RECEPTOR-MEDIATED DNA-BASED THERAPEUTICS DELIVERY

DISSERTATION

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By

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ABSTRACT

The overall objective of this dissertation was to develop and evaluate receptor-mediated non-viral delivery systems for DNA-based therapeutics. Novel strategies, such as covalent vector stabilization and PEGylation of the vector, might prove critical for the in vivo performance of receptor-targeted vectors. Continued efforts in optimization of receptor-mediated delivery systems will likely lead to the development of tumor-specific vehicles for DNA-based therapeutics delivery and promote the advancement of clinical translation of cancer gene therapy.

In Chapter 2, a non-viral, PEI-based, HER2-targeted gene transfer vector was developed. The anti-HER2 antibody (trastuzumab, Herceptin®) was conjugated to PEI and polyplexes were shown to selectively deliver plasmids to HER2-overexpressing cells with high resistance to serum. Herceptin/PEI polyplexes exhibited promising HER2 receptor-specific gene transfer properties.

In Chapter 3, a modified ethanol dilution method for the preparation of ODN was developed. DC-Chol was chosen as the cationic lipid due to its unique charge property in different pH. Combining with PEG-derivatives, this formulation can avoid some problems including rapid circulation elimination occurred on cationic ODN-liposomal formulations. This method provides a suitable platform to prepare ODN-containing
liposomes. The small size, near-neutral charge, low toxicity, and, more importantly, high encapsulation efficiency of ODNs at optimized conditions are important characteristics for the development of DNA-based therapeutics delivery systems. In the next two chapters, similar method was applied to other antisense delivery systems including ODNs and siRNAs with a high molecular weight ligand.

The aim of Chapter 4 was to develop a targeted ODN(G3139)-containing liposome formulation that can efficiently and specifically delivery ODNs to leukemias. Transferrin receptors were overexpressed in many tumor and leukemia cells, especially those rapidly proliferating tumor cells. A Tf-targeted liposomal formulation of antisense G3139 was evaluated in K562 leukemia cells, which exhibited excellent characteristics in terms of particle size, loading efficiency, colloidal stability, and vehicle toxicity. Furthermore, this formulation was very efficient in antisense delivery, showing excellent bcl2 downregulation efficiency and TfR specificity.

In Chapter 5, similar strategy was applied to siRNA delivery. DFO was used to upregulate TfR in K562 cells. The data demonstrated that DFO pretreatment increased the uptake of TfR-targeted siRNA in K562 cells and exhibited higher luciferase downregulation effect. Tf-targeted siRNA formulation with DFO pretreatment was a highly efficient delivery vehicle for siRNA for leukemias that express TfR. This formulation provides the prospect of more selective targeting effect in association with increased intracellular concentrations in target cells. More future studies such as optimization and in vivo studies are needed for this formulation to work clinically.
Dedicated to My Parents
ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 DNA-based Therapeutics

DNA-based therapeutics include plasmids containing transgenes for gene therapy, oligodeoxyribonucleotide (ODN) for antisense applications, small interference RNAs (siRNAs), etc. Although most DNA-based therapeutics are still in early stages of clinical development, they have emerged as promising candidates for a wide range of diseases such as cancer, AIDS, cardiovascular diseases, and neurological disease including Alzheimer disease and Parkinson’s disease. One of the advantages of DNA-based therapeutics over currently available drugs is their selective recognition of molecular targets and pathways, which contribute tremendous specificity to their actions. For example, for DNA-based therapeutics designed for gene ablation, only selected genes would be switched off, which assures the specificity of disease treatment and decreases the side effects in other cells types. Despite some favorable characteristics and some success in preclinical studies, the introduction of DNA-based therapeutics into human could be described as limited, with rare success [1]. Up to date, there is only one DNA-
based therapeutic (vitravene, antisense ODN) approved by FDA and several other are in Phase 3 clinical trials (Table 1.1). The inertia in the development of DNA-based therapeutics could be, in part, ascribed to their inefficient cellular uptake and unfavorable pharmacokinetic profile in vivo. Under normal conditions, the internalization of DNA-based therapeutics alone is minimal due to their physicochemical properties such as molecular weight and charges. Furthermore, DNA molecules that do manage to enter cells are subject to intracellular degradation as well as restricted nuclear access. Therefore, in the past decades, viral- and non-viral gene transfer vectors were developed to improve the delivery of DNA-based therapeutics into cells. A schematic representation of delivery of DNA-based therapeutics using delivery vectors is shown in Figure 1.1.

Three types of DNA-based therapeutics including plasmids, antisense ODNs, and siRNAs were used in this dissertation and will be briefly introduced in the following sections.

