TWO-IN-ONE: COMBINED TARGETED NANOSCALE–BASED CHEMO AND GENE THERAPY FOR TUMOR SUPPRESSION AND PREVENTION OF METASTASES

by

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A Dissertation submitted to the Graduate School-New Brunswick Rutgers, The State University of New Jersey in partial fulfillment of the requirements for the degree of Doctor of Philosophy Graduate Program in Pharmaceutical Science written under the direction of Professor Tamara Minko, Ph.D.

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ABSTRACT OF THE DISSERTATION

Two-in-One: Combined Targeted Nanoscale–Based Chemo and Gene Therapy for Tumor Suppression and Prevention of Metastases

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Tamara Minko

The efficiency of siRNA is limited by its low resistance against enzymatic degradation, limited permeability across cell membrane, and substantial liver and renal clearance. Therefore, in order to exploit potential therapeutic applications of siRNA, the effective delivery of siRNA to the site of action and into targeted cells is required. We are proposing to design, characterize and test in vitro the efficient siRNA delivery systems in order to treat human ovarian carcinoma. Internally quaternized poly(amidoamine) dendrimer (PAMAM) with different surface modifications and poly(amidoamine)-poly(ethylene glycol)-poly-L-lysine conjugates were prepared and successfully delivered siRNA into A2780 ovarian cancer cells.

To effectively treat primary ovarian tumor, prevent the development of intraperitoneal metastases and limit side effects of therapy, we proposed a combination of receptor-mediated targeted chemotherapy with genotherapy. To this end, we used
tumor-targeted complex liposomal delivery systems containing: (1) two anticancer drugs with different mechanisms of action (doxorubicin and cisplatin); (2) suppressors of two different mechanisms of cellular resistance - two antisense oligonucleotides (ASO) targeted to MDR1 and BCL2 mRNA; and (3) a targeting moiety specific to cancer cells – LHRH peptide (ligand specific to LHRH receptors that are overexpressed in plasma midbrain of ovarian cancer cells). The targeted combinatorial treatment of primary aggressive ovarian tumor led to the substantial regression of the growth of primary tumor, prevention of the development of intraperitoneal metastases and limitation of the adverse side effects of chemotherapy on healthy organs.
DEDICATION

To my wonderful parents,

Who always gave me moral support and instilled the importance of hard work and higher education into my mind.

To my supervisor, Professor Tamara Minko,

Who has always given me valuable suggestions and generous encouragement.

To my beloved husband, Qiang,

Whose love supported me and enabled me to complete this work.
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1 INTRODUCTION

Nanoparticles are emerging as a class of therapeutic agents for cancer treatment. In general, nanoparticles therapeutics are comprised of therapeutic entities, such as small molecule drugs, proteins, nucleic acids and peptides, and components that assemble with the therapeutic entities, such as polymers and lipids, to form nanoparticles (1, 2). These nanoparticles can have improved therapeutic effects when comparing with therapeutic entities they contain because of active intercellular delivery and more specific targeting to tumor tissues. These properties are dependent on the particle size, surface properties (including the presence of targeting moieties) and some other characteristics of the nanoparticles (1, 3-5).

In the past two decades, there has been a progressive increase in the number of commercially available nanoparticles as therapeutic products. Based on a global survey conducted by the European Science and Technology Observatory in 2006, more than 150 companies are developing nanoscale therapeutics. Among those products approved for clinical use, liposomal drugs and polymer-drug conjugate are two dominate classes (2). Dendrimers represent a new class of polymer, which has well-defined structures with precise control of size and shape as well as terminal group functionality (6-9). Polycationic dendrimers such as poly(amidoamine) (PAMAM) dendrimers have been extensively investigated as efficient vehicles for the delivery of genes and therapeutic
drugs (10-12). Liposomes are spherical vesicles consisting of an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayers configuration (13). Liposomes can be used for great variety of molecules such as small drug molecules, proteins, nucleotides and even plasmids (14-16).

Ovarian cancer is the cancer that forms in tissues of the ovary (one of a pair of female reproductive glands in which the ova, or eggs, are formed). It is one of the most common causes of cancer death from gynecologic malignancy in the industrialized world due to the invasiveness, insidious progression, and rapid development of resistance to chemotherapy. Ovarian cancer may spread to the lining of the abdominal cavity as intraperitoneal metastases (carcinomatosis) and leads to ascites (17-20). The incidence of ascites in women presenting with ovarian cancer ranges from 45% to 75%. Ovarian cancer ascites fluid contains ovarian cancer, lymphoid and mesothelial cells. It also harbors growth factors, bioactive lipids, cytokines and extracellular matrix constituents. These factors may promote cell growth, survival and invasion; therefore, ascites play an active role in ovarian cancer progression rather than a passive one (17, 19, 20).

Doxorubicin (DOX) and cisplatin (CIS) are FDA approved anti-cancer chemotherapy drugs and widely used to treat various types of cancers. Oligonucleotides are short (13-25 nucleotides), chemically modified or unmodified single-stranded DNA molecules. In general, antisense oligonucleotides refer to synthetic oligonucleotides that are complementary in sequence and hybridize (at least in theory) to a unique sequence in the
total pool of targets present in cells (21-23). Short or small interfering RNA (siRNA) is a class of short double-stranded RNA molecules composed of 20-25 nucleic acids. siRNA is involved in the RNA interference (RNAi) pathway to inhibit gene expression of a specific gene (24-26).

In these studies, PAMAM dendrimer-based siRNA delivery system will be developed for 

*in vitro* study. The delivery system was expected to deliver the siRNA into cells efficiently and suppress the specific genes. Due to the limitations of the unmodified PAMAM dendrimer as drug delivery system, we modified the surface of dendrimers by using different strategies and a series of experiments have been conducted to characterize and examine those surface modified dendrimers. The results showed that modified PAMAM dendrimer provided higher transfection efficiency of siRNA than the unmodified PAMAM dendrimer. Meanwhile, the cytotoxicity of PAMAM dendrimer has been significantly decreased and the stability of siRNA in serum has been improved.

To treat invasive ascites tumor, we utilized liposome as drug/ASO delivery system. Two anticancer drugs (Doxorubicin and Cisplatin), two ASO targeted to MDR1 and BCL2 mRNA and Luteinizing hormone-releasing hormone (LHRH) peptide as targeting moiety have been co-delivered. The results showed that the targeted combinatorial treatment of primary aggressive ovarian tumor led to the substantial regression of the growth of primary tumor, prevention of the development of intraperitoneal metastases and
limitation of the adverse side effects of chemotherapy on healthy organs. That makes the developed tumor-targeted delivery systems highly promising for clinical applications.
2 BACKGROUND AND SIGNIFICANCE

2.1 Delivery systems

2.1.1 Poly(amidoamine) dendrimers (PAMAM)

Polymers have been widely studied as carriers of both covalently bound and physically entrapped drug molecules to improve their water solubility, increase the permeability, decrease the cytotoxicity, protect them from possible enzymatic degradation or hydrolysis, and/or increase the site-specific delivery of drugs. However, the application of conventional classes of polymers has been limited by their properties, such as their high polydispersity. Dendrimers represent a new class of polymer, which has well-defined structures with precise control of size and shape as well as terminal group functionality (6-9). Polycationic dendrimers such as poly(amidoamine) (PAMAM) dendrimers have been extensively investigated as efficient vehicles for the delivery of genes and therapeutic drugs (10-12). As shown in Fig. 2.1, dendrimers are a type of regular and highly branched, monodisperse, spherical nanomaterials with dense peripheral groups that can be functionalized with drugs, targeting moieties, and other biologically active components (27). Dendrimers can be synthesized by either a divergent or a convergent approach. In the divergent method, dendrimers are synthesized radially from a central core through a stepwise repetitive reaction sequence. Increasing growth is defined in
terms of ‘generation number’, which was characterized by size, shape, molecular weight and number of surface functional groups. In contrast, the convergent method involves construction of branched subunits (dendrons) followed by their attachment to a multifunctional core. Both approaches lead to a dendritic structure with properties amenable to modifications of shape, size, polarity, internal structure and surface properties. PAMAM dendrimers usually are prepared by the divergent approach and the branching amidoamine dendritic structure is built up from a central core, which can be either an ethylenediamine or ammonia core. An iterative process involving Michael addition followed by amidation can be used to synthesize PAMAM. The first iteration results in the formation of zero generation (G0) dendrimer and the subsequent addition-amidation cycles each lead to growth and the formation of a higher generation. Currently, PAMAM dendrimers up to G10 are commercially available (7, 28).

As drug delivery systems, dendrimers can either encapsulate drugs within the dendritic structure or interact with drugs at their terminal functional groups via electrostatic or covalent bonds. Several groups have also investigated that PAMAM and other dendrimers can be used for controlled drug release (29, 30). A number of studies have demonstrated the potential of dendrimers to improve the intracellular delivery of drugs. It is clear that the cellular pharmacokinetics will play a major part in determining biological properties including toxicity; therefore, the rate of internalization by particular cell types and the intracellular fate are important. Endocytosis, both clathrin- and
caveolae-dependent pathways, were found to be the dominant pathway that the delivery system undergoes (7). Tajarobi et al. investigated the transport of fluorescently labeled cationic dendrimer generations (G0-G4) across Madin-Darby canine kidney cells and the rank order of permeability was of G4 >> G1 ≈ G0 > G3 > G2. Therefore, it was concluded that transepithelial transport was both a function of dendrimer size and interaction with the cells (7, 31). A great number of studies focused on the surface modification of dendrimers to improve the permeability across cell membrane (7, 30, 32-34). However, dendrimer cytotoxicity is another critical issue as drug delivery system. Many studies have examined dendrimer toxicity in different cell lines with different incubation time and various assay methods, and some general trends were found. Dendrimers bearing –NH$_2$ termini display concentration- and usually generation-dependent cytotoxicity (28, 35). Dendrimer toxicity is dependent on the chemistry of the core, but it is most strongly influenced by the surface. It was shown that cationic dendrimer is more toxic than anionic or PEGylated dendrimers (36). Lee et al. quaternized PAMAM-OH dendrimers (QPAMAM-OH) and QPAMAM-OH showed lower level of cytotoxicity than PAMAM-NH$_2$ because of the shielding of the internal cationic charges by surface hydroxyl groups (34). In another study, PAMAM G4 surface was modified with lysine or arginine and the toxicity increased in HEP G2 cells or 293 cells. This can be explained by increased charge density and molecular weight (37). In addition, low generation dendrimers showed higher toxicity because they have more accessible surface terminal groups and these become sterically hindered due to crowding.
at higher generation. To create less toxic dendrimers, increased branching (generation) and a greater surface coverage with biocompatible terminal groups like PEG are being widely used (28).

Biodistribution of parenterally administered dendrimers has been widely and it showed that the higher molecular mass and/or more branched dendrimers exhibited longer circulation half-lives due to slower excretion into the urine when compared with lower molecular mass carriers, less branched dendrimers, or linear polymers (38). There is also increasing interest in the use of dendrimers as carriers for targeted drug delivery. Besides, to target the macromolecular anticancer agents to angiogenic solid tumors, passive tumor localization by enhanced permeability and retention (EFR) effect arising from hyperpermeable tumor vasculature has been widely used for in vivo and clinical study. Dendrimers such as poly(amidoamine) and poly(propylenimine) possess cationic primary amine groups at the surface, which participate in the DNA binding process and increase the cellular uptake of DNA by transforming the entire complex into nanoscale polyplexes (10, 12, 39-41). However, these highly efficient delivery systems have been less explored for siRNA delivery. It has been shown that PAMAM dendrimers or their conjugates are more efficient in delivering antisense oligonucleotides or plasmid DNA than siRNA (33). Therefore, in this proposal, one of our purposes is to design, synthesize and validate surface-modified PAMAM dendrimers as nanocarriers for enhanced intracellular delivery of siRNA.
2.1.2 Liposomes

Liposomes are spherical vesicles consisting of an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayers configuration (Fig. 2.2) (13). Liposomes can be produced from cholesterol, non-toxic surfactants, sphingolipids, glycolipids, long chain fatty acids and even membrane proteins. Since liposomes were discovered more than 40 years ago by A.D. Bangham, they have become the versatile tool in biology, biochemistry and medicine today. In 1960s, liposomes have been used to deliver many compounds in its aqueous compartment. Currently, as drug carriers, liposomes can be used for great variety of molecules such as small drug molecules, proteins, nucleotides and even plasmids (14-16). Since liposomes were discovered over 40 years ago, a lot of efforts have been invested to develop liposomal formulations and several formulations of liposome-based anthracyclines have been approved by the FDA. Up to date, virtually all traditional anti-cancer drugs have been encapsulated in liposomes and a number of them have been included in clinical trials as anti-cancer therapeutics (42).

Liposomes can be prepared by using several groups of phospholipids including phospholipids from natural source, phospholipids modified from natural source, semi synthetic phospholipids, fully synthetic phospholipids and phospholipids with natural
head groups. Dioleoyl phosphotidyl ethanolamine (DOPE), Dilauryl phosphotidyl glycerol (DLPG), Distearoyl phosphotidyl serine (DSPS), Dilauryl phosphotidyl choline (DLPC), Dimyristoyl phosphotidyl choline (DMPC), Dipalmitoyl phosphotidyl choline (DPPC), Distearoyl phosphotidyl choline (DSPC), Dioleolyl phosphotidyl choline (DOPC), Dilauryl phosphotidyl ethanolamine (DLPE), Dimyristoyl phosphotidyl ethanolamine (DMPE), and Distearoyl phosphotidyl ethanolamine (DSPE) are the commonly used phospholipids for liposome preparation. Cholesterol can be added to the bilayers mixture as a fluidity buffer and intercalator with phospholipids molecules and it can restrict the transformation of Trans to gauche conformation (13, 16).

Liposomes can be prepared through three different strategies including mechanical methods, methods based on replacement of organic solvent and methods based on size transformation or fusion of prepared vesicle. In mechanical methods, film method and ultrasonic methods are the two main types. In the methods based on replacement of organic solvent, lipids are co-solvated in organic solution, which is then dispersed into aqueous phase containing materials to be entrapped in liposome. This method includes reverse phase evaporation and ether vaporization method. Methods based on size transformation or fusion of prepared vesicle includes two main types: freeze thaw extrusion method and the dehydration-rehydration methods. Each method has the advantages and disadvantages and the purpose of liposomes should be the major concern to choose the method for liposome preparation (13, 16).
The size of liposomes may be from nanometers to micrometers and plays an important role on their fate i.e. for which application they can be used for. The therapeutic applications of liposome are dependent on stability and physical integrity of lipid bilayers structure. Therefore, the procedure of liposome production must be predictable and reproducible with particle size distribution. Drug loading is another crucial parameter to evaluate the liposome as drug delivery system. It can be achieved either actively (i.e. after liposome formation) or passively (i.e. the drug is encapsulated during liposome formation). Hydrophobic drugs can be directly incorporated into liposomes during vesicle formation and the extent of uptake and retention is governed by drug-lipid interactions. The trapping efficiency can often achieve 100%, but is dependent on the solubility of the drug in liposome membrane. Passive encapsulation of water-soluble drugs is dependent of the ability of liposomes to trap aqueous buffer containing a dissolved drug during liposome formation (13, 16).

Liposomes can be administered by various routes, including intravenously, orally, ocularly or intranasally. The tendency of liposomes to be cleared from circulation by reticuloendothelial system (RES) is the major limitation of liposomal DDS. Therefore, many liposomal formulations or strategies have been developed to avoid RES trapping and prolong circulation time in vivo (43-45). Several different modifications of liposomal membrane were proposed to mimic cells circulating in the blood to escape host
recognition as foreign substances. Also, poly(ethylene glycol) (PEG) is widely used to cover liposome in order to escape recognition (46). In addition, to avoid the side effects and increase the delivery efficiency, targeting moiety, such as antibody, protein and ligand, can be attached to the surface of liposome and the drug carriers will deliver drug to the site of action (47). Our lab has successfully utilized liposome as the carrier of anti-cancer drugs and ASO to some carcinoma cell lines (42, 48, 49). In this proposal, we plan to use liposome to delivery combinatorial anti-cancer drug and antisense oligonucleotides (ASO) with targeting moiety to treat more invasive ovarian cancer in vivo. This cell line will be described in the following sections.

2.2 Ovarian cancer and multidrug resistance mechanisms

2.2.1 Ovarian cancer and ascites

Ovarian cancer is the cancer that forms in tissues of the ovary (one of a pair of female reproductive glands in which the ova, or eggs, are formed). It is one of the most common causes of cancer death from gynecologic malignancy in the industrialized world due to the invasiveness, insidious progression, and rapid development of resistance to chemotherapy. The estimated new cases and deaths from ovarian cancer in the United Stated is 2010 are 21,880 and 13,850 cases respectively. Most of the ovarian cancers are either ovarian epithelial carcinomas (cancer that begins in the cells on the surface of the
ovary) or malignant germ cell tumors (cancer that begins in egg cells). Ovarian epithelial cancer is a disease in which malignant cells form in the tissue covering the ovary. Ovarian germ cell tumor is a disease in which malignant cells form in the germ (egg) cells of the ovary. There are four stages to be used for these two types of ovarian cancers. In stage I, cancer is found in one or both of the ovaries; in stage II, cancer is found in one or both ovaries and has spread into other areas of the pelvis; in stage III, cancer is found in one or both ovaries and has spread to other parts of the abdomen; and in stage IV, cancer is found in one or both ovaries and has metastasized (spread) beyond the abdomen to other parts of the body, such as lungs, liver, lymph nodes, or bones (17, 19, 20, 48, 50-54).

Ovarian cancer may spread to the lining of the abdominal cavity as intraperitoneal metastases (carcinomatosis) and leads to ascites. The incidence of ascites in women presenting with ovarian cancer ranges from 45% to 75%. Ovarian cancer ascites fluid contains ovarian cancer, lymphoid and mesothelial cells. It also harbors growth factors, bioactive lipids, cytokines and extracellular matrix constituents. These factors may promote cell growth, survival and invasion; therefore, ascites play an active role in ovarian cancer progression rather than a passive one (17, 19, 54, 55).

2.2.2 Mechanisms of multidrug resistance
The main mechanisms of multidrug resistance are common to most cancers and include “pump” and “nonpump” resistance (48, 50-53). Cells express mechanisms of resistance that confer simultaneous resistance to many different structurally and functionally unrelated drugs. This phenomenon is known as multidrug resistance and can result from changes that limit accumulation of drugs within cells by limiting intracellular uptake, enhancing efflux, or affecting membrane lipids. These changes can block (1) the programmed cell death (apoptosis) that is activated by most anticancer drugs, (2) the activation of general response mechanisms that detoxify drugs and repair damage to DNA, and (3) the alterations in the cell cycle and checkpoints that render cells relatively resistant to the cytotoxic effects of drugs.

2.2.2.1 Drug efflux

Drug accumulation in cells results from a balance between drug entry and exit mechanisms. Drug can enter cells in various ways, such as diffusion and transport. One of the exit mechanisms in multidrug resistant cancer cells is the expression of an energy-dependent drug efflux pump, known alternatively as P-glycoprotein (P-gp) or the multidrug transporter. P-glycoprotein, the product of the *MDR1* gene in the human, was one the first members described of the ATP-binding cassette (ABC) family. P-gp is widely expressed in many human cancers, including cancers of the gastrointestinal (GI) tract (liver, small and large intestine, pancreatic cancer), cancers of the hematopoietic system,
cancers of the genitourinary system (kidney, ovary, testicle), and childhood cancers. P-gp is a 170,000-dalton-molecular weight phosphoglycoprotein and consists of two ATP binding cassettes and two transmembrane regions, each of which contains six transmembrane domains. P-gp can detect and bind a large variety of drugs as they enter the plasma membrane. These drugs include many anticancer drugs such as doxorubicin, daunorubicin, vinblastine and vincristine and taxol, as well as many pharmaceuticals ranging from antiarrhythmics and antihistamines to cholesterol-lowering statins and HIV protease inhibitors. Binding of these drugs causes activation of one of the ATP-binding domains, and the hydrolysis of ATP results in a major change in P-gp shape, which results in release of the drug into the extracellular space. Therefore, the intracellular drug concentration and thereby the efficacy of the treatment are decreased (56).

2.2.2 Anti-apoptosis

The main mechanism of nonpump resistance is an activation of cellular antiapoptotic defense. After internalization into cancer cells, most anticancer agents act by inducing apoptosis, however, they also concurrently activate antiapoptotic cellular defense. The inability of cancer cells to initiate apoptosis in response to the stimuli significantly limits the efficacy of anticancer drugs (57). The BCL-2 protein family includes both pro- and anti-apoptotic molecules and plays a crucial role in the cellular protection against apoptosis. Antiapoptotic molecules help to maintain the integrity of mitochondrial
membrane and prevent the leakage of cytochrome c into the cytoplasm, limit the activation of executors of apoptosis, caspases and therefore protect cells from apoptotic cell death. In contrast, proapoptotic molecules stimulate apoptosis mainly by suppression of the activity of antiapoptotic BCL-2 family proteins. The ratio between these two subsets helps determine the susceptibility of cells to a death signal (18, 57, 58). BCL-2 family members possess up to four conserved BCL-2 homology (BH) domains (BH1, BH2, BH3 and BH4), which are critical to the functions of these proteins, including their impact on cell survival and their ability to interact with other family members and regulatory proteins. The pro-apoptotic molecules usually display less sequence conservation of the first α-helical segments, BH4, while many anti-apoptotic members display sequence conservation in all four domains. BCL-2 homology 3 (BH3) domain of pro-apoptotic proteins of BCL-2 family is responsible for the induction of apoptosis (57, 58). Figure 2.3 shows the summary of anti-apoptotic and pro-apoptotic BCL-2 members (57).

