In vivo* comparison of body and organ distribution of nanocarrier-based delivery systems after intravenous and intratracheal administration.

5. *In vivo* evaluation of efficacy of intratracheal vs. intravenous treatment of lung tumor.

6. *In vitro* employment of a noninvasive adjuvant therapy that overcomes cellular resistance (MRP, BCL-2, etc.).
4. Receptor - targeted nanocarriers for tumor specific treatment and imaging

4.1. Introduction

Nanotechnology products are most often used as pharmaceutical nanocarriers for delivering drugs or imaging agents to the site of the action in desired quantities and for releasing therapeutic loads with a specific time profile. Linear and branched polymers, dendrimers, quantum dots, nanoparticles, nanospheres, nanotubes, nanocrystals, nanogels, liposomes, micelles, as well as other types of nanocarriers are being employed in different fields of medicine for diagnostics, imaging, treatment and prophylactics of many pathological conditions, including cancer\textsuperscript{1-21}. In contrast to earlier developed nanotherapeutics, which had a relatively simple two-component drug-carrier composition, modern nanocarriers often include other active ingredients that perform different functions for enhancing cellular uptake and efficiency of the main drug, therefore preventing adverse side effects, providing drug release with a predetermined profile in the certain compartment of an organ, tissue or cell, and preventing the development and/or suppression of the existent drug resistance, etc. The increase in complexity and performed functions of nanocarriers actually converts them into multifunctional nanotherapeutical products\textsuperscript{22}.

While nanocarriers are currently being extensively used for delivering imaging and cytotoxic agents to tumors, there are no studies which compare different types of carriers containing the same active ingredients in similar experimental conditions in both \textit{in vitro} and \textit{in vivo} settings. The present study fills this gap and is aimed at investigating the three most widely used types of nanocarriers with different architecture, size and molecular mass (linear polymer, branched star-like dendrimer and liposomes) containing a near-
infrared cyanine Cy5.5 dye (imaging agent) and/or paclitaxel (anticancer drug with low aqueous solubility). In addition, a synthetic analog of luteinizing hormone-releasing hormone (LHRH peptide) was attached to the nanocarriers and used as a targeting moiety (ligand to LHRH receptors that are overexpressed in the plasma membrane of several types of cancer cells and are not expressed detectably in normal visceral organs). Analysis of experimental *in vitro* and *in vivo* data at the first time revealed a very important phenomenon that can potentially have a significant impact on drug delivery in general and cancer chemotherapy in particular. We found that targeting drug carriers to extracellular receptors overexpressed in the plasma membrane of cancer cells, eliminated the differences between the carriers in terms of their internalization by cancer cells, cytotoxicity, tumor and organ distribution, adverse side effects and antitumor activity. Specific targeting of nanocarriers to cancer cells substantially enhanced cytotoxicity and antitumor efficacy of all delivery systems to the comparable and exceptionally high level and limited their adverse side effects despite of the considerable differences in the size, molecular mass of the components, composition and architecture of the carriers. The present study sends an important message that the architecture, composition, size and molecular mass of the receptor - targeted drug nanocarriers can be selected based on other than anticancer efficacy considerations (cost, type of active ingredients, difficulties in production, stability, patient compliance, etc.) ensuring that the high efficacy and low adverse side effects could be achieved automatically by tumor targeting.

4.2. Methods

4.2.1. Nanocarriers
Three types of delivery systems were designed and prepared based on different nanocarriers: linear polymer, branched star-like dendrimer and liposomes (Fig. 1a). All systems contained at least one of the following components: (1) a synthetic analog of luteinizing hormone-releasing hormone (LHRH) decapptide as a targeting moiety; (2) near-infrared cyanine Cy5.5 fluorescent dye as an imaging agent and (3) paclitaxel (TAX) as an anticancer drug. LHRH peptide provided targeting of an entire DS to the receptors overexpressed in many types of cancer cells. TAX was selected as an example of a highly effective chemotherapeutic agent with low aqueous solubility. Cy5.5 represents a class of very effective dyes currently used as cancer imaging agents. The systems were prepared and characterized using procedures previously developed in our laboratory7, 23, 24, 29, 34-36. Based on the results of dynamic light scattering and atomic force microscope measurements, the average size of dendrimers, PEG polymers, and liposomes were about 5, 30 and 100 nm respectively. The preparation of each DS is briefly summarized below.

**Linear PEG polymers.** Cy5.5-PEG conjugate was prepared by the coupling of Cy5.5 NHS ester activated by triethanolamine with Bis-diamine PEG. The resulting compound with one free amine group at the terminal was coupled with succinic acid to form Cy-5.5-PEG-COOH complex, which further reacted with LHRH-NH₂ to form an amide conjugate. To synthesize PEG-TAX conjugate, α,ω bis PEG-citric acid containing carboxyl end groups at the terminals was coupled with hydroxyl groups in paclitaxel to form an ester conjugate. On each stage of the synthesis, the resulting solution was filtered and the filtrate was dialyzed extensively with dimethyl sulfoxide (DMSO) using a dialysis membrane (molecular mass cut off = 2,000 Da) for 24 hours to remove unreacted
low molecular mass components. Furthermore, the conjugate was purified using a size exclusion Sephadex G10 column and dried under the vacuum at room temperature.

**Dendrimers.** Polyamidoamine (PAMAM) generation four hydroxyl dendrimer conjugate with hydroxyl functional group was conjugated with carboxylic group of boc glycine.HCl and further conjugated with Cy5.5 to form dendrimer-glycine-Cy5.5 conjugate. The latter was conjugated with succinic acid to form a dendrimer-glycine-Cy5.5-carboxylic acid conjugate, which in turn was coupled with LHRH. TAX was attached to succinic acid, which is reacted on an equimolar basis with hydroxyl group in paclitaxel to form a paclitaxel-succinic acid conjugate leaving one free carboxyl group for further conjugation with hydroxyl terminal of the dendrimer. The solution of each dendrimeric complex was filtered and the filtrate was dialyzed extensively with anhydrous DMSO for 24 hours to remove unreacted components. The dendrimer was further purified using a size exclusion Sephadex G10 column.

**Liposomes.** DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoetanol amine-N-aminopolyethylene glycol-Mₐ~2000 ammonium salt-polyethylene glycol) was conjugated with LHRH and Cy5.5 as described above. Egg phosphotidyl choline, cholesterol, DSPE-PEG-Cy5.5 and/or DSPE-PEG-LHRH conjugates were dissolved in chloroform, evaporated to a thin film in rotary evaporator, rehydrated with 0.9 % NaCl and gradually extruded through 200 nm and 100 nm pore size polycarbonate filters with an extruder apparatus. The final phospholipid concentration was 20 mM. Paclitaxel was diluted in methanol (50 mg/ml) and added to the liposome suspension in a ratio of 10%/90% v/v. The non-encapsulated drug was separated from liposomes by extensive dialyses with saline. The amount of TAX incorporated into liposomes was determined by
high-performance liquid chromatography (HPLC) using C18 column. The encapsulation efficacy of TAX ranged from 55 to 60 % in different series of experiments. For the determination of the stability of liposomal TAX formulations, drug release from liposomes suspended in a micro-dialysis system was monitored by periodic withdrawals of drug released into the neutral buffer, and detected by HPLC as described above. At 4 °C, leakage from both targeted and non-targeted liposomes was minimal, with less than 5% of TAX lost after 1 month, which is convenient for storage of vesicles.

4.2.2. In vitro experiments

The experiments were carried out on human small H69 and A549 non-small lung cancer cells. The expression of a targeted LHRH receptor was measured in mRNA isolated from cell lysates and commercially available mRNA from healthy human organs by the reverse transcription polymerase chain reaction (RT-PCR) as previously described23, 25, 29. In part of the experiments, LHRH peptide was labeled with rhodamine as previously described24 and incubated within 48 h with lung cancer cells. Cells were fixed, washed, and labeled LHRH was visualized by a fluorescent microscope. In this series of experiments, cellular nuclei were labeled with Hoechst 33258 fluorescent dye. Cellular internalization, accumulation, and distribution of different conjugates labeled with Cy5.5 were analyzed by a confocal microscope. To assess intracellular distribution of the substances, ten optical sections, known as a z-series, were scanned sequentially along the vertical (z) axis from the top to the bottom of the cell. The intensity of fluoresce in each scan was compared with that in the first (top of the cell) image. Average intensity through all 10 images was used to characterize the penetration and accumulation of the analyzed substance in the cell. Cellular viability was analyzed by the modified MTT assay as
previously described\textsuperscript{37, 38} after 48 h incubation with different DS and appropriate controls. Based on the results of the test, the IC\textsubscript{50} doses (the concentrations which kill 50\% of cells) were calculated for free paclitaxel and TAX-containing DS.

4.2.3. \textit{In vivo experiments}

Experiments were carried out on nude mice bearing subcutaneous rapidly grown xenografts of human lung cancer cells as previously described\textsuperscript{39, 40}. Each experimental group consisted of 8-10 animals. According to the approved institutional animal use protocol, the tumor was measured by a caliper every day and its volume was calculated as \(d^2 \times D/2\) where \(d\) and \(D\) are the shortest and longest diameter of the tumor in mm, respectively. When the tumor reached a mean size of 500 mm\(^3\), mice were treated intravenously (via the tail vein) with different DS and appropriate control substances. The paclitaxel concentration was equal to 2.5 mg/kg for free TAX and all TAX-containing DS. At the end of the experiments, apoptosis induction in the tumor and other organs was assessed by measuring the enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in tissue homogenates as previously described\textsuperscript{23}. No group of mice showed any significant differences in mass change. In a separate series of the experiments, different labeled DS were injected intravenously to untreated mice with tumor size about 1500 mm\(^3\) and their distribution in the entire body of mice, excised tumor and other organs was analyzed in anesthetized animals by \textit{in vivo} imaging using IVIS Xenogen imaging system.

4.2.4. \textit{Statistics}
The results are expressed as mean ± SD from 4-8 independent measurements. Statistical analysis was performed as a one-way analysis of variances (ANOVA) and comparisons among groups were performed by independent sample t-tests.

4.3. Results

4.3.1. Different nanocarriers

Three different types of widely used nanocarriers and delivery systems (DS) were prepared and characterized in the present study (Fig. 4.1a). The first DS consisted of a linear poly(ethylene glycol) (PEG) polymer as a carrier, LHRH peptide as a targeting moiety and paclitaxel (TAX) as an anticancer drug or near-infrared cyanine dye Cy5.5 as an imaging agent. The second and third systems employed a branched PAMAM polymer or PEGylated liposome respectively as carriers in combination with LHRH peptide, Cy5.5, and/or TAX. In all systems, LHRH peptide and Cy5.5 dye were conjugated to PEG or PAMAM polymer via a non-biodegradable amide bond, while TAX was conjugated to a polymer via biodegradable ester bond or was incorporated in the phospholipid bilayer of liposomal membrane (Fig. 4.1a). Drug concentration in different DS was measured by HPLC. Structure, size and molecular mass of the designed DS were confirmed by atomic force microscope, MALDI-TOFF mass spectrometry, 1H NMR spectroscopy, dynamic light scattering and molecular modeling. The prepared three types of nanocarrier-based DS covered a wide range of characteristics of nanocarriers including size (5-200 nm), molecular mass of polymers (from ~3 KDa of linear PEG to ~15 KDa of dendrimer) and architecture (linear, branched and vesicular).

4.3.2. LHRH receptors are overexpressed in the plasma membrane of cancer cells and are not expressed detectably in normal visceral organs
Two series of experiments were performed to show the localization and expression of targeted LHRH receptors in cancer cells. In the first series, drug sensitive and multidrug resistant human lung cancer cells were incubated with LHRH peptide labeled by rhodamine (red fluorescence). Cellular nuclei were labeled with nuclear dye Hoechst 33258 (blue fluorescence). Fluorescence was analyzed by a fluorescent microscope and photographed. Light and both fluorescence filters (red and blue) images were digitally combined. Typical representative images of A549 human lung cancer cells are shown in Fig 4.1b. It is clear from the images, that LHRH peptide (ligand) bound to the corresponding LHRH receptors is localized predominately in the plasma membrane of cancer cells. The measurement by RT-PCR of the expression of genes encoding LHRH receptors in different human lung cancer cells and in various healthy human organs (liver, kidney, spleen, heart, lung) showed that LHRH receptors are overexpressed in human lung cancer cells and not detectably expressed in healthy lungs and other visceral organs. These data confirmed our previous findings in ovarian, breast, and prostate cancer cells as well as cancerous and healthy reproductive organs\textsuperscript{23, 25} and provides the rationale of using LHRH peptide as a targeting moiety/penetration enhancer to target different DS specifically to tumors and facilitate their uptake by cancer cells.

4.3.3. Receptor-based targeting to cancer cells improves penetration, accumulation and intracellular distribution of delivery nanocarriers.