1.1.1 Plasmids

Plasmids are high molecular weight, double strand DNA constructs containing transgene encoding specific proteins [2]. Plasmids have been used to introduce transgene into cells that inherently lack an ability to produce specific proteins. Plasmids can be used to displace or to repair a defective gene that is leading to disease, such as cystic fibrosis, hemophilia, and sickle cell anemia, or to provide a new or changed function to a cell such as introducing resistance to HIV or programming an immune cell to attack cancer.
The mechanism of action of plasmids requires DNAs to enter the nucleus after entering cytoplasm. Due to its high molecular weight (1-100 kb), the entry into cells and to the nucleus via nuclear membrane pores are extreme challenges in the gene delivery field [3]. While viral vectors can only carry plasmids smaller than 10 kb, non-viral gene transfer vectors, theoretically, have no limitations in the size of plasmids that they can deliver. Combined with other favorable characteristics such as low immunogenicity, a non-viral gene transfer vector was therefore chosen in the current study.

Because the physicochemical properties of plasmids are primarily determined by their size and topology and the properties of gene transfer vectors are independent of the DNA sequence carried, some reporter genes are commonly chosen to allow rapid evaluation of gene transfer efficiency in designing novel vector formulations. After the desired pharmacokinetic and pharmacodynamic profiles of gene expression in the target tissues have been established, the reporter gene construct can be substituted by the therapeutic gene construct. Commonly used reporter genes include β-galactosidase (β-gal), firefly luciferase (luc), and green fluorescence protein (GFP).

1.1.2 Antisense oligodeoxyribonucleotide (AS-ODN)

Antisense oligodeoxyribonucleotide (AS-ODN) are short (typically 15-20 bases) single-stranded DNA that can selectively inhibit the expression of a single protein [4]. For antisense applications, AS-ODNs interact and form a duplex with mRNAs or pre-mRNAs and then inhibit their translation, which consequently inhibits protein synthesis.
On the molecular level, several mechanisms of antisense actions have been proposed for antisense drugs. These include inhibition of transcription, inhibition of splicing and inhibition of mRNA maturation, etc [5]. Among them, activation of RNase H is by far one of the most recognized theories accepted by many researchers. After entering cells, ODNs hybridize to the target mRNA and form a sense-antisense RNA-DNA duplex. The formation of the duplex initiates recruitment of an endogenous nuclease, the RNase H enzyme. RNase H will degrade the target mRNA by degrading the RNA strand of RNA-DNA duplex, which consequently inhibits target mRNA expression. AS-ODNs will move on to another target RNA molecule and begin another cycle [6].

Design of AS-ODNs is essential for the clinical efficacy of antisense therapy. Several factors including length, chemistry, conformation, and hybridization capacity should be considered. The suggested length of therapeutic AS-ODNs ranges from 12 to 25 bases (12-25 mers). Very short sequences of AS-ODNs are likely to be nonspecific. As the length increases, hydrogen bonding and interactions between base pairs increase, leading to an increase in overall binding affinity between AS-ODNs and the target mRNA. However, as length increases, ODNs become more difficult to internalize to cells due to their size and the tendency to self-hybridize, which, therefore, may affect their cellular uptake.

AS-ODNs with the endogenous phosphodiester backbones (PO-ODNs) are susceptible to degradation by nucleases and therefore have limited clinical use [6]. Various chemical modifications has been attempted to (a) improve the stability of ODNs
and the affinity to target mRNA, (b) facilitate recruitment of RNase H to efficiently
cleave target mRNA, and (c) reduce or eliminate the toxicity of oligonucleotides (Figure
1.2). PO-ODNs are capable of recruiting and activating RNase H; however, they are
sensitive to nucleases and exhibit short half-lives in circulation and intracellularly. The
most common modifications include the introduction of phosphorothioate (PS-ODNs)
and methyl phosphonate linkages into the backbone (Figure 1.2). PS-ODNs, also known
as first-generation AS-ODNs, have significantly increased half-lives in vitro and in vivo
and still retain the ability to recruit RNase H to degrade mRNA/PS-ODN hybrids [5].
More importantly, this modification is much more resistant to nucleases than the parent
PO-ODN [7]. Some other chemical modifications of AS-ODN are shown in Figure 1.2.
Most of them are still in early stages of development.

For therapeutic purposes, AS-ODN can be used to selectively block the
expression of specific proteins that are essential to some disease development [8]. For
example, fomivirsen sodium (Vitravene, ISIS Pharmaceuticals, Carlsbad, CA), the first
and only FDA-approved DNA-based therapeutic agent, was designed to inhibit human
cytomegalovirus for the treatment of cytomegalovirus retinitis in AIDS patients.
Genasense (oblimersen sodium, G3139), developed by Aventis and Genta, is being
investigated in phase 3 clinical trials in combination with other chemotherapeutic agents
for various cancers including malignant melanoma, chronic lymphocytic leukemia, and
multiple myeloma [7]. Genasense is an 18-mer antisense oligonucleotide designed to
specifically bind the first six codons of human Bcl2 mRNA, thereby resulting in a
decrease in Bcl-2 protein translation [9, 10]. Two other drug candidates, Affinitak (ISIS
Pharmaceutics) and Alicaforsen (ISIS Pharmaceutics), also showed good progress and currently are in phase 3 clinical trials (Table 1.1).