2.2.2.3 Treatments for ovarian cancer

Standard treatments for ovarian cancer involve aggressive cytoreductive (debulking) surgery followed by chemotherapy. In general, since carcinomatosis and ascites indicate a more advanced stage of the disease, that usually requires extensive high dose chemotherapy (17, 59, 60). Furthermore, tumor cells from malignant ascites are more
invasive and resistant to chemotherapy when compared with primary ovarian tumors (20, 61, 62). The precise mechanisms underlying the formation of ascites in ovarian cancer are unknown. However, it is known that the success of chemotherapeutic treatment of primary ovarian cancer and especially tumor cells growing in ascitic fluid is limited by the intrinsic and acquired resistance of cancer cells to chemotherapy (20, 61-64). Such resistance requires the use of multiple chemotherapeutic agents thus increasing the rate of severe adverse side effects of therapy on healthy organs and tissues. Therefore, we hypothesized that effective treatment of advanced multidrug resistant primary ovarian tumors and their intraperitoneal metastases is possible only by suppressing simultaneously at least two main types of cellular resistance and by inducing cell death using several anticancer agents with different mechanisms of action. Such an objective could be best achieved if several anticancer agents are simultaneously delivered specifically to the tumor in combination with other active components that perform different functions for enhancing cellular uptake and efficiency of drugs in cancer cells, limiting adverse side effects, and preventing the development of drug resistance and metastases. Our results proved that the proposed targeted combinatorial treatment of primary aggressive ovarian tumor led to the substantial regression of the growth of primary tumor, prevention of the development of intraperitoneal metastases and limitation of the adverse side effects of chemotherapy on healthy organs.
2.3 Therapeutic entities

2.3.1 Anti-cancer drugs (Doxorubicin and Cisplatin)

2.3.1.1 Doxorubicin

Doxorubicin (DOX) and cisplatin (CIS) are FDA approved anti-cancer chemotherapy drugs and widely used to treat various types of cancers. Doxorubicin is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius*. It consists of a naphthacenequinone nucleus linked through a glycosidic bond to an amino sugar, daunosamine. The chemical formula of DOX is C$_{27}$H$_{29}$NO$_{11}$ with the molecular weight of 543.52 (65, 66). The structure is as follows (67):

![Doxorubicin structure](image)

Doxorubicin is commonly used to treat some leukemias and Hodgkin’s lymphoma, as well as cancers of breast, stomach, bladder, ovaries, lung, thyroid, soft tissue sarcoma, multiple myeloma, etc. It can cause acute adverse effects, including nausea, vomiting,
heart arrhythmias, neutropenia, and complete alopecia. When the cumulative dose reaches 550 mg/m$^2$, the risks of developing cardiac side effects, including congestive heart failure, dilated cardiomyopathy and death, increase dramatically.

Doxorubicin is usually given by intravenous injection (IV) in the form of hydrochloride salt. The brand names include Adriamycin PFS, Adriamycin RDF and Rubex (68, 69). Following administration, DOX is known to interact with DNA by specific intercalation and inhibition of macromolecular biosynthesis. It also stabilizes the topoisomerase II complex after it breaks the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the replication process. Moreover, the molecule is amphoteric, containing acidic functions in the ring phenolic groups and a basic function in the sugar amino group and it binds to cell membranes and plasma proteins (68, 70). As an anti-cancer drug, DOX shows high cytotoxicity on malignant cells, however, it also causes toxic effects on various organs which may be a result of non-specific intercalation and cell membrane binding. In addition, most cancer cells have the developed multidrug resistance, e.g. pump resistance caused by membrane transporters to pump out the anticancer agents (42, 69, 71). Thus, a delivery system, especially a targeted delivery system, becomes necessary to avoid the side effects to normal tissues and deliver the drug directly into the cancer cells.
2.3.1.2 Cisplatin

Cisplatin, (*cis*-diamminedichloroplatinum(II), CIS) is a platinum-based chemotherapy drug which is used to treat cancers including sarcoma, small cell lung cancer, germ cell tumors, lymphoma and ovarian cancer. Cisplatin is platinum-based and was the first medicine developed in that drug class. It is actually first created in the mid 19th century and went into clinical trials for cancer therapy in 1971. It is still used today although many newer chemotherapy drugs developed over the past decades. The other names for cisplatin are DDP, cisplatinum, and *cis*-diamminedichloridoplatinum(II) (CDDP) The chemical formula of cisplatin is Pt(NH$_3$)$_2$Cl$_2$ with the molecular weight of 300.05 (72, 73). The structure is as follows:

![Cisplatin Structure](http://www.cisplatin.org)

As an anti-cancer chemotherapy drug, cisplatin also shows a number of side effects. Those may include nephrotoxicity, neurotoxicity, nausea and vomiting, ototoxicity,
alopecia, electrolyte imbalance, decrease of blood cells in bone marrow, thrombocytopenia, leucopenia, myelosuppression, and so on. The toxicity is widely believed to result from the formation of lesions that block polymerases or disrupt the integrity of the genome and those side effects are generally reversible and subside when treatment ends (72, 74, 75).

Cisplatin is soluble in water and can be administered intravenously. The shape of the cisplatin molecule is flat and square. Its covalent bonds can exchange with other ligands, including water. In the aqueous environment of the body, the chlorine atom in the cisplatin molecule can be easily replaced by a hydroxyl group, and later by part of the DNA. The way the cisplatin operates is by forming a platinum complex inside of a cell which binds to and causes crosslinking of DNA. When DNA is cross-linked in this manner, it ultimately triggers apoptosis. One of the methods that it uses to cause apoptosis through cross-linking is by damaging the DNA so that the repair mechanisms for DNA are activated. Once the repair mechanisms are activated and the cells are found not to be salvageable, the death of those cells is triggered instead (76, 77). However, the cisplatin-resistance is usually developed in the majority of cancer patients (71, 74). The proposed mechanisms of cisplatin-resistance include changes in cellular uptake and efflux of the drug, increased detoxification of the drug, inhibition of apoptosis and increased DNA repair (78). Therefore, a lot of efforts were invested to develop the efficient delivery system which can deliver the drug to the site of action and directly into
the tumor cells to avoid the side effects and resistance (75, 79-81).

2.3.2 Anticancer oligonucleotides (ASO)

Oligonucleotides are short (13-25 nucleotides), chemically modified or unmodified single-stranded DNA molecules. In general, antisense oligonucleotides refer to synthetic oligonucleotides that are complementary in sequence and hybridize (at least in theory) to a unique sequence in the total pool of targets present in cells (21-23).

There are multiple mechanisms that can be exploited to inhibit the function of the RNA when the ASO bind to the target RNA. The best characterized mechanism causes cleavage of the targeted RNA by endogenous cellular nucleases, such as RNase H or the nuclease associated with the RNA interference mechanism. Beside this, ASO may also inhibit expression of target gene by non-catalytic mechanisms, such as modulation of splicing or translation arrest. Fig. 2.4 shows the RNase H dependent inhibition mechanism. Antisense oligonucleotides containing a native DNA or chemically modified DNA segment bind to the target mRNA and form an RNA/DNA heteroduplex, which is a substrate for endogenous cellular RNase H. RNase H cleaves the RNA/DNA duplex region of the mRNA thus preventing protein translation and thereby blocks gene expression (21-23, 82-84).
ASOs have proven to be powerful tools to inhibit gene expression levels both in vitro and in vivo. However, the use of ASOs is limited as they are rapidly degraded by the intracellular endonucleases and exonucleases, usually via 3'→5' activity, although it is not a complicated matter to synthesize phosphodiester oligonucleotides. In addition, the degradation products, dNMP mononucleotides, may be cytotoxic and also exert antiproliferative effects. Therefore, many chemical modifications have been successfully developed to attempt to overcome these problems and enhance the properties. The key issues that medicinal chemists have focused on are to enhance the potency of ASOs, decrease toxicities, change the pharmacokinetics and lower manufacturing costs. The modified antisense oligonucleotides include phosphorothioate-modified ASO, methylphosphonate-modified ASO, N3'→P5' phosphoramidate-modified ASO, P-ethoxy-modified ASO, etc. Recently, due to these improvements in design and chemistry of ASOs, this technology has become a routinely used tool in basic research, target validation, genomics and drug discovery (21, 83).

However, so far, the FDA has only approved one antisense-based drug although there are around 30 different oligonucleotides being evaluated in different clinical trials. There are several reasons why it is so challenging to use ASOs in clinics. These are essentially their instability in biological media, their poor penetration into cells, and fast elimination through liver and kidney. Although modification of nucleic acid structure has provided some advances, these molecules require effective delivery systems to become useful.
therapeutic agents. A lot of research efforts have been invested on developing efficient
delivery systems for ASO to overcome the problems. Among them, lipid-based and
polymer-based systems have made enormous contribution to this field (3, 82).

2.3.3 Small interfering RNA (siRNA)

Short or small interfering RNA (siRNA) is a class of short double-stranded RNA
molecules composed of 20-25 nucleic acids. siRNA is involved in the RNA interference
(RNAi) pathway to inhibit gene expression of a specific gene (24-26).

As shown in Fig. 2.5, dicer, an RNAse III nuclease, cleaves double stranded RNA into
small interfering RNA (siRNA) of about 20-25 nucleotides long. siRNA then binds to the
RNA induced silencing complex (RISC), whose catalytic component argonaute, an
endonuclease is capable of degrading messenger RNA (mRNA) whose sequence is
complementary to that of the siRNA guide strand. In addition, siRNA also acts in
RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin
structure of a genome. Despite several potential therapeutic applications, the efficiency of
siRNA in vivo is limited by its low resistance against enzymatic degradation, limited
permeability across cell membrane, and substantial liver and renal clearance (84).

Therefore, in order to exploit potential therapeutic applications of siRNA, the effective
delivery of siRNA to the site of action and into targeted cells is required. While considerable efforts have recently been made to develop new siRNA carriers, the research on the delivery of siRNA is still in a preliminary stage (85-91). One of the most common methods used for the delivery of charged nucleic acids involves their electrostatic interaction with cationic carriers. Several types of cationic carriers such as liposome/lipids, dendrimers, and polymeric amines have been successfully used for the delivery of plasmid DNA and oligonucleotides (40, 41). Even though siRNA is structurally similar to plasmid DNA with negatively charged anionic phosphodiester backbones, electrostatic interaction of siRNA largely differs from that of plasmid DNA (39). The molecular weight and molecular topography of siRNA and plasmid DNA play an important role in their electrostatic interaction with cationic agents. Nonviral vectors, and especially polymers, form looser complexes with siRNA than with plasmid DNA, and incomplete encapsulation of the nuclear acid leads to the exposure of siRNA to enzymatic or physical degradation prior to delivery to the targeted cells. Consequently, carriers that are successfully used for the delivery of plasmid DNA cannot be efficiently employed for the delivery of siRNA, and a different approach is required.

Herein, to deliver siRNA into cancer cells more efficiently, we developed PAMAM dendrimer-based delivery system. By using different surface modification, the properties of PAMAM dendrimer were enhanced and cytotoxicity was decreased. Effects of different quaternization degree on siRNA delivery were also evaluated. Moreover, the
triblock PAMAM-PEG-PLL siRNA delivery system was prepared and examined. siRNA were successfully delivered into cells and efficiently knocked down the target gene. For \textit{in vivo} study to treat ascites tumors, we hypothesized that effective treatment of advanced multidrug resistant primary ovarian tumors and their intraperitoneal metastases is possible only by suppressing simultaneously at least two main types of cellular resistance and by inducing cell death using several anticancer agents with different mechanisms of action. Such an objective can be best achieved if several anticancer agents are simultaneously delivered specifically to the tumor in combination with other active components that perform different functions for enhancing cellular uptake and efficiency of drugs in cancer cells, limiting adverse side effects, and preventing the development of drug resistance and metastases.

2.3.4 Targeting moiety (Luteinizing hormone-releasing hormone)

Luteinizing hormone-releasing hormone (LHRH) peptide is a targeting ligand to LHRH receptors that are over-expressed in the plasma membrane of several types of cancer cells and are not expressed detectably in normal visceral organs. Our previous findings in ovarian, breast, and prostate cancer cells provided the rationale of using LHRH peptide as a targeting moiety/penetration enhancer to target different drug delivery systems to tumors and facilitate their uptake by cancer cells (92-94).
The use of LHRH peptide provides for two main advantages. Firstly, in contrast to non-targeted dendrimer-based drug delivery system (DDS) that accumulates almost equally in a tumor, liver, and kidney, LHRH directs the entire DDS specifically to the tumor and prevents the accumulation in healthy tissues. Secondly, interacting with receptors in the plasma membrane of cancer cells, LHRH peptide enforces the internalization of DDS by cancer cells via receptor-mediated endocytosis. This mechanism of nanocarrier internalization by cancer cells is much more efficient when compared with penetration of free anticancer drugs or non-targeted DDS by a “simple” diffusion or endocytosis, respectively. Such a switch of mechanisms substantially enhances intracellular internalization and the anticancer efficacy of the delivered drug and other active components of DDS (51, 92-96).
Figure 2.1 Schematic structure of a generation 3 PAMAM dendrimer.

(http://www.informaworld.com/smpp/content~db=all~content=a758573859~tab=content~order=title)
\textbf{Figure 2.2} Schematic structure of liposomes.

(http://www.myvisiontest.com/newsarchive.php?id=816)
Figure 2.3 Summary of anti-apoptotic and pro-apoptotic BCL-2 family members. BCL-2 homology regions (BH1-BH4) are denoted as the carboxy-terminal hydrophobic (TM) domain (57).
**Figure 2.4** RNase H-mediated recognition of mRNA:oligonucleotide hybrid.

Figure 2.5 Mechanism of siRNA silencing.

(http://www.gene-quantification.de/si-rna.html)
3 SPECIFIC AIMS

Specific Aim 1: To develop efficient PAMAM-based drug/gene delivery system through surface modification and internal quaternization.

RNA interference is a natural process of sequence-specific, posttranscriptional gene silencing mediated by short double stranded RNA. Given the ability to interfere with the disease-causing proteins at an early stage of gene expression, short interfering RNAs (siRNAs) have generated considerable attention as potential therapeutic agents for the treatment of cancer and other related diseases. However, low resistance against enzymatic degradation, limited permeability across cell membranes, and substantial liver and renal clearance has restricted therapeutic applications of siRNA in vivo.

Polycationic dendrimers such as poly(amidoamine) (PAMAM) dendrimers have been extensively studied as efficient vehicles for the delivery of genes and therapeutic drugs. Dendrimers are a type of regular and highly branched, monodisperse, spherical nanomaterials with dense peripheral groups that can be functionalized with drugs, targeting moieties, and other biologically active components. Biodistribution studies showed that the higher molecular mass and/or more branched dendrimers exhibited longer circulation half-live due to slower excretion into the urine when compared with lower molecular mass carriers, less branched dendrimers, or linear polymers. Dendrimers
such as poly(amidoamine) and poly(propylenimine) possess cationic primary amine groups at the surface, which participate in the DNA binding process and increase the cellular uptake of DNA by transforming the entire complex into nanoscale polyplexes. However, these highly efficient delivery systems have been less explored for siRNA delivery. It has been shown that PAMAM dendrimers or their conjugates are more efficient in delivering antisense oligonucleotides or plasmid DNA than siRNA.

Therefore, the proposed dendrimer as a nanocarrier possesses the following advantages: (1) modified neutral surface of the dendrimer for low cytotoxicity and enhanced cellular internalization; (2) existence of cationic charges inside the dendrimer (not on the outer surface) resulting in highly organized compact nanoparticles, which can potentially protect nucleic acids from degradation. Our hypothesis is that the surface modification (surface acetylation) and internal quaternization of PAMAM-NH$_2$ and or PAMAM-OH may provide advantages over the unmodified PAMAM dendrimers.

**Specific Aim 2: To explore the effect of the degree of quaternization and specific cancer targeting of PAMAM-OH dendrimers for efficient siRNA delivery.**

To improve the siRNA delivery using the QPAMAM-OH dendrimer, we plan to investigate two independent strategies: 1) degree of quaternization for proton sponge or buffering effect; and 2) targeting ligand as a penetration enhancer. Polyethyleneimine
(PEI) and poly(amidoamine) (PAMAM) dendrimers, exhibit high transfection efficiency
due to buffering or the so-called proton sponge effect resulting from low pKa of tertiary
amines. It is hypothesized that substitution of tertiary amines in poly(amidoamine)
(PAMAM-OH) dendrimers with permanently charged quaternary amines may perhaps
obstruct the transfection efficiency of siRNA. We anticipate improved transfection
efficiency by decreasing the degree of quaternization that would enable tertiary amines
for buffering effect and quaternary amines for the complex with siRNA.

Previously, in our lab, we have successfully used a synthetic analog of Luteinizing
Hormone-Releasing Hormone (LHRH peptide) for the effective targeting of anticancer
drugs, therapeutic peptides, and different complex delivery systems to cancer cells \textit{in}
\textit{vitro} and \textit{in vivo} (93, 95-97). LHRH peptide is a targeting ligand to LHRH receptors that
are over-expressed in the plasma membrane of several types of cancer cells and are not
expressed detectably in normal visceral organs. Our previous findings in ovarian, breast,
and prostate cancer cells provided the rationale of using LHRH peptide as a targeting
moiety/penetration enhancer to target different drug delivery systems to tumors and
facilitate their uptake by cancer cells. Resulting from this success, an introduction of a
targeting LHRH peptide to the QPAMAM-OH dendrimer was envisaged to enhance the
intracellular delivery of siRNA by a receptor mediated endocytosis pathway. This study
is aimed at designing, synthesizing, and validating different cancer-targeted
QPAMAM-OH dendrimers as nanocarriers for the enhanced intracellular delivery of
siRNA to cancer cells. The impact of two independent factors (degree of quaternization and LHRH targeting ligand) is also examined.

Specific Aim 3: To design, synthesize, and evaluate a triblock delivery system (PAMAM-PEG-PLL) that provides solutions for major problems in siRNA delivery i.e. poor cellular uptake, low endosomal escape, and facile enzymatic degradation.

A novel triblock poly(amideamine)-poly(ethylene glycol)-poly-L-lysine (PAMAM-PEG-PLL) nanocarrier will be designed, synthesized, and evaluated for the delivery of siRNA. The design of the nanocarrier is unique and provides a solution to most of the common problems associated with the delivery and therapeutic applications of siRNA. We hypothesize that every component in the triblock nanocarrier plays a significant role and performs multiple functions: (1) tertiary amine groups in PAMAM dendrimer work as proton sponge and play a vital role in the endosomal escape and cytoplasmic delivery of siRNA, (2) PEG, a linker connecting PLL and PAMAM dendrimer renders nuclease stability and protects siRNA in human plasma, (3) PLL provides primary amines to form polyplexes with siRNA through electrostatic interaction and also acts as penetration enhancer, and (4) conjugation to PEG and PAMAM reduces toxicity of PLL and the entire triblock nanocarrier PAMAM-PEG-PLL.

Specific Aim 4: To develop a targeted multifunctional liposomal delivery system to
treat multidrug resistant primary ovarian tumor and prevent the development of metastases.

Ovarian cancer is one of the most common causes of cancer death from gynecologic tumors all over the world. Standard treatment involves aggressive cytoreductive (debulking) surgery followed by chemotherapy. Ovarian cancer may also spread to the lining of the abdominal cavity and lead to the buildup of fluid inside the abdomen, called ascites. In general, ascites indicate a more advanced stage of the disease and usually require extensive high dose chemotherapy (17, 60). It was also found that malignant metastatic ascites are more invasive and resistant to chemotherapy when compared with primary ovarian tumors (20, 61, 62). The success of chemotherapeutic treatment of primary ovarian cancer and especially tumor cells growing in ascitic fluid is limited by the intrinsic and acquired resistance of cancer cells to chemotherapy (20, 61-63). Such resistance requires high doses of chemotherapeutic agent(s) thus increasing severe adverse side effects of therapy on healthy organs and tissues. The main mechanisms of multidrug resistance are common to most cancers and include “pump” and “nonpump” resistance (48, 50-53). Pump resistance is caused by membrane transporters that pump out the anticancer agents from cells, decreasing the intracellular drug concentration and therefore the efficacy of the treatment. The main mechanism of nonpump resistance is an activation of cellular antiapoptotic defense. Based on the results of our previous study and literature data, we hypothesized that effective treatment of advanced multidrug
resistant primary ovarian tumors and intraperitoneal metastases is possible only by simultaneous suppression of at least two main types of cellular resistance and cell death induction by several anticancer agents with different mechanisms of action. Liposomes are the kind of widely used drug/gene carriers for cancer therapy and a number of liposome-based formulations are commercially available or are currently undergoing clinical trials (14, 15, 98-100). Therefore, liposome will be selected as our delivery system to treat ascites cells and xenograft tumors. Our proposed delivery system contains Doxorubicin (DOX) or Cisplatin (CIS) as anticancer drugs, a synthetic analog of Luteinizing Hormone-Releasing Hormone (LHRH) as tumor targeting moiety, Antisense Oligonucleotides (ASO) targeted to MDR1 and BCL2 mRNA as suppressors of pump and nonpump resistance.
4 SURFACE MODIFIED AND INTERNALLY CATIONIC POLY(AMIDOAMINE) DENDRIMERS FOR EFFICIENT SIRNA DELIVERY

4.1 Introduction

Short or small interfering RNA (siRNA), is a class of short double stranded RNA molecules composed of 20-25 nucleic acids. siRNA is involved in the RNA interference (RNAi) pathway to inhibit gene expression of a specific gene (24, 101, 102). In addition, siRNA also acts in RNAi-related pathways, e.g. as an antiviral mechanism or in shaping the chromatin structure of a genome. Despite several potential therapeutic applications, the efficiency of siRNA in vivo is limited by its low resistance against enzymatic degradation, limited permeability across cell membrane, and substantial liver and renal clearance (84). Therefore, in order to exploit potential therapeutic applications of siRNA, the effective delivery of siRNA to the site of action and into targeted cells is required. While considerable efforts have recently been made to develop new siRNA carriers, the research on delivery of siRNA is still in a preliminary stage (85-91).

One of the most common methods used for the delivery of charged nucleic acids involves their electrostatic interaction with cationic carriers. Several types of cationic carriers such as liposome/lipids, dendrimers, and polymeric amines have been successfully used for
the delivery of plasmid DNA and oligonucleotides (41, 103). Even though siRNA is structurally similar to plasmid DNA with negatively charged anionic phosphodiester backbones, electrostatic interaction of siRNA largely differs from that of plasmid DNA (39). The molecular weight and molecular topography of siRNA and plasmid DNA play an important role in their electrostatic interaction with cationic agents. Nonviral vectors, and especially polymers, form looser complexes with siRNA than with plasmid DNA and incomplete encapsulation of the nucleic acid leads to the exposure of siRNA to enzymatic or physical degradation prior to delivery to the targeted cells. Consequently, carriers that are successfully used for the delivery of plasmid DNA cannot be efficiently employed for the delivery of siRNA, and a different approach is required.