Cellular uptake and intracellular distribution of fluorescent dye and different nanocarriers labeled with Cy5.5 were studied by confocal fluorescence microscopy using viable A549 human lung cancer cells. The concentration of fluorescent dye in the incubation media was the same for free dye and dye delivered by various carriers. It was found that
incubation of cancer cells with free Cy5.5 led to the accumulation of only a trace amount of the applied dye in cancer cells (Fig 4.2., panel 1, bar 1). Moreover, the distribution of the dye throughout the body of the cells was highly inhomogeneous with almost a 5-fold difference in the dye concentration from the top to the bottom of the cells (curve 1 in Fig. 4.1c). This distribution reflects the poor penetration ability of free Cy5.5 from the medium contacted with the top of the cell to the bottom of the cell attached to a base of the incubation dish. In contrast to the free dye, labeled non-targeted carriers (DS without LHRH peptide) showed significantly higher penetration ability and accumulation in cancer cells. However, different carriers demonstrated substantial variations in their accumulation in cancer cells (Fig 4.1b). While the labeled linear PEG polymer showed the lowest accumulation, liposomes demonstrated the highest penetration ability and accumulation inside cancer cells (compare bars 2, 4 and 6 in Fig. 4.1b). The concentration difference between the top and bottom of the cancer cells was about 40% for all non-targeted carriers. Targeting to cancer cells by LHRH peptide significantly improved penetration and accumulation of all carriers and decreased variations in their concentrations through the cell (compare bars and curves 3, 5, and 7 in Fig. 4.1 b and c). It should also be stressed that targeting substantially decreased the differences in the accumulation between different carriers. While the difference in the total accumulation inside cancer cells between different carriers reached 3-folds for non-targeted carriers, cancer targeting by LHRH peptide decreased this difference to only 10-20%.

4.3.4. LHRH peptide enhances cytotoxicity of anticancer drug.

Cytotoxicity of free TAX and different DS containing paclitaxel was measured by the MTT assay with appropriate controls. It was found that all non-targeted and cancer
targeted DS by themselves (without an anticancer drug) were not toxic (Fig. 3a). The toxicity of different non-targeted carriers containing TAX varied more than 100-times. While linear PEG polymer-TAX conjugate had the lowest cytotoxicity, PAMAM-TAX dendrimer showed the highest toxicity (Fig. 4.3b). The incorporation of LHRH peptide as a cancer targeting moiety dramatically (5-800 times) enhanced toxicity of all DS and substantially eliminated differences in IC₅₀ doses between the different targeted DS (compare bars 11, 13 and 15 in Fig. 4.3b).

4.3.5. Receptor targeting leads to the preferential accumulation of nanocarriers in the tumor and limits adverse side effects on healthy organs

Body distribution of different non-targeted and tumor-targeted nanocarriers was studied by in vivo imaging after intravenous injections of the systems labeled with Cy5.5 to mice bearing xenografts of A549 human lung cancer cells. Animals were anesthetized with Isoflurane and photographed in visible and fluorescent light using IVIS imaging systems 48 h after the injection. The intensity of the fluorescence was represented on composite light/fluorescent images by different colors with blue color reflecting the lowest fluorescence intensity and red color – the highest intensity. After photographing, animals were euthanized, tumor and organs were excised, photographed, and processed similarly to the images of an entire animal. Typical representative images and histograms of fluorescence distribution in mice obtained in this series of the experiments are presented in Fig 4.4. The data showed that a substantial fraction of all injected non-targeted nanocarriers was accumulated in the tumor. However, significant amounts of non-targeted nanocarriers were found in healthy organs. In addition to the tumor, substantial amounts of linear PEG were found in the liver and kidney, while liposomes and PAMAM
dendrimer accumulated in significant quantities in the liver, spleen, and kidney respectively. Tumor targeting by LHRH peptide significantly changed body distribution of all tumor-targeted nanocarriers increasing their accumulation in the tumor and decreased their build up in healthy organs.

Adverse side effects of different DS containing paclitaxel were assessed by measuring apoptosis induction in the tumor and different organs (Fig. 4.5). Similar to cytotoxicity, the delivery of the anticancer drug by nanocarriers substantially enhanced their ability to induce apoptosis in the tumor but did not prevent adverse side effects of the treatment. In general, apoptosis induction in the tumor and organs correlated with organ distribution of nanocarriers. Tumor targeting dramatically improved the situation leading to an increase in the apoptosis induction in the tumor and almost complete elimination of drug adverse side effects. It should be stressed, that similar to the aforementioned results, tumor targeting also decreased the variations in cell death induction between different DS (compare bar 1 for LHRH-PEG-TAX, LHRH-PAMAM-TAX and LHRH-Lip-TAX in Fig. 4.5.).

4.3.6. Targeting to LHRH receptors enhances antitumor activity of anticancer delivery systems.

Antitumor activity of free TAX and different non-targeted and tumor targeted DS were compared in experiments on nude mice bearing xenografts of A549 human lung cancer cells. It was found that even non-targeted DS containing paclitaxel were significantly more effective in limiting tumor growth when compared with free drug (Fig. 4.6.). We also found differences between various DS: dendrimeric DS was the least effective, while PEG-polymer-based DS was the most effective in terms of the suppression of tumor
growth (compare curves 6 in Figs 6a-c). The average tumor size at the end of the treatment was 839 ± 25, 684 ± 37 and 625 ± 16 mm$^3$ for non-targeted dendrimeric, liposomal, and polymeric DS respectively (Means ± SD, P<0.05 between all series). Targeting of cancer cells by LHRH peptide significantly enhanced antitumor activity of all systems and leveled down the differences between various nanocarriers. Finally, all tumor targeted DS containing paclitaxel caused significant and comparable tumor shrinkage. The average tumor size at the end of the treatment was 4.03 ± 0.21, 4.07 ± 0.39 and 3.93 ± 0.29 folds smaller for targeted dendrimeric, liposomal, and polymeric DS respectively when compared with non-targeted systems (Means ± SD, P>0.05 between all series).

4.4. Discussion

In the present study, at the first time we characterized and compared based on similar experimental conditions, three different types of non-targeted and tumor-targeted delivery systems carrying a similar payload. We selected DS with linear PEG polymer, star-like PAMAM dendrimer and liposomal carriers. The selection of such systems was based on the following considerations. First, these nanocarriers represent the main classes of modern types of nanocarriers which are being used for cancer imaging and chemotherapy$^{26-28}$. Second, these delivery systems employ three of the most widely used types of nanocarrier architecture: linear polymers, branched polymers, and liposomes. Third, they also cover almost the entire range of most frequently exploited nanocarrier sizes (5-200 nm). Four, the same anticancer drug delivered by such different nanocarriers possesses substantially different properties including cellular internalization,
cytotoxicity, tumor and body distribution, adverse side effects on healthy organs, and antitumor activity.

Each of these nanocarriers was targeted specifically to the tumor by adding a targeting moiety – a synthetic analog of luteinizing hormone-releasing hormone. LHRH peptide is a ligand for the receptors that are overexpressed in plasma membrane of breast, ovarian and prostate cancer cells\textsuperscript{25, 29, 30} and, as found in the present study, in some types of lung cancer cells. In contrast, the expression of these receptors in healthy organs in most cases is non-detectable\textsuperscript{23, 31}. It should be stressed, that, in contrast to the therapeutic applications of LHRH hormone, we do not use LHRH peptide as a drug to directly treat hormone-dependent breast, ovarian cancer, and gynecological malignancies\textsuperscript{32, 33}. Instead, we are using the analog of this peptide as a targeting moiety to extracellular LHRH receptors for the direct targeting of cancer cells in concentrations which do not possess neither therapeutic nor cytotoxic effects.

The rationale for this research was ten-fold. First, by employing the tumor-specific targeting moiety, we expected to improve body distribution of different non-targeted nanocarrier-based delivery systems and increase the sensitivity and specificity of imaging and/or treatment of cancer and limit adverse side effects of the treatment on healthy organs. Second, we anticipated that targeting nanocarriers to cancer cells would enhance cellular internalization and distribution, cytotoxicity and antitumor activity of an encapsulated anticancer drug. Third, we hypothesized that tumor-targeting will be the most valuable for the least efficient types of nanocarriers, therefore diminishing the differences between DS of various architecture, size and molecular mass in terms of anticancer efficacy and adverse side effects of the treatment on healthy tissues.
We found that targeting of DS to extracellular LHRH receptors overexpressed in the plasma membrane of many types of cancer cells substantially improved internalization and intracellular distribution of the entire DS and incorporated imaging agent. Targeting also enhanced overall accumulation of a nanocarrier by cancer cells and increased the fraction of the applied dose which actually penetrates cancer cells and therefore has the potential to effectively kill the cells or increase the sensitivity of their detection by a fluorescent or other labeling. Enhanced accumulation by cancer cells of tumor-targeted DS containing an anticancer agent led to the substantial increase in the cytotoxicity of the drug and formed the basis for its high anticancer efficacy. Moreover, high antitumor efficacy of tumor-targeted anticancer DS was accompanied by preferential accumulation of such DS specifically in tumor cells which provided low adverse side effects of cytotoxic DS on healthy organs and increased the specificity and sensitivity of cancer detection by fluorescent or other imaging agents. It is also very important that tumor-specific targeting minimized the differences between nanocarriers of distinct types of architecture, size, mass, and composition.

The results of the present study exceeded our expectations. Experimental data demonstrated that targeting to cancer cells by LHRH peptide enhanced antitumor activity of all tested DS to the exceptionally high level comparable for all types of nanocarriers. Simultaneously, targeting to tumor-specific receptors prevented serious adverse side effects of the treatment on healthy organs. Consequently, our data showed that the internalization and intracellular distribution of DS, but not size, molecular mass, composition or architecture of the carrier, play a critical role in anticancer effect of tumor-targeted chemotherapy. This conclusion could have a broad impact on the cancer
drug delivery and imaging. In particular, it means that tumor specific receptor-targeting of nanocarriers could provide for a high antitumor therapeutic activity and imaging efficacy with low adverse side effects on healthy organs for practically any type of anticancer/imaging DS. At the same time, other parameters of nanocarriers, including size, composition, architecture, etc. can be selected based on other considerations, such as type of imaging or therapeutic agents, their aqueous solubility, electric charge, chemical structure, etc. This conclusion is made for the first time in the present study and has the theoretical and practical potential for drug delivery in general and for nanoparticle cancer imaging and chemotherapy, in particular.
4.5. References


4.6. Figure Legends

Figure 4.1. Tumor specific receptor - targeted delivery systems for cancer imaging and treatment. a, Three different types of tumor-targeted delivery systems (linear PEG polymer, branched PAMAM dendrimer and PEGylated liposomes). A synthetic analog of LHRH decapetide, near-infrared cyanine dye Cy5.5 and paclitaxel (TAX) were used as a tumor targeting moiety, imaging agent, and anticancer drug respectively. b-c, Luteinizing hormone-releasing hormone (LHRH) receptors are overexpressed in the plasma membrane of cancer cells and are not expressed detectably in normal visceral organs. b, Human non-small A549 lung carcinoma cells were incubated with LHRH peptide labeled with Rhodamine (red fluorescence). Nuclei of the cells were labeled with Hoechst 33258 dye (blue fluorescence). Labeled LHRH peptide was localized predominantly in the plasma membrane. c, The expression of LHRH receptors was measured by reverse transcription polymerase chain reaction (RT-PCR) in human drug sensitive H69 (1) and multidrug resistant H69/AR (2) small-cell lung cancer cells and A549 non-small cell lung cancer cells (3) as well as in mRNA isolated from healthy visceral organs (4-lung, 5-liver, 6-heart, 7-kidney, 8-spleen, 9-muscle, 10-thymus).

Figure 4.2. LHRH peptide as a tumor-targeting moiety enhances penetration and normalizes intracellular distribution of different nanocarriers in cancer cells. a, Confocal microscopy fluorescent images of human A549 lung carcinoma cells incubated for 24 h with free near infrared dye Cy5.5 (red fluorescence), non-targeted and cancer-targeted different delivery systems labeled with Cy5.5 (z-series from the top to the
Quantitative analysis of the accumulation and distribution inside the cells of labeled delivery systems.

**Figure 4.3. LHRH peptide significantly increases the cytotoxicity of paclitaxel incorporated into different nanoscale-based delivery systems.**  
*a,* Components of delivery systems are not toxic for human A549 lung carcinoma cells.  
*b,* IC₅₀ doses for free paclitaxel (TAX), non-targeted and cancer cell-targeted delivery systems. Means ± SD are shown.  
*P < 0.05* when compared with free paclitaxel.  
*†P < 0.05* when compared with corresponding non-targeted system.