1.1.3 RNA interference (RNAi)

RNA interference was first discovered in the nematode *C. elegans*, where treatment with dsRNA resulted in a sequence-specific gene silencing effect [11]. Since then, it has been recently shown that unexpectedly small double strand RNA fragments appear to be very efficient agents to inhibit gene expression in mammalian cells.

The pathway of RNA interference can be broken down into two main phases. In the first phase, long double-stranded RNA (dsRNA) is recognized and digested by the endogenous endonuclease Dicer, an RNase III enzyme, to generate small interfering RNAs (siRNAs). siRNAs are short double-stranded RNA segments with typically 21 to 23 nucleotide bases that are complementary to the mRNA sequence of the target protein [12]. Exogenous synthetic siRNAs or endogenously expressed siRNAs can bypass the requirement for dsRNA processing by Dicer, and also be incorporated into the RNA-induced silencing complex (RISC) – the main molecule in the second phase. For synthetic siRNAs, the second phase starts from passage through the cell membrane and entrance to the cytoplasm. Duplex siRNAs are incorporated into RISC and are unwound by the helicase in RISC. The unwound antisense strand binds to the target mRNA and forms a new sense-antisense duplex. An RNase within RISC, an endogenous nuclease, degrades the target mRNA at sites not bound by the antisense strand of siRNA, which
thereby inhibits target mRNA expression [4]. The composition of RISC is still not completely known.

siRNAs are designed to downregulate disease-causing genes through the above mechanism. Although the use of siRNA as a therapeutic agent is still in its infancy, the therapeutic applications of RNAi are potentially enormous. Since the complementarity between the antisense strand and the target mRNA governs the sequence-specificity of gene silencing, genetic polymorphisms-related disease or tumors that can be targeted for degradation without affecting wild-type RNAs are some most obvious applications. Possible applications of RNAi therapy include cancer therapy for specific oncogene silencing or multidrug resistance, acute liver failure, and some infectious disease such as HIV, influenza viruses, Hepatitis B and C viruses, Herpesviruses, etc [13, 14].

The purposes of AS-ODN and siRNA treatment are similar but the underlying mechanisms are different. It has been reported that RNAi may be more potent than antisense RNA in human cell lines. Bertrand et. al [12] compared efficiencies of a nuclease resistance AS-ODN and of siRNA in HELA cells and in xenografted mice. Their results showed that siRNAs were more efficient and their effect longer lasting in cell culture. In mice studies, only activities of siRNA were observed. More and more promising results shed light on the future of RNAi therapy.

The MWs of siRNAs are around 15,000 – 20,000 Daltons, which is about 2 -3 fold the MW of AS-ODNs but still much smaller than plasmids. Both siRNA and AS-ODN are anionic. Therefore, from formulation and delivery point of view, siRNA and AS-ODNs have similar physicochemical properties. Similar to other DNA-based
therapeutics, delivery is probably still the biggest obstacle for the development of RNAi therapy. Main issues regarding to delivery of siRNAs include stability in circulation, protection from nuclease attack before reaching target cells, cellular uptake, stability in cytoplasm, etc. One important advantage of siRNAs over plasmids is that the action site of siRNAs is in cytoplasm; therefore, nuclear uptake of siRNAs is not a concern while nuclear uptake is clearly an important factor for plasmid DNA delivery. In summary, optimized siRNA delivery with high efficacy and selectivity and rational siRNA design for RNAi may lead to the success of RNAi therapy.

1.2 Barriers to efficient delivery of DNA-based therapeutics

There are many factors that may affect the extent and duration of gene expression following administration of DNA-based therapeutics. Among them, an efficient delivery with the aid of optimized gene transfer vectors could be an essential step for a successful gene therapy. In order to design an efficient gene transfer vehicle, the following biological barriers have to be considered.

(a) Stability in blood circulation. Biological instability is the first barrier to consider when delivering DNA or RNA-based therapeutics. The existence of abundant nucleases in the blood results in a rapid degradation of plasmid after intravenous administration [15]. This can be partially overcome either by condensing DNA with modified polycations or by incorporating another polycationic component [16, 17]. Second, negatively-charged blood components such as serum albumin and lipoproteins may facilitate the aggregation of cationic vectors or even compete for the binding of
DNA to cationic polymers or lipids [18, 19]. Incorporation of polyethylene glycol (PEG) can provide a steric barrier against aggregation, which has proven to be effective for both cationic polymers [20] and lipids [21].