Polycationic dendrimers such as poly(amidoamine) (PAMAM) dendrimers have been extensively studied as efficient vehicles for the delivery of genes and therapeutic drugs (10-12). Dendrimers are a type of regular and highly branched, monodisperse, spherical nanomaterials with dense peripheral groups that can be functionalized with drugs, targeting moieties, and other biologically active components (4, 27). Biodistribution studies showed that the higher molecular mass and/or more branched dendrimers exhibited longer circulation half-life due to slower excretion into the urine when compared with lower molecular mass carriers, less branched dendrimers, or linear polymers (38). Dendrimers such as poly(amidoamine) and poly(propylenimine) possess cationic primary amine groups at the surface, which participate in the DNA binding
process and increase the cellular uptake of DNA by transforming the entire complex into nanoscale polyplexes (10, 12, 39, 41, 103). However, these highly efficient delivery systems have been less explored for siRNA delivery. It has been shown that PAMAM dendrimers or their conjugates are more efficient in delivering antisense oligonucleotides or plasmid DNA than siRNA (33).

Lee et al have demonstrated synthesis of internally quaternized PAMAM-OH dendrimers and their efficiency in the delivery of plasmid DNA (34, 104). Although the internally charged PAMAM dendrimers formed stable polyplexes with plasmid DNA, their transfection ability was far lower than polyplexes of the poly(ethyleneimine) or PAMAM dendrimer. It is generally believed that the neutral surface of a dendrimer results in low transfection efficiency. In contrast, cationic dendrimers with positive surface charge interact with negatively charged cell membranes and permeate more easily than the neutral or anionic dendrimers. However, such a positive surface charge renders cytotoxicity to the cationic dendrimers, which limits their clinical applications. Recently, it has been shown that modification of surface amine groups of PAMAM dendrimer reduced their cytotoxicity (104, 105).

Herein, we describe synthesis of surface modified and an internally quaternized PAMAM dendrimer that possesses low cytotoxicity and at the same time offers internal positive charges for electrostatic interaction with negatively charged siRNA. A novel
QPAMAM-NHAc dendrimer in which surface amine groups were modified with acetyl group and internal tertiary nitrogens were quaternized was prepared and evaluated for siRNA delivery.

4.2 Materials and methods

4.2.1 Materials

Generation four PAMAM-NH₂ (Mw ~ 14214 Da, 64 amine end groups), generation four PAMAM-OH dendrimer (Mw ~ 14277 Da, 64 hydroxyl end groups), and acetic anhydride and methyl iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO). Spectra/Pore dialysis membrane with the molecular weight cutoff of 2000 Da was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Fluorescent RNA duplex - siRNA labeled with Pierce NuLight™ DY-547 fluorophores (siGLO Red Transfection Indicator, red fluorescence) was obtained from Applied Biosystems (Ambion, Inc., Foster City, CA). All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ).

4.2.2 Cell line

The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton
(Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Fisher Scientific, 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.

4.2.3 Synthesis of internally quaternized QPAMAM-OH dendrimer (Fig. 4.1A)

A slightly modified previously described procedure was used for synthesis of QPAMAM-OH dendrimer (34). Briefly, PAMAM-OH generation four (172 mg, 0.012 mmol) was dissolved in N, N'-dimethyl formamide (DMF, 1 mL) and excess methyl iodide (MeI, 0.5 mL) was added. The reaction mixture was sealed and stirred at 50 °C for 48 h. The reaction mixture was then precipitated into diethyl ether to obtain solid, which was dried under a vacuum and redissolved in water (1 mL). The resulting solution was dialyzed against 2 M NaCl and deionizer water successively using dialysis membrane (molecular mass cut off 2000 Da) and then lyophilized to afford white solid.

4.2.4 Synthesis of acetylated PAMAM-NHAc dendrimer (Fig. 4.1B)

Triethylamine (0.17 mL, 1.2 mmol) was added to a stirred solution of PAMAM-NH₂ generation four (172 mg, 0.012 mmol) dissolved in anhydrous methanol (10 mL) followed by the addition of excess acetic anhydride (0.1 mL, 0.96 mmol). The resulting
mixture was stirred at room temperature for 24 h. Methanol was evaporated under reduced pressure and the resulting residue was dissolved in water (2 mL). Further purification by extensive dialysis against deionizer water using dialysis membrane (molecular mass cut off 2000 Da) and freeze-drying afforded acetylated PAMAM dendrimer.

4.2.5 Synthesis of acetylated and internally quaternized QPAMAM-NHAc dendrimer

PAMAM-NHAc (100 mg, 0.0059 mmol) was dissolved in DMF (1 mL) and excess methyl iodide (0.5 mL) was added. The reaction mixture was sealed and stirred at 50 °C for 48 h. The reaction mixture was then precipitated into diethyl ether to obtain solid, which was dried under a vacuum and redissolved in water (1 mL). The resulting solution was dialyzed against 2 M NaCl and deionizer water successively using dialysis membrane (molecular mass cut off 2000 Da) and then lyophilized to afford white solid.

4.2.6 Proton nuclear magnetic resonance spectroscopy (¹H NMR)

¹H NMR was performed on a Varian VNMRS 400 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA). The chemical shift was expressed as parts per million (ppm) and a solvent peak was used as reference (D2O, 4.8 ppm). The following abbreviations are used
in the results section to identify multiplicities: s, singlet; m, multiplet; br, broad.

4.2.7 Atomic force microscopy

The samples of siRNA/dendrimer condensates were imaged with a tapping mode atomic force microscope (Nanoscope III A, Veeco Digital Instruments, Chadds Ford, PA). During imaging, 125 μm long rectangular silicon cantilever/tip assembly was used with a spring constant of 40 N/m, resonance frequency of 315-352 kHz and a tip radius of 5-10 nm. The images were generated by the change in amplitude of the free oscillation of the cantilever as it interacts with the sample. The height differences on the surface are indicated by the color code: lighter regions indicate higher heights. In order to image siRNA condensates, 5 μL of dendrimer/siRNA solutions were deposited on freshly cleaved mica. After 3-5 minutes of incubation, the mica surface was rinsed with 3 drops of deionized water 4 times and dried under a flow of nitrogen.

4.2.8 Gel electrophoresis

The complexes of dendrimers (PAMAM-NH₂, QPAMAM-OH and QPAMAM-NHAc) and siRNA were prepared in water at N/P (Nitrogen to Phosphate, which reflects positive to negative charge ratio) charge ratios ranging from 0-1.5 and incubated at room temperature for 30 min. The charge ratio was calculated by relating the number of
positive charges on dendrimer (primary amine groups of PAMAM-NH$_2$ and quaternary amine groups of QPAMAM-OH and QPAMAM-NHAc) with the number of negatively charged phosphate groups of siRNA. Dendrimer-free siRNA was used as the control. Double-stranded RNA ladder (New England Biolabs) with the smallest base pairs at 21 was used as a size reference. The samples were further diluted with DPBS buffer and electrophoresed with ethidium bromide in 4% agarose gel at 100 V for 50 min in Tris-Borate-EDTA buffer. siRNA bands on the gel were visualized under ultraviolet light.

4.2.9 Dynamic Light Scattering (DLS) analysis

Three types of complexes with N/P charge ratios of 1, 1.5 and 3 were prepared from dendrimers (PAMAM-NH$_2$, QPAMAM-OH and QPAMAM-NHAc) and siRNA in water. The resulting complexes were incubated for 30 min and the size was determined using the DynePro-MS800 dynamic light scattering/molecular sizing instrument with argon laser wavelength $\lambda=830$ nm, a detector angle 90°, and typical sample volume of 20 $\mu$L. Each light scattering experiment consisted of 20 or more independent readings, each 10 s in duration. Data analysis was conducted using DynaPro Instrument Control Software for molecular Research DYNAMICS (version 5.26.60). The obtained DLS data represents the average of three runs.
4.2.10 Cellular internalization of siRNA and dendrimer/siRNA complex

To analyze cellular internalization and intracellular localization of siRNA, fluorophore labeled siRNA (siGLO Red, red fluorescence) either free or complexed with dendrimer (N/P = 3) was incubated 75 min with living A2780 human ovarian cancer cells. Fluorescence and its distribution within the cell were examined every minute using a confocal microscope. Fluorescent images were digitally scanned and fluorescence inside cells (that reflects cellular accumulation of labeled siRNA) was expressed in arbitrary units.

4.2.11 In vitro cytotoxicity

A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cytotoxicity of three dendrimers as previously described (106). To measure cytotoxicity, cells were separately incubated in a microtiter plate with different concentrations of PAMAM-NH$_2$, QPAMAM-OH and QPAMAM-NHAc dendrimers. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. On the basis of these measurements, cellular viability was calculated for each dendrimer concentration. A decrease in the cellular viability indicated an increase in dendrimer toxicity.
4.2.12 Statistical analysis

Data obtained was analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value ± standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student’s test and considered $P$ value of less than 0.05 as statistical significant.

4.3 Results

4.3.1 Synthesis and characterization of internally quaternized PAMAM dendrimers (QPAMAM-OH)

The internal quaternization of PAMAM-OH dendrimer was carried out according to a previously described procedure (34) with a slight modification (Fig. 4.1A). Excess methyl iodide was added to ensure complete quaternization of internal tertiary amine groups. Analysis of dendrimer by $^1$H NMR (400 MHz, D$_2$O) revealed the following major peaks (Fig. 4.2): $\delta$ 2.83-2.98 (br m, CH$_2$CONH), 3.16 (br s, N$^+$CH$_3$), 3.32-3.38 (br m, CH$_2$CH$_2$OH), 3.46-3.57 (br m, CONHCH$_2$CH$_2$N$^+$), 3.62-3.78 (br m, N$^+$CH$_2$CH$_2$CONH and CH$_2$OH). The degree of quaternization was confirmed by $^1$H-NMR spectroscopy. Proton peaks arising from the (-CH$_2$CH$_2$N-) methylene group adjacent to tertiary nitrogen were completely shifted, suggesting almost complete
quaternization. Furthermore, the exact degree of quaternization (97%) was determined by comparing the integrated peak area of newly introduced methyl group (N<sup>+</sup>-CH<sub>3</sub>) at δ 3.16 to that of the unmodified methylene protons (–CH<sub>2</sub>CH<sub>2</sub>OH) at δ 3.32-3.38.

4.3.2 Synthesis and characterization of surface modified and internally quaternized PAMAM dendrimers (QPAMAM-NHAc)

The surface modified and internally quaternized dendrimer QPAMAM-NHAc was synthesized in two steps (Fig. 4.1B). During the first step, the primary amine groups of PAMAM-NH<sub>2</sub> dendrimer were modified with acetyl group to yield fully acylated PAMAM-NHAc (34). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) spectral data for this dendrimer are (Fig. 4.2): δ 1.98 (s, COCH<sub>3</sub>), 2.40-2.50 (br m, CH<sub>2</sub>CONH), 2.62-2.73 (br m, CONHCH<sub>2</sub>CH<sub>2</sub>N), 2.82-2.92 (br m, NCH<sub>2</sub>CH<sub>2</sub>CONH), 3.26-3.37 (m, CONHCH<sub>2</sub> and CH<sub>2</sub>NHCOCH<sub>3</sub>). The degree of acetylation was confirmed by <sup>1</sup>H-NMR spectra by relating the integrated peak area of signal appeared at δ 1.98 ppm (-NHCOCH<sub>3</sub>) to that of methylene protons of PAMAM dendrimer (δ 2.40-3.37). During the second step, internal tertiary amine groups of the surface modified PAMAM-NHAc dendrimer were quaternized by treatment with excess methyl iodide in N,N'-dimethylformamide at 50 °C. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) spectral data for this dendrimer are (Fig. 4.2): δ 2.00 (s, COCH<sub>3</sub>), 2.81-2.99 (br m, CH<sub>2</sub>CONH), 3.16 (br s, N<sup>+</sup>CH<sub>3</sub>), 3.29-3.38 (br m, CONHCH<sub>2</sub> and CH<sub>2</sub>NHCOCH<sub>3</sub>), 3.47-3.58 (br m, CONHCH<sub>2</sub>CH<sub>2</sub>N<sup>+</sup>), 3.66-3.81 (br m,
N<sup>+</sup>CH<sub>2</sub>CH<sub>2</sub>CONH). The degree of quaternization was estimated using <sup>1</sup>H-NMR spectroscopy by comparing the integrated peak area of newly introduced methyl group (N<sup>+</sup>-CH₃) at δ 3.16 to that of the unmodified methylene protons (CONHCH<sub>2</sub> and CH<sub>2</sub>NHCOCH<sub>3</sub>) at δ 3.29-3.38.

4.3.3 Analysis of dendrimer/siRNA complex formation by agarose gel electrophoresis

Three types of dendrimers PAMAM-NH₂, QPAMAM-OH and QPAMAM-NHAc, were mixed with siRNA in water at various N/P charge ratios and were subjected to electrophoresis in agarose gel (Fig. 4.3a). The complex formation was observed for all three dendrimers as evidenced by oligonucleotide bands disappearance from agarose gels. Interestingly, QPAMAM-OH and QPAMAM-NHAc dendrimers displayed a comparable ability to form complexes with siRNA and no significant difference was observed in their band intensities.

4.3.4 Analysis of particle size of dendrimer/siRNA complexes

The particle size of each of the dendrimer/siRNA complexes was determined by dynamic light scattering. Measured size of dendrimer/siRNA complexes at charge ratio ranging from 1 to 3 rel. units are shown in Fig. 4.3b. The complexes formed using PAMAM-NH₂
and QPAMAM-OH dendrimers exhibited a decrease in particle size as the charge ratio was increased. These results are in agreement with previous studies by Lee et al on dendrimer/pDNA polyplexes, where the particle size decreased systematically as the charge ratio was increased (34). The complexes formed with QPAMAM-NHAc dendrimer showed an initial decrease in average particle size from 165 nm to 72 nm as N/P ratio increased from 1 to 1.5 rel. units. However, the particle size did not decrease further with the increase in the N/P ratio to 3 rel. units.

4.3.5 Atomic force microscopy (AFM)

The size and morphology of all three dendrimer/siRNA complexes at N/P ratio 3 rel. units were also determined by atomic force microscopy (Fig. 4.4). It was found that well-condensed spherical particles were formed with internally quaternized QPAMAM-OH and QPAMAM-NHAc dendrimers, while the PAMAM-NH₄ dendrimer resulted in nano-fiber shaped structures. The particle size distribution was consistent with the dynamic light scattering data.

4.3.6 In vitro cytotoxicity

The cytotoxicity evaluation of each dendrimer at various concentrations revealed a relationship between dendrimer surface charge and cellular cytotoxicity (Fig. 4.5). An
increase in surface charge led to the increase in cytotoxicity of the dendrimer. The PAMAM-NH₂ dendrimer showed highest toxicity, while significant decrease in toxicity was observed for QPAMAM-OH dendrimer. Notably, among all three dendrimers, QPAMAM-NHAc was the least toxic even at higher concentrations.

4.3.7 Cellular uptake of siRNA

The cellular uptake of fluorophore labeled siRNA (siGLO Red, red fluorescence) either naked or complexed with different dendrimers (N/P = 3) was analyzed using confocal fluorescent microscopy. In this series, A2780 human ovarian cancer cells were separately incubated 80 min with naked siRNA or dendrimer-siRNA complexes and fluorescence distribution within the cell was examined every minute using a confocal microscope. The naked siRNA without any complexation with cationic dendrimers failed to internalize cells, which was evident by absence of measurable red fluorescence in the cell cytoplasm and nuclei (Fig. 4.6). Similarly, PAMAM-NH₂/siRNA and QPAMAM-OH/siRNA complexes did not penetrate into cellular cytoplasm within the incubation time. In contrast, the QPAMAM-NHAc-siRNA complex was internalized by the cells and distributed uniformly inside the cytoplasm and nuclei by the end of the incubation period.
4.4 Discussion

Given the ability to knock down any gene of interest, siRNAs has generated a considerable interest as a novel and very effective tool for basic research and applied medicine. However, poor cell penetration ability and facile enzymatic degradation of siRNA, limits its effective use (39). The presence of a hydroxyl group at 2’ position of the pentose ring in the RNA favors hydrolysis of phosphodiester backbone of nucleic acid by serum nucleases (107). Consequently, the development of efficient delivery systems which will protect siRNA from degradation during the voyage in the bloodstream and organs and facilitate its uptake by the targeted cells is one of the major challenges in the therapeutic application of siRNA.

Similar to the delivery of plasmid DNA, electrostatic interaction of siRNA with cationic carriers is expected to enhance cellular uptake and protect it from enzymatic degradation. Cationic liposomes and dendrimers are considered as two of the most promising nanocarriers for the effective delivery of plasmid DNA, antisense oligonucleotides, and siRNA. Cationic liposomes interact with siRNA leading to a relatively large size of complexes due to uncontrolled and incomplete encapsulation of nucleic acid thereby exposing siRNA to enzymatic degradation prior to delivery to cell (108). Dendrimers have been extensively studied for the efficient delivery of plasmid DNA and antisense oligonucleotides. However, they have been less explored for the delivery of siRNA.
Moreover, the relatively high cytotoxicity of cationic PAMAM-NH₂ dendrimer due to non-specific interaction of primary amine groups with cell membrane is the main concern for their potential applications as nanocarriers.

In the present study, two different approaches have been used to reduce the cytotoxicity of PAMAM dendrimers. First, an internally quaternized PAMAM dendrimer (QPAMAM-OH) that forms less toxic complexes with negatively charged plasmid DNA (34) was prepared and tested as a nanocarrier to deliver siRNA. Secondly, the surface primary amine groups of the PAMAM-NH₂ dendrimer were modified by acetylation followed by internal quaternization resulting in novel internally quaternized QPAMAM-NHAc dendrimer. While most traditional cationic nanocarriers have an electrical charge on their surface, both of the developed dendrimers possess neutral outer surfaces with internal positive charges. Therefore, one can hypothesize that siRNA could be internalized (at least partially) inside the dendrimer. This hypothesis is based on a report by Lee et al. (34) that large plasmid DNA can be incorporated in the inner space of dendrimers. Since siRNA are smaller than DNA, we suggest that it can easily penetrate between dendrimer branches. It is possible that siRNA was partially accommodated inside the dendrimer branches and the remaining part of siRNA was internalized in another molecule of dendrimer. Thus, at N/P ratio 1, an interpolyelectrolyte complex would be formed which is supported by the fact that siRNA/dendrimer complex at N/P charge ratio 1 showed a larger diameter. On the other
hand, at higher N/P charge ratio, several dendrimer molecules were available to accommodate siRNA in parts and thus exhibited a decrease in diameter. These results might suggest that siRNA is partially internalized in dendrimer branches and is covered by several dendrimers thus limiting the exposure of siRNA to nucleases. Therefore, siRNA is likely to be shielded by the dendrimer surface which in turn limited the exposure of siRNA to a hazardous environment. This could potentially improve the stability of the entire system during its journey towards the site of action. Present experimental data confirmed that acetylated quaternized dendrimers containing siRNA are taken up to a higher extent than other dendrimers or siRNA alone.

Agarose gel retardation studies clearly indicated that both synthesized QPAMAM-OH and QPAMAM-NHAc dendrimers, as well as commercially available PAMAM-NH2 dendrimer interacted with siRNA irrespective of internal or surface positive charges. As anticipated, surface modification of PAMAM-NH2 dendrimers significantly reduced their cytotoxicity. It was found that the QPAMAM-NHAc dendrimer showed the least toxicity amongst all three tested nanocarriers. These results are consistent with previously published data (105) and confirm that prevention of non-specific nanocarrier-cell interactions caused by surface amine group decreases the cytotoxicity of PAMAM dendrimer. Furthermore, dynamic light scattering studies revealed condensation of siRNA into particles of nanometer dimensions when complexed with the dendrimers. The particle size dramatically decreased with an increase in N/P, which is in good agreement
with the assumption that the dendrimer acts as a cross linker, and the particles size increases if the complexation is not complete. It is interesting that hydroxyl terminated dendrimers have lower average diameter at 1.5 and 3 N/P ratio. These results are consistent with observations made by Lee et al. (34). It is believed that at N/P ratio 1 dendrimer acts as a cross linker leading to a large size interpolyelectrolyte complex. Such a mechanism is supported by the fact that siRNA/dendrimer complex at N/P charge ratio 1 showed larger diameter. On the other hand, at higher N/P charge ratio, a higher number of dendrimers are available to form complexes with siRNA. Therefore, the net number of dendrimer/siRNA would be considerably reduced in the complex, which in turn decreased the diameter of the particles. The reason for slight increase in particle size of QPAMAM-NHAc/siRNA complex at charge ratio 3 rel. units is unknown. However, all dendrimer/siRNA complexes were small enough to be internalized by cells via an adsorptive nonspecific endocytosis pathway (109).

The particle size distribution of synthesized nanocarriers was further supported by atomic force microscope experiments, which also revealed very interesting morphological features of studied dendrimer/siRNA complexes at charge N/P ratio of 3.0 rel. units. The internally quaternized and surface neutral dendrimers QPAMAM-OH and QPAMAM-NHAc formed complexes with siRNA resulting into a more compact spherical nanometer dimension particles. In contrast, the PAMAM-NH₂ dendrimer possessing cationic amino groups at surface formed ribbon-like nanofibers, probably
originated from the interpolyelectrolyte complexation with siRNA. Previous studies on understanding the condensation mechanism of DNA and dendrimers proposed a model that involves binding of one dendrimer molecule to two or more DNA chains (110). Apparently, such complexation leads to formation of nanofiber shaped structures (111), however, the dendrimer generation and charge ratio could also contribute in determining the morphology of the complexes. Based on these observations, we believe that internal positive charges in QPAMAM-OH or QPAMAM-NHAc dendrimer plays a significant role in controlling the morphology of the complexes by shielding siRNA with dendrimer surface. Apparently, at high N/P ratio, siRNA could be encapsulated by several QPAMAM-OH or QPAMAM-NHAc dendrimers to produce compact and spherical particles. On the other hand, surface charged PAMAM-NH$_2$ probably are involved in linking two or more siRNA molecules to form higher molecular weight polymer chains.