**Figure 4.4. Correlation between tumor size and fluorescence intensity of near infrared dye Cy5.5**  
*a,* serial dilutions of Cy5.5 starting with 1 nmole in a black well plate.  
*b,* side view picture of a mouse labeled with the near-infrared fluorescent dye Cy5.5, image obtained using the IVIS system (Xenogen, Inc.)  
*c, d,* graphs depicting the relationship between tumor size and fluorescence intensity.  
*e,* mice bearing s.c. lung carcinoma and injected with the fluorescent dye cy5.5.  
*f,* bars depicting the linear relationship between tumor volume, mass, and fluorescence intensity of the Cy5.5 dye.

**Figure 4.5. LHRH peptide as a tumor-specific targeting moiety increases accumulation of different delivery systems in the tumor of mice bearing xenografts of human A549 lung carcinoma and limits their buildup in healthy organs.** The nanocarriers were labeled with near infrared dye Cy5.5, injected intravenously into the
mice. The distribution of labeled nanocarriers was analyzed in live anesthetized animals 48 h after injection using IVIS Xenogen imaging system. The intensity of fluorescence is expressed by different colors with blue color reflecting the lowest intensity and red – highest intensity. After measuring the distribution of fluorescence in the entire animal, tumor and healthy organs were excised and their fluorescence were registered and processed by the imaging system.

**Figure 4.6. Targeting by LHRH peptide enhances apoptosis induction in the tumor and prevents adverse side effects on healthy organs of different delivery systems containing paclitaxel.** Mice bearing xenografts of human A549 lung carcinoma were treated with free paclitaxel and different delivery systems with the same dose of TAX (2.5 mg/kg). The enrichment of histone-associated DNA fragments (mono- and oligonucleosomes) in the tumor and organs of control animals was set to unit 1, and the degree of apoptosis was expressed in relative units. Means ± SD are shown. *P < 0.05 when compared with control (animals treated with saline).

**Figure 4.7. Tumor targeting by LHRH peptide enhances antitumor activity of paclitaxel delivered by different nanocarriers.** Mice bearing xenografts of human A549 lung carcinoma were treated with free paclitaxel and different delivery systems with the same dose of TAX (2.5 mg/kg). Tumor volume was measured by a caliper every day for 7 days after the treatment. Means ± SD are shown.
Figure 4.1.

Lung Cancer Cells
1–H69/AR
2–H69
3–A549

Healthy Organs
4–lung
5–liver
6–heart
7–kidney
8–spleen
9–muscle
10–thymus
Figure 4.2.
Figure 4.3.
Figure 4.5.

**a. PEG Polymer**

**b. PAMAM Dendrimer**

**c. Liposomes**
Figure 4.6.
Figure 4.7.
5. Development of a reproducible orthotopic mice model of human lung cancer using both intravenous (Tail Vein) and intratracheal administration of lung cancer cell suspensions

5.1. Introduction

Model systems of lung cancer that reflect different aspect of the disease are important for understanding of the disease. Such models are also necessary for the development of new therapies for lung cancer.

Examples of lung cancer models include:

(1) Spontaneous or chemical induced lung cancer models\textsuperscript{1-3}:

Unfortunately, these tumors are measurable very late in their course, and their response to therapy is poor. Therefore, spontaneous or chemical induced model systems can only be used for studying the early stages of carcinogenesis.

(2) Transgenic lung cancer models\textsuperscript{4, 6-9}:

Early events, such as initiation and carcinogenesis, cannot be studied with these models but testing of new therapeutic strategies can be done with these models.

(3) Human lung tumor xenografts:

The hosts for these models are immunosuppressed laboratory animals such as nude mouse mutants (nu/nu homozygotes), severe combined immunodeficient (SCID) mice and Rowett nude rats. Implantation of xenografts is done using subcutaneous
injection. The procedure is simple and the site, usually the dorsal lateral flank, is easily accessible\textsuperscript{3,5}.

Unfortunately, disadvantages of these models include, tumor growth in an unusual site (the subcutis), which might affect study results\textsuperscript{10-12}.

In orthotopic models, human tumors are implanted directly into the appropriate organ or tissue of origin in the laboratory animal. Advantages include improved tumor uptake and enhanced metastatic properties\textsuperscript{13,14}.

Examples of orthotopic implantation of tumors include: colon carcinoma cells grown in the cecal wall, and bladder carcinoma in the bladder, and they all yield more metastases than when grown subcutaneously\textsuperscript{15,16}.

An organ-specific site provides tumor cells with the most appropriate environment for local growth and metastasis, thereby supporting Paget's hypothesis that malignant cells have special affinity for growth in the environment of their origin (The seed and soil theory)\textsuperscript{17}. Also, orthotopic tumors are more virulent, they give a more realistic approach in studying lung cancer, and animal survival is shorter than subcutaneous models but it must be noted that they tend to be much more complex and costly than subcutaneous models.

Orthotopic lung cancer models are described using endobronchial, intrathoracic or intravenous injection of tumor cell suspensions\textsuperscript{18-22} and by surgical implantation of fresh tumor tissue\textsuperscript{23,24}. 

Due to the many advantages of orthotopic lung cancer models, we aim in this study to develop a reproducible orthotopic mice model of human lung cancer using both intravenous (Tail Vein) and intratracheal administration of lung cancer cell suspensions.

5.2. **Materials and Methods**

5.2.1. **Cell Culture**

Orthotopic model of lung cancer was achieved by the intratracheal and intravenous (Tail Vein) injection of \( (1 \times 10^7) \) of A549-luc-C8 cells (Xenogen, Inc.).

5.2.2. **Animal Models**

Both nude female mouse mutants (nu/nu homozygotes) and severe combined immunodeficient (SCID) mice beige SCID aging between 6-8 weeks (Taconic Farms, Inc.) were used in this study for comparison of the results.

5.2.3. **In Vivo Imaging**

Imaging was done using the IVIS® Imaging System 100 Series by Xenogen Corporation 2004. The imaging set –up consisted of a light - tight box equipped with a 150-W halogen lamp. Bioluminescence was detected by a 12-bit monochrome CCD camera, exposure time was set for 1 minute with large binning, and F/stop of F2. Photographic images were exposed for 0.2 seconds with medium binning, and an F/Stop of F8. Images were acquired digitally as PNG files.

5.2.4. **Morphologic Studies**

Morphologic evaluation of lung tissue was done by tissue fixation in 10% neutral formalin. Slides were prepared using ten-micrometer tissue stained with hematoxylin-eosin and images of the slides were captured using a microscope.
5.3. Results

5.3.1. Success rate of intratracheal A549-luc-C8 tumor cells uptake was similar with both strains of mice used

Success of tumor uptake rate was similar in both strains (approximately 30% of those intratracheally injected with lung tumor developed and maintained lung cancer in their lungs).

Figure 5.1. is a photographic picture of the intratracheal installation procedure. Figure 5.2. illustrates our results using female SCID mice. The Right Panel of this figure depicts organs excised from a SCID mouse (red arrow) on day 55 of intratracheal implantation of lung tumors. The length of this period was sufficient for the mouse to develop brain metastasis (bioluminescence is clearly shown in the lungs and the brain but not in liver, kidneys, heart, or spleen).

Figure 5.3. depicts our results for intratracheal implantation of A549-luc-C8 tumor cells obtained with female Nude mouse mutants (nu/nu homozygotes).

5.3.2. Intratracheal implantation of lung cancer cells has a much higher uptake rate than intravenous tumor injection

Although intratracheal and tail vein injections tend to be invasive to some extent, our results show that intratracheal implantation of tumor cells had a much higher uptake rate by the lungs than with tail vein. Also, the risks associated with tail vein injection outweigh their benefits. For instance, there is a risk of developing tumors on the tail itself, which tends to be very painful for the mouse. Also, with tail vein injection, tumor cells tend to have a longer circulation time in the bloodstream and hence, a lesser chance of growing in the lungs, a problem avoided with intratracheal implantation.
Figure 5.4. is an illustration of our results using intravenous injections of tumor cells through the tail vein. Results for week 1, week 3, and week 5 after tumor implantation are depicted. Lower panel (left) is a photographic picture showing tumor developing on the tail itself. Lower panel (right) illustrates organs excised from one mouse (red arrow); tumor cells are seen in the tail and the lungs but not in liver, kidneys, heart, spleen, or brain.

Figure 5.5. depicts our results of intrateacheal tumor injection. Upper panel show week 1, week 3, and week 5 after intratracheal tumor implantation. Lower panel (left and middle) illustrates microscopic examination of hematoxylin-eosin stained lung tissue obtained from one healthy (lower panel, left) and another cancerous (lower panel, middle) mice, respectively (red arrows). Lower right panel depicts organs excised from one mouse with lung tumor, bioluminescence is seen in the lungs and also in the brain (metastasis).

5.4. Discussion

Model systems of lung cancer are crucial in understanding the disease and for the development of new therapies. Subcutaneous models of lung cancer do not provide accurate representation of the disease and its progression.

By developing orthotopic models of lung cancer, we are able to have an improved tumor uptake and enhanced invasive properties. The orthotopic models are also more virulent and more realistic in studying lung cancer.

To this aim, 2 methods of tumor implantation are employed; namely by 1) intratracheal and 2) intravenous injection of tumor cells. We believe the intratracheal
method of tumor implantation is more successful and has fewer risks. Both mouse strains used (SCID and homozygous nude) had a similar uptake rate of tumor cells (approximately, 30%).

5.5. Conclusions

A reproducible orthotopic mice model of human lung cancer was developed to give an accurate representation of the disease, a step necessary for the development of new therapies for lung cancer.
5.6. References


Intratracheal Injection of Lung Cancer Cells

Figure 5.1. Photographic image depicting Intratracheal Installation Procedure
Figure 5.2. Bioluminescence in SCID mice
Figure 5.3. Bioluminescence in nude mice
Figure 5.4. Intravenous Injection of Human Lung Cancer cells
Figure 5.5. Intratracheal Injection of Human Lung Cancer cells
6. Intratracheal versus intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug.

6.1. Introduction.

Every year, close to 400,000 Americans die of lung disease, making it the third most frequent cause of death in the USA (1 in 7 deaths). An additional 35 million Americans are living with chronic lung diseases\(^1\). Therefore, the development of effective methods of drug delivery to the lungs is an immediate and important task of modern medicine. Traditionally, three main routes are being used to deliver therapeutic ingredients to the lungs: oral, systemic intravenous, and pulmonary. Pulmonary drug delivery to the lung enhances drug retention by lung cells, increases drug concentration inside the cells, decreases the need for high drug dose, and limits the adverse side effects of the treatment on healthy organs. In addition, inhalatory local delivery directly to the lung avoids the destruction and decrease in the stability of the active ingredients in gastro-intestinal tract and during first-pass liver metabolism associated with oral delivery and in the blood after systemic parenteral administration. However, even the most effective modern drugs cannot be delivered directly to the lungs by inhalations and require a special delivery system. Liposomes, small lipid vesicles with a composition close to lung surfactant, have been successfully used for the delivery of different therapeutic agents to the lung\(^2\text{-}^9\). However, distribution of different types of liposomal carriers and their payloads through the body after the inhalation as well as their ability to deliver diverse active ingredients inside lung cells have not been studied in detail. These aspects are especially important when liposomes are used for the delivery of potentially toxic ingredients (antibiotics,
anticancer drugs, etc.) and/or other highly effective therapeutic components (proteins, antisense oligonucleotides, DNA, RNA, etc).

Liposomes have been widely investigated since 1970 as drug carriers for improving the delivery of therapeutic agents to specific sites in the body and almost immediately were explored for cancer treatment\textsuperscript{10, 11}. One of the serious obstacles limiting therapeutic applications of liposomes is short circulation residence time as a result of their elimination from the blood stream by Reticuloendotelial System (RES) after systemic administration\textsuperscript{12, 13}. This problem was partially solved by covering the liposome surface with phosphoetanolamine derivatives of poly(ethylene glycol) (PEG). These “neutral” and sterically stabilized liposomes (Stealth liposomes) used as carriers for hydrophilic anticancer drugs (doxorubicin, vincristine etc.) exhibited really long circulation time in the vascular system\textsuperscript{14-17} and showed enhanced accumulation of drugs in tumor tissues. On the other hand, circulation time and efficacy of chemotherapy can be improved by changing liposomal size, charge, and route of their administration.

During the past 20 years, a variety of techniques have been developed for delivery by liposomes. Both conventional drugs and new genetic drugs including plasmid DNA-containing therapeutic genes, antisense oligonucleotides (ASO), and small interfering RNA (siRNA)\textsuperscript{9, 18-29} have been utilized. The delivery of different types of payloads in turn requires different properties of carriers, including their surface charge. Lipophilic and hydrophilic drugs can be incorporated into the lipid membrane or inner aqueous space of liposomes respectively. While modified uncharged nucleotides can be delivered by neutral or slightly charged liposomes, native negatively charged ASO, siRNA, or DNA molecules required cationic liposomes. The practical application of different types
of liposomes for inhalatory local delivery of various therapeutic components is limited by the almost complete absence of data describing body distribution of different liposomes after their local intratracheal delivery.