(b) Extravasation limited by particle size. Although tumor blood vessels are relatively permeable compared to normal blood vessels, the particle size of the vector still limits the rate of extravasation [22]. Extravasation is a rate-limiting step or macromolecules and, therefore, constraining the particle size to below 300 nm is needed for sufficient endothelial escape of gene transfer vectors to the tumor interstitium.

(c) Targeting of DNA to particular cell types. Selective binding and internalization of vectors to specific cells can be enhanced by the attachment of targeting ligands to gene transfer vectors. For example, in the current study, Herceptin as a HER2-specific ligand will be incorporated into PEI-based polyplexes to facilitate the uptake of DNA in HER2-overexpressing cells.

(e) Endosomal escape of vectors. The inability of vectors to escape from the endosomal compartment presumably results in the trafficking via late endosomes to lysosomes where the DNA is degraded. Therefore, efficient endosomal escape is essential for a successful gene delivery. For example, PEI can effectively buffer the endosomal compartment, which eventually results in osmolytic lysis of endosomes.

(f) Cytoplasmic stability of DNA. Significant cytoplasmic degradation of naked DNA could severely limit the total amount of DNA delivered to the nucleus. It has been proposed that cytosolic nucleases may be responsible for the degradation of naked DNA in the cytoplasm [23]. Therefore, protecting plasmid DNA from nucleases attack is one
of the important characteristics for an ideal gene transfer vector. Pollard et al [24] reported that cationic polymers but not cationic lipids can promote gene delivery from the cytoplasm to the nucleus.

(e) Nuclear uptake. The nuclear membrane is a barrier that prevents the uptake of macromolecules into the nucleus. Poor access of plasmid DNA to the nucleus is one pitfall of non-viral gene delivery [25]. Nuclear transport may be improved through the attachment of nuclear localization signal peptides which can redirect intracellular protein transport to the nucleus [26, 27].

1.3 Non-viral delivery systems

Early efforts in vector design have focused primarily on genetically engineered viruses, such as retrovirus, adenovirus, and adeno-associated virus [28-30]. However, inherent obstacles associated with these vectors, such as immunogenicity and safety concerns have limited their clinical adoption [31]. Non-viral synthetic vectors are, therefore, being developed as alternatives to viral vectors [31-34]. These vectors provide formulation design flexibility and can be tailored to the size and topology of the DNA cargo as well as a specific route of vector administration. Compared to viral vectors, non-viral vectors are potentially less immunogenic, are relatively easy to produce in clinical quantities, and are associated with fewer safety concerns [31, 33, 34]. Non-viral vectors designed for parenteral administration encompass a wide range of formulations, including unmodified (naked) DNA [35], cationic polymer-DNA complexes (polyplexes) [36], cationic lipid-DNA complexes (lipoplexes) [37], and polymer-lipid-DNA ternary
complexes (lipopolyplexes) [37-39], etc. While unmodified DNA is designed for direct intra-tissue injection, most other types of non-viral vectors are designed for systemic or airway administration [19, 40-46].

1.3.1 Cationic polymers

Polyplexes are formed by electrostatic interactions between negatively-charged plasmid DNA and a cationic polymer. DNA condensation by cationic polymers is an essential step for non-viral gene delivery since DNA itself is almost immobile in extracellular matrix. Poly-L-lysine (PLL, Figure 1.3(A)) [47] and polyethylenimine (PEI, Figure 1.3(B)) are the two most commonly used cationic polymers.

PLL, the first cationic polymer to be developed, can efficiently condense plasmid DNA but it lacks endosomal-lytic activity, which prevents the escape of DNA and results in low transfection activity [48]. Gottschalk et al. [49] reported that folate-PLL carrying CMV/β-gal showed relatively low transfection activity in KB cells. However, when the transfection complex was co-incubated with a replication-defective adenovirus, a 1000-fold increase of β-gal activity was observed. These results suggested that endosomolytic activity, which was provided by the viral particles, was essential for efficient DNA delivery.