In addition to enzymatic degradation, low cellular uptake of naked siRNA is one of the serious problems that limit their potential applications. Since siRNAs are negatively charged in physiological range of pH, the cellular internalization of naked siRNA through a negatively charged cell membrane is considerably low. In fact, our confocal microscope studies demonstrated that naked siRNA are not internalized by cells. Similar to plasmid DNA, complexation of siRNA with cationic agents such as polymers, liposomes, or dendrimers increases cell permeability. Alternatively, the backbone of all bases in oligonucleotides (DNA and RNA) can potentially be modified to neutralize their
electric charge, enhance nuclease resistance, and increase the incorporation efficacy into liposomes or other neutral nanocarriers (112-114). Previously, we successfully used both approaches utilizing neutral and cationic liposomes for effective delivery of P-ethoxy antisense oligonucleotides and siRNA respectively (52, 115-117). In the present study, we exploited complexation of siRNA to the cationic outer surface or inner core of dendrimers in order to facilitate the uptake of siRNA by cells. However, our data showed that the complexation of siRNA with surface charged PAMAM-NH₂ and internally charged QPAMAM-OH dendrimers could not improve the siRNA cell permeability. Although the exact reason for inefficiency of these types of dendrimers is unknown, we believe that size and morphology of siRNA/PAMAM-NH₂ complex and neutral surface of QPAMAM-OH dendrimers contributed to the registered low cellular uptake. In contrast, the surface modified and internally quaternized QPAMAM-NHAc showed excellent cell permeability resulting in a substantial accumulation of siRNA in cells within 80 min. These results are in agreement with the observations made by Kolhatkar and co-authors that surface acetylated PAMAM dendrimers showed good permeability across the cell membrane (105). The authors suggested a paracellular transport route for increased permeability of acetylated dendrimers due to reduced nonspecific interaction of dendrimers with cell membrane leading to an increase in concentration of dendrimers across the apical side of the membrane. It is possible that a similar mechanism was responsible for a higher uptake of acetylated dendrimers. Cellular uptake is a complex mechanism and involves several important factors that include concentration of
penetrating material across cell membrane, surface charge, and hydrogen bonding with cell membrane. Increased permeability of surface modified dendrimers due to reduced nonspecific interaction of dendrimers with cell membranes leading to an increase in concentration of dendrimers across the apical side of the membranes was suggested (105). Alternatively, amide groups on the surface of dendrimer may also offer hydrogen bonding with the cell membranes to enhance the cellular uptake of the complex. Wender et al. demonstrated the importance of hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells (118). It was found that charge is necessary, but not sufficient for cellular uptake and hydrogen bonds with H-bond acceptor functionality on the cell surface increased uptake significantly. It is possible that these mechanisms could also be responsible at least in part for higher penetration ability of QPAMAM-NHAc/siRNA complex. However, further studies are required to elucidate the exact mechanism for cell permeability of acetylated and internally quaternized QPAMAM-NHAc dendrimer and its complex with siRNA.

4.5 Conclusions

In summary, we have demonstrated surface modified and internally quaternized PAMAM dendrimers as novel siRNA delivery systems. Three types of dendrimers were examined for their cytotoxicity and siRNA delivery in A2780 ovarian cancer cells. The modification of surface amine groups of PAMAM dendrimers to amide and internal
quaternization proved to play a critical role for the reduced cytotoxicity and efficient cell permeability of the dendrimer-siRNA complex.
**Figure 4.1** Synthesis of dendrimers. a, PAMAM-OH dendrimer was internally quaternized to form QPAMAM-OH dendrimer with inner cationic charges. b, PAMAM-NH$_2$ dendrimer was acetylated to form PAMAM-NHAc dendrimer. Surface acetylated PAMAM-NHAc dendrimer was internally quaternized to form QPAMAM-NHAc dendrimer possessing internal cationic charges.
Figure 4.2 Typical proton nuclear magnetic resonance (1H NMR) spectra of different dendrimers.
Figure 4.3 Influence of negative to positive charge ratios on dendrimer-siRNA complex formation and particle size. a, Typical agarose gel electrophoresis images of the siRNA-dendrimer complexes at different N/P (Nitrogen to Phosphate, which reflects positive to negative charge ratio) charge ratios. The dendrimer-siRNA complex formation led to the disappearance of siRNA bands from agarose gels. b, Average particle size of different siRNA-dendrimer complexes determined by dynamic light scattering. Means ± SD are shown. *P < 0.05 when compared with N/P ratio equal 1.
Figure 4.4 Typical atomic force microscope images of the condensates formed by siRNA-in the presence of different dendrimers. The images were made in a tapping mode. The height differences on the surface are indicated by the color code: lighter regions indicate higher heights.
Figure 4.5 Cytotoxicity of dendrimers. Cellular viability was evaluated by the modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Means ± SD are shown.
**Figure 4.6** Cellular internalization of free siRNA and siRNA delivered by different dendrimers. Typical confocal microscopy images of living A2780 cells incubated with fluorophore labeled siRNA (siGLO Red, red fluorescence) and dendrimer-siRNA complexes and time course of fluorescence intensity of siGLO accumulated inside cells. Means ± SD are shown.
5 INTERNALLY CATIONIC POLY(AMIDOAMINE) PAMAM-OH DENDRIMERS FOR SIRNA DELIVERY: EFFECT OF THE DEGREE OF QUATERNIZATION AND CANCER TARGETING

5.1 Introduction

RNA interference is a natural process of sequence-specific, posttranscriptional gene silencing mediated by short double stranded RNA (119). Given the ability to interfere with the disease-causing proteins at an early stage of gene expression, short interfering RNAs (siRNAs) have generated considerable attention as potential therapeutic agents for the treatment of cancer and other related diseases (24, 25, 102, 120). However, low resistance against enzymatic degradation, limited permeability across cell membranes, and substantial liver and renal clearance has restricted therapeutic applications of siRNA in vivo (84). Consequently, the development of efficient delivery systems which will protect siRNA from the degradation during the voyage in the bloodstream and organs and facilitate its uptake by the targeted cells is one of the major challenges in the therapeutic application of siRNA. Recently, we reported novel, surface neutral, and internally cationic poly(amidoamine) (QPAMAM) generation four dendrimers for the efficient intracellular delivery of siRNA, including surface acetylated QPAMAM-NHAc and hydroxyl-terminated QPAMAM-OH dendrimers (121). These dendrimers as nanocarriers possess the following advantages: (1) neutral surface of the dendrimer for low
cytotoxicity; (2) existence of cationic charges inside the dendrimer (not on the outer surface) resulting in highly organized compact nanoparticles, which can potentially protect nucleic acids from degradation. Noteworthily, surface modified QPAMAM-NHAc dendrimer demonstrated enhanced cellular uptake of siRNA when compared with the internally cationic QPAMAM-OH dendrimer (degree of quaternization 97%). In the present study, to improve the siRNA delivery using the QPAMAM-OH dendrimer, we investigated two independent strategies: 1) degree of quaternization for proton sponge or buffering effect; and 2) targeting ligand as a penetration enhancer. Polyethyleneimine (PEI) and poly(amidoamine) (PAMAM) dendrimers, exhibit high transfection efficiency due to buffering or the so-called proton sponge effect resulting from low pKa of tertiary amines (122, 123). It was hypothesized that substitution of tertiary amines in poly(amidoamine) (PAMAM-OH) dendrimers with permanently charged quaternary amines may perhaps obstruct the transfection efficiency of siRNA (124). We anticipated improved transfection efficiency by decreasing the degree of quaternization that would enable tertiary amines for buffering effect and quaternary amines for the complexation with siRNA.

Besides the proton sponge effect, conjugation of cell penetrating peptides to the macromolecular cargo represents another attractive method that is known to facilitate delivery of genetic materials across cell membranes with high efficiency (125-127). In particular, cell penetrating peptides greatly improved internalization and effectiveness of
antisense oligonucleotides into the mammalian cells (128, 129). Cell penetrating peptides are positively charged short peptides that improve cellular uptake of different payloads including negatively charged chunks of DNA/RNA and their neutral complexes with macromolecules. While cell penetrating peptides possess distinct advantages in antisense oligonucleotides delivery, they were substantially less effective in enhancing the delivery of siRNA and siRNA-nanocarrier complexes (33, 130, 131). Additionally, cell penetrating peptides enhance cellular uptake by virtually all cells in the body, not only by targeted cells, e.g. cancer cells in case of cancer treatment. Therefore, other penetration enhancers that are specific for targeted cells are required (132).

Previously, we have successfully used a synthetic analog of Luteinizing Hormone-Releasing Hormone (LHRH peptide) for the effective targeting of anticancer drugs, therapeutic peptides, and different complex delivery systems to cancer cells in vitro and in vivo (93, 95-97, 133). LHRH peptide is a targeting ligand to LHRH receptors that are over-expressed in the plasma membrane of several types of cancer cells and are not expressed detectably in normal visceral organs. Our previous findings in ovarian, breast, and prostate cancer cells provided the rationale of using LHRH peptide as a targeting moiety/penetration enhancer to target different drug delivery systems to tumors and facilitate their uptake by cancer cells. Resulting from this success, an introduction of a targeting LHRH peptide to the QPAMAM-OH dendrimer was envisaged to enhance the intracellular delivery of siRNA by a receptor mediated endocytosis pathway. The present
study is aimed at designing, synthesizing, and validating different cancer-targeted QPAMAM-OH dendrimers as nanocarriers for the enhanced intracellular delivery of siRNA to cancer cells. The impact of two independent factors (degree of quaternization and LHRH targeting ligand) is also examined.

5.2 Materials and methods

5.2.1 Materials

Generation four PAMAM-OH dendrimer (Mw ~ 14277 Da, 64 hydroxyl end groups, 1,2-diaminoethane dendrimer core), N,N’-dimethylformamide, succinic anhydride, 4-(methylamino)pyridine and methyl iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fluorescein isothiocyanate (FITC) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC·HCl) were obtained from Fluka (Allentown, PA). Synthetic analog of LHRH, Lys6-des-Gly10-Pro9-ethylamide (Gln-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et), having a reactive amino group only on the side chain of the lysine at position 6 was synthesized according to our design by American Peptide Company, Inc. (Sunnyvale, CA). Spectra/Pore dialysis membranes with the molecular weight cutoff of 2000 and 500 Da were obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Fluorescent RNA duplex - siRNA labeled with Pierce NuLight™ DY-547 fluorophores
(siGLO Red Transfection Indicator, red fluorescence) was obtained from Applied Biosystems (Ambion, Inc., Foster City, CA). siRNA targeted to BCL2 mRNA was synthesized by Applied Biosystems (Ambion, Inc., Foster City, CA). The sequence of siRNA was 5'-GUGAAGUCAACAUGCCUGCTT-3'. All other chemicals were purchased from Fisher Scientific (Fairlawn, NJ).

5.2.2 Cell line

The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Fisher Scientific, 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.

5.2.3 Synthesis of internally quaternized QPAMAM-OH dendrimer

QPAMAM-OH dendrimer with 75% degree of quaternization was prepared using the previously described procedure (121). Briefly, PAMAM-OH generation four (172 mg, 0.012 mmol, Fig. 5.1) was dissolved in N,N’-dimethylformamide (DMF, 1 mL) and methyl iodide (MeI, 50 μL) was added. The reaction mixture was sealed and stirred at room temperature for 24 h. The reaction mixture was then precipitated into diethyl ether.
to obtain solid, which was dried under a vacuum and redissolved in water (1 mL). The resulting solution was dialyzed against 2 M NaCl and deionizer water successively using a dialysis membrane (molecular mass cut off 2000 Da) and then lyophilized to afford the QPAMAM-OH dendrimer (Fig. 5.1) as white solid. Internally quaternized PAMAM dendrimers with various degree quaternization were synthesized by using appropriate mole equivalents of methyl iodide. To enforce a higher degree of quaternization, excess methyl iodide was used and the reaction was conducted at an elevated temperature.

5.2.4 Synthesis of PAMAM-OH-LHRH conjugate

Succinic anhydride (5 mg, 0.05 mmol) was added to a stirred solution of LHRH peptide (50 mg, 0.036 mmol) in anhydrous pyridine (1 mL). The reaction mixture was stirred at room temperature for 24 h. Evaporation of solvents under reduced pressure and purification by extensive dialysis against deionizer water using dialysis membrane (molecular mass cut off 500 Da) followed by freeze-drying afforded LHRH-hemisuccinate as white solid. The obtained conjugate was directly subjected to further reaction with the PAMAM-OH dendrimer. Briefly, PAMAM-OH generation four (86 mg, 0.006 mmol, Fig. 5.1) and LHRH-hemisuccinate (17.2 mg, 0.012 mmol) were dissolved in anhydrous dichloromethane (5 mL) and anhydrous dimethyl sulfoxide (5 mL). EDC·HCl (2.5 mg, 0.013 mmol) was added to the above solution as a condensing agent and DMAP (1 mg) was used as a catalyst and the reaction mixture was allowed to
stir for 24 h. After evaporation of the solvent, the resulting conjugate was purified by extensive dialysis against deionizer water using dialysis membrane (molecular mass cut off 2000 Da) and passing through a Sephadex column and then lyophilized to afford PAMAM-OH-LHRH (Fig. 5.1) conjugate as pale yellow solid. One potential challenge of the design of our DDS is that the LHRH was conjugated to the dendrimer carrier via ester bond. Such a structure might be susceptible to esterases in circulation when applied in vivo. However our previous studies provided evidence that the conjugation of LHRH peptide through succinate ester to dendrimer or polymer did not affect biological efficiency in vivo (49, 97, 133). Moreover, preliminary data showed that delivered by targeting DDS biologically active products, including anticancer drug, antisense oligonucleotides or siRNA, are substantially less effective if non-biodegradable amide bond is used to conjugate LHRH peptide to the carrier.

5.2.5 Synthesis of internally quaternized QPAMAM-OH-LHRH conjugate

Methyl iodide (0.5 mL) was added to a stirred solution of PAMAM-OH-LHRH conjugate (50 mg, Fig. 5.1) dissolved in N,N’-dimethylformamide (1 mL). The reaction mixture was sealed and stirred at room temperature for 24 h. The reaction mixture was then precipitated into diethyl ether to obtain solid, which was dried under a vacuum and redissolved in water (1 mL). The resulting solution was dialyzed against 2 M NaCl and deionizer water successively using dialysis membrane (molecular mass cut off 2000 Da)
and then lyophilized to afford QPAMAM-OH-LHRH conjugate (Fig. 5.1).

5.2.6 Synthesis of fluorescein labeled internally quaternized QPAMAM-OH-FITC dendrimer

Fluorescein labeled PAMAM-OH dendrimer was prepared using our procedure that was previously reported (115). The resulting PAMAM-OH-FITC was then internally quaternized using the protocol described above.

5.2.7 Proton nuclear magnetic resonance spectroscopy ($^1$H NMR)

$^1$H NMR was performed on a Varian VNMRS 400 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA). The chemical shift was expressed as parts per million (ppm) and a solvent peak was used as reference ($D_2O$, 4.8 ppm). The following abbreviations are used in the results section to identify multiplicities: s, singlet; m, multiplet; br, broad.

5.2.8 Atomic force microscopy

The samples of siRNA-dendrimer condensates were visualized with a tapping mode atomic force microscope (Nanoscope III A, Veeco Digital Instruments, Chadds Ford, PA). During imaging, a 125 μm long rectangular silicon cantilever/tip assembly was used with
a spring constant of 40N/m, resonance frequency of 315-352 kHz and a tip radius of 5-10 nm. The images were generated by the change in amplitude of the free oscillation of the cantilever as it interacts with the sample. In order to image siRNA condensates, 5 μL of dendrimer-siRNA solutions were deposited on freshly cleaved mica. After 3-5 minutes of incubation, the mica surface was rinsed with 3 drops of deionized water 4 times and dried under a flow of nitrogen.

5.2.9 In vitro cytotoxicity

A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cytotoxicity of the three dendrimers. To measure cytotoxicity, cells were separately incubated in a microtiter plate with different concentrations of QPAMAM-OH and QPAMAM-OH-LHRH dendrimers. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. On the basis of these measurements, cellular viability was calculated for each dendrimer concentration as previously described (93, 115, 134). A decrease in the cellular viability indicated an increase in dendrimer toxicity.

5.2.10 Gel electrophoresis

The complexes of dendrimers (QPAMAM-OH and QPAMAM-OH-LHRH) and siRNA
were prepared in water at N/P (Nitrogen to Phosphate, which reflects positive to negative charge ratio) ratios ranging from 0 to 3 relative units and incubated at room temperature for 30 min. The charge ratio was calculated by relating the number of positive charges on a dendrimer (quaternary amine groups) with the number of negatively charged phosphate groups of siRNA. Dendrimer-free siRNA was used as the control. The samples were further diluted with DPBS buffer and subjected to agarose gel electrophoresis and staining with ethidium bromide in 4% agarose gel at 100 V for 50 min in Tris-Borate-EDTA buffer. siRNA bands on the gel were visualized under ultraviolet light and digitally photographed.

5.2.11 Dynamic Light Scattering (DLS) analysis and zeta potential

Three types of siRNA complexes with N/P charge ratios of 1, 1.5 and 3 were prepared from dendrimers (QPAMAM-OH and QPAMAM-OH-LHRH) and siRNA in water. The resulting complexes were incubated for 30 min and the size was determined using the DynePro-MS800 dynamic light scattering/molecular sizing instrument with argon laser wavelength $\lambda=830$ nm, a detector angle 90°, and typical sample volume of 20 $\mu$L. Each light scattering experiment consisted of 20 or more independent readings, each 10 s in duration. Data analysis was conducted using DynaPro Instrument Control Software for molecular Research DYNAMICS (version 5.26.60). The obtained DLS data represents an average of three runs. 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 5 times, and average values were calculated.

5.2.12 Cellular internalization

Two types of experiments were carried out to analyze cellular internalization and intracellular localization of siRNA. In the first experiment, fluorophore labeled siRNA (siGLO Red, red fluorescence) either free or complexed with dendrimers QPAMAM-OH and QPAMAM-OH-LHRH (N/P = 3) was incubated for 17 h with living A2780 human ovarian cancer cells. Fluorescence distribution within the cell from the top to the bottom cell surfaces (z-sections) was examined using a confocal microscope. In another series of experiments, cellular internalization of FITC-labeled dendrimer (green fluorescence) and its complex with fluorophore labeled siRNA (siGLO Red, red fluorescence) were studied in living A2780 human ovarian cancer cells by confocal microscopy. Cells were incubated with non-targeted and targeted dendrimer-siRNA conjugates within 100 min and images were taken with 25 min intervals. Superposition of green (dendrimer) and red (siRNA) fluorescence images allows for detecting co-localization of siRNA with the dendrimer resulting in yellow color.
5.2.13 Gene expression

siRNA targeted to the BCL2 mRNA was used to study the gene silencing efficacy of siRNA delivered by the dendrimers. In these experiments, the final siRNA concentration in the complexes was 1 μM. A2780 cells were incubated with dendrimer-siRNA complexes and appropriate controls within 24 h and total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) procedure was used for the analysis of gene expression as previously described (95). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) with 2 μg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences). After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using GenAmp PCR System 2400 (Perkin-Elmer, Shelton, CT). β2-microglobulin was used as an internal standard. The following pairs of primers were used: BCL2: 5′-GGA TTG TGG CCT TCT TTG AG-3′ (sense), 5′-CCA AAC TGA GCA GAG TCT TC-3′ (antisense); β2-microglobulin (β2-m) -ACC CCC ACT GAA AAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR regimen was as follows: 94°C for 5 minutes; 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 41 cycles; and 60°C for 10 minutes. PCR products were separated in 4% NuSieve 3:1 Reliant-agarose gels (Lonza, Basel, Switzerland) in 1x Tris-borate EDTA buffer (0.089 mol/L Tris-borate, 0.002 mol/L EDTA, pH 8.3; Research Organics Inc., Cleveland, OH) by submarine
electrophoresis. The gels were stained with ethidium bromide and digitally photographed.

5.2.14 Statistical analysis

Data obtained was analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value ± standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student’s test and considered P value of less than 0.05 as statistical significant.

5.3 Results

5.3.1 Synthesis and characterization of internally quaternized PAMAM dendrimers (QPAMAM-OH)

The internal quaternization of a PAMAM-OH dendrimer was carried out according to the described procedure (Fig. 5.1). Analysis of the dendrimer by $^1$H NMR (400 MHz, D$_2$O) revealed the following major peaks (Fig. 5.2, A): δ 2.46-2.56 (br m, COCH$_2$CH$_2$N), 2.66-2.74 (br m, CONHCH$_2$CH$_2$N) 2.83-2.98 (br m, COCH$_2$CH$_2$N$^+$), 3.16 (br s, N$^+$CH$_3$), 3.32-3.38 (br m, CH$_2$CH$_2$OH), 3.46-3.57 (br m, CONHCH$_2$CH$_2$N$^+$), 3.62-3.78 (br m, N$^+$CH$_2$CH$_2$CONH and CH$_2$OH). The degree of quaternization was confirmed by $^1$H NMR spectroscopy. Proton peaks arising from the (-CH$_2$CH$_2$N-) methylene group
adjacent to tertiary nitrogen indicated downfield chemical shift. Furthermore, the exact degree of quaternization (75%) was determined by comparing the integrated peak area of newly introduced methyl group (N$^+$-CH$_3$) at δ 3.16 to that of the unmodified methylene protons (–CH$_2$CH$_2$OH) at δ 3.32-3.38.

5.3.2 Synthesis of targeting ligand (LHRH peptide) conjugated and internally quaternized PAMAM dendrimer (QPAMAM-OH-LHRH)

The targeted and internally quaternized PAMAM dendrimer (QPAMAM-OH-LHRH) was synthesized in three simple steps (Fig. 5.1). During the first step, LHRH analog, Lys$^6$-des-Gly$^{10}$-Pro$^9$-ethylamide (Gln-His-Trp-Ser-Tyr-D-Lys[D-Cys]-Leu-Arg-Pro-NH$_2$-Et), having a reactive primary amine group only on the side chain of the lysine at position 6 was reacted with succinic anhydride to form a LHRH-hemisuccinate. In the second step, PAMAM-OH-LHRH conjugate was synthesized by reacting the PAMAM-OH dendrimer with LHRH-hemisuccinate using EDC·HCl as coupling agent. Finally, the internal quaternization of the PAMAM-OH-LHRH dendrimer was carried out according to the previously described procedure to yield QPAMAM-OH-LHRH conjugate. $^1$H NMR (400 MHz, D$_2$O) spectral data for this conjugate was similar to that of described for QPAMAM-OH and the exact degree of quaternization was determined to be 85%. Since it was difficult to detect LHRH peptide by proton NMR due to low concentration, a well
known BCA protein assay method was used. The presence and concentration of LHRH peptide in targeted dendrimers was detected by a colorimetric method using Pierce Bicinchoninic Acid (BCA) protein assay (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacturer recommendations. Typical UV-visible spectra of colored reaction product are presented in Fig. 5.2, B. The spectra of the product corresponding to free LHRH and all the conjugates containing LHRH have well defined absorbance maximum around 560 nm corresponding to the absorbance of the BCA/copper complex formed as a result of the reaction of BCA reagent with the cuprous cation produced from the reduction of Cu^{2+} to Cu^{1+} by the LHRH peptide. This maximum is absent in the assay spectra of the QPAMAM-OH dendrimer that does not contain LHRH. The average estimated concentration of LHRH peptide in working PAMAM-OH-LHRH and QPAMAM-OH-LHRH dendrimer solution (50 µM) was about 50 µg/ml (38 µM). Based on this value one can estimate that the targeted DDS contained an average one LHRH peptide per one dendrimer molecule.