In the present study, traditional “neutral” PEGylated and 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) cationic liposomes were investigated. DOX and neutral P-ethoxy-ASO were used as payloads for “neutral” liposomes while DOTAP was used to deliver negatively charged siRNA. The study was carried out to fulfill three specific aims. First, internalization of liposomes with different sizes and charges into lung carcinoma cells was compared in vitro. Second, two distinct routes of administration, systemic intravenous and local intratracheal, were compared in vivo on mice using similar “neutral” and cationic liposomes with respective payload. Third, the effectiveness of intravenous and intratracheal treatment of lung cancer with liposomal DOX were compared on nude mice with an orthotopic model of human lung human A549 non-small cell lung carcinoma.

6.2.1. Cell line. A549-luc-C8 light producing cell line derived from A549 human non-small cell lung carcinoma cells by stable transfection of the North American Firefly Luciferase gene expressed from the CMV promoter was obtained from Xenogen Corporation, (Alameda, California). Cells were cultured in RPMI medium, supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ (v/v) in air. All experiments were performed on cells in the exponential growth phase.

6.2.2. Animals. Athymic nu/nu mice 6-8 weeks old were obtained from Taconic (Hudson, NY). All mice were maintained in micro-isolated cages under pathogen free conditions in the animal maintenance facilities of Rutgers, The State University of New Jersey.

6.2.3. Liposomal compositions of doxorubicin, antisense oligonucleotides and siRNA. PEGylated “neutral” liposomes were used for delivery of electrically neutral DOX and P-ethoxy ASO. Negatively charged siRNA was delivered by cationic DOTAP liposomes. ASO targeted to BCL2 mRNA were synthesized and labeled with fluorescein isothiocyanate (FITC) by Oligos Etc. (Wilsonville, OR) according to our design. The DNA backbone of all bases in oligonucleotides was P-ethoxy modified to eliminate an electrical charge and increase incorporation efficacy into “neutral” liposomes. A siGLO Red transfection indicator (RNA duplex labeled with Pierce NuLight DY-547 fluorophore) was purchased from Dharmacon Inc. (Chicago, IL) and was used to study siRNA delivery and cellular uptake. Doxorubicin was purchased from Sigma (St. Louis, MO).
To prepare “neutral” liposomes, lipids (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, evaporated to a thin film layer in a rotary evaporator, and rehydrated with 300 mM citrate buffer, pH 4. The lipid mole ratio for this formulation was 51:44:5 egg phosphatidylcholin: cholesterol: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-aminopolyethylene glycol – Mw - 2000 ammonium salt respectively. ASO was loaded into liposomes by dissolving in rehydration buffer in concentration 0.5 mM. Liposomes were extruded gradually using polycarbonate membranes 200 nm and 100 nm at room temperature using an extruder device from Northern Lipids, Inc. (Vancouver, BC, Canada). Obtained liposomes were dialyzed overnight against 0.9% NaCl at 4 °C, and loaded with DOX (5 mg/mL) at 37 °C. Liposomes were separated from free DOX by dialysis against 100 volumes 0.9% NaCl. The encapsulation efficacy of DOX and ASO was ~ 95% and ~ 50% respectively. The final phospholipids concentration was 10 mg/ml. Mean liposomes diameter was 100-140 nm. Cationic liposomes were prepared from positively charged dioleoyl-2-trimethylammonium propane - cationic (Avanti Polar Lipids, Alabaster, AL) in concentration 5 mg/mL using thin layer procedure described above and followed by extrusion through 100 nm polycarbonate membrane. siGLO Red was dissolved in RNAse free solution to final concentration 200 µM. DOTAP liposomes were mixed with siGLO in ratio 6:1 v/v and incubated at room temperature for 15 min before use. Mean DOTAP:siGLO complexes size was >500 nm.

Aliquots of each liposomal formulation were labeled with near infrared fluorescent dye Cy5.5 Mono NHS Ester (GE Healthcare, Amersham, UK) or fluorescein isothiocyanate FITC. A fluorescent dye was dissolved together with lipids in chlorophorm.
6.2.4. Liposome size and zeta potential. Particle size was measured by dynamic light scattering using 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY). Aliquot of 40 µL of each sample was diluted in 2 mL of its external buffer. Zeta potential was measured on PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY). Samples were taken as is and their volume was 1.5 mL. All measurements were carried out at room temperature. Each parameter was measured in triplicate, and average values were calculated.

6.2.5. Atomic force microscopy. The shape of cationic, “neutral” conventional and PEGylated liposomes were studied by atomic force microscopy (AFM) imaging using the previously described procedure. Briefly, 50 µl of liposome suspension in water was deposited on pre-cut (~ 25x25 mm²) and pre-cleaned Plain Premium microscope slides (Fisher Scientific Co, Pittsburgh, PA), kept for 10 min at 100% humidity to achieve particles precipitation. Water was removed by dry nitrogen flow and dried samples were subjected to imaging with an atomic force microscope (Nano-R AFM Pacific Nanotechnology Instrument, PNI, Santa Clara, CA) in close contact (tapping) mode using tapping mode etched OMCL-AC160TS silicon probes (Olympus Optical Co. Tokyo, Japan). The captioning was performed in the height mode. The images were processed, and the measurements were performed with Femtoscan software v. 2.2.85(5.1) (Advanced Technologies Center, Moscow, Russia). For statistics, no less than 50 objects of each sample were analyzed.

6.2.6. Confocal microscopy. Cellular internalization of liposomes, DOX, ASO and siRNA were monitored in A549 lung cancer cells by confocal microscopy. Three series of the experiments were carried out: (1) FITC- labeled “neutral” liposomes (green
fluorescence) contained DOX (intrinsic red fluorescence); (2) “neutral” liposomes labeled with Cy5.5 (red fluorescence) contained ASO labeled with FITC (green fluorescence); (3) FITC-labeled cationic liposomes (green fluorescence) contained siGLO Red (red fluorescence). Cells were separately incubated 24 h at 37 °C with each liposomal formulation. Red and green fluorescence images were photographed and digitally overlaid. Superposition of images allows for detecting of co-localization of labeled liposomes and their payload (yellow color).

6.2.7. Body distribution of liposomes. The distribution of different liposomes and their payload was examined on nude mice. Labeled, empty “neutral” and cationic liposomes as well as different liposomes containing DOX, labeled ASO or siRNA were delivered by intratracheal or intravenous administrations. Injected volume of liposomes was 100 µL for each formulation; DOX concentration was 2 mg/kg. Lungs, heart, liver, spleen, and kidneys were excised 30 min, 1 hour, and 24 hours after the treatment and fluorescence was registered by IVIS imaging system (Xenogen Corporation, Alameda, CA). Animals were anesthetized with isoflurane, euthanized, organs were excised and visible light and fluorescence images were taken and overlaid by an imaging system. The intensity of fluorescence was represented on composite light/fluorescent images by different colors with blue color reflecting the lowest fluorescence intensity and red color – the highest intensity. Images of each organ were then scanned and total fluorescence intensity was calculated by a special computer program originally developed for our laboratory by Dr. V. P. Pozharov. Preliminary experiments showed a strong linear correlation between the total amount of labeled substance accumulated in the organ and calculated total fluorescence intensity (data are not shown). The fluorescence was expressed in arbitrary
units with 1 units represented approximately $2 \times 10^{10}$ photons/$s/sr/cm^2$. The method allows a quantitative comparison of the accumulation of the same fluorescent dye between different series of the experiments.

6.2.8. Orthotopic lung tumor model and tumor treatment. Orthotopic engraftment of tumor was established by intratracheal (i.t.) instillation of $5.0 \times 10^6$ A549 non-small cell lung carcinoma cells into athymic nu/nu mice. Tumor growth was monitored once a week by real-time bioluminescence *in vivo* with an imaging system using luciferase expressing lung cancer cells (Xenogen Bioscience, Cranbury, NJ). To compare the anticancer effectiveness of local (inhalatory) and systemic (intravenous) administration of liposomal drugs, the following experiments have been performed. Six weeks after tumor instillation, the mice were divided into five groups (5 animals per group). The mice from the first group were considered a control group. Lung cancer cells were injected into lungs of the second, third, fourth and fifth groups of animals. The animals from the second group were not treated (untreated tumor control). Five weeks after tumor instillation, experimental group #3 was treated once by intravenous injection of 2 mg/kg of DOX. Animals from group #4 received the same dose of liposomal DOX intravenously and mice from group #5 were treated with intratracheal instillation of the same dose of liposomal DOX. The dose of 2 mg/kg was estimated in preliminary experiments as a maximum tolerated dose for liposomal DOX. Twenty four hours after the treatment, animals from all groups were sacrificed, lung tissue samples from each animal were fixed in 10% buffered formalin. Each tissue sample was trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. The evaluation was performed by a veterinary pathologist. 10 slices per each sample were
evaluated under the same magnification. The number of cancer cells in each microscopic field of view was calculated and presented as percent of untreated tumor control (group # 2).

6.2.9. Endotracheal instillation of lung cancer cells and liposomes. Mice were anesthetized through intraperitoneal injection with 80 mg/kg ketamine and 10-12 mg/kg xylazine. Once anesthetized, the mouse was placed on the titling rodent work stand (Hallowell EMC, Pittsfield, MA) in supine position and restrained in position by an incisor loop. The tongue was then extruded using atraumatic forceps or via rotation with a cotton tip applicator. The larynx was visualized using a modified 4 mm ear speculum attached to an operating head of an ophthalmoscope (Welch Allyn, Skaneateles Falls, NY). The modified speculum, acting as a laryngoscope blade, in an inverted fashion provided dorsal displacement of the tongue and magnification of the laryngeal opening. One hundred micro liters of cells or liposomes in suspension were instilled within the trachea using a 40 mm length of Clay Adams Intramedic polyethylene tubing (Diagnostic Systems, Sparks, MD, PE-10, I.D 0.28 mm, O.D. 0.61mm), attached to a 27 gauge hypodermic needle. The tubing was advanced approximately 10 mm past the epiglottis. The tubing and speculum were withdrawn immediately after instillation. The mouse was then removed from the work stand and held in a vertical position by the scruff until multiple normal respirations were observed.

6.2.10. Statistical analysis. Data were analyzed using descriptive statistics, single-factor analysis of variance (ANOVA), and presented as mean values ± the standard deviation from four to eight independent measurements. The difference between variants is considered significant if P < 0.05.
6.3. Results.

6.3.1. Particle size and zeta potential measurements.

Table 6.1 shows the size and zeta potential of liposomal compositions used in the study. As can be seen, “neutral” liposomes have a slight negative charge. The finding that the zeta potential of lipid assemblies containing PEG-DSPE is negative is in agreement with our previously reported data\textsuperscript{31}. It also supports the "hidden charge effect" that was suggested for liposomes sterically stabilized through grafting of mPEG-DSPE with PEG moiety $\geq 0.75$ kDa\textsuperscript{31,32}. Similar to their conventional counterpart, these liposomes can be considered “neutral”. Inclusion of DOX and ASO into “neutral” liposomes did not change their size and zeta potential. The diameter of positively charged DOTAP liposomes after extrusion was about 100-140 nm (same as for “neutral” liposomes). Mixing with siRNA in ratio 6:1 v/v lipid:siRNA respectively led to formation of rather large DOTAP:siRNA complexes and decreased surface charge likely due to the electrostatic interactions between positively charged lipid and negatively charged siRNA.

6.3.2. Atomic force microscopy. Atomic force microscopy (AFM) was widely used during last decade to characterize morphology of nanoparticles. AFM topographical images of the liposome preparations revealed convex meniscus shaped particles that were uniformly distributed on the mica surface (Fig.6.1). No substantial presence of liposome aggregates were observed as expected from physico-chemical properties of uniformly charged particles. Since liposomes flattening on the mica surface after deposition and drying results in distortion of their actual shape in suspension, we calculated the value of reconstructed diameter ($d$) from the liposomes volume measured by AFM ($V$) under the assumption that liposomes adopt the spherical shape in aqueous solutions, using the
equation $d = (6V/\pi)^{1/3}$ as previously described\textsuperscript{33}. The complexation of liposomes with siRNA resulted in more than 2.5-fold increase of their $d$ to over 500 nm. Further, liposomes modification with siRNA makes their appearance “fuzzy”, thus hiding object topology.