PEI—a stable, easy to handle, and inexpensive cationic polymer with average MW ranging from 800 Da to 100,000 Da [50]—has been shown to be among the most efficient non-viral gene delivery vectors per se both in vitro [51-54] and in vivo [55-57]. In contrast to PLL, PEI has both DNA condensing and endosomolytic activities due to its
buffering capacity at mildly acidic pH. PEI alone forms polyplexes with DNA that exhibit high transfection activities, especially at high nitrogen-to-phosphate (N/P) ratios. Unlike PLL, the high positive charge density of PEI facilitated the binding of anionic DNA within the physiological pH range \([51, 58]\). Therefore, PEI is able to form smaller complexes with DNA \([59]\). Since every third atom of PEI is a nitrogen atom that can be protonated, PEI/DNA complexes upon internalization could cause proton accumulation in endosomes via a “proton-sponge” effect \([51, 60]\), which may result in swelling and disruption of endosomes, and consequently endosomal escape of DNA to the cytoplasm \([51, 60]\). Moreover, PEI contains primary amino-groups, through which a desired targeted ligand may be attached. Taken together, these properties make PEI a notable vector for non-viral gene delivery. The transfection efficiency of PEI-based polyplexes was affected by the molecular weight of PEI \([58]\), the particle size of polyplexes \([61]\), and, most importantly, the charge ratio of PEI to DNA (N/P ratio).

1.3.2 Lipidic delivery systems

Liposomes have emerged as one of the most versatile tools for the delivery of DNA therapeutics and many other drugs \([62]\). Many different lipidic systems have been developed as vectors for DNA-based therapeutics \textit{in vitro} and \textit{in vivo}. In early studies, DNA was encapsulated in neutral or anionic liposomes without changing the structures of the liposomes. Cationic liposomes, similar to cationic polymers (polyplexes), interact with DNA through charge interaction and extensive lipid rearrangement occurred during
complex formation. Therefore, the term “lipoplex”, referred to DNA-cationic liposomes-complex, was accepted by many researchers [63].

Cationic liposomes generally consist of mixtures of cationic and zwitterionic lipids. Commonly used cationic lipids are 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 3β(N-(N’N’-dimethylaminoethane)-carbamoyl) cholesterol (DC-Chol), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), etc. The most commonly used zwitterionic lipids, also known as helper lipids, is dioleylphosphatidylethanolamine (DOPE). Cationic lipids in liposomal formulation serve the same function as cationic polymers to form a complex with anionic DNA or RNA and also enhance complex association due to the positive charges. The zwitterionic lipids help in membrane fusion and perturbation. There are several ready-to-use cationic liposomes in the market with different lipid compositions, e.g., Lipofectamine (Invitrogen, Carlsbad, CA) and Lipofectin (Invitrogen).

1.4 Receptor-mediated drug and gene delivery

1.4.1 Herceptin and HER2 receptor

Human epidermal growth factor receptor-2 (HER2, also known as ErbB2, HER-2/Neu, or p185HER2) belongs to a four-member HER receptor family (EGFR, HER2, ErbB3, and ErbB4) involved in signal transduction pathways that regulate cell growth and differentiation [64]. HER2 receptor is a 185 kDa transmembrane glycoprotein consisting of an extracellular domain, a transmembrane domain, and an intracellular domain with intrinsic tyrosine kinase activities [65]. The amplification of HER2/neu
gene and overexpression of HER2 protein have been observed in a number of tumors, especially in breast cancer and ovarian cancers [66], and have been known to remain stable in all tumor stages [67]. The combination of overexpression of HER2 receptors in specific tumors and low expression of HER2 receptors in normal tissues provides a suitable therapeutic window for therapeutic agents targeting HER2, thereby minimizing damage to normal cells. In addition, the presence of an extracellular domain, high expression in tumors yet low in normal tissues, makes HER2 receptor a good candidate as a cellular marker for receptor-mediated cancer therapy.

However, there is no known high-affinity ligand available for HER2 receptor [64], monoclonal antibodies directed against HER2 thus offers a potential strategy for HER2-targeted delivery. Anti-HER2 immunoliposomes have been investigated as a targeted drug delivery vehicle [68-70]. Herceptin (rhuMAb, also known as trastuzumab), a recombinant humanized monoclonal HER2 antibody approved by FDA for the treatment of breast tumors, can specifically bind to the juxtamembrane region of HER2 receptor [71] with a high binding affinity (Kd = 0.1-1 nM) [68, 72] and exert an inhibitory effect on the growth of HER2-overexpressing breast cancer cells [73-75].

1.4.2 Folate (FA) and folate receptor (FR)

1.4.2.1. The folate receptor as a tumor marker

The folate receptor (FR) is a 38 kDa glycosyl phosphatidylinositol (GPI)-anchored glycoprotein that exists in three isoforms, two membrane bound (α and β) [76, 77] and one truncated (γ) [78, 79]. FR expression in normal tissues is highly restricted
and its physiological role is clear only in specific instances [80]. Transmembrane transport of folate co-enzymes in most normal tissues is primarily mediated by a low affinity reduced folate carrier with $K_t$ in the $\mu$M range [81].