5.3.3 Dynamic Light Scattering (DLS), zeta potential and Atomic force microscopy

The particle size of each dendrimer-siRNA complex was determined by dynamic light scattering at charge ratio ranging from 1 to 3 relative units as shown in Fig. 5.2, C. Similar to our previous studies, the complexes formed by using QPAMAM-OH and QPAMAM-OH-LHRH dendrimers exhibited a decrease in particle size as the charge ratio
was increased (121). Furthermore, atomic force microscopy at N/P ratio 3 relative units revealed formation of well-condensed spherical particles (Fig. 5.2, D). The average potential of dendrimer-siRNA complexes was 0.11±0.88 mV. Therefore, the dendrimer-siRNA complexes can be considered neutral.

5.3.4 In vitro cytotoxicity

In general the cytotoxicity of a dendrimer is greatly influenced by the surface charge of the dendrimer and cytotoxicity increases with an increase in surface charge. The analysis of cytotoxicity of each dendrimer at various concentrations by the MTT assay revealed that the surface neutral and internally charged dendrimers QPAMAM-OH and QPAMAM-OH-LHRH does not lead to the death of more than 5-10% of cells even at relatively high concentrations up to 12.5 µM.

5.3.5 Analysis of dendrimer/siRNA complex formation by agarose gel electrophoresis

Dendrimers QPAMAM-OH and QPAMAM-OH-LHRH, were mixed with siRNA in water at various N/P ratios and were subjected to electrophoresis in agarose gel (Fig. 5.2, E). The complex formation was observed for both the non-targeted QPAMAM-OH and targeted QPAMAM-OH-LHRH dendrimers as evidenced by oligonucleotide bands
disappearance from agarose gels. It should be stressed that at N/P ratios higher than 1 relative unit siRNA band completely disappeared.

5.3.6 Cellular uptake of siRNA

Two series of experiments were carried out to analyze cellular internalization and intracellular localization of free siRNA and siRNA-dendrimer complexes. In the first series, living A2780 cells were separately incubated with free and complexed with dendrimers fluorophore labeled siRNA (siGLO Red, red fluorescence) within 100 min. The dendrimers were labeled with FITC (green fluorescence). The fluorescence of each fluorophore (green and red) was registered with a confocal microscope every 25 min and digitally photographed. Green and red fluorescence images of a dendrimer and siRNA respectively were then digitally overlaid. The results of these experiments are shown in Fig. 5.3. The data obtained showed that naked siRNA failed to penetrate cancer cells. In contrast, both the dendrimer and dendrimer-siRNA complex were internalized by cancer cells and distributed uniformly in cellular cytoplasm and nucleus resulting in homogenous yellow color on overlaid pictures (Fig. 5.3, bottom panel). The comparison of cellular internalization of siRNA delivered by dendrimers with different degree of quaternization showed that dendrimers with lower degree of quaternization (20-30%) delivered siRNA more efficiently when compared with those with higher degree (70-85%) of quaternization (data not shown). One can speculate that a uniform distribution of
fluorescence on two-dimensional fluorescent images may not reflect a distribution of a fluorescence substance within the cell. Theoretically, a dendrimer and/or siRNA might stick to the surface of the cells and create the impression that these substances are internalized by the cells and distributed through the cytoplasm and nuclei. In order to reject such an assumption and to study the distribution of siRNA delivered by dendrimers in the third dimension (from the top to the bottom of the cell), a special king of measurement, so-called “z-sections”, were performed by a confocal microscope using cells incubated with dendrimers-siRNA complexes and washed out from the excess of unbound substances by free media. In this series, A2780 human ovarian cancer cells were incubated 17 h with dendrimer-siRNA complexes, washed out by fresh media and subjected to confocal microscopy. Data showed that siRNA delivered by both QPAMAM-OH and QPAMAM-OH-LHRH dendrimers were distributed from the top to the bottom of the cell (Fig. 5.4). A comparison of the left and right panels on Fig. 5.4 clearly shows that targeting the dendrimer to cancer cells by LHRH peptide substantially enhanced internalization of siRNA conjugated with the QPAMAM-OH-LHRH dendrimer leading to a considerably higher concentration of delivered siRNA inside cancer cells.

### 5.3.7 Gene expression

The gene knockdown efficiency of siRNA delivered by non-targeted QPAMAM-OH and targeted QPAMAM-OH-LHRH dendrimer was investigated using quantitative RT-PCR.
We selected BCL2 protein responsible for cellular antiapoptotic defense as a target for siRNA. The results of these experiments are shown in Fig. 5.5. It was found that siRNA delivered by all non-targeted QPAMAM-OH dendrimers with different degree of quaternization slightly, but statistically significantly, lowered the expression of the targeted gene (Fig. 5.5, bars 2-4). Targeted to cancer cells QPAMAM-OH-LHRH-siRNA complex led to a significant suppression of the expression of the BCL2 gene.

5.4 Discussion

In recent years, dendrimers have emerged as novel cationic nanocarriers for exogenous gene transfer into mammalian cells mainly due to their well-defined structure, nanometer size, and relatively easy structural modifications (136). Among them, different generations of poly(amidoamine) dendrimers have been successfully tested as promising nanocarriers for the delivery of plasmid DNA and antisense oligonucleotides that exhibited high transfection efficiency (137-139). However, PAMAM dendrimers offered only a limited success in the delivery of short oligonucleotides (siRNA) (33). Recently, we developed, designed, and evaluated a novel surface modified and internally cationic PAMAM dendrimer (QPAMAM-NHAc) with a high degree of quaternization (~90%) (121). This dendrimer efficiently delivered siRNA into cancer cells. In contrast, unmodified PAMAM-NH₂ as well as internally cationic, hydroxyl terminated and almost completely quaternized dendrimers (QPAMAM-OH) failed to internalize siRNA into the
ovarian cancer cells. Interestingly, both internally quaternized dendrimers QPAMAM-NHAc and QPAMAM-OH formed compact and spheroid nanoparticles with siRNA, while the PAMAM-NH\textsubscript{2} dendrimer exhibited nanofiber shaped structures. These results clearly indicated the importance of surface modification and internal quaternization for intracellular delivery of siRNA. We figured out that the size and morphology of the PAMAM-NH\textsubscript{2}-siRNA complex and neutral surface of the QPAMAM-OH dendrimer with a high degree of quaternization are the major obstacles for the cellular uptake and efficiency of siRNA.

The mechanism by which dendrimers efficiently deliver genes has been extensively investigated (137-139). A PAMAM dendrimer possess several internal tertiary amine groups, which are believed to play a critical role in the suppression of lowering the pH in endosomes and lysosomes. These tertiary amine groups are known to induce osmotic swelling of the endosome due to endosomal buffering that leads to rupture of endocytic vesicles and subsequent release of their payload. Such an effect of tertiary amine in a dendrimer or polymer is called a proton sponge effect. We envisioned that almost complete quaternization of tertiary amine groups in a QPAMAM-OH dendrimer could hamper the proton sponge effect and might be one of the reasons for the low efficacy of siRNA. Therefore, in order to increase the transfection efficiency of siRNA, a partially quaternized PAMAM dendrimer (QPAMAM-OH) with approximately 75\% of quaternization was synthesized and evaluated as a nanocarrier for siRNA delivery. The
evaluation of cellular uptake and transfection efficacy of siRNA delivered by this dendrimer showed the following. With the decrease in the degree of quaternization enhanced cellular uptake of the QPAMAM-OH-siRNA complex, the resulting efficacy of the delivered siRNA in terms of the suppression of the expression of targeted gene was very low. Based on these results one can suggest that despite the decrease in the quaternization resulting in the increase in cellular uptake of an entire dendrimer-siRNA complex, the aforementioned suppression of the proton sponge effect was still sufficient to prevent the release of free siRNA from the endosome.

To further increase the cellular uptake of dendrimer-siRNA complexes, we added a cancer cell targeting moiety to the dendrimeric delivery system. A synthetic analog of natural LHRH peptide conjugated and cancer-targeted internally quaternized PAMAM dendrimer (QPAMAM-OH-LHRH) was synthesized for this purpose. The rationale for using LHRH peptide was based on the following main considerations (96): 1) The receptors for this peptide are over-expressed in several types of cancer cells, including ovarian, breast, endometrial, and prostate cancers, 2) The receptors are less expressed in normal cells, 3) LHRH peptide targets the conjugated nanocarrier specifically to cancer cells and facilitates cellular uptake using over-expressed LHRH receptors through receptor-mediated endocytosis. 4) LHRH-conjugated nanocarriers preferentially accumulated in tumors, limiting side effects on healthy organs. In our previous experiments, we extensively studied targeting mechanisms of LHRH peptide in cancer
cells, including experiments that involve competitive binding (93). It was clearly shown that LHRH peptide works through specific LHRH receptors which are overexpressed in many types of cancer cells and specific targeting is due to LHRH molecules.

The surface neutral and internally cationic QPAMAM-OH dendrimer was prepared by a known method using controlled internal quaternization of a PAMAM dendrimer. A two-step synthetic protocol was used for the synthesis of the QPAMAM-OH-LHRH dendrimer. In the first step, the PAMAM-OH dendrimer was reacted with LHRH-hemisuccinate to produce PAMAM-OH-LHRH conjugate. During the second step, PAMAM-OH-LHRH was internally quaternized to afford the QPAMAM-OH-LHRH dendrimer. The degree of quaternization in both of the dendrimers was estimated by $^1$H-NMR spectroscopy. Unfortunately, $^1$H-NMR was not useful in this case to detect the presence of LHRH peptide due to its low concentration in the conjugate. Consequently, the presence of LHRH peptide in QPAMAM-OH-LHRH was confirmed by BCA protein assay. Furthermore, the concentration of LHRH peptide in the QPAMAM-OH-LHRH conjugate was estimated to be around 50 μg/ml based on the calibration curve for standard LHRH peptide using BCA protein assay.

Among several advantages, the low cytotoxicity of internally quaternized dendrimer makes them most suitable nanocarriers for the safe delivery of genes and other therapeutic drugs. In the present study, both the dendrimers, QPAMAM-OH and
QPAMAM-OH-LHRH, showed low toxicity even at higher concentrations. Low cytotoxicity is particularly important when the high loading of nanocarrier is required and it has been shown that the higher dendrimer-siRNA ratio results in smaller particles for relatively easy internalization (115, 121). It has been documented that a higher dendrimer-siRNA (N/P) ratio also gives superior results in the in vitro gene expression studies (140).

The oligonucleotide band disappearance in agarose gel electrophoresis studies suggested that both the dendrimers had almost identical ability in forming a complex with siRNA and a stable complex was formed at N/P ratio 1 and above.

Dynamic Light Scattering analysis was in good agreement with our previous studies and the particle size decreased with an increase in the N/P (Nitrogen to Phosphate, positive to negative charge ratio) ratio (121). However, the particle size in partially quaternized dendrimers was larger when compared to that previously obtained for a completely quaternized PAMAM dendrimer (121). Atomic Force Microscope studies revealed compact and spherical particles for QPAMAM-OH-siRNA complex and the presence of LHRH marginally influenced the morphology of QPAMAM-OH-LHRH-siRNA complex. The size of dendrimer-siRNA complexes used in the present study varied between different dendrimers and was in most cases relatively higher when compared with that registered by other investigators (140-142). It is known that the size of dendrimer-RNA
complexes, their stability and uniformity critically depend on the size of the RNA molecule, the dendrimer generation, and the charge ratio between the dendrimer and the RNA (141). Larger RNA molecules, higher generations of dendrimers, and larger dendrimer-to-RNA charge ratios usually form stable, uniform nanoscale RNA/dendrimer complexes. Previously, we reported that the complexation of PAMAM generation 4 dendrimer with siRNA produced very large size nanofibers (121). It has been shown that dendrimer generation greatly affects the particle size and a higher generation dendrimer could lead to smaller particles. The formation of relatively large dendrimer-siRNA complexes in the present study could most likely be explained by their low resulting positive charge where cationic charges of dendrimers were hindered by dendrimer surface. This probably leads to the formation of less condensed complexes. An increase in N/P ratio led to the condensation of complexes and decrease in their diameter. At N/P ratio about 3 relative units, atomic force microscopy revealed formation of well condensed nanoparticles along with a small amount of larger particles. Such a broad size diversity of dendrimer-siRNA complexes have previously been registered in independent studies (115, 121, 140, 141). It should also be stressed that AFM images have a tendency to overestimate the size particles (134). The average size of complexes at N/P ratio equal to 3 relative units (the ratio which we used for cellular uptake study) determined by dynamic light scattering technique was about 150 nm. Our previous investigations clearly showed that delivery systems of such size with a low surface charge provide for an effective intracellular internalization of anticancer drugs, antisense oligonucleotides,
and siRNA. Moreover, targeting of DDS carriers specific to cancer cells substantially enhanced this internalization (49, 96, 133). Our new data support the previous findings and show that cancer targeted QPAMAM-OH-LHRH-siRNA with 85% of quaternization and N/P ratio equal to 3 relative units provide for an enhanced cytoplasmic delivery of siRNA, uniform distribution of delivered siRNA within the cytoplasm, and led to the effective suppression of the targeting gene.

Next, the role of degree of quaternization and targeting ligand as a penetration enhancer was investigated in the cellular uptake of siRNA by human ovarian cancer A2780 cells. Indeed, the degree of quaternization influenced the intracellular uptake of siRNA and relatively less quaternized dendrimer QPAMAM-OH improved the internalization of siRNA when compared with the previously studied QPAMAM-OH dendrimer with almost complete quaternization (121). The cellular uptake of siRNA was significantly improved when the targeting ligand was conjugated to the dendrimer, even though the degree of quaternization of this dendrimer was slightly higher than QPAMAM-OH (85% vs. 75%). These studies clearly indicate the importance of the degree of quaternization and targeting ligand LHRH for the enhanced intracellular delivery of siRNA using nontoxic nanocarriers. Although the exact reason for improved cellular internalization of siRNA using QPAMAM-OH is unknown, two possible contributing factors are suggested (1) more efficient interaction of free tertiary amines with negatively charged cell membrane than permanently charged quaternary amines, and (2) tertiary amines assisted
the endosomal escape of siRNA to cytoplasm by the so-called proton sponge effect. On the other hand, the receptor mediated endocytosis pathway was considered a reason for the higher uptake of siRNA by the QPAMAM-OH-LHRH dendrimer. A series of our previous experiments clearly demonstrated such a phenomenon for the enhancement of other various drug delivery systems when LHRH peptide was used as targeting ligand (49, 95-97, 133).

In order to exploit the effect of lower degree of quaternization for effective siRNA delivery, a QPAMAM-OH dendrimer with approximately 30% of quaternization and its complex with siRNA were prepared. The preliminary testing showed that such a complex possesses good cell penetrating activity (data not shown). Unfortunately, the less quaternized dendrimers degraded in water over a period of time presumably through Hofmann elimination or a retro Michael type of reaction (143), which precluded their further use in the siRNA delivery.

Having been encouraged by the higher cellular uptake of siRNA by QPAMAM-OH and QPAMAM-LHRH-OH dendrimers with relatively low degree of quaternization, we further compared gene silencing efficiency for BCL2 gene of siRNA complexes with dendrimers of different degree of quaternization. The delivery of siRNA by the non-targeted QPAMAM-OH dendrimers with different degrees of quaternization led to only a mild decrease in the expression of the targeted gene. In contrast, targeted
QPAMAM-OH-LHRH-siRNA complex significantly suppressed the expression of the BCL2 gene in cancer cells. The result suggests that degree of quaternization to some extent is important for the cellular uptake but not sufficient to achieve a gene silencing effect. In contrast, delivery of siRNA by a cancer-targeted dendrimer substantially enhances its gene silencing effectiveness. These data show that an effective intracellular delivery of siRNA by dendrimers does not guarantee its high gene silencing activity. In order to successfully decrease the expression of targeted mRNA, siRNA should be delivered into the cellular cytoplasm, released from the delivery system with preserved silencing activity, and enter the RNA interference pathway. For instance, it was found that poor endosomal escape of the carrier and inefficient cytoplasmic decoupling of the complexed nucleic acid may be a critical step in limiting gene silencing activity of siRNA delivery systems (144). Recently, Hollins et al (145) reported that PAMAM dendrimeric drug delivery systems, differing only in their structural architecture, elicit opposing effects on the expression of the gene targeted for silencing by siRNA. Despite providing similar improvements in siRNA uptake, dendrimer formulations with comparable efficiency of cytoplasmic delivery led to an approximately 10-fold variation in the expression of targeted mRNA. A relative independence of the efficiency of cellular uptake of different DDS and their gene silencing activities was reported in other independent studies (144, 146-148). In addition, it was found that some dendrimers without siRNA can influence gene expression (148). Similarly, in the present study, three non-targeted QPAMAM-OH-siRNA complexes induced a comparable decrease in the
expression of BCL2 mRNA despite different efficiency in delivering of siRNA in the cytoplasm of cancer cells. Although mechanisms of such phenomenon require more detailed study, one can suggest that intracellular internalization of a dendrimer-siRNA complex by receptor-mediated endocytosis provides an effective delivery to the place of action and allows for preserving the activity of complicated siRNA. The present data show that cancer-targeted delivery system down regulated the expression of targeted mRNA 4-4.5 times more effectively when compared with non-targeted dendrimers.

The present experimental data support our previous conclusion that targeting of nanocarriers to cancer minimized the influence of the architecture, composition, size, and molecular mass of nanocarriers on the efficacy of their payload. Previously, we experimentally supported this important statement for the efficacy of the delivered anticancer drug (49). In the present publication, we provide experimental support of the phenomenon for siRNA delivered by internally quaternized dendrimers. This finding shows that the observation probably has general character, which in turn can potentially produce a high impact on nanocarrier-based drug delivery of cancer therapeutics. This conception implies that one can design nanocarrier architecture with specific composition, size, molecular mass, and other characteristics based solely on the effective encapsulation of active ingredient(s), desired their release profile, intracellular distribution, cost, and other factors ensuring that the high specific efficacy of the delivered agent(s) could be achieved automatically by cancer targeting.
5.5 Conclusions

Two internally cationic poly(amidoamine) dendrimers were designed and evaluated for siRNA delivery into cancer cells. The result suggested that a lesser degree of quaternization improved the cellular uptake of siRNA but did not considerably increase its gene silencing activity. In contrast, targeting of the dendrimer specifically to the plasma membrane of cancer cells by LHRH peptide further improved internalization of siRNA by cancer cells and significantly enhanced its intracellular activity leading to a substantial suppression of the expression of a targeted gene. Data obtained show the high potential of targeted internally cationic dendrimers as nanocarriers for efficient delivery of siRNA to cancer cells and their possible use in cancer chemotherapy.
Figure 5.1 Synthesis of internally quaternized non-targeted QPAMAM-OH and targeted QPAMAM-OH-LHRH dendrimers with 75% and 85% of quaternization.
**Figure 5.2** Characterization of synthesized dendrimers. (A) $^1$H-NMR chart for the QPAMAM-OH dendrimer. (B) UV-visible spectra of different dendrimers. (C) Average particle size of different siRNA-dendrimer complexes determined by dynamic light scattering. (D) Atomic force microscopy images of dendrimer-siRNA complexes. The height differences on the surface are indicated by the color code: lighter regions indicate higher heights. (E) Agarose gel electrophoresis of dendrimer-siRNA complexes.
**Figure 5.3** Cellular internalization of free siRNA and siRNA delivered by the internally quaternized dendrimer. Typical confocal microscopy images of living A2780 cells incubated with fluorophore labeled siRNA (siGLO Red, red fluorescence, A), a dendrimer labeled with FITC (green fluorescence, B) and a dendrimer-siRNA complex (C). Superposition of green and red fluorescence images (C) allows for detecting of co-localization of the dendrimer and siRNA in the solution and inside cells (yellow color).
**Figure 5.4** Confocal microscopy images of human A2780 ovarian carcinoma cells incubated for 17 hours with non-targeted (A) and cancer targeted (B) dendrimer-siGLO complexes (z-series, from the top of the cell to the bottom).
**Figure 5.5** Suppression of the expression of the BCL2 gene by complexes of siRNA with dendrimers of different degree of quaternization and N/P ratio equal to 3 relative units. Typical images of RT-PCR products of genes encoding BCL2 protein and β2-microglobulin (β2-m, internal standard) in human ovarian cancer cells. 1 – Control (cells incubated with fresh media); 2 – Cells incubated with QPAMAM-OH-0.20-siRNA (20% of quaternization); 3 – Cells incubated with QPAMAM-OH-0.70-siRNA (70% of quaternization); 4 – Cells incubated with QPAMAM-OH-siRNA (97% of quaternization); 5 – Cells incubated with QPAMAM-OH-LHRH-siRNA (85% of quaternization).
6 MULTIFUNCTIONAL TRIBLOCK NANOCARRIER (PAMAM-PEG-PLL) FOR THE EFFICIENT INTRACELLULAR SIRNA DELIVERY AND GENE SILENCING

6.1 Introduction

Design and creation of novel nanometer-size carriers for the safe delivery of small interfering RNA (siRNA) towards their potential applications in cancer therapy is one of the challenging and rapidly growing areas of research. RNA interference (RNAi) is a conservative biological response to siRNA that regulates the expression of protein coding genes (24, 25, 119, 120, 149). However, the broad therapeutic applications of siRNA are limited by major delivery problems (84). The efficient in vivo gene knock down requires a delivery system that would overcome the following limitations: (1) low cellular uptake, (2) poor endosomal escape, (3) substantial liver and renal clearance, (4) facile enzymatic degradation in the blood and extracellular environment and (5) inefficient gene silencing.