6.3.3. Cellular uptake of liposomes, ASO, DOX, and siRNA. The delivery of ASO, DOX and siRNA by liposomes into cells was studied \textit{in vitro} using confocal microscopy. A-549 human lung carcinoma cells were incubated with “neutral” liposomes containing ASO or DOX, cationic liposomes labeled with Cy5.5 or DOTAP:siRNA complexes and then intracellular liposomes distribution was examined. Results are shown on Fig.6.2. As can be seen, after 24 hours incubation, “neutral” and cationic liposomes were detected in the cellular cytoplasm and nucleus. Fluorescence of the payload of “neutral” liposomes (DOX or ASO) was also registered both in the cellular cytoplasm and nucleus. It is known that liposomes quench the fluorescence of any loaded agent\textsuperscript{34}. Therefore, the visualization of fluorescence strongly suggests that by 24 hours of incubation ASO and DOX were completely released from liposomes and localized in the cellular plasma and nucleus. The results obtained also show that after 24 hours of incubation, substantial amount of complexes DOTAP:siRNA penetrated into cellular cytoplasm area (Fig. 6.2, bottom panel) despite their relatively big size (>500 nm). In order to prove that liposomes were not adhered on the cell surface but actually penetrated the cells, we analyzed their distribution in different cellular layers from the upper to the lower of the fixed cells (z-sections, results are not presented). The data obtained show that the distribution of either small (100-140 nm) “neutral” and cationic liposomes or large (>500 nm) DOTAP:siRNA complexes in cytoplasm and nucleus was very similar in different
cell layers. Therefore, both systems can be used for delivery of therapeutic agents with biological activity into the cellular cytoplasm and nuclei.

6.3.4. Body distribution of “neutral” and cationic liposomes after intravenous and intratracheal injection. Body distribution of “neutral” and cationic liposomes possessed several common and distinct features (Fig. 6.3 and 6.4). First, after intravenous (i.v.) injection both types of liposomes accumulated predominately in liver, kidneys and lungs. However, the total amount of “neutral” liposomes accumulated in these organs was higher when compared with cationic liposomes. Intratracheal injection (i.t.) of both types of liposomes substantially increased their accumulation in the lungs and decreased the accumulation in other organs. Twenty four hours after i.t. injection, a substantially higher amount of labeled “neutral” liposomes was found in the lungs when compared with cationic liposomes.

6.3.5. Body distribution of free and liposomal DOX.

During the first 0.5 hours after i.v. injection, a comparable amount of DOX was found in all investigated organs (Fig. 6.5). However, at the later time-periods, the distribution was substantially changed toward preferential accumulation of free DOX in liver and kidney. Only trace amounts of DOX were found in the lungs 24 hours after i.v. injection.

Delivery of DOX by “neutral” liposomes substantially increased its accumulation in all studied organs (Fig. 6.6 and 6.7). However, the tendency in the dynamic of the body distribution of DOX delivered by liposomes was similar to that of free DOX. During the first hour after its i.v. systemic delivery by liposomes, DOX accumulated predominately
in the liver, kidneys and lungs. The amount of DOX in the lungs declined 24 hours after treatment. Intratracheal local delivery of liposomal DOX substantially changed the distribution of delivered DOX between the studied organs. After i.t. delivery, a predominant amount of the drug accumulated in the lungs during all studied time periods after injection.

6.3.6. **Body distribution of ASO and siRNA delivered by “neutral” and cationic liposomes after intravenous and intratracheal injection.**

Body distribution of ASO and siRNA delivered by “neutral” and cationic liposomes was similar to the distribution of liposomal DOX (please compare Figs. 6.6-6.8). Intravenous delivery led to predominant accumulation of ASO and siRNA in the liver, kidneys and lungs. In most cases, 24 hours after the i.v. injection liposomal payloads were accumulated mainly in the liver and kidney, while their amount retained in the lungs substantially decreased when compared with earlier periods after the treatment. It is interesting, that an amount of relatively large DOTAP:siRNA complexes (>500 nm) accumulated in the lungs was higher in the later stages of the experiment (1-24 hours), while the quantity of smaller cationic liposomes without siRNA (10-140 nm) was higher in the earlier stages (0.5-1 hour).

6.3.7. **Intratracheal and intravenous treatment of lung tumor.** Human lung tumor-bearing mice were injected with free DOX and liposomal DOX intravenously and intratracheally respectively. The results of these experiments are shown on the Fig. 6.9. It was found that treatment with intravenous injection of free DOX slightly, but appreciably decreased the number of cancer cells in the lungs (bar 2). Intravenous treatment with liposomal DOX led to the more substantial decrease in the number of tumor cells (bar 3).
However, the most significant positive effect was found after the local delivery of liposomal DOX directly to the lungs by intratracheal injection (bar 4). In this case, after local intratracheal delivery of liposomal DOX, the number of cancer cells was decreased by 45% when compared with untreated tumor, by 38% when compared with treatment by intravenous of free DOX, and by 30% when compared with intravenous injection of liposomal DOX.
6.4. Discussion.

It is well known that liposomal drug formulations are more efficient in terms of their cellular internalization, specific activity, and adverse side effects when compared with free drugs. Liposomes of different sizes and surface electric charges are currently being used for the delivery of encapsulated drugs or complexated large molecules. In addition to systemic intravenous injection, different routes of liposome administration including pulmonary have been proposed. While the high effectiveness of liposomal formulations is widely acknowledged, several aspects of liposomal drug delivery are not clearly understood. It is not apparent yet what is an optimum of size and charge of liposomal drugs in terms of their successful intracellular uptake, body distribution, and treatment efficacy after administration via different routes. We address the problem in the present study by investigating in vitro cellular uptake and intracellular localization along with in vivo body distribution after intravenous or intratracheal administration of small slightly negative (“neutral”) and positively charged liposomes as well as their relatively large complexes with siRNA.

We used small “neutral” PEGylated and positively charged DOTAP liposomes. DOX and uncharged P-ethoxy ASO were used as a payload for neutral liposomes while siRNA was complexated to cationic liposomes. Atomic force microscopy and light scattering data showed that empty liposomes of both types represent homogenous spherical structures with diameter of 100-140 nm. Loading of “neutral” liposomes with DOX or neutral modified ASO did not change their average size and homogeneity. In contrast, complexation of cationic liposomes with siRNA led to dramatic (up to 10-times) increase in their diameter. Investigation of cellular internalization, localization, and distribution of
liposomes showed that despite the differences in size, both small empty and loaded liposomes as well as relatively large complexes were able to deliver their payload to the cellular cytoplasm and even nuclei. Moreover, labeled “neutral” liposomes, as well as their payload, were found inside cellular nuclei. These data confirm our previous transmission electron and fluorescence microscopy findings for the nuclear penetration of conventional and “neutral” liposomes with average size of 100-200 nm both in vitro and in vivo\textsuperscript{22,23,33}. It seems that, in contrast to small liposomes, relatively large DOTAP:siRNA complexes were not able to penetrate cellular nuclei. Therefore, liposomes and liposomal complexes of different sizes (100-1000 nm) used in this study were able to provide an efficient delivery of different biologically active substances, including antibiotics, antisense oligonucleotides and small interfering RNA into the cellular cytoplasm.

Systemic administration of drugs, especially for treatment of lung diseases has several disadvantages including, but not limited to, unfavorable body distribution and decrease in specific drug activity as a result of drug deactivation during systemic circulation and high possibility of adverse side effects on healthy organs. Delivery of biologically active substances as liposomal prodrugs can partially improve the situation especially in case of using “neutral” “stealth” liposomes as carriers. However, our data showed that intratracheal administration of liposomes substantially limit their accumulation in the reticuloendothelial system and sustain higher concentrations of liposomes and their payload in the lungs for at least 24 hours after injection. Once again, similarly to in vitro data, the size of liposomes and liposomal complexes with siRNA played a secondary role, while the route of administration served as a primary determinant of body
distribution of liposomes and their payload. These data clearly show potential advantages of local lung delivery of different liposomal therapeutics for the treatment of lung diseases and confirm that liposomes can be used for intratracheal delivery of drugs, antisense oligonucleotides and siRNA into the lungs.

The efficient delivery of therapeutics to the site of action, however, does not automatically guarantee successful treatment of a disease. In order to confirm that intratracheal delivery of anticancer drugs can enhance the effectiveness of treatment of lung diseases, we used the orthotopic mice model of human lung cancer. Mice bearing human cancer cells in their lungs were treated with free and liposomal doxorubicin delivered intravenously and intratracheally. It was found that intratracheal treatment was much more efficient in limiting the growth of lung cancer cells in the lungs when compared with free and liposomal DOX delivered via the systemic route. Therefore, the present study showed advantages of local intratracheal delivery of liposomal drugs for the treatment of lung cancer when compared with systemic administration of the same drug.
**Abbreviations**

AFM - Atomic Force Microscopy

ASO - Antisense Oligonucleotides

DOTAP - 1,2-Dioleoyl-3-Trimethylammonium-Propane

DOX - Doxorubicin,

DSPE-PEG - 1,2-distearoyl-sn-glycero-3-phosphoethanol amine-N-Aminopolyethylene glycol-M_w~2000 ammonium salt

FITC - Fluorescein Isothiocyanate

i.t - intratracheal

i.v. - intravenous

PEG - Poly(Ethylene Glycol)

siRNA - small interfering RNA

RES - Reticuloendotelial System
6.5. References.


34. Tsukioka Y., Matsumura Y., Hamaguchi T., Koike H., Moriyasu F., Kakizoe T., Pharmaceutical and biomedical differences between micellar doxorubicin (NK911) and liposomal doxorubicin (Doxil), Jpn J Cancer Res, 93, 1145-1153 (2002).


Table 6.1. Size and zeta potential of liposomes at room temperature

<table>
<thead>
<tr>
<th>Liposomes/Parameter</th>
<th>Size (nm)\textsuperscript{a}</th>
<th>Zeta potential (mV)\textsuperscript{a}</th>
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<tr>
<td>EPC/Chol/DSPE-PEG</td>
<td>120 ± 20</td>
<td>-10 ± 2</td>
</tr>
<tr>
<td>EPC/Chol/DSPE-PEG loaded with DOX</td>
<td>130 ± 10</td>
<td>-10 ± 2</td>
</tr>
<tr>
<td>EPC/Chol/DSPE-PEG loaded with ASO</td>
<td>130 ± 10</td>
<td>-10 ± 2</td>
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<tr>
<td>DOTAP:siRNA complexes</td>
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<td>+4 ± 2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Means ± SD are shown.
6.6. Legends to Figures

Figure 6.1. Assessment of different liposomes by atomic force microscopy. Typical images of neutral conventional and “neutral” liposomes, cationic liposomes, and complexes of cationic liposomes with siRNA are shown. Liposome suspensions were applied on freshly cleaved mica, kept for 10 min at 100% humidity atmosphere to achieve deposition and dried under nitrogen flow to remove external water. Panoramic views were captured in <dem> mode (phase contrast).

Figure 6.2. Intracellular localization of different liposomes and their payload. A-549 lung cancer cells were incubated with substances indicated and visible light and fluorescent images (red and green fluorescence) were taken by a confocal microscope. Visible light image was digitally overlaid with both fluorescence images to obtain a composite image showing co-localization of liposomes and their payload (in yellow color). The upper panel contains representative images of “neutral” liposomes labeled with FITC (green fluorescence) containing doxorubicin (DOX, intrinsic red fluorescence). The middle panel shows representative images of complexes of “neutral” liposomes labeled with near infrared dye Cy5.5 (red fluorescence) containing FITC labeled antisense oligonucleotides (ASO, green fluorescence). The bottom panel contains representative images of complexes of FITC labeled cationic liposomes (green fluorescence) with siRNA labeled with DY-547 dye (red fluorescence). Superposition of
images allows for detecting of co-localization of liposomes and their payload inside cells (yellow color).

**Figure 6.3. Body distribution of “neutral” liposomes.** PEGylated liposomes were labeled with near infrared Cy5.5 dye and delivered intravenously or intratracheally into the mice. The distribution was measured using IVIS Xenogen imaging system. The upper panel (a) contains representative images of different organs excised 0.5, 1 and 24 h after injection. The intensity of fluorescence is expressed by different colors with blue color reflecting the lowest intensity and red color – highest intensity. The bottom panel (b) demonstrates average organ accumulation of labeled liposomes. Means ± SD are shown.

**Figure 6.4. Body distribution of cationic liposomes.** Liposomes were labeled with near infrared dye Cy5.5 and injected intravenously or intratracheally into the mice. The distribution was measured using IVIS Xenogen imaging system. The upper panel (a) contains representative images of different organs excised 0.5, 1 and 24 h after injection. The intensity of fluorescence is expressed by different colors with blue color reflecting the lowest intensity and red color – highest intensity. The bottom panel (b) demonstrates average organ accumulation of labeled liposomes. Means ± SD are shown.
Figure 6.5. Body distribution of free doxorubicin (DOX). DOX was injected intravenously into the mice. The distribution of DOX possessing intrinsic fluorescence was measured using IVIS Xenogen imaging system. The upper panel (a) contains representative images of different organs excised 0.5, 1 and 24 h after injection. The intensity of fluorescence is expressed by different colors with blue color reflecting the lowest intensity and red – highest intensity. The bottom panel (b) demonstrates average organ accumulation DOX. Means ± SD are shown.

Figure 6.6. Body distribution of “neutral” liposomes containing doxorubicin (DOX). PEGylated liposomes were delivered intravenously or intratracheally into the mice. Intrinsic fluorescence of DOX was measured using IVIS Xenogen imaging system. The upper panel (a) contains representative images of different organs excised 0.5, 1 and 24 h after injection. The intensity of fluorescence is expressed by different colors with blue color reflecting the lowest intensity and the red color reflecting the highest intensity. The bottom panel (b) shows average organ accumulation of labeled liposomes. Means ± SD are shown.