FR expression is frequently amplified in a variety of human cancers and in tumor cell lines [82-84]. Specifically, type $\alpha$ FR is often overexpressed among epithelial-lineage tumors [83] whereas both acute and chronic myelogenous leukemias express type $\beta$-FR [84]. Garin-Chesa et al. demonstrated, by histochemical staining using an anti-FR-$\alpha$ antibody, elevated FR expression in ovarian, endometrial, colorectal, breast, lung, and renal cell carcinomas, as well as in brain metastases [83]. Increased FR expression has also been identified by RT-PCR analyses in choriocarcinomas, meningiomas, uterine sarcomas, osteosarcomas, non-Hodgkin lymphomas, and choroid plexus tumors [84]. FR expression in non-mucinous ovarian carcinomas has been shown to approach 100% [85]. Furthermore, high FR expression in ovarian cancer has been associated with an increase in the percentage of S-phase cells indicating aggressive tumor growth [85].

1.4.2.2. Targeted drug/gene delivery via the FR

The consistent overexpression of FR in ovarian cancers suggests its potential utility as a cellular target for drug delivery. Radiolabeled monoclonal antibodies against FR-$\alpha$, MOv18 and MOv19, have been evaluated as potential agents for radioimaging and radiotherapy in ovarian cancer patients [86, 87]. In addition, chimeric and bispecific constructs of anti-FR antibodies have been evaluated as potential immunotherapy agents against ovarian cancer [88, 89].
Covalent coupling of folic acid to a drug or drug carriers has been evaluated as an alternative strategy for targeting the FR. Folic acid (Figure 1.4) is a high affinity ligand of FR (Kd \( \sim 10^{-10}\)M). Covalent derivatization of the \( \gamma \)-carboxyl of folic acid has been shown to result in only a moderate reduction in receptor binding affinity [90]. Following receptor binding, folate conjugates are internalized by cells via receptor-mediated endocytosis which appears to follow a non-degradative pathway [91].

Folate conjugation, therefore, presents a potential strategy for targeting diagnostic and therapeutic agents non-destructively to FR+ tumor cells. This approach has been explored in the targeted delivery of protein toxins [91-93], prodrug converting enzyme [94], cytokine [95], anti-T-cell antibody [88], radionuclide chelates [96, 97], chemotherapeutics [98, 99], starburst dendrimers [100], liposomes [98, 101, 102], and gene transfer vectors [38, 49, 103]. Lee et. al. was the first to show FR targeting of liposomes [101]. They discovered that, although folate can be directly linked to low molecular weight agents or macromolecules while retaining its high affinity for the FR, targeting of relatively large particles such as liposomes requires the introduction of a long PEG linker [101].

Recently, a variety of gene transfer vectors have also been conjugated to folate for FR-mediated tumor cell-specific delivery, including adenoviral particles [104], polyplexes [49, 105-112], lipoplexes [113, 114] and lipopolypoplexes [38, 115-117]. FR-targeted drug delivery has recently been reviewed in [105, 118-122].

Folic acid (Mw 441) has several important advantages over macromolecular ligand [105, 118, 119, 121-123]: (i) unlimited availability, (ii) superior functional
stability, (iii) relatively simple and defined conjugation chemistry, providing easy incorporation into a gene transfer vector, and (iv) presumed lack of immunogenicity. In addition, the FR endocytosis pathway is reportedly non-destructive, allowing the receptor to recycle and to continuously accumulate folate conjugates. Moreover, the high frequency of FR amplification in a variety of human tumors makes this delivery strategy widely applicable.

Potential obstacles for the use of folic acid in tumor targeting are: (i) binding interference from circulating folates in the plasma and (ii) FR expression in the apical membrane of kidney proximal tubules. Competition from the endogenous form of folate, N-5-methyltetrahydrofolate (at 1-50 nM in human plasma), should not significantly impede FR binding of multivalent folate conjugates such as folate-derivatized gene transfer vectors. Although low molecular weight folate conjugates have been shown to accumulate at high levels in the kidneys [97], the size of high molecular weight drug carriers, such as folate-derivatized gene transfer vectors, prevents glomerular filtration thus eliminating the potential for FR-mediated kidney accumulation [124].

A phase I/II clinical trial has been completed at the MD Anderson Cancer Center evaluating the use of 111In-DTPA-folate for the diagnostic imaging of ovarian carcinomas. The γ-scintigraphy images of patients showed high radionuclide localization in malignant tumors but not in benign tumors. For detection of malignant ovarian carcinomas, a sensitivity of 92% and a specificity of 83% were achieved in this study. No significant accumulation of the folate conjugate has been found in any major organs.
beside the tumor and the kidneys. These findings show that using folate conjugation to target therapeutics is feasible in humans.

In summary, FR-targeted formulations are attractive vectors for DNA-based therapeutics, given the inherent endosomalytic activity provided by the lipidic components. Their utility for tumor-selective delivery in vivo should be further validated in animal model studies.