Recent investigations in the area of nanomaterials for RNA delivery, including the works in our laboratory, provided solutions to some of the major siRNA delivery problems (5, 94, 121, 150-155). However, the developed delivery approaches address only selected siRNA delivery problems lacking optimal balanced delivery system that includes a solution for all the major aforementioned challenges. For example, a biodegradable
polymer poly-L-lysine (PLL) is being used for gene delivery and its polyplexes are taken up into cells efficiently. However, transfection efficiencies of PLL-siRNA complexes remain several orders of magnitude lower when compared with other transfection agents. One potential reason for inefficient transfection has been identified as the lack of amino groups with a pKa ~5-7 for so called “proton sponge effect” that offers endosomolysis and subsequent release of siRNA. The desired transfection effect was achieved by structural modification of PLL using a targeting ligand or endosomolytic agents like chloroquine or fusogenic peptides (156, 157). A significant improvement in transfection efficiency was observed when histidine or imidazole moieties were attached to the PLL (158, 159).

Another major challenge in the safe delivery of siRNA is its facile enzymatic degradation in cytoplasm due to the presence of nucleases that dramatically reduce siRNA half-life. Previously, we have reported that internally quaternized and cancer-targeted poly(amidoamine) (PAMAM) dendrimers provide for the efficient cellular uptake and excellent gene silencing (154). It was shown that surface modification and internal quaternization of dendrimers reduced their cytotoxicity and substantially improved the cellular uptake while targeting of the dendrimers to cancer cells initiated receptor mediated endocytosis and led to the efficient gene knock down. We have also demonstrated the importance of free tertiary amine groups in dendrimers for endosomal escape. The present study explores a different approach and is aimed at the design,
synthesis, and evaluation of a triblock delivery system that provides solutions for major problems in siRNA delivery i.e. poor cellular uptake, low endosomal escape, and facile enzymatic degradation. A novel triblock nanocarrier PAMAM-PEG-PLL has been designed to combine individual features of PAMAM dendrimer, polyethylene glycol (PEG) and poly-L-lysine. PAMAM dendrimer provides tertiary amines for endosomal escape, PEG covers up siRNA protecting it from enzymatic degradation while PLL offers cationic amine groups for electrostatic interaction with negatively charged siRNA.

6.2 Materials and methods

6.2.1 Materials

Generation four PAMAM-NH₂ dendrimers (Mw ~ 14,214 Da, 64 amine end groups), PLL·HBr (Mw ~12,000, degree of polymerization equal to 57), 4-(methylamino)pyridine and methyl iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO). α, ω-Bis(2-carboxyethyl)polyethylene glycol (Mw ~3,000 Da) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride were obtained from Fluka (Allentown, PA). Spectra/Pore dialysis membranes were obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Ethidium Bromide (EtBr) solution was purchased from Promega (Madison, WI). The sequence of siRNA targeted to BCL2 mRNA custom synthesized by Ambion (Austin, TX), was: 5'-GUG AAG UCA ACA
UGC CUG C-dTdT-3’ (sense strand) and 5’-GCA GGC AUG UUG ACU UCA C-dTdT-3’
(antisense strand). Non-specific siRNA used as a negative control (sense strand, 5’-CCU
CGG GCU GUG CUC UUU U-dTdT-3’; antisense strand, 5’-AAA AGA GCA CAG CCC
GAG G-dTdT-3’) was received from Dharmacon Inc. (Lafayette, CO). Fluorescent RNA
duplex - siRNA labeled with Pierce NuLight™ DY-547 fluorophores (siGLO Red
Transfection Indicator, red fluorescence) was obtained from Applied Biosystems
(Ambion, Inc., Foster City, CA). All other chemicals were purchased from Fisher
Scientific (Fairlawn, NJ).

6.2.2 Cell line

The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton
(Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St.
Louis, MO) supplemented with 10% fetal bovine serum (Fisher Scientific, 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.

6.2.3 Synthesis of surface modified PAMAM dendrimer (PAMAM-NHAc)

The surface modified and partially acetylated PAMAM-NHAc dendrimer was prepared
using a previously reported procedure (121) (Fig. 1). Briefly, triethylamine (0.11 mL,
0.82 mmol) was added to a stirred solution of PAMAM-NH$_2$ generation four dendrimer (172 mg, 0.012 mmol) dissolved in anhydrous methanol (10 mL) followed by the addition of excess acetic anhydride (0.08 mL, 0.72 mmol). The resulting mixture was stirred at room temperature for 24 h. Methanol was evaporated under reduced pressure and the resulting residue was dissolved in water (2 mL). Further purification by extensive dialysis against deionizer water using dialysis membrane (molecular mass cut off 2,000 Da) and freeze-drying afforded acetylated PAMAM dendrimer. The degree of acetylation was confirmed by proton nuclear magnetic resonance ($^1$H NMR).

### 6.2.4 Synthesis of PAMAM-PEG-COOH conjugate

α, ω-Bis(2-carboxyethyl)polyethylene glycol (15 mg, 5 μmol, Mw ~3000 Da) and PAMAM-[(NHAc)$_{38}$(NH$_2$)$_6$] dendrimer (83 mg, 5 μmol) were dissolved in the mixture of anhydrous solvents methylene chloride (5 mL) and dimethyl sulfoxide (5 mL) (Fig. 1). After stirring for 10 min at room temperature, N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC·HCl) (1 mg, 5.3 μmol) and 4-(methylamino)pyridine (DMAP) (0.5 mg) were added to the reaction mixture. The resulting mixture was stirred for an additional 36 h at room temperature and solvents were removed under reduced pressure. The residue was dissolved in water and purified by extensive dialysis using Spectra/Por dialysis membrane (molecular weight cutoff, MWC = 6,000 Da) against deionized water. The conjugate was further purified by
passing through a sephadex G10 column using water as eluent and lyophilized to obtain PAMAM-PEG-COOH as a white solid.

6.2.5 Synthesis of PAMAM-PEG-PLL conjugate

Triethylamine (0.2 mL) was added to a stirred solution of poly-L-lysine hydrobromide (22 mg, 1.83 μmole, Mw = ~12,000, degree of polymerization equal to 57) in anhydrous dimethyl sulfoxide (3 mL) (Fig. 1). The reaction mixture was further diluted with anhydrous methylene chloride (5 mL) followed by the addition of PAMAM-PEG-COOH conjugate (22 mg, 1.14 μmol) and stirred at room temperature for 15 min. EDC·HCl (1.5 mg, 7.8 μmol) and DMAP (0.5 mg) were added to the reaction mixture. The resulting solution was stirred for an additional 36 h at room temperature. The side product carbodiimide urea was filtered off and solvents were removed under reduced pressure. The residue was dissolved in water and purified by extensive dialysis using Spectra/Por dialysis membrane (MWC = 25,000 Da) against deionized water. The conjugate was further purified by passing through a Sephadex G10 column using water as eluent and lyophilized to obtain PAMAM-PEG-PLL as a hygroscopic white solid.

6.2.6 Synthesis of PEG-PLL conjugates

NHS-PEG-OMe (15.6 mg, 3.1 μmole, Mw =~5000) in phosphate buffer (pH 8.4) was
added to a stirred solution of poly-L-lysine hydrobromide (22 mg, 1.83 µmole, Mw = ~12000, degree of polymerization equal to 57) in phosphate buffer (pH 8.4). The resulting solution was stirred for 6 h at room temperature. The resulting reaction mixture was then dialyzed against 1N HCl for 12 h and subsequently extensively dialyzed against deionized water using dialysis membrane Spectra/Por (MWC = 8,000 Da). Further purification by passing through sephadex G10 column using water as eluent and freeze drying afforded PEG-PLL conjugate.

6.2.7 Synthesis of PAMAM-PEG-PLL-Cy5.5

Cy 5.5 mono NHS ester (1.5 mg, 1.32 µmol) dissolved in anhydrous dimethyl sulfoxide (1 mL) was added to a stirred solution of PAMAM-PEG-PLL (9 mg, 0.32 µmol, Mw ~28,000) in 0.1 mM NaHCO₃ (1 mL). The resulting mixture was stirred in the dark at room temperature for 6 h. Extensive dialysis using Spectra/Por dialysis membrane (MWC = 25,000) against deionized water was carried out to remove unreacted Cy 5.5. Additionally, the conjugate was purified by passing through sephadex column. The concentration of Cy 5.5 dye attached to the PAMAM-PEG-PLL nanocarrier was estimated by measuring its fluorescence (Excitation 675 nm, emission 694 nm) using Cy 5.5 NHS ester as standard.
6.2.8 Proton nuclear magnetic resonance spectroscopy (\(^1\text{H NMR}\))

\(^1\text{H NMR was performed on a Varian VNMRS 400 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA). The chemical shift was expressed as parts per million (ppm) and a solvent peak was used for reference (D}_2\text{O, 4.8 ppm). The following abbreviations are used in the results section to identify multiplicities of spectra peaks: s, singlet; m, multiplet; br, broad.}

6.2.9 In vitro cytotoxicity

A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cytotoxicity of the following nanocarriers PEG, PLL, PEG-PLL, PAMAM-NH\(_2\), PAMAM-OH, PAMAM-NHAc, and PAMAM-PEG-PLL as previously described (48, 132). To measure cytotoxicity, cells were separately incubated in a microtiter plate with different concentrations of PLL, PEG-PLL and PAMAM-PEG-PLL. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. On the basis of these measurements, cellular viability was calculated for each nanocarrier concentration. A decrease in the cellular viability indicated an increase in the toxicity.
6.2.10 Characterization of siRNA complexation with nanocarriers

The complexes of cationic nanocarriers (PLL, PEG-PLL and PAMAM-PEG-PLL) and siRNA were prepared in water at N/P (amine/phosphate) charge ratios ranging from 0 to 1.5 and incubated at room temperature for 30 min. The charge ratio was calculated by relating the number of cationic primary amine groups on nanocarrier with the number of negatively charged phosphate groups of siRNA. Dendrimer-free siRNA was used as the control. Double-stranded RNA ladder (New England Biolabs, Ipswich, MA) with the smallest base pairs at 21 was used as a size reference. The samples were further diluted with DPBS buffer and electrophoresed in 4% agarose gel at 100 V for 50 min in Tris-Borate-EDTA buffer containing ethidium bromide. siRNA bands on the gel were visualized under ultraviolet light and photographed. Complex formation was also quantified by measuring fluorescence of ethidium bromide in the sample at 530 nm excitation and 590 nm emission wavelengths (160). The fluorescence intensity at N/P charge ratio equal to 0 was set to 100%.

6.2.11 Dynamic Light Scattering (DLS) analysis and zeta potential

PAMAM-PEG-PLL-siRNA complex was prepared by mixing PAMAM-PEG-PLL and siRNA in water at N/P ratio equal to 3. The resulting complex was incubated for 30 min and the size was determined using the DynePro-MS800 dynamic light
scattering/molecular sizing instrument with argon laser wavelength $\lambda=830$ nm, a detector angle 90°, and typical sample volume of 20 μL. Each light scattering experiment consisted of 20 or more independent readings, 10 s in duration each. Data analysis was conducted using DynaPro Instrument Control Software for molecular Research DYNAMICS (version 5.26.60). The obtained DLS data represents the average of three runs. 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 5 times, and average values were calculated.

6.2.12 Cellular internalization

Cellular uptake and intracellular localization of siRNA was investigated using a confocal microscopy. In this experiment, living cancer cells were incubated with fluorophore labeled naked siRNA (siGLO Red, red fluorescence) and siRNA complexed with cationic nanocarrier PAMAM-PEG-PLL ($N/P=3$). Cellular uptake studied substances was monitored in living cells placed in a chamber at 37 °C within 1 h. Cellular localization of siRNA was examined on fixed and washed cells after the incubation for 24 h with the substances. Fluorescence and its distribution within the cell were examined using a confocal microscope.
6.2.13 Gene expression

Reverse transcription-polymerase chain reaction (RT-PCR) was used for the analysis of gene expression as described previously (48). The cationic nanonarriers (PLL, PLL-PEG-OMe and PAMAM-PEG-PLL)-siRNA (for BCL2 gene) complexes were added to the cells with the final concentration of siRNA equal to 1 μM. After 24 h, total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Piscataway, NJ) with 2 μg of total cellular RNA and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences, Piscataway, NJ). After synthesis, the reaction mixture was immediately subjected to polymerase chain reaction (PCR), which was carried out using GenAmp PCR System 2400 (Perkin-Elmer, Shelton, CT). β2-microglobulin (β2-m) was used as an internal standard. The following pairs of primers were used: BCL2: 5′-GGA TTG TGG CCT TCT TTG AG-3′ (sense), 5′-CCA AAC TGA GCA GAG TCT TC-3′ (antisense); β2-m (internal standard)-ACC CCC ACT GAA AAA GAT GA (sense), ATC TTC AAA CCT CCA TGA TG (antisense). PCR regimen was as follows: 94°C for 5 minutes; 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 41 cycles; and 60°C for 10 minutes. PCR products were separated in 4% NuSieve 3:1 Reliant-agarose gels (Lonza, Basel, Switzerland) in 1x Tris-borate EDTA buffer [0.089 mol/L Tris-borate, 0.002 mol/L EDTA (pH 8.3); Research Organics Inc., Cleveland, OH] by submarine electrophoresis. The gels were stained with EtBr and
6.2.14 Serum stability of siRNA and PAMAM-PEG-PLL-siRNA complex

Serum stabilities of naked siRNA and siRNA complexed with PAMAM-PEG-PLL nanocarrier were investigated by incubating siRNA or PAMAM-PEG-PLL-siRNA complex in 50% human serum at 37 °C. Ten samples were prepared by mixing siRNA (30 nmol) in water with PAMAM-PEG-PLL (74.8 nmol, N/P = 3) solution in water and separately incubated for 30 min at room temperature. In the case of naked siRNA, equal volume of RNAase free water was used instead of nanocarrier. To each of these samples 50% human plasma was added (final siRNA concentration was 1.43 nM) and incubated at 37 °C. Samples were removed at an indicated time intervals (0, 0.25, 0.5, 0.75, 1, 3, 6, 12, 24 and 50 h) and analyzed using a gel electrophoresis (4% agarose gel at 100 V for 50 min) in Tris-Borate-EDTA buffer containing EtBr. siRNA bands on the gel were visualized under ultraviolet light. PAMAM-PEG-PLL-siRNA samples were pre-treated with polymethacrylic acid to release free siRNA from cationic nanocarriers. 100 µL of polymethacrylic acid solution (4µM) was added to the complexes (triblock nanocarrier/siRNA=3) and incubated at 37 °C with 50% human plasma. The released siRNA was then analyzed by a gel electrophoresis.
6.2.15 Statistical analysis

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value ± standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student’s t test and considered P values of less than 0.05 as statistically significant.

6.3 Results

6.3.1 Synthesis of triblock PAMAM-PEG-PLL nanocarrier

A three step synthetic route was used for the preparation of PAMAM-PEG-PLL nanocarrier (Fig. 6.1). In the first step, PAMAM dendrimer was partially acetylated to afford PAMAM-[(NHAc)$_{58}$(NH$_2$)$_6$] dendrimer. Second step involved synthesis of PAMAM-PEG-COOH by reacting one of the acid group of α,ω-bis(2-carboxyethyl)polyethylene glycol (Mw = ~3000) with one of the primary amine of PAMAM-[(NHAc)$_{58}$(NH$_2$)$_6$] dendrimer. During the third step, terminal free acid group of PAMAM-PEG-COOH was reacted with PLL using EDC as a coupling reagent.

6.3.2 $^1$H NMR spectra
All proton NMR spectra were recorded in solution of studied compounds in D\textsubscript{2}O using a 400 MHz NMR spectrometer. The chemical shift (\(\delta\)) was expressed as parts per million (ppm). The data obtained confirmed the structures of synthesized substances.

**PAMAM-NHAc** \(^1\)H NMR spectral data are shown in Fig. 6.1a. The following peaks were identified: \(\delta\) 1.98 (s, CO\textsubscript{2}CH\textsubscript{3}), 2.40-2.50 (br m, CH\textsubscript{2}CONH), 2.62-2.73 (br m, CONHCH\textsubscript{2}CH\textsubscript{2}N), 2.82-2.92 (br m, NCH\textsubscript{2}CH\textsubscript{2}CONH), 3.26-3.37 (m, CONHCH\textsubscript{2} and CH\textsubscript{2}NHCOCH\textsubscript{3}). The degree of acetylation (~90%) was confirmed from the proton NMR spectra (Fig. 6.1a) by calculation the ration between the integrated peak area of signal appeared at \(\delta\) 1.98 ppm (-NHCOCH\textsubscript{3}) to that of methylene protons of PAMAM dendrimer (\(\delta\) 2.40-3.37).

**PAMAM-PEG-COOH** \(^1\)H NMR spectrum (Fig. 6.1b) showed the following peaks: \(\delta\) 2.00 (s, COCH\textsubscript{3}), 2.40-2.50 (br m, CH\textsubscript{2}CONH), 2.64-2.75 (br m, CONHCH\textsubscript{2}CH\textsubscript{2}N), 2.82-2.92 (br m, NCH\textsubscript{2}CH\textsubscript{2}CONH), 3.30-3.38 (m, CONHCH\textsubscript{2} and CH\textsubscript{2}NHCOCH\textsubscript{3}), 3.74 (s, -CH\textsubscript{2}CH\textsubscript{2}O). The spectra showed mono acylation of dendrimer with \(\alpha, \omega\)-bis(2-carboxyethyl)polyethylene glycol leaving another –COOH group free for conjugation with poly-L-lysine (PLL). The \(^1\)H-NMR spectra for this compound confirmed the presence of both polyethylene glycol and dendrimer protons. Further mono functionalization was determined by calculating the area under proton peaks arising from polyethylene glycol (Mw = ~3000, -CH\textsubscript{2}CH\textsubscript{2}O-, ~270 H) and dendrimer (-COCH\textsubscript{3}, 174 H) appeared at \(\delta\) 3.74 and \(\delta\) 2.00, respectively. **PAMAM-PEG-PLL** \(^1\)H-NMR spectral data are shown in Fig. 6.2c. The following peaks were identified: \(\delta\) 1.30-1.50 (br m, PLL), 1.60-1.90 (br m, PLL), 1.98 (s, PAMAM), 2.40-2.50 (br m, PAMAM), 2.60-2.70
(br m, **PAMAM**), 2.78-2.88 (br m, **PAMAM**), 2.90-3.05 (br m, **PLL**), 3.26-3.37 (m, **PAMAM**), 3.70 (s, **PEG**), 4.25-4.35 (br m, **PLL**). The spectra confirmed the formation of PAMAM-PEG-PLL nanocarrier showing the presence of proton peaks arising from dendrimer (PAMAM), polyethylene glycol (PEG) and poly-L-lysine (PLL). **PEG-PLL**

1H-NMR spectrum (Fig. 6.1d) showed the following peaks: δ 1.38-1.60 (br m, -CH₂-CH₂-CH₂-), 1.68-1.90 (br m, -CH₂-CH₂-CH(CH(NH₂)) & -CH₂-CH₂-CH₂NH₂-), 2.90-3.10 (br m, -CH₂-CH₂NH₂-), 3.70 (s, -CH₂CH₂OPEG), 4.30-4.40 (br m, -CH₂-CH₂-CH(CO)NH₂-).

### 6.3.3 In vitro cytotoxicity

The measurement of viability of cells incubated with different concentrations of PEG, PLL, PEG-PLL, PAMAM-NHAc-PEG and PAMAM-NHAc-PEG-PLL compounds showed their relatively low cytotoxicity (Fig. 6.2a). No substantial differences were found between different nanocarriers under the concentrations of the compounds lower than 4 µM. However, the concentrations exceed 4 µM, PEG-PLL demonstrated higher cytotoxicity when compared with PLL alone and PAMAM-NHAc-PEG-PLL. Therefore, the toxicity of PEG-PLL was reduced when PEG-PLL was conjugated to PAMAM-NHAc dendrimer.
6.3.4 Analysis of dendrimer-siRNA complex formation by agarose gel electrophoresis

The nanocarrier-siRNA complex formation and optimal N/P ratio was determined by agarose gel electrophoresis. The PLL, PEG-PLL and PAMAM-PEG-PLL nanocarriers were mixed with siRNA in water at various N/P charge ratios and were subjected to electrophoresis in agarose gel (Fig. 6.2b). The numbers of cationic primary amine groups in PLL, PEG-PLL and PAMAM-PEG-PLL were calculated based on PLL Mw (~8,000) and degree of polymerization. All three nanocarriers showed the complex formation at N/P ratio 1 and above as evidenced by oligonucleotide bands disappearance from agarose gels.

6.3.5 Nanocarrier-siRNA particle size

The hydrodynamic diameter of PAMAM-PEG-PLL-siRNA complex was determined by dynamic light scattering at charge ratio ranging from 1 to 3 rel. units. The PAMAM-PEG-PLL/siRNA particle size slightly decreased with increasing the charge ratio to 3 relative units (Fig. 6.2c).

6.3.6 Cellular uptake of siRNA
The cellular uptake of naked and complexated fluorophore labeled siRNA (siGLO Red, red fluorescence) was studied in living (not washed and fixed) cells using confocal microscopy. A2780 human ovarian cancer cells were incubated with free siRNA and PAMAM-PEG-PLL-siRNA complex, and were subjected to confocal microscopy. Consistent with our previous findings (121, 154), naked siRNA did not penetrate the cancer cells (Fig. 6.3a). In contrast, siRNA complexated with a PAMAM-PEG-PLL cationic nanocarrier provided excellent cellular uptake (Fig. 6.3b). Moreover, optical sections z-series of a single living cell showed the homogenous and uniform distribution of siRNA-dendrimer complex in different cellular layers from the top of cell to the bottom (Fig. 6.3c).