Figure 6.7. Body distribution of antisense oligonucleotides (ASO) delivered by “neutral” liposomes. P-ethoxy neutral ASO were labeled with fluorescein isothiocyanate (FITC), encapsulated into “neutral” liposomes and delivered intravenously or intratracheally into the mice. The distribution was measured using IVIS Xenogen imaging system. The upper panel (a) contains representative images of different organs
excised 0.5, 1 and 24 h after injection. The intensity of fluorescence is expressed by
different colors with blue color reflecting the lowest intensity and red color reflecting the
highest intensity. The bottom panel (b) demonstrates average organ accumulation of
labeled siRNA. Means ± SD are shown.

Figure 6.8. Body distribution of siRNA delivered by cationic liposomes. siRNA
labeled with near infrared dye (siGLO red) was complexated with cationic liposomes
and delivered intravenously or intratracheally into the mice. The distribution was
measured using IVIS Xenogen imaging system. The upper panel (a) contains
representative images of different organs excised 0.5, 1 and 24 h after injection. The
intensity of fluorescence is expressed by different colors with blue color reflecting the
lowest intensity and red color reflecting the highest intensity. The bottom panel (b)
demonstrates average organ accumulation of labeled siRNA. Means ± SD are shown.

Figure 6.9. Influence of intravenous free DOX (2), intravenous liposomal DOX (3)
and inhalatory liposomal DOX (4) delivery on the number of cancer cells in mice
lungs. The number of cells was expressed as percent of untreated control (1).
* P < 0.05 when compared with untreated control.
† P < 0.05 when compared with free DOX (i.v. injection).
‡ P < 0.05 when compared with intravenous liposomal DOX (i.v. injection).
Figure 6.1.
Figure 6.2.
Figure 6.3.
Figure 6.4.
Figure 6.5.
Figure 6.6.
Figure 6.7.
Figure 6.8.
Figure 6.9.
7. **Co-delivery of siRNA and an anticancer drug for treatment of multidrug resistant cancer**

7.1. **Introduction**

Lung cancer is the leading cause of cancer death in the United States. Small Cell Lung Carcinoma (SCLC) is the most aggressive type of lung cancer and is responsible for high mortality. Due to the size and distribution of small cell lung cancer, cytoreductive surgery is not very effective for this disease and therefore chemotherapy and/or radiation are the treatment of choice. However, the efficacy of chemotherapy in lung cancer is limited by the development of cancer cell resistance during the treatment. To overcome this resistance, higher doses of the toxic anticancer drug is administered, thus producing adverse side effects upon healthy organs. Two main mechanisms are responsible for the observed resistance: pump and nonpump resistance (Fig. 7.1.)\(^1\), \(^2\). Pump resistance is caused by membrane efflux pumps that decrease the anticancer drug concentration inside the cells\(^3\). The main transporters responsible for the pump resistance in small cell lung cancer cells are the members of the so-called “multidrug resistance associated proteins” (MRP)\(^2\), \(^4\), \(^5\). P-glycoprotein efflux pump plays a less important role in drug resistance of SCLC. Nonpump drug resistance is primarily attributed to the mechanisms responsible for the activation of antiapoptotic cellular defense, and BCL2 protein is a key player in this defense\(^6\)-\(^11\).

Considerable efforts have recently been made to suppress multidrug resistance and/or antiapoptotic cellular defense\(^12\)-\(^14\). Synthetic analogs of the BCL2 homology 3 (BH3) domain of pro-apoptotic members of the BCL2 protein family, including BAK, BAX, and BAD\(^7\), \(^10\), \(^15\)-\(^20\), antisense oligonucleotides and small interfering RNA (siRNA)
targeted to BCL2, MDR1 and MRP mRNA\textsuperscript{21-23}, c-Jun NH2-terminal kinase\textsuperscript{24}, ribozymetraditional drugs\textsuperscript{25}; several drug groups from a traditional mitomycin C\textsuperscript{26} and the exotic plant stress hormones family of jasmonates\textsuperscript{27} have been used. However, these attempts have not demonstrated a very high efficiency in terms of their anticancer effect. In our opinion, three main deficiencies in the previous approaches are primarily responsible for their relatively low efficacy in treating multidrug resistant cancers in general and SCLC in particular. First, in most cases, drug efflux pumps and antiapoptotic cellular defense are being suppressed separately. However, the inhibition of only one contributor to cellular resistance is usually not sufficient for overcoming all mechanisms of cancer cell resistance to chemotherapy. For instance, we found that an increase in intracellular drug concentration as a result of the suppression of drug efflux pumps usually leads to almost proportional activation of antiapoptotic cellular defense. As a result, such increase in the concentration does not result in a proportional increase in cell death. Similarly, a suppression of only antiapoptotic cell death is not sufficient for overcoming multidrug resistance. Secondly, suppression of drug efflux pumps alone without simultaneous induction of cell death signal is not sufficient for the effective killing of resistant cancer cells. Thirdly, even when suppressors of both types of resistance are used in combination with an anticancer drug, these components are delivered separately to cancer cells. However, in order to maximize the efficacy of the treatment all cell death inducer(s) and suppressor(s) of both types of cellular drug resistance should be simultaneously delivered inside the cancer cell and active components should be released with a comparable profile. Such spatial-temporal
synchronization requires one complex system simultaneously encapsulating all the above mentioned active components.

Based on this analysis, we hypothesize that only simultaneous suppression of both pump and nonpump cellular resistance in combination with cell death induction by an anticancer drug is able to significantly increase the efficacy of chemotherapy against potentially resistant lung cancers. Such an objective can only be achieved if an anticancer agent will be simultaneously delivered in one multifunctional nanocarrier-based system in combination with other active ingredients that perform different specific functions for enhancing cellular uptake and efficiency of the main drug specifically in cancer cells and preventing the development and/or suppression of the existent drug resistance. In the proposed study, we plan to apply nanotechnology approaches to the development and evaluation of such multifunctional nanotherapeutics. We constructed a novel multifunctional Nanocarrier-based Delivery System (NDS) which provides co-delivery of an anticancer drug simultaneously with suppressors of pump and nonpump cellular resistance. NDS contains the following components (Fig. 7.1.): (1) cationic liposomes as carrier; (2) doxorubicin (DOX) as a cell death inducer/anticancer agent; (3) siRNA targeted to MRP1 mRNA as a suppressor of drug efflux pump (pump resistance) in SCLC and (4) siRNA targeted to BCL2 mRNA as a suppressor of cellular antiapoptotic defense (nonpump resistance). The present paper describes the results of the evaluation of the efficacy of the proposed drug delivery system.
7.2. Material & methods

7.2.1. Cell line

The human multidrug resistant H69AR small cell lung cancer cell line was obtained from ATCC (Manassas, VA). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ (v/v) in air. All experiments were performed on cells in the exponential growth phase.

7.2.2. Drug and small interfering RNA

Doxorubicin was obtained from Sigma (St. Louis, MO). The sequences of the small interfering RNA (siRNA) targeted to BCL2 and MRP1 mRNA were: 5'-GUGAAGUCAACAUGCCUGCTT -3' and 5'-GGCUACAUUCAGAUGACACTT -3', respectively. siRNA was synthesized by Applied Biosystems/Ambion (Austin, TX).

7.2.3. Multifunctional nanocarrier-based delivery systems

Cationic liposomes were prepared from positively charged 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP, Avanti Polar Lipids, Alabaster, AL) using ethanol injection method. Briefly, dry lipid was dissolved in 98% ethanol (10% from final volume) at room temperature and the dissolved mixture was added to 0.9% NaCl to final lipid concentration of 10 mg/ml. Obtained liposomes were extruded gradually using 200 nm and 100 nm polycarbonate membranes at room temperature using extruder device from Northern Lipids Inc. (Vancouver, BC, Canada) and loaded with DOX (5 mg/mL) at 37°C. Liposomes were separated from free drug by dialysis against 100 volumes 0.9% NaCl overnight at 4 °C. The encapsulation efficacy of DOX was ~ 70%. The
concentration of doxorubicin in liposomes was determined by High-Performance Liquid Chromatography (HPLC) using symmetry C18 column (150 mm x 4.6 mm, Waters Corporation, Milford, MA) operated at room temperature. The mobile phase consisted of 0.1% Trifluoroacetic Acid (TFA) in water/acetonitrile 25:75 v/v; the flow rate was set to 1.0 ml/min, wavelength 480 nm. The chromatographic installation consisted of a Model 1525 pump (Waters Corporation, Milford, MA, USA), a Model 717 Plus auto-injector (Waters Corporation, Milford, MA, USA) and a Model 2487 variable wavelength UV detector (Waters Corporation, Milford, MA, USA) connected to the Millennium software. The siRNA (stock concentration, 370 µM) was prepared by dissolving in RNAse free water at room temperature. Cationic liposomes:siRNA complexes with +/− charge ratio 4:1 were formed by mixing appropriate amounts of siRNA and cationic liposomes in 0.9% NaCl\textsuperscript{29, 30}. This mixture was vortexed and incubated at room temperature for 30 min. The +/− charge ratio is calculated as the total number of positive charges on the DOTAP molecules, divided by the total number of negative charges on the siRNA (with 1 siRNA molecule containing 42 negatively charged phosphate groups). Final siRNA concentration in the delivery system was 123 µM when MRP1 and BCL2 siRNA were used separately and 61.6 µM of each siRNA when they were used in combination.

7.2.4. Liposome size and zeta potential.

Particle size was measured by dynamic light scattering using 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY). Aliquot of 40 µL of each sample was diluted in 2 mL of its external buffer. Zeta potential was measured on PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY). Samples were taken as is and their volume was 1.5 mL. All measurements were carried out at room
temperature. Each parameter was measured in triplicate, and average values were calculated.

7.2.5. Intracellular localization of liposomes, siRNA and DOX

To analyze cellular internalization of liposomes, aliquots of liposomal formulations were labeled with fluorescein isothiocyanate FITC. A fluorescent dye was dissolved together with lipids in chlorophorm. A siGLO red transfection indicator (RNA duplex labeled with Pierce NuLight DY-547 fluorophore) was purchased from Dharmacon Inc. (Chicago, IL) and was used to study siRNA delivery and cellular internalization. Cell nuclei were stained by Hoechst 33258 nuclear dye (Sigma, St. Louis, MO). The fluorescent labels were visualized using a fluorescent microscope (Olympus, Center Valley, PA).

7.2.6. Cytotoxicity

The cellular cytotoxicity of all studied formulations was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described\textsuperscript{31-33}. To measure cytotoxicity, cells were separately incubated in 96-wells microtiter plate with different concentrations of all possible combinations of our NDS components which resulted in a total of 13 separate series of experiments: (1) control (fresh media); (2) empty cationic liposomes; (3) free DOX; (4) free siRNA targeted to BCL2 mRNA; (5) free siRNA targeted to MRP1 mRNA; (6) free siRNA targeted to BCL2 mRNA mixed with free siRNA targeted to MRP1 mRNA; (7) cationic liposomes containing BCL2 siRNA; (8) cationic liposomes containing MRP1 siRNA; (9) cationic liposomes containing BCL2 siRNA and MRP1 siRNA; (10) cationic liposomes containing DOX; (11) cationic liposomes containing DOX and siRNA targeted to BCL2
mRNA; (12) cationic liposomes containing DOX and siRNA targeted to MRP1 mRNA; (13) cationic liposomes containing DOX and siRNA targeted to BCL2 and MRP1 mRNA in the cell growth medium. Control cells received an equivalent volume of fresh medium. The duration of incubation was 48 h. Based on these measurements, IC$_{50}$ doses of free and liposomal formulations of all delivery systems (the concentrations of active ingredients necessary to inhibit the cell growth by 50%) were calculated as previously described$^{34}$.

7.2.7. **Gene Expression**

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used for the analysis in H69AR cell line expression of genes encoding MRP1 and BCL2 protein as previously described$^{1, 31}$. Cells were separately incubated for 48 h with the following formulations: (1) control (fresh media); (2) cationic liposomes; (3) free DOX; (4) cationic liposomes containing DOX; (5) cationic liposomes containing MRP1 or BCL2 siRNA; (6) cationic liposomes containing DOX and MRP1 or BCL2 siRNA. Concentrations of DOTAP, DOX and siRNA were 0.07 mM, 34.5 µM and 123 µM respectively. RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). The following pairs of primers were used (5’ to 3’): *MRP1* - ATG TCA CGT GGA ATA CCA GC (sense), GAA GAC TGA AC T CCC TTC CT (antisense); *BCL2* - GGA TTG TGG CCT TCT TTG AG (sense), CCA AAC TGA GCA GAG TCT TC (antisense); *β$_2$-microglobulin (β$_2$-m, internal standard)* - ACC CCC ACT GAA AAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR products were separated in 4% NuSieve 3:1 Reliant® agarose gels (BMA, Rockland, ME) in 1 × TBE (Tris/Borate/EDTA) buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research
Organics Inc., Cleveland, OH) by submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA). Gene expression was calculated as the ratio of mean band density of analyzed RT-PCR product to that of the internal standard ($\beta_2$-m).