1.4.3 Transferrin (Tf) and transferrin receptor (TfR)

Transferrin (Tf), an iron-transporting 80 kDa glycoprotein, is efficiently taken up into cells by transferrin receptor (TfR)-mediated endocytosis [125, 126]. TfR, a dimeric transmembrane glycoprotein of 180 kDa, are found on the surface of most proliferating cells and tumor cells frequently carry elevated numbers of TfR compared with the corresponding normal cells [127, 128]. The abundant presence of transferrin receptors on the surface of proliferating cells could provide a simple mechanism to preferentially deliver antisense oligonucleotides into tumor cells. Transferrin-conjugated polymers [129, 130] and liposomes [131-137] have been evaluated for tumor cell selective delivery of therapeutic agents including plasmid DNA via the TfR.

1.5 Summary and dissertation overview

To achieve successful targeted gene delivery in vivo, an ideal gene transfer vector should have the ability to protect DNA-based therapeutics from enzyme degradation in
the extracellular matrix, to efficiently and selectively deliver genes to target cells, and, most importantly, to preserve the functionality of genes inside the cells. Although non-viral vectors have advantages over viral vectors in terms of safety, design flexibility, unlimited size in nucleic acids to be carried, cost, and ability to repeated administration, first generation non-viral vectors rely only on electrostatic interaction for cellular uptake, which, therefore, results in ineffective and non-selective gene delivery \textit{in vivo}. Insertion of a molecular conjugate such as a ligand or an antibody into gene transfer vectors may overcome these problems.

Among many other problems we may encounter, the efficacy of DNA-based therapeutics depends heavily on efficient delivery into targeted cells. Therefore, selection of the appropriate target and delivery system is crucial to the success of gene therapy.

The main purpose of the current study is to develop a non-viral delivery system that can efficiently and specifically deliver gene-related products to specific cell types. In this dissertation, I developed tumor-specific gene transfer vectors which were designed to target to three different receptors, including HER2 receptors (Chapter 2), folate receptors (Chapter 3), and transferrin receptors (Chapters 4 and 5), which overexpress in different tumor types, respectively. In Chapter 2, trastuzumab, a recombinant humanized monoclonal HER2-specific antibody approved by FDA, was covalently linked to PEI to deliver plasmid DNAs to HER2-overexpressing breast cancer cells. In Chapter 3, folate was pegylated and then incorporated into liposomes to achieve a targeting effect in KB cells, an FR-overexpressing cell line. In Chapters 4 and 5, transferrin proteins were
applied as the target ligand to deliver antisense oligonucleotides or siRNAs to leukemia cells. Due to the difference in properties of each targeting moiety, various strategies were applied to incorporate the target ligands into each formulation. Physicochemical and biological properties of each formulation were evaluated and discussed in these chapters.
<table>
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Table 1.1 DNA based therapeutics currently approved or in phase 3 clinical trials
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<td>Antigen HER2 receptor</td>
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<tr>
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<td>EGF receptor</td>
<td>Cancer cells</td>
<td>Cancer</td>
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**Table 1.2** Lists of examples of receptor-mediated targeting pairs
Figure 1.1 Schematic representation of delivery of DNA-based therapeutics using non-viral delivery vectors. (1) complexation or encapsulation of DNA with delivery vectors; (2) interaction of DNA/vector complex with cell membrane; (3) internalization via receptor- or non-receptor-mediated endocytotic processes; (4) endosomal breakdown; (5) cytoplasmic release of DNA alone or NDA/vector complex; (6) dissociation of DNA from DNA/vector complex; (7) nuclear uptake.
Figure 1.2 Chemical modifications of antisense oligonucleotides
Figure 1.3

(A) Poly-L-lysine

(B) Polyethylenimine

poly-L-lysine (PLL) and (B) polyethylenimine (PEI)
Figure 1.4 Structure of folic acid
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CHAPTER 2