6.3.7 Serum stability

The stability of siRNA in the blood serum was determined by incubating siRNA either naked or complexed with PAMAM-PEG-PLL nanocarrier in the human blood serum (Fig. 6.4). As expected, naked siRNA started to degrade after 1 h of incubation and completely degraded within 12 hours. In contrast, complexation of siRNA to PAMAM-PEG-PLL nanocarrier protected siRNA from the nuclease degradation; even 48 h after the incubation of complexated siRNA with human blood serum, siRNA remained nondegraded. Therefore the proposed complexation of siRNA with PAMAM-PEG-PLL prevents the degradation of siRNA in the plasma.
6.3.8 Gene expression

The gene knockdown efficiency of siRNA delivered by Poly-L-Lysine (PLL), PEG-PLL, PAMAM, PAMAM-PEG and PAMAM-PEG-PLL nanocarriers with appropriate controls (fresh media, naked specific siRNA, naked non-specific siRNA with scrambled sequence and non-specific siRNA delivered by PAMAM-PEG-PLL nanocarrier) was investigated using quantitative RT-PCR. We selected BCL2 protein responsible for cellular antiapoptotic defense as a target for siRNA. The results of these experiments are shown in Fig. 6.5. It was found that siRNA delivered by PAMAM, PAMAM-PEG, PLL, and PEG-PLL nanocarriers lowered the expression of the targeted gene approximately up to 70-50% from its control value (Fig. 6.5, bars 2, 3, P < 0.05). In contrast, delivery of siRNA by a PAMAM-PEG-PLL triblock nanocarrier led to a significant suppression of the expression of the targeted BCL2 gene down to 20% from the control value (P < 0.05). The decrease in gene expression after incubation with PAMAM-PEG-PLL-siRNA was statistically significant (P < 0.05) when compared with either PLL-siRNA or PEG-PLL-siRNA complexes.

6.4 Discussion

Delivery of siRNA into the cytoplasm of cancerous cells where it triggers sequence
specific mRNA degradation has recently emerged as a powerful tool in the gene therapy. The major obstacles in safe transportation of siRNA have been extensively investigated and condensation to nanoparticles has now been recognized as the most efficient method for facile transport of siRNA. Therefore, designing a nanocarrier that enables effective and safe transfer of siRNA into mammalian cells is a task of great interest. We are actively involved in the design, synthesis, and evaluation of various multifunctional nanoparticles as siRNA delivery carriers that include liposomes, surface modified dendrimers, mesoporous silica nanoparticles and surface engineered Superparamagnetic Iron Oxide (SPIO) nanoparticles, etc (94, 115, 121, 150, 154, 161-164). In general, combining multiple functions in a single delivery system is a difficult task and requires laborious synthetic efforts. In continuation with our previous studies on developing multifunctional nanomedicines, herein we describe design, synthesis, and evaluation of a synthetically simple yet novel triblock multifunctional nanocarrier PAMAM-PEG-PLL that effectively combines three functionalities which are otherwise ineffective when tested individually. The triblock nanonarrier PAMAM-PEG-PLL serves three distinct functions: (1) PLL provides cationic primary amine groups for electrostatic interaction with negatively charged siRNA (2) PAMAM dendrimer offers necessary tertiary amine groups for proton sponge effect, while (3) PEG confers nuclease stability in blood serum.

The synthesis of triblock nanocarrier was achieved in two simple steps that involved: (1) selective mono coupling on PEG-(COOH)2 to PAMAM-NHAc to afford
PAMAM-PEG-COOH and (2) coupling PAMAM-PEG-COOH with primary amine of PLL afforded the PAMAM-PEG-PLL. The formation of PAMAM-PEG and PAMAM-PEG-PLL was confirmed by proton magnetic resonance that revealed presence of all signals derived from PAMAM, PEG and PLL. The selective mono functionalization was confirmed by relating the area under peak of signals arising from polyethylene glycol (Mw 3000, -CH2CH2O-, ~270 H) and dendrimer (-COCH3, 174 H). The area under peak for the signals originated from PLL in the triblock PAMAM-PEG-PLL was also in good agreement with the assigned structure. For comparison studies a control polymer PLL-PEG was prepared by treating NHS-PEG-OMe with PLL. Introduction of single PEG was again confirmed using 1H-NMR by relating area under peak for PEG (–CH2CH2-O–), with that of PLL proton signals.

Studies on PLL as a cationic nanocarrier for gene transfection efficiency revealed that PLL alone provides relatively low gene knockdown, which is attributed to the lack of tertiary amine groups for the so called proton sponge effect. It is believed that this effect plays a substantial role in endosomal escape of siRNA inside cells after endocytosis (165-167). Nevertheless, PLL in combination with proton sponge ligands such as imidazole or histidine effectively reduced the gene expression; however, the toxicity of PLL dramatically decreased when imidazole or histidines were attached to PLL (158, 159). In the present investigation, we propose a combination of PLL with a nontoxic
PAMAM-NHAc dendrimer that possess several internal tertiary amine groups. It is expected that these groups will induce osmotic swelling of the endosome due to endosomal buffering and lead to the rupture of endocytotic vesicles and subsequent release of their payload. Furthermore, polyethylene glycol (PEG) was included in the nanocarrier to enhance siRNA stability against nuclease enzymes during the voyage in the human blood stream. We also achieved a decrease in cytotoxicity of PLL by attaching a nontoxic PAMAM-NHAc dendrimer and polyethylene glycol.

The ability of PLL, PEG-PLL and PAMAM-PEG-PLL to form complex with siRNA was compared using agarose gel electrophoresis method. All three nanocarriers formed a stable complex at N/P ratio 1 and above. The numbers of cationic primary amine groups were calculated based on PLL molecular weight and degree of polymerization (~8000 Da and 57, respectively). Each PAMAM-PEG-PLL carrier (calculated Mw 27650 Da) contained approximately 56 primary amine groups. Similarly, cationic groups for PEG-PLL (calculated Mw 11,000 Da, DP 57) and PLL (Mw 8000 Da, DP 57) were calculated as 56 and 57 respectively. As expected, the agarose gel electrophoresis data showed that PAMAM-PEG-PLL showed similar to PEG-PLL and PLL ability to form complexes with siRNA. Dynamic light scattering data revealed an average size around 150 nm of the resulting complexes of the proposed nanocarriers with siRNA. This size of the resulting nanoparticles and possible impact of PLL as penetration enhancer resulted in the efficient cellular uptake of triblock nanocarrier PAMAM-PEG-PLL-siRNA
complexes by human cancer cells.

However, effective uptake of siRNA by cells does not automatically ensure effective silencing of its targeted mRNA. For instance, previously, we have shown that an effective intracellular delivery of siRNA by dendrimers does not guarantee its high gene silencing activity (121, 154). Down regulation of specific gene by siRNA can be controlled by two possible contributing factors (1) effective cellular internalization of siRNA and (2) endosomal escape of the payload to perform the task. Some cationic polymers used for siRNA delivery including PLL polymer show an excellent penetration into the cells, while demonstrating a relatively weak gene knockdown due to poor endosomal release of the siRNA payload (168). The PAMAM dendrimer unit in the triblock of the proposed nanocarrier PAMAM-PEG-PLL provides the required tertiary amines for proton sponge effect and subsequent endosomal release of the siRNA. We would like to note that proton sponge effect is only one possible mechanism of the release of siRNA from the complex. The following mechanisms can potentially be involved in the intracellular release of siRNA. First, siRNA-carrier complex enters the cells by endocytosis in membrane limited endosomes that eventually fuse with lysosomes. This leads to the sharp decrease in pH disrupting electrostatic interactions between the nucleic acid and carrier and ultimately leading to the siRNA release. Secondly, lysosomal enzymes and the acidic environment can either degrade or swell polymers stimulating the release siRNA from the nanoparticle (39). Thirdly, polymers can themselves possess
some membrane disruptive properties. They can swell and burst the endosome through protonation of excess amine groups (169). The exact mechanisms of intracellular release of siRNA require further more detailed investigation.

Thus PAMAM-PEG-PLL nanocarrier fulfills both the requirements of an effective delivery system of improved penetration and delivery of siRNA to the cytoplasm to achieve desired gene knockdown. The role of the PAMAM dendrimer was confirmed by comparing the gene silencing efficiency of BCL2 gene of siRNA complexed with triblock PAMAM-PEG-PLL, PLL and PEG-PLL nanocarriers. Indeed the triblock nanocarrier PAMAM-PEG-PLL-siRNA showed maximum suppression of the expression of targeted BCL2 gene while PLL alone or in combination with poly(ethylene glycol) (PLL-PEG) led to a substantially lower decrease in the expression of this gene.

After confirming the role of PLL and PAMAM in the triblock nanocarrier PAMAM-PEG-PLL, we further explored the role of PEG to protect the siRNA during the voyage in the human blood stream. Nuclease enzyme degradation of siRNA in the blood serum is one of the major obstacles for the in vivo therapeutic applications of the siRNA. We and others have reported that PEGylation of siRNA or nanocarriers greatly improved the stability of the siRNA in the human blood serum (89, 170-173). Though the exact mechanisms of such stabilization are not clear, one can assume that siRNA is shielded by a linear polymer polyethylene glycol and thus minimizes its exposure to the nuclease
enzymes. This assumption is based on the following considerations. Although, PEG is a middle block of the nanocarrier, it is also a hydrophilic segment and therefore one can expect a micelle like geometry of the complex. Though we don’t have any evidence, we believe that the triblock nanocarrier on complexation with siRNA forms micelle wherein the hydrophilic region (PEG) encapsulates PLL/siRNA complex. As expected, siRNA complexed with the proposed triblock nanocarrier PAMAM-PEG-PLL showed excellent siRNA stability in human blood serum. In fact, complexated siRNA was stable in the human serum more than 48 h, while naked siRNA degraded in less than 6 h.

6.5 Conclusions

A triblock nanocarrier was designed, synthesized, and evaluated for the efficient delivery of siRNA. The multifunctional triblock nanocarrier is synthetically simple to prepare and provide a solution to several obstacles involved in therapeutic applications of siRNA.
Figure 6.1 Synthesis of triblock PAMAM-PEG-PLL nanocarrier.
Figure 6.2 Representative 1H-NMR spectra in: a, D2O of PAMAM-NHAc; b, PAMAM-PEG-COOH; c, PAMAM-PEG-PLL; d, PLL-PEG-OMe.
Figure 6.3 Characterization of different nanocarriers and their complexes with siRNA: a, viability of human cancer cells incubated with carriers indicated.
Figure 6.4 Cellular uptake and localization of naked siRNA and PAMAM-PEG-PLL-siRNA complexes. Representative confocal microscopy images of cancer cells incubated with fluorophore labeled siRNA (siGLO Red, red fluorescence): a, naked siRNA; b, PAMAM-PEG-PLL-siRNA; c, optical sections z-series of cells incubated with PAMAM-PEG-PLL-siRNA.
Figure 6.5 Serum stability of naked and complexated siRNA. a, representative images of agarose gel electrophoreses of naked siRNA and PAMAM-PEG-PLL-siRNA complexes; b, Quantitative analysis of band intensity. Means ± SD are shown.
Figure 6.6 Expression of BCL2 gene in A2780 human ovarian cancer cells incubated with: 1, control (fresh media); 2, naked BCL2 siRNA; 3, naked non-specific siRNA; 4, PAMAM-BCL2 siRNA; 5, PAMAM-PEG-BCL2 siRNA; 6, PLL-BCL2 siRNA; 7, PEG-PLL-BCL2 siRNA; 8, PAMAP-PEG-BCL2 siRNA; 9, PAMAP-PEG-PLL-BCL2 non-specific siRNA. Means ± SD are shown. *P < 0.05 when compared with control. †P < 0.05 when compared with PLL-siRNA. ‡P < 0.05 when compared with PAMAM-siRNA.
7 SUPPRESSION OF TUMOR GROWTH AND PREVENTION OF METASTASES BY RECEPTOR-TARGETED COMBINED CHEMO AND GENE THERAPY

7.1 Introduction

Liposomes are another kind of widely used drug/gene carriers for cancer therapy and a number of liposome-based formulations are commercially available or are currently undergoing clinical trials (14, 15, 98-100). Our group has developed the complex liposomal drug delivery system containing a traditional drug doxorubicin (DOX) as an apoptosis inducer in combination with antisense oligonucleotides targeted to MDR1 and BCL2 mRNA as suppressors of pump and nonpump cellular resistance in cancer cells (42). In that study, human multidrug resistant ovarian carcinoma A2780/AD cell line was used to evaluate the cytotoxicity and uptake of this liposomal drug delivery system. The combination of DOX with ASO targeted to MDR1 and BCL2 mRNA in one liposomal drug delivery system was more toxic in vitro for A2780/AD cells when compared with free and liposomal DOX and liposomal DOX combined only with one type of ASO. In the in vivo study, nude mice bearing xenografts of human ovarian carcinoma A2780 cells were treated intraperitoneally with different formulations. The results showed that liposomal DOX combined with ASO targeted to MDR1 and BCL2 mRNA suppressed tumor growth more effectively than free and liposomal DOX (48, 50-53). Taken together, data suggested that this liposomal drug delivery system could not only deliver drug to the
desired site but also deliver therapeutic agents with biological activity. Therefore, liposome was selected as our delivery system to treat ascites cells and xenograft tumors. As we mentioned above, ascites cells are very invasive cells and resistant to the anti-cancer drugs. We hypothesized that effective treatment of advanced multidrug resistant primary ovarian tumors and their intraperitoneal metastases is possible only by suppressing simultaneously at least two main types of cellular resistance and by inducing cell death using several anticancer agents with different mechanisms of action. Such an objective can be best achieved if several anticancer agents are simultaneously delivered specifically to the tumor in combination with other active components that perform different functions for enhancing cellular uptake and efficiency of drugs in cancer cells, limiting adverse side effects, and preventing the development of drug resistance and metastases. To this end, we constructed a tumor-targeted liposomal drug delivery system (DDS) containing doxorubicin (DOX) or cisplatin (CIS) as anticancer drugs, a synthetic analog of Luteinizing Hormone-Releasing Hormone (LHRH) as tumor targeting moiety, and Antisense Oligonucleotides (ASO) targeted to MDR1 and BCL2 mRNA as suppressors of pump and nonpump resistance, respectively. We tested the DDS in a mouse xenograft model of human metastatic ovarian cancer with intraperitoneal metastases. Primary tumors were developed by subcutaneous injection of cancer cells isolated from fresh malignant ascites of patients with advanced ovarian carcinoma.
7.2 Materials and methods

7.2.1 Materials

Egg phosphatidylcholin (EPC), Cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-aminopolyethelyenglycol – Mw -2000 ammonium salt (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL); P-ethoxy modified antisense oligonucleotides targeted to MDR1 (5’–TTC AAG ATC CAT CCC GAC CTC GCG –3’) and BCL2 (5’–CAG CGT GCG CCA TCC TTC CC–3’) mRNA were synthesized according to our design (48, 52, 174) by Oligos Etc. (Wilson, OR); cisplatin and doxorubicin were purchased from Sigma (St. Louis, MO). A synthetic analog of LHRH, Lys6–des-Gly10–Pro9-ethylamide (Gln–His–Trp–Ser–Tyr–DLys–Leu–Arg–Pro–NH–Et) was synthesized according to our design (49, 95-97, 133, 162, 174) by American Peptide (Sunnyvale, CA). For in vitro experiments, LHRH peptide was labeled by Rhodamine (Invitrogen Molecular Probs, Carlsbad, CA) as previously described (133).

7.2.2 Drug formulations

PEGylated liposomes were prepared as previously described (42). Briefly, lipids: EPC, Chol, and DSPE-PEG were dissolved in chloroform, evaporated to a thin film layer using rotary evaporator Rotavapor® R-210/R-215 (BUCHI Corp., New Castle, DE, USA) and rehydrated with the appropriate buffer to the final lipid concentration 40 mM. The lipid
mole ratio for all formulations was 51:44:5 EPC: Chol: DSPE-PEG respectively. To prepare tumor-targeted liposomes, DSPE-PEG was conjugated with LHRH peptide as previously described (49) and added together with regular DSPE-PEG. To prepare CIS loaded liposomes, thin film layer was rehydrated in 0.9 % NaCl containing 2 mg/ml of CIS. ASO targeted to BCL2 and MDR1 mRNA were dissolved in rehydration buffer in concentrations 0.25 mM of each ASO (42). Free cisplatin and ASO were separated from liposomes by dialyses against 100 volumes of 0.9 % NaCl during 24 hours. To prepare DOX loaded liposomes, thin film layer was rehydrated with 300 mM citrate buffer (pH = 4) followed by overnight dialysis against 0.9% NaCl at 4°C, and incubated with DOX solution (5 mg/ml) at 37°C during 40 min (162). Liposomes were separated from free DOX by overnight dialysis against 100 volumes 0.9% NaCl. All liposomal samples were gradually extruded through 200 nm and 100 nm pore size polycarbonate filters with an extruder apparatus (Northern Lipids Inc., Vancouver, BC, Canada). The size of liposomes (~100 nm) was measured using Particle Sizer Analyzer (Brookhaven Instruments, New York, NY).

Formulations that we used to treat mice with included: (1) Saline (untreated control); (2) Liposomes (Lip); (3) LHRH; (4) DOX; (5) Lip-DOX; (6) Lip-DOX-BCL2 ASO; (7) Lip-DOX-MDR1 ASO; (8) Lip-DOX-BCL2-MDR1 ASO; (9) LHRH-Lip-DOX-BCL2-MDR1 ASO; (10) CIS; (11) Lip-CIS; (12) Lip-CIS-BCL2 ASO; (13) Lip-CIS-MDR1 ASO; (14) Lip-CIS-BCL2-MDR1 ASO; (15)
LHRH-Lip-CIS-BCL2-MDR1 ASO; (16) DOX + CIS; (17) Lip-DOX + Lip-CIS; (18) Lip-DOX-BCL2 ASO + Lip-CIS-BCL2 ASO; (19) Lip-DOX-MDR1 ASO + Lip-CIS-MDR1 ASO; (20) Lip-DOX-BCL2-MDR1 ASO + Lip-CIS-BCL2-MDR1 ASO; (21) LHRH-Lip-DOX-BCL2-MDR1 ASO + LHRH-Lip-CIS-BCL2-MDR1 ASO.

7.2.3 Cancer cells

Discarded anonymous pathological materials obtained from the Cancer Institute of New Jersey were used to isolate cancer cells from tissues obtained from patients with ovarian carcinoma. The fluid with cancer cells was obtained from the peritoneum area of the patients with ovarian cancer. The samples were centrifuged for 20 minutes at 2000 g; the supernatant was discarded and cell pellets were consequently resuspended. The resuspended cells were cultured in RPMI media supplemented with fetal bovine serum, 2.5 μg/ml insulin and 1.2 ml/100 ml penicillin-streptomycin. In order to visualize cells and document tumor growth, ascitic cells were transfected for 24 hr in a 6-well plate with pMetLuc-Control vector (Clontech, Mountain View, CA) with neomycin resistance gene using Lipofectamine™ 2000 (Invitrogen™, Carlsbad, CA) according to the manufacture’s recommendation.

7.2.4 Animal model and in vivo antitumor activity

To create animal model, briefly, human ascitic cells were subcutaneously transplanted
into the flanks of female athymic nu/nu mice (Taconic, Hudson, NY). When the tumors reached a size of about 0.3 cm\(^3\) (15-20 days after transplantation), mice were treated intraperitoneally with all the 21 formulations as listed above. The doses of DOX (2.5 mg/kg) and CIS (2.5 mg/kg) in formulations were corresponded to the maximum tolerated dose of these drugs estimated in separate experiments as previously described (92, 96, 97, 133). The animals were treated maximum 8 times over four weeks and the development of primary tumor and intraperitoneal metastases was monitored by IVIS Lumina Imaging System (Xenogen, Alameda, CA) and Vevo 2100 Ultrasound System (VisualSonics, Toronto, Canada). To initiate bioluminescence of cancer cells, 1X substrate/reaction buffer (Clontech, Mountain View, CA) was injected to each mouse and the bioluminescent images were taken after a certain time. The size of primary tumor was measured by a caliper. At the end of the experiments, tumors and ascites were excised and their mass was measured. Animal weight was evaluated every day during the treatment period. Changes in tumor size were used as an overall marker for antitumor activity. According to the protocol approved by the Rutgers University Animal Care and Facilities Committee, animals were euthanized when tumor volume reached approximately 2,000 mm\(^3\) (about 10% of body weight) or when body weight significantly changed when compared with the control untreated group.

7.2.5 Expression of targeted genes and proteins
The expression of MDR1 and BCL2 genes was measured using a quantitative RT-PCR as previously described (42). Gene expression was calculated as a percent of internal standard (β2-microglobulin). To identify the presence of BCL2, P-glycoprotein and Caspase 3 (CASP3) proteins, the immunohistochemical staining of paraffin embedded tumor tissue slides was carried out. For BCL2 and CASP3, slides were deparaffinized in xylene for 5 min followed by progressive rehydration in 100%, 95%, 70% and 50% ethanol for 3 min during each step. Endogenous peroxidase activity was blocked by incubating slides in 3% H2O2 solution in methanol at room temperature for 10 min and washing in 300 ml PBS two times for 5 min. Then slides were stained with anti-mouse monoclonal antibodies for BCL2 conjugated with FITC (BioLegend, San Diego, CA) and for CASP3 conjugated with Alexa Flour 647 (BioLegend, San Diego, CA) by incubating for an hour, washed in 300 ml PBS two times for 5 min and analyzed by fluorescent microscopy. To identify the expression of P-glycoprotein, after deparaffinization and rehydration, the slides were stained using Vector® M.O.M. Immunodetection Kit (Vector Laboratories, Inc., Burlingame, CA). Mouse monoclonal antibody to P-glycoprotein obtained from Abcam (Cambridge, MA) was used as primary antibody for the detection of P-glycoprotein. Biotinylated anti-mouse IgG Reagent and HSP-Streptavidine Detection System in combination with DAB substrate kit (Vector Laboratories, Inc., Burlingame, CA) for peroxidase were used for visualization. After staining, the slides were visualized by light microscopy and photographed.
7.2.6 Histopathologic analysis

After sacrificing an animal, the tumors and organs were extracted and immediately fixed in 10% phosphate-buffered formalin. Samples were subsequently dehydrated and embedded in Paraplast®. Five micrometer slides were cut and stained with hematoxylin-eosin as previously described (175) and analyzed.

7.2.7 Apoptosis

The apoptosis induction was analyzed by the measurement of the enrichment of histone-associated DNA fragments (mono- and oligo-nucleosomes) in homogenates of the tumor, malignant ascites and other organs (liver, kidney, lung, heart and brain) using anti-histone and anti-DNA antibodies by a cell death detection ELISA Plus kit (Roche, Nutley, NJ) as previously described (48, 96, 175).

7.2.8 Statistical analysis

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as a mean value ± standard deviation (SD) from five independent measurements. We analyzed data sets for significance with Student’s test and considered P value of less than 0.05 as statistical significant.
7.2.9 Veterinary care

Veterinary care was provided by Rutgers University Laboratory Animal Services. All procedures were performed according to the guidelines set by the National Institute of Health Guide and Use of Animals and the Institutional Animal Care and Use Committee at Rutgers University, NJ.