7.2.8. Protein Expression

To confirm RT-PCR data, the expression of MRP1 and BCL2 proteins were analyzed. The identification of the above proteins was made by Immunocytochemical (ICC) staining of the human multidrug resistant H69AR small cell lung cancer cells. Cells were treated for 48 h with the following compositions: (1) control (fresh media); (2) empty cationic liposomes; (3) DOX; (4) cationic liposomes containing DOX; (5) cationic liposomes containing siRNA targeted to MRP1 mRNA; (6) cationic liposomes containing siRNA targeted to BCL2 mRNA; (7) cationic liposomes containing DOX and siRNA targeted to MRP1 mRNA; (8) cationic liposomes containing DOX and siRNA targeted to BCL2 mRNA. Concentrations of DOTAP and DOX were 0.07 mM and 34.5 µM respectively. Concentrations of siRNA were 123 µM when MRP1 and BCL2 siRNA were used separately and 61.6 µM of each siRNA when they were used in combination. Before staining, cells were washed three times in ice cold PBS, then fixed with ice cold acetone at -20°C for 7 min. After that, the slide chambers were washed again three times with PBS and stained using R.T.U. VECTASTAIN® Universal Elite ABC-Peroxidase Kit (Catalog #PK7200, Vector Laboratories, Inc., Burlingame, CA). Mouse monoclonal antibody to MRP-1 (ab24102, 1:20 dilution) and rabbit polyclonal antibody to BCL2 protein (ab62468, 1:200 dilution) obtained from Abcam (Cambridge, MA) were used as primary antibodies for the detection of MRP1 and BCL2 proteins, respectively.
Biotinylated anti-mouse IgG Reagent prediluted (Vector Laboratories, Inc., Burlingame, CA) and horseradish peroxidase (HRP) - Streptavidin Detection System ready-to-use stabilized ABC reagent (Vector Laboratories, Inc., Burlingame, CA) in combination with ImmPACT 3,3’-dichlorobenzidine (DAB) substrate kit for peroxidase (Catalog #SK-4105, Vector Laboratories, Inc., Burlingame, CA) were used for visualization. Nuclear staining was done with VECTOR Hematoxylin QS (Catalog#H-3404, Vector Laboratories, Inc., Burlingame, CA). The slides were cleared by washing in 95% ethanol, 100% ethanol, and then xylene. Mounting was done with VectaMount permanent mounting medium (Catalog#H-5000, Vector Laboratories, Inc., Burlingame, CA). After staining the slides were analyzed by a light microscope (Olympus, Center Valley, PA) and photographed.

**7.2.9. Apoptosis**

The analysis of apoptosis was based on the detection of single- and double-stranded DNA breaks (nicks) by an *in situ* cell death detection kit (Roche, Nutley, NJ) using terminal deoxynucleotidyl transferase mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described\(^35\), \(^36\). Briefly, cells were fixed, permeabilized and incubated with the TUNEL reaction mixture. The label incorporated at the damaged sites of the DNA was visualized by a fluorescence microscope.

**7.2.10. Statistical analysis**

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA), and presented as mean value ± standard deviation (S.D.) from four to eight independent measurements in separate experiments.
7.3. Results

7.3.1. Characteristics of multifunctional nanocarrier-based delivery system

The size of positively charged DOTAP liposomes after extrusion was about 10-140 nm, and zeta potential was about +20 mV. Inclusion of doxorubicin did not change the size and charge of the liposomes. Mixing of cationic liposomes with negatively charged siRNA in the ratio of 6:1 (lipid:siRNA v/v) led to the formation of larger DOTAP:siRNA complexes with the size about 500 nm and decreased the resulting surface charge of the entire complex to +4 mV due to the electrostatic interactions between lipids and siRNA.

7.3.2. Intracellular localization of liposomes, siRNA and DOX

To evaluate the penetration of cationic liposomes into cancer cells and intracellular localization of delivered siRNA and DOX, we separately labeled each non-fluorescent component of NDS (cationic liposomes and siRNA) with different fluorescent dyes (FITC and DY-547 respectively). DOX by itself possesses an intrinsic red fluorescence. Multidrug resistant human H69AR lung cancer cells were separately incubated with free siRNA, cationic liposomes containing siRNA, and similar cationic liposomes containing DOX. In addition, cell nuclei were stained with nuclear-specific dye Hoechst 33258 (blue fluorescence). Fluorescence was registered with a fluorescent microscope and images were digitally overlaid in order to analyze intracellular co-localization of liposomes and their active components (Fig. 7.2.). Superposition of green and red fluorescence gives yellow color and allows for detecting cytoplasmic co-localization of cationic liposomes and siRNA or DOX. Superposition of green and red with blue fluorescence allows us for detecting nuclear localization of liposomes (cyan), DOX or siRNA (pink) or nuclear co-
localization of liposomes with DOX or siRNA (white color). An absence of red fluorescence in cancer cells after incubation with free labeled siRNA (data not shown) supports our previous finding and shows that free siRNA is unable to penetrate the plasma membrane of cancer cells\textsuperscript{37, 38}. Cationic liposomes were able to penetrate into cancer cells. They effectively delivered their payload into the cytoplasm (siRNA and DOX) and nuclei (DOX). These data clearly show that proposed NDS can be used for co-delivery of anticancer drugs and siRNA into cancer cells.

\subsection*{7.3.3. Suppression of targeted mRNA and proteins}

Two methods have been used to estimate the effectiveness of the suppression of targeted MRP1 and BCL2 proteins. First, the expression of targeted mRNA in multidrug resistant human lung cancer cells was measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The results of these measurements are shown on Fig. 7.3. As expected, both MRP1 and BCL2 mRNA were expressed in these multidrug resistant cancer cells (Fig. 7.3., A and B, bar 1). Exposure with empty cationic liposomes did not significantly change the expression of both type of mRNA (Fig. 7.3., A and B, bar 2). It was found that treatment of the cells with free DOX (Fig. 7.3., A and B, bar 3) and liposomal DOX (Fig. 7.3., A and B, bar 4) led to the significant overexpression of both MRP1 and BCL2 mRNA. These data support our previous findings that chemotherapy with an anticancer drug leads to the activation of both types of resistance: pump and nonpump resistance\textsuperscript{1, 2, 32, 39, 40}. While after the delivery of DOX with cationic liposomes both types of mRNA still reminded overexpressed, the dynamic of changes in the expression was opposite for MRP1 and BCL2 mRNA (compare bars 3 and 4 on Fig. 7.3., A and B). Whereas, the expression of MRP1 mRNA after treatment with liposomal DOX...
was further elevated, the expression of BCL2 was decreased when compared with treatment by free DOX. Delivery of siRNA by cationic liposomes led to the substantial suppression of targeted mRNA: MRP1 (Fig. 7.3., A, bar 5) and BCL2 (Fig. 7.3., B, bar 5). The combination of one type of siRNA targeted to MRP1 or BCL2 in one delivery system with DOX significantly decreased the overexpression of corresponding mRNA when compared with free and liposomal DOX without siRNA (compare bar 6 with bars 3 and 4 on Fig. 7.3., A and B). In fact, inclusion of siRNA targeted to MRP1 mRNA in the delivery system containing DOX led to the decrease in the expression of MRP1 in 2.5 and 3.1 times when compared with free and liposomal DOX respectively ($P < 0.05$ in both cases). Similarly, BCL2 siRNA decreased the expression of targeted mRNA in 3.3 and 2.7 times when compared with free and liposomal DOX respectively ($P < 0.05$ in both cases).

Analysis of protein expression (Fig. 7.4.) supports data obtained using the RT-PCR method (Fig. 7.3.). Again, treatment with the anticancer drug (DOX) induced overexpression of proteins responsible for active drug efflux (MRP1) and cellular antiapoptotic defense (BCL2). Incubation of multidrug resistant small lung cancer cells with nanocarrier-based delivery system containing the same concentration of DOX but in the presence of siRNA targeted to MRP1 mRNA (suppressor of pump resistance) and BCL2 mRNA (suppressor of nonpump resistance) led to the suppression of MRP1 and BCL2 proteins respectively.

7.3.4. Apoptosis induction
Apoptosis was studied in cells incubated for 48 h with different formulations and stained by TUNEL (Fig. 7.5.). It was found that cationic liposomes containing only MRP1 or BCL2 siRNA or their combination were able to induce cell death by apoptosis in multidrug resistant human lung cancer cells (Fig. 7.5., upper panel). It is interesting that the degree of apoptosis provoked by the combination of both types of siRNA in one liposomal delivery system was comparable with that induced by free DOX. Delivery of DOX by liposomes enhanced its cell death inducing ability. The delivery of DOX by liposomes in combination with siRNA targeted to MRP1 or BCL2 enhanced the degree of apoptosis. Simultaneous cell death induction by DOX in combination with the suppression of pump and nonpump cellular resistance led to the induction of apoptosis to a level that cannot be achieved by each component of the complex delivery system applied separately.

7.3.5. Cytotoxicity

Cytotoxicity of different formulations was analyzed by a modified MTT assay (Fig. 6). It was found that all liposomal delivery systems that do not contain DOX were nontoxic except cationic liposomes containing both types of siRNA targeted to MRP1 and BCL2 mRNA (Fig. 7.6., A). A moderate (approximately on 22%), but statistically significant decrease in viability of multidrug resistant human lung cancer cells was found after their incubation within 48 h with cationic liposomes contained a combination of MRP1 and BCL2 siRNA (Fig. 7.3., A, bar 10). As expected, delivery of DOX by liposomes slightly (about on 10%) but statistically significantly increased its toxicity (Fig. 7.6., B, bar 7). A further improvement in toxicity of liposomal DOX was achieved by incorporating MRP1 or BCL2 siRNA in the same delivery system. IC_{50} of liposomal
DOX combined with MRP1 or BCL2 siRNA was approximately 60% and 40% less than free DOX respectively ($P < 0.05$ in both cases). The maximal enhancement in cytotoxicity was achieved under a concurrent cell death induction by DOX and simultaneous suppression of pump (by MRP1 siRNA) and nonpump (by BCL2 siRNA) resistance (Fig. 7.6., B and C). The IC$_{50}$ dose of this combination was only about 20% of that in free DOX (compare bar 13 with bar 7.6. in Fig. 7.6., B, and corresponding curves in Fig. 7.6., C). In other words, cytotoxicity of liposomes containing DOX and two types of siRNA targeted against MRP1 and BCL2 mRNA was almost 4.5 times higher than that of free DOX, 4.1 times higher than liposomal DOX, 1.8 to 2.7 times higher than liposomal DOX formulation containing only one type of siRNA.
7.4. Discussion

The effectiveness of chemotherapy is often constrained by the limited accumulation of active ingredients in multidrug resistant cancer cells. The penetration of low molecular weight anticancer drugs is restricted by the activation of drug efflux pumps in multidrug resistant cancer cells. In contrast, cellular uptake of relatively high molecular weight components including antisense oligonucleotides, small interfering RNA and other nucleotide-based therapeutics is limited by their intrinsic characteristics (i.e. size, charge, etc.). To simultaneously solve both problems and enhance the efficacy of chemotherapy of drug resistant cancers, we are proposing a co-delivery of an anticancer drug with siRNA-based suppressors of pump and nonpump cellular resistance. We constructed a multifunctional nanocarrier-based delivery system which consisted of (1) cationic liposomes as a carrier; (2) Doxorubicin (DOX) as a cell death induces/anticancer agent; (3) siRNA targeted to MRP1 mRNA as a suppressor of pump resistance and (4) siRNA targeted to BCL2 mRNA as a suppressor of nonpump resistance. This NDS was evaluated on multidrug resistant lung cancer cells. The results of experimental testing of this multifunctional system showed that cationic liposomes were able to efficiently deliver siRNA and DOX inside multidrug resistant lung cancer cells. Simultaneous delivery of suppressors of pump and nonpump resistance in combination with the anticancer drug led to the effective apoptosis induction and killing of drug resistant lung cancer cells. The data obtained also support our hypothesis that simultaneous suppression of both pump and nonpump cellular resistance in combination with cell death induction by an anticancer drug is able to effectively kill multidrug resistant lung cancers.
MRP proteins (including MRP1) are members of the superfamily of ATP-Binding Cassette (ABC) transporters that transport various molecules across extra- and intracellular membranes. MRP proteins are mainly responsible for the pump resistance in small cell lung cancer cells\(^2,^4,^5\). The main role of MRP1 protein in the development of multidrug resistance in small cell lung cancer is active efflux of anticancer drugs out from the cells decreasing their intracellular concentration thus limiting their cell death inducing activity. However, MRP proteins can play other roles related to drug metabolism and drug detoxification inside cancer cells. In particular, they can transfer conjugated after enzymatic inactivation and degradation drugs outside the cells or into cellular organelles for further degradation\(^41-45\). Similar mechanisms also protect cells from endogenously generated cytotoxic substances. One can hypothesize, that the suppression of MRP1 protein by disturbing such cellular protective mechanisms can potentially induce apoptosis in cancer cells even in the absence of an anticancer drug. The results of the present study support such assumption and show that siRNA targeted to MRP1 mRNA delivered into lung cancer cells by cationic nanocarrier was able to induce apoptosis in small cell lung cancer cells. However, the level of apoptosis induced by the suppression of MRP1 protein alone was substantially lower when compared with the degree of apoptosis induced by liposomal doxorubicin or complex delivery system that includes DOX in combination with one or two types of siRNA (i.e. liposomal DOX combined with siRNA targeted to BCL2 mRNA or/and siRNA targeted to MRP1 siRNA).

A major function of BCL2 protein in the cellular antiapoptotic defense includes the prevention of cytochrome \(c\) release from the mitochondrion\(^8,^11,^46,^47\). Consequently, the suppression of BCL2 protein and associated leakage of cytochrome \(c\) into the cytosol
leads to the formation of apoptosome – the combination of cytochrome c, procaspase 9, dATP, and apoptotic protease activating factor-1 (APAF1). This converts inactive procaspase 9 into its active form. Active caspase 9 initiates a cascade of downstream caspases, and these caspases, in turn, activate apoptosis. Therefore, the suppression of BCL2 protein alone even without anticancer drug is able to induce apoptosis most probably by triggering the outlined above caspase-dependent mechanism initiated by the release of mitochondrial cytochrome c into the cytosol. However, as can be seen from the present experimental results, the level of apoptosis achieved by this mechanism is significantly lower when compared with cell death induced by the combination of siRNA targeted to BCL2 mRNA with the anticancer drug.

As expected, free and liposomal DOX were able to induce cell death in multidrug resistant lung cancer cells. However, this induction was accompanied by the simultaneous activation of pump and nonpump resistance associated with overexpression of MRP1 and BCL2 proteins. The activation of both types of cellular resistance decreased the efficiency of DOX as an anticancer drug. In contrast, the suppression of pump and nonpump resistance enhanced its anticancer activity and led to the effective induction of cell death in multidrug resistant cancer cells.

Therefore, we were able to verify our hypothesis and show that cell death induction by an anticancer drug in combination with suppression of both pump and nonpump resistance is required for effective killing of multidrug resistant cancer cells. Although each active ingredient (DOX, MRP1 and BCL2 siRNA) alone delivered by liposomes was capable of inducing cell death in multidrug resistant cancer cells, only the co-delivery of all active components in one multifunctional nanocarrier-based delivery
System improved cellular drug uptake and led to the simultaneous cell death induction and suppression of drug cellular resistance and substantially enhanced the efficacy of chemotherapy of multidrug resistant cancer cells.
7.5. Conclusion

A simultaneous co-delivery of doxorubicin as a cell death inducer/anticancer agent with siRNA targeted to MRP1 mRNA as a suppressor of drug efflux pumps (pump resistance) and siRNA targeted to BCL2 mRNA as a suppressor of cellular antiapoptotic defense (nonpump resistance) by cationic nanocarrier enhanced efficacy of chemotherapy to a level that cannot be achieved by separate treatment with anticancer drug or siRNA alone.
Abbreviations:

ANOVA - Single Factor Analysis Of Variance

BCL2 - B-cell CLL/lymphoma 2 Protein

BH3 - BCL2 Homology 3

DAB - 3,3'-dichlorobenzidine

DOTAP - 1,2-Dioleoyl-3-Trimethylammonium-Propane

DOX - Doxorubicin

EDTA - Ethylenediaminetetraacetic Acid

HPLC - high-Performance Liquid Chromatography

HRP - Horseradish Peroxidase

IC$_{50}$ dose - the concentrations of active ingredients necessary to inhibit the cell growth by 50%

MDR - Multidrug Resistance

MRP - Multidrug Resistance Associated Protein(s)

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NDS - Nanocarrier-based Delivery System

RT-PCR - Reverse Transcription-Polymerase Chain Reaction

SCLC - Small cell lung carcinoma

siRNA - Small Interfering RNA

TBA - Tris/Borate/EDTA
TFA - Trifluoroacetic Acid

TUNEL - Terminal Deoxynucleotidyl Transferase Mediated dUTP-Fluorescein Nick End Labeling
7.6. References


7.7. Figure Legends

Figure 7.1. Co-delivery in one liposomal drug delivery system of DOX simultaneously with siRNA targeted to MRP1 and BCL2 mRNA enhances cell death induction by increasing intracellular DOX concentration and suppression of cellular antiapoptotic defense.

Figure 7.2. Intracellular localization of cationic liposomes, siRNA and the anticancer drug DOX. Typical images of multidrug resistant H69AR human lung cancer cells incubated with cationic liposomes containing DOX or siRNA. Liposomes were labeled with FITC (green fluorescence), siRNA was labeled with DY-547 (red fluorescence), DOX possesses an intrinsic red fluorescence. In addition, cell nuclei were stained with nuclear-specific dye Hoechst 33258 (blue fluorescence). Superposition of images allows for detecting of cytoplasmic co-localization of liposomes with DOX or siRNA (yellow) and nuclear localization of liposomes (cyan) or DOX (pink). Nuclear co-localization of liposomes and DOX gives white color.

Figure 7.3. Typical images of gel electrophoresis of the RT-PCR products and mRNA levels for MRP1 and BCL2 in H69AR human lung cancer cells. Cells were incubated for 48 hours with the indicated formulations. β2-microglobulin (β2-m) was used as an internal standard. Means ± SD are shown.
A: 1 – Control (fresh media); 2 – Cationic liposomes; 3 – Free DOX; 4 – Cationic liposomes-DOX; 5 – Cationic liposomes-MRP1 siRNA; 6 – Cationic liposomes-DOX-MRP1 siRNA.

B: 1 – Control (fresh media); 2 – Cationic liposomes; 3 – Free DOX; 4 – Cationic liposomes-DOX; 5 – Cationic liposomes-BCL2 siRNA; 6 – Cationic liposomes-DOX-BCL2 siRNA.

*P < 0.05 when compared with control.

Figure 7.4. Typical images of in multidrug resistant H69AR human lung cancer cells stained with antibody against MRP1 (top panel) and BCL2 (bottom panel) proteins. Dark color indicates high protein concentration. Cells were incubated for 48 hours with the indicated formulations.

Figure 7.5. Apoptosis induction in multidrug resistant H69AR human lung cancer cells. Typical fluorescent microscopy images of cancer cells incubated for 48 hours with the indicated formulations and stained by TUNEL.

Figure 7.6. Viability of multidrug resistant H69AR human lung cancer cells incubated 48 h with the indicated formulations. A, cytotoxicity of formulations that do not contain DOX; B, cytotoxicity of formulations that contain DOX; C, actual dose-response curves of formulations that contain DOX. The concentration of siRNA and composition of cationic liposomes in all formulations were the same. Means ± SD are
shown. 1 – Control (fresh media); 2 – Empty cationic liposomes; 3 – Free BCL2 siRNA; 4 – Free MRP1 siRNA; 5 – Free BCL2 siRNA-MRP1 siRNA; 6 – Free DOX; 7 – Cationic liposomes-DOX; 8 – Cationic liposomes-BCL2 siRNA; 9 – Cationic liposomes-MRP1 siRNA; 10 – Cationic liposomes-MRP1 siRNA-BCL2 siRNA; 11 – Cationic liposomes-DOX-BCL2 siRNA; 12– Cationic liposomes-DOX-MRP1 siRNA; 13 – Cationic liposomes-DOX-BCL2 siRNA-MRP1 siRNA.

*P < 0.05 when compared with control; †P < 0.05 when compared with free DOX.
Figure 7.1.
Figure 7.2.
Figure 7.3.
Figure 7.4.
Figure 7.5.
Figure 7.6.
8. Conclusions

By employing tumor targeted drug delivery systems (DDS), we were able to improve body distribution of different non-targeted nanocarrier-based delivery systems and increase the sensitivity and specificity of imaging and/or treatment of cancer and limit adverse side effects of the treatment on healthy organs. We also found that tumor-specific targeted chemotherapy, but not size, molecular mass, composition or architecture of the nanocarrier, plays a critical role in anticancer effect. This conclusion is made for the first time in this research project and could have a broad impact on the cancer drug delivery and imaging.

We then developed a successful orthotopic lung cancer model using the intratracheal method of tumor implantation. This is a crucial preliminary step for understanding of the disease, its progression, and the development of new successful therapies for lung cancer.

Third, since several aspects of liposomal drug delivery are not clearly understood, we sought to investigate in vitro cellular uptake and intracellular localization along with in vivo body distribution after intravenous or intratracheal administration of small slightly negative ("neutral") and positively charged liposomes.

We used small “neutral” PEGylated and positively charged DOTAP liposomes. DOX and uncharged P-ethoxy ASO were used as a payload for neutral liposomes while siRNA was complexated to cationic liposomes. In all cases, we found that systemic administration (i.v.) of our drug delivery systems (DDS) for the treatment of lung cancer, has several disadvantages including, unfavorable body distribution and decrease in
specific drug activity as a result of drug deactivation during systemic circulation and high adverse side effects on healthy organs. Our data showed that intratracheal administration of liposomes substantially limit their accumulation in the reticuloendothelial system (RES) and sustain higher concentrations of liposomes and their payload in the lungs for at least 24 hours after injection. We found that the size of liposomes and liposomal complexes with siRNA played a secondary role, while the route of administration served as a primary determinant of body distribution of liposomes and their payload. These data clearly show potential advantages of local lung delivery of different liposomal therapeutics for the treatment of lung diseases and confirm that liposomes can be used for intratracheal delivery of drugs, antisense oligonucleotides and siRNA into the lungs.

Fourth, since the efficient delivery of therapeutics to the site of action does not necessarily guarantee successful treatment of a disease, we sought to confirm that intratracheal delivery of anticancer drugs can enhance the effectiveness of treatment of lung diseases, hence, we used the orthotopic mice model of human lung cancer. Mice bearing human cancer cells in their lungs were treated with free and liposomal doxorubicin delivered intravenously and intratracheally. It was found that intratracheal treatment was much more efficient in limiting the growth of lung cancer cells in the lungs when compared with free and liposomal DOX delivered via the systemic route (i.v.). Therefore, we concluded that local intratracheal delivery of liposomal drugs is much more effective for the treatment of lung cancer when compared with systemic administration of the same drug.

Last, but not least, targeted cancer therapy alone is not always successful because the targeted drug still has to overcome many types of resistance, including pump (active
drug efflux) and non pump resistance. Hence, we employed small interfering RNA (siRNA) to suppress the overexpression of: 1) Multidrug resistance associated protein transporter 1 (MRP-1), hereby inhibiting pump resistance and 2) BCL-2, a protein family that functions in the cellular protection against apoptosis. The aim of that particular investigation was to test a novel liposomal drug delivery system which contains a traditional anticancer drug doxorubicin (DOX) as an apoptosis inducer in combination with small interfering RNA (siRNA) targeted to MRP1 and BCL2 mRNA as suppressors of pump and nonpump cellular resistance in cancer cells. Taken together, our results suggest that liposomal drug delivery system containing an anticancer drug as an apoptosis inducer in combination with small interfering RNA (siRNA) targeted to MRP1 and BCL2 mRNA as suppressors of pump and nonpump resistance, respectively has a high potential as a novel antitumor therapeutic agent. Therefore, we suggest administration of our proposed drug delivery system (DDS) through inhalatory treatment, using a nebulizer, for example.
9. CURRICULUM VITA

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EDUCATION

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PUBLICATIONS

PAPERS

1. Saad M, Garbuzenko O. B., Ber E, Chandna P, Khandare JJ, Pozharov VP, Minko T. Receptor -

   V. A., Pozharov V. P., Minko T. Non-viral nanoscale-based delivery of antisense oligonucleotides
   targeted to HIF1 alpha enhances the efficacy of chemotherapy in drug resistant tumor, Clin.


4. Zalipsky S, Saad M, Kiwan R, Ber E, Minko T. Antitumor activity of new liposomal prodrug of

5. Pakunlu RI, Wang Y, Saad M, Khandare JJ, Starovoytov V, Minko T. In vitro and in vivo
   intracellular liposomal delivery of antisense oligonucleotides and anticancer drug. J Control


7. Bhardwaj RK, Herrera-Ruiz D, Eltoukhy N, Saad M, Knipp GT. The functional evaluation of
   human peptide/histidine transporter 1 (hPHT1) in transiently transfected COS-7 cells. Eur J