TUMOR-TARGETED GENE DELIVERY VIA ANTI-HER2 ANTIBODY
(TRASTUZUMAB, HERCEPTIN®) CONJUGATED POLYETHYLENIMINE

2.1. Abstract

A series of novel nonviral vectors targeting the HER2/neu gene product human epidermal growth factor receptor-2 (HER2) were constructed and evaluated in breast cancer cell lines to optimize vector formulation for receptor-specific gene transfer. These vectors were DNA/polycation complexes (polyplexes) prepared by mixing, at varying ratios, plasmid DNA carrying a luciferase reporter gene to HerPEI, which is a conjugate of linear polyethylenimine (PEI), a cationic polymer, and trastuzumab (Herceptin®), a HER2-specific monoclonal antibody. Transfection studies were carried out in both high HER2 expression Sk-Br-3 and low HER2 expression MDA-MB-231 breast cancer cells. The HerPEI polyplexes showed significantly greater transfection activity up to 20 fold than non-derivatized PEI-based polyplexes in the Sk-Br-3 cells. The transfection efficiency of targeted polyplexes depended on the trastuzumab:PEI ratio and can be blocked by excess free trastuzumab, suggesting HER2-mediated gene delivery. In
contrast, no significant difference in transfection activities was observed between HER2-targeted and non-targeted polyplexes in the HER2 low-expression MDA-MB-231 cells. The transfection efficiency of HerPEI polyplexes was retained in serum containing medium. In summary, HerPEI polyplexes have shown promising HER2 receptor-specific gene transfer properties and warrant further evaluation as a tumor targeted vector for gene therapy.
2.2. Introduction

Gene therapy is an emerging therapeutic modality for the treatment of cancer. However, lack of safe, efficient, and well-characterized gene transfer vectors has limited its clinical application. Early efforts in vector design have primarily concentrated on genetically engineered virus, such as adenovirus, retrovirus, and adeno-associated virus [1]. Despite relatively high transfection efficiency, the therapeutic application of viral vectors has been hindered by inherent problems such as immunogenicity and toxicity [2, 3]. Non-viral vectors including cationic polymer-DNA complexes (polyplexes) [4, 5], cationic lipid-DNA complexes (lipoplexes), and polymer-lipid-DNA ternary complexes (lipopolyplexes) [6, 7] have thus been explored as alternatives to viral vectors [2, 8]. Non-viral vectors are potentially less immunogenic, are relatively easy to prepare in clinical quantities, and are associated with fewer safety concerns than their viral counterpart [8, 9].

Polyethylenimine (PEI), a cationic polymer, has been shown to be among the most efficient non-viral gene delivery vectors both *in vitro* [10-13] and *in vivo* [14-16]. The high positive charge density of PEI can facilitate efficient electrostatic interactions with the anionic DNA [11, 17]. Since nitrogen atoms in PEI have an overall mildly acidic pKₐ, PEI/DNA complexes, when internalized by endocytosis, could mediate proton and chloride influx into endosomes, resulting in a “proton-sponge” effect [11, 18] associated with the disruption of endosomes and escape of DNA into the cytoplasm [13, 18].
Tumor-targeted gene delivery is a promising strategy for enhancing the efficacy of cancer gene therapy [19]. The main objective of the present study was to investigate the utility of trastuzumab-PEI conjugates (HerPEI) as targeted gene delivery vectors. Human epidermal growth factor receptor-2 (HER2) is a 185 kDa transmembrane glycoprotein consisting of an extracellular domain, a transmembrane domain, and an intracellular domain with intrinsic tyrosine kinase activity [20]. The amplification of her2 gene and the overexpression of HER2 protein have been observed in a number of tumors, especially in breast and ovarian cancers [21], and have been shown to be associated with poor clinical outcome [22]. The combination of overexpression of HER2 receptors in tumors and low expression of HER2 receptors in normal tissues provides a potential therapeutic window for agents targeting the HER2. In addition, the presence of an extracellular domain makes HER2 receptor a cellular marker suitable for receptor-mediated targeted delivery in tumors. There is no known high-affinity ligand for HER2 receptors. Nonetheless, monoclonal antibodies directed against HER2 offer a potential strategy for HER2-targeted delivery. Trastuzumab (Herceptin®), a recombinant humanized monoclonal anti-HER2 antibody approved for clinical use, can specifically bind to the juxtamembrane region of HER2 receptor [23] with a high affinity (Kd = 0.1-1 nM) [24, 25], and can exert an inhibitory effect on the growth of HER2-overexpressing breast cancer cells [26-28].

In this study, a series of HerPEI polyplex formulations containing plasmid DNA encoding the firefly luciferase reporter gene were prepared and their physicochemical properties were characterized. The transfection activities in HER2 high-expression and low-expression cells were determined and compared. Our results indicated that HER2-
targeted PEI polyplexes can efficiently and selectively deliver plasmid DNA to HER2 overexpressing cells. In summary, HerPEI polyplexes have shown promising HER2 receptor-specific gene transfer properties and warrant further evaluation as a tumor targeted vector for gene therapy.
2.3. Materials and methods

2.3.1. Materials

Polyethylenimine (PEI, linear, MW ~ 25 kDa) was purchased from Polysciences, Inc. (Warrington, PA). Bovine serum albumin (BSA) protein standards, ethidium bromide, heparin, human IgG (IgG), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dithiobis (succinimidylpropionate) (DSP) and bicinechinonic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, IL). Luciferase assay reagents, recombinant luciferase, and RNase-Free DNase (DNase I) were purchased from Promega (Madison, WI). All tissue culture media and supplies were