7.3 Results

7.3.1 Development of primary solid tumors and intraperitoneal metastases

To create a mouse model of human ovarian carcinoma, cells isolated from human malignant ascites obtained from patients with advanced ovarian cancer were transfected with luciferase or green fluorescent protein (Fig. 7.1a) and subcutaneously injected in the flank of nude mice (Fig. 7.1b). Transfection of cancer cells allowed for their visualization in live anesthetized animals using a bioluminescence IVIS imaging system. In untreated animals, the growth of primary solid subcutaneous tumors in 80% of animals was accompanied by the development of malignant intraperitoneal ascites and carcinomatosis (Fig. 7.1c). In addition to bioluminescence visualization, the existence of intraperitoneal metastases and malignant ascites was also confirmed in live animals using ultrasound Vevo imaging system (Fig. 7.1d, f).

To deliver anticancer drugs specifically to tumor cells we used the LHRH peptide as a
targeting moiety. LHRH receptors (LHRHR) are overexpressed in many types of cancer cells and are not expressed in a detectable level in visceral organs (Fig. 7.1h). Although the expression of these receptors was found in tissues of healthy non-tumorous reproductive organs, the expression of LHRHR in tumor cells was significantly higher when compared with corresponding healthy tissue taken from the same organ of the same patient (Fig. 7.1i). To determine whether LHRHR are overexpressed in cells isolated from human malignant ascites, we incubated ascitic cells with LHRH peptide labeled with Rhodamine and registered its fluorescence by fluorescence microscopy. The fluorescence of labeled LHRH peptide was observed predominantly in the plasma membrane of cancer cells (Fig. 7.1e) where LHRH receptors are localized. In addition, we measured the expression of mRNA encoded LHRHR in cells isolated from human malignant ascites and found that these receptors were overexpressed in malignant ascites (Fig. 7.1g). Consequently, LHRH peptide can be used for targeting cancer cells in both primary tumors and intraperitoneal metastases. We constructed a complex tumor-targeted proapoptotic drug delivery system that contained PEGylated liposomes as carriers, LHRH peptides conjugated to distal ends of PEG polymers as targeting moieties, CIS or DOX as anticancer drugs-cell death inducers, and MDR1 and BCL2 antisense oligonucleotides (ASO) as suppressors of pump and nonpump cellular resistance, respectively (Fig. 7.1j). CIS, as a substance with low aqueous solubility, was located in the phospholipid bilayer of the liposomal membrane, while water-soluble DOX and P-ethoxy-modified electrically neutral ASO were located in the inner aqueous space of
neutral liposomes.

7.3.2 Histology of tumor and malignant ascites

Solid primary tumors developed from cells obtained from patients with malignant ascites demonstrated at least three distinct histological types (Fig. 7.2, left panel). The predominant type consisted of solid sheets and nests of markedly pleomorphic, undifferentiated tumor cells with large, irregular nuclei and 1-2 prominent basophilic nucleoli. The cytoplasm was lightly eosinophilic and cell margins were not identifiable. The tumor cells displayed numerous mitotic figures, many of which were atypical. Multinucleated tumor giant cells were common. The margins of the tumor nests were surrounded by fine fibrovascular connective tissue. The second distinct histological type, occupying approximately 30% of the tumor, consisted of ribbons and nests of well-differentiated tumor cells forming identifiable glands. The epithelium of the glands was columnar, usually with a single row of circular to elongated nuclei. The nuclei were moderately pleomorphic and clear, with one or more nucleoli. The glandular element showed a high level of mitotic activity. The third tumor type consisted of aggregates of extremely large, pleomorphic cells with multiple large basophilic nuclei. Many of these cells contain abundant, brightly eosinophilic cytoplasm. The cytoplasm ranged in consistency from dense to vacuolated. This phase of the tumor is primarily localized to the margins of the tumor mass, adjacent to
the skeletal muscle and may represent a degenerative stage of the cancer. There were suggestions of squamous differentiation, including cytokeratin and keratin pearls. Infrequent psammoma bodies were identified diffusely within the tumor mass. Psammoma bodies are predominately seen in ovarian serous adenocarcinomas (176-178).

Histologically untreated intraperitoneal metastases consisted of numerous spherical tumor nodules adherent to white adipose tissue (Fig. 7.2, middle panel). The nodules were comprised primarily of poorly differentiated cancer cells, some displaying several squamous characteristics. These cells were eosinophilic, round to polygonal in shape, and demonstrated considerable nuclear pleomorphism and a high mitotic rate. Many of the mitotic figures were atypical, with tripolar or tetrapolar divisions easily identified. In discrete regions, the cell membrane showed distinct “prickle” attachments characteristic of squamous differentiation. The tumor cell nuclei were large and showed variable amounts of heterochromatin and 1-3 basophilic nucleoli. At the center of some tumor nodules were cystic spaces containing a fibrovascular core. The tumor nodules were enclosed by a layer of flattened to cuboidal epithelial cells. The flattened cells showed clear squamous differentiation, while the cuboidal cells were without discernable lineage differentiation. The fat to which the nodules were attached was focally infiltrated with polymorphonuclear neutrophilic leukocytes and lymphocytes.
Histological analysis showed that mice with subcutaneous tumors treated with combination of two DDS (LHRH-Lip-DOX-BCL2-MDR1 ASO and LHRH-Lip-CIS-BCL2-MDR1 ASO) underwent both apoptosis and necrosis (Fig. 7.2, right panel). Many of the tumor nests displayed central necrosis and glassy-eosinophilic cytoplasmic swelling. Clusters of brightly eosinophilic tumor cells were noted; these contained ample, swollen and degenerating cytoplasm with well-developed zones of tumor necrosis. Treated tumor nodules consisted of necrotic tumor tissues and showed a fibroblastic reparative response. The majority of the nodules contained ‘ghosts’ of dead tumor cells, hyalinized connective tissue, and a mild mixed inflammatory response. Only rare viable malignant cells were identified within the nodules, and these were represented by single or small clusters of cells, some of which are undergoing degeneration. The degree of tumor killing was >99% of the cells. Macrophages containing a granular, light gold material were present within the dead tumor mass and abundantly within the lymph node. No ascites development was registered in mice with primary tumor treated with the aforementioned combination of DDS.

7.3.3 Expression of targeted genes and proteins

Analysis of in vivo expression of targeted genes responsible for cellular drug resistance showed that empty liposomes and LHRH peptide alone (controls) did not change significantly the expression of both MDR1 and BCL2 genes (Fig. 7.3a, b, bars 1-3).
Treatment of mice bearing xenografts of human malignant ascites with free DOX, CIS and their combination led to statistically significant overexpression of both genes (Fig. 7.3a,b, bars 4, 10, 16). Liposomal formulations of DOX and CIS induced overexpression of BCL2 mRNA (Fig. 7.3b, bars 5, 11, 17). However, the delivery of drugs as liposomal formulations led to the decrease in the expression of the MDR1 mRNA when compared with free drugs (Fig. 7.3a, compare bars 4 and 5, 10 and 11, 16 and 17). Simultaneous delivery of anticancer drugs with ASO targeted to MDR1 mRNA decreased the expression of the MDR1 gene (Fig. 7.3a, bars 7-9, 13-15, 19-21). Similarly, simultaneous delivery of anticancer drugs with ASO targeted to BCL2 mRNA decreased the expression of the BCL2 gene (Fig. 7.3b, bars 6, 8, 9, 12, 14, 15, 18, 20, 21). Direct measurement of the expression of corresponding proteins (Fig. 7.4) supports the results of gene expression analysis. In fact, the expression of P-glycoprotein (encoded by the MDR1 gene) and BCL2 protein in xenografts of human ovarian cancers was increased after the treatment of mice with free and liposomal forms of the drugs (Fig. 7.4, panels a-b, #2-5), while incorporation of ASO into the liposomal delivery system suppressed these proteins (Fig. 7.4, panels a-b, #6-10). It should be stressed that targeting of liposomal DDS to tumor cells by LHRH peptide led to a more complete suppression of the expression of both P-glycoprotein and BCL2 (Fig. 7.4, compare #8-10 with #6-7 on panels a-b).
We studied apoptosis induction in tumor, malignant ascites, and healthy organs (liver, kidney, spleen, heart, lung and brain) after the treatment of mice with different drug formulations by measuring the expression of apoptosis executor - caspase 3 (Fig. 7.4, panel c) and by immunochemical determination of histone-complexed DNA fragments (mono- and oligonucleosomes) (Fig. 7.5). Empty liposomes and LHRH did not induce detectable levels of apoptosis either in tumor, ascites, or healthy organs (Fig. 7.5, bars 1, 2, 3). Free DOX, CIS and their combination activated caspase 3 and induced apoptosis in tumor and malignant ascites (Fig. 7.4, panel c, #2; Fig. 7.5, bars 1, 4, 10, 16). However, in addition to inducing apoptosis in tumor and malignant ascites, treatment of mice with free drugs led to the substantial apoptosis induction in the liver, kidney, spleen, heart and lung. Incorporation of drugs into the liposomes substantially enhanced their ability to induce apoptosis in solid tumors and accompanying intraperitoneal metastases and limited apoptosis induction in kidney, spleen, heart and lung (Fig. 7.5, bars 5, 11, 17). However, apoptosis in the liver remained augmented after the treatment of mice with all non-targeted liposomal DDS (Fig. 7.5, bars 5-8, 11-14, 17-20). Suppression of both types of cellular resistance by ASO targeted to MDR1 and BCL2 mRNA substantially increased the expression of caspase 3 (Fig. 7.4, panel c, #6) and apoptosis induction (Fig. 7.5, bars 6-8, 12-14, 18-20). Targeting of DDS containing anticancer drugs and suppressors of pump and nonpump resistance specific to cancer cells by LHRH peptide...
led to several positive consequences. First, it increased the level of suppression of targeted proteins and therefore augmented the activation of caspase 3 and apoptosis itself in cancer cells (Fig. 7.4, panel c, #8-10; Fig. 7.5, bars 9, 15, 21). Second, the delivery of drugs and other active components specifically to cancer cells prevented the induction of apoptosis in the liver as well as all other studied healthy organs (Fig. 7.5, bars 9, 15, 21). Third, incorporation of a targeting moiety in DDS led to the prevention of the development of intraperitoneal metastases and malignant ascites (Fig. 7.5, bars 9, 15, 21 and Fig. 7.6, bars 9, 15, 21).

7.3.5 Antitumor effect

The antitumor effect of all studied formulations was estimated by the measurement of the volume of the primary tumor and total mass of the intraperitoneal metastases. Treatment of mice with saline, empty liposomes, and LHRH peptide (controls) did not influence the growth of primary tumor or the development of intraperitoneal metastases (Fig. 7.6 lines and bars 1-3). Free and liposomal DOX slowed down the growth of the primary subcutaneous tumor but was not effective in preventing the formation of malignant ascites or intraperitoneal metastases and did not statistically significantly decrease their mass (Fig. 7.6, lines and bars 4, 5). Free and liposomal CIS was more effective in limiting the growth of both primary tumor and malignant ascites when compared to corresponding DOX formulations (compare lines and bars 10, 11 with 4, 5 in Fig. 7.6).
The combination of two drugs (free and liposomal forms) was more effective when compared with formulations containing only one drug (compare lines and bars 16, 17 with 4, 5 and 10, 11 in Fig. 7.6). Inclusion of ASO targeted to MDR1 and/or BCL2 mRNA into the liposomal drug formulations led to the more significant suppression of the growth of the primary tumor, carcinomatosis and ascites (Fig. 7.6, lines and bars 6-8, 12-14, 18-20). Targeting of DDS specifically to cancer cells by LHRH peptide further increased antitumor activity of liposomal DDS and prevented the development of intraperitoneal metastases and ascites (Fig. 7.6, lines and bars 9, 15, 21).

7.4 Discussion

The present work was aimed at examining the antitumor effect of a liposomal tumor-targeted proapoptotic anticancer drug delivery system on the primary tumor and intraperitoneal metastases mouse model developed after subcutaneous inoculation of cancer cells isolated from malignant ascites of patients with ovarian carcinoma. In fact, inoculation of these cancer cells provoked, in untreated mice, the development of two models of cancer. The first one represented a subcutaneous model of aggressive primary tumor. The second model of intraperitoneal metastases with ascites developed in approximately 80% of untreated mice. This mouse model of human intraperitoneal carcinomatosis with ascites is different from traditionally used orthotopic models when established cancer cells, primary tumor isolates, or cells from human malignant ascites are
intraperitoneally injected into nude mice (179-183). In our opinion, this model more adequately showed the natural progression of ovarian cancer with the development of carcinomatosis and ascites. It is interesting that this model can be effectively employed only when the cells that are used for the initiation of primary subcutaneous tumor are very invasive and multidrug resistant. Retrospectively analyzing our previous experimental data, we found that intraperitoneal metastases never developed when drug sensitive ovarian cancer cells were injected subcutaneously. When established human multidrug resistant ovarian cancer cell lines or primary tumor isolates were used, malignant ascites accompanied only approximately 10% of primary tumors. Consequently, one can conclude that cancer cells isolated from human ovarian malignant ascites are substantially more invasive when compared with established multidrug resistant cancer cell lines. Such an assumption is also supported by the data of Veatch et al (62) who found that “…ascites cells were 4-fold more invasive than solid tumor cells.” In addition, the development of multidrug resistance accompanied by overexpression of the MDR1 gene was observed in the laboratory of Dr. Scotto in tumors of patients treated with DOX (184). Taking into account that cells from ovarian malignant ascites belong to a multidrug resistant phenotype, we hypothesized that effective therapy of intraperitoneal metastases and ascites requires drugs with different mechanisms of action and the simultaneous suppression of both cellular pump and nonpump resistance. Ideally, any cytotoxic treatment should preferentially target cancer cells sparing healthy tissues. We were successful in accomplishing this by targeting the LHRH receptor (96, 97, 185). The
experimental verification of our hypothesis showed several advantages of the proposed approach.

The first advantage is the simultaneous suppression of both major types of cellular resistance. We found that antisense oligonucleotides targeted to MDR1 and BCL2 mRNA and delivered by PEGylated liposomes effectively suppressed both the major drug efflux pump (P-glycoprotein) and the antiapoptotic cellular defense (BCL2 protein) leading to the inhibition of cellular pump and nonpump resistance, respectively. Second, such suppression led to the substantial enhancement of cell death and increased efficiency of standard cytotoxic drugs to levels that could not be achieved using conventional therapy by free or liposomal forms of the drugs. Third, effective cell death induction in primary tumor and intraperitoneal metastases led to the substantial regression of tumor growth and decrease in total mass of intraperitoneal metastases. Fourth, targeting of DDS specifically to ovarian cancer cells significantly reduced the adverse side effects of the treatment on healthy organs. This effect is explained by the specific body distribution of the tumor targeted delivery system, where the major part of intravenously injected tumor-targeted DDS is accumulated in tumor cells, while only trace amounts of DDS can be found in healthy organs (96, 97). The fifth advantage of the proposed approach includes the prevention of the development of detectable intraperitoneal metastases and ascites after the treatment of an aggressive primary ovarian tumor with the targeted DDS. Moreover, we found that each targeted DDS containing either one drug alone or a drug
combination prevented the formation of carcinomatosis and ascites. In contrast, similar non-targeted systems containing the same components (drug, ASO targeted to MDR1 and ASO targeted to BCL2 mRNA) did not produce such an effect. These results reinforce the importance of targeting anticancer therapy specifically to ovarian cancer cells (by a ligand to extracellular receptors overexpressed in cancer cells) for the prevention of the development of intraperitoneal metastases and ascites. The aforementioned advantages of the proposed targeted combinatorial treatment of primary aggressive ovarian tumor and prevention of the development of intraperitoneal metastases make the proposed approach utilizing tumor-targeted delivery systems ideal for clinical applications.

7.4.1 Conclusions

The targeted combinatorial treatment of primary aggressive ovarian tumor led to the substantial regression of the growth of primary tumor, prevention of the development of intraperitoneal metastases and limitation of the adverse side effects of chemotherapy on healthy organs. That makes the developed tumor-targeted delivery systems highly promising for clinical applications.
**Figure 7.1** Experimental model and treatment. a, Representative image of cells isolated from human malignant ascites.
from human malignant ascites and transfected with luciferase or green fluorescent protein. b-d, f, Cells isolated from human malignant ascites were injected into the flanks of nude mice resulting in the formation of solid primary tumor (b) and intraperitoneal metastases (c, d, f). Typical bioluminescent (b, c, IVIS imaging system) and ultrasound (d, f, Vevo 2100 imaging system) images of a live anesthetized mouse with primary and metastatic tumors. e, Expression of LHRH receptors (LHRHR) in the plasma membrane of cells isolated from human malignant ascites obtained from patients with ovarian carcinoma. The cells were incubated with LHRH peptide labeled by Rhodamine (red fluorescence). g, Expression of LHRHR and MDR1 gene encoding P-glycoprotein in cells isolated from human malignant ascites. h-i, Representative images of gel electrophoresis and quantitation of RT-PCR products of gene encoding LHRHR. Means ± S.D. are shown. *P < 0.05 when compared with healthy tissues from the same patient. j, Multifunctional tumor-targeted liposomal delivery system.
Figure 7.2 Histology of tumor and intraperitoneal metastases. Treatment of aggressive subcutaneous tumor with combination therapy (LHRH-Lip-DOX-BCL2-MDR1 ASO + LHRH-Lip-CIS-BCL2-MDR1 ASO) led to the significant changes in histopathological pattern of the developed solid subcutaneous and metastatic tumors. Upper panel: typical IVIS images of mice bearing subcutaneous xenografts of human malignant ascites. The tumor was accompanied by the development of intraperitoneal metastases. Bottom panel: typical microscopy images of tumor tissues and intraperitoneal metastases stained with hematoxylin-eosin.
**Figure 7.3** Gene expression in subcutaneous tumors. a, MDR1 gene expression. b, BCL2 gene expression. Mice bearing xenografts of human malignant ascites were treated 8 times within 30 days with the following substances: (1) Saline (untreated control); (2) Liposomes (Lip); (3) LHRH; (4) Doxorubicin (DOX); (5) Lip-DOX; (6) Lip-DOX-BCL2 ASO; (7) Lip-DOX-MDR1 ASO; (8) Lip-DOX-BCL2-MDR1 ASO; (9) LHRH-Lip-DOX-BCL2-MDR1 ASO; (10) Cisplatin (CIS); (11) Lip-CIS; (12) Lip-CIS-BCL2 ASO; (13) Lip-CIS-MDR1 ASO; (14) Lip-CIS-BCL2-MDR1 ASO; (15) LHRH-Lip-CIS-BCL2-MDR1 ASO; (16) DOX + CIS; (17) Lip-DOX + Lip-CIS; (18)
Lip-DOX-BCL2 ASO + Lip-CIS-BCL2 ASO; (19) Lip-DOX-MDR1 ASO + Lip-CIS-MDR1 ASO; (20) Lip-DOX-BCL2-MDR1 ASO + Lip-CIS-BCL2-MDR1 ASO; (21) LHRH-Lip-DOX-BCL2-MDR1 ASO + LHRH-Lip-CIS-BCL2-MDR1 ASO. Means ± S.D. are shown. *P < 0.05 when compared with untreated control.
**Figure 7.4** Protein expression in subcutaneous tumors. Typical images of tumor tissue sections stained with antibody against P-glycoprotein (a), BCL2 (b) and Caspase 3 (c) proteins. High intensity of the color indicates high protein concentration. Mice bearing xenografts of human malignant ascites were treated 8 times within 30 days with the following substances: (1) Saline (untreated control); (2) DOX+CIS; (3) Lip-DOX; (4) Lip-CIS; (5) Lip-DOX + Lip-CIS; (6) Lip-DOX-BCL2-MDR1-ASO; (7) LHRH-Lip-CIS-BCL2-MDR1-ASO; (8) LHRH-Lip-DOX-BCL2-MDR1-ASO; (9) LHRH-Lip-CIS-BCL2-MDR1-ASO; (10) LHRH-Lip-DOX-BCL2-MDR1-ASO + LHRH-Lip-CIS-BCL2-MDR1-ASO.
Apoptosis induction in subcutaneous tumors and intraperitoneal metastases.

Mice bearing xenografts of human malignant ascites were treated 8 times within 30 days with the following substances: (1) Saline (untreated control); (2) Liposomes (Lip); (3) LHRH; (4) DOX; (5) Lip-DOX; (6) Lip-DOX-BCL2 ASO; (7) Lip-DOX-MDR1 ASO; (8) Lip-DOX-BCL2-MDR1 ASO; (9) LHRH-Lip-DOX-BCL2-MDR1 ASO; (10) CIS;
Figure 7.6 Treatment with tumor-targeted complex delivery systems containing two anticancer drugs with different mechanisms of action and suppressors of pump and nonpump cellular drug resistance substantially inhibits the growth of subcutaneous tumor and prevents the development of intraperitoneal metastases. Cancer cells were isolated
from malignant ascites obtained from patients with ovarian carcinoma and injected subcutaneously into the flanks of nude mice. When the tumors reached a size of about 0.3 cm³ (15-20 days after transplantation), mice were treated maximum 8 times within 30 days with substances indicated. Upper panel represents tumor growth during the treatment; bottom panel represents the mass of intraperitoneal metastases at the end of the treatment. (1) Saline (untreated control); (2) Liposomes (Lip); (3) LHRH; (4) Doxorubicin (DOX); (5) Lip-DOX; (6) Lip-DOX-BCL2 ASO; (7) Lip-DOX-MDR1 ASO; (8) Lip-DOX-BCL2-MDR1 ASO; (9) LHRH-Lip-DOX-BCL2-MDR1 ASO; (10) Cisplatin (CIS); (11) Lip-CIS; (12) Lip-CIS-BCL2 ASO; (13) Lip-CIS-MDR1 ASO; (14) Lip-CIS-BCL2-MDR1 ASO; (15) LHRH-Lip-CIS-BCL2-MDR1 ASO; (16) DOX + CIS; (17) Lip-DOX + Lip-CIS; (18) Lip-DOX-BCL2 ASO + Lip-CIS-BCL2 ASO; (19) Lip-DOX-MDR1 ASO + Lip-CIS-MDR1 ASO; (20) Lip-DOX-BCL2-MDR1 ASO + Lip-CIS-BCL2-MDR1 ASO; (21) LHRH-Lip-DOX-BCL2-MDR1 ASO + LHRH-Lip-CIS-BCL2-MDR1 ASO. Means ± S.D. are shown. *P < 0.05 when compared with untreated tumor (saline).
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\section*{Publications}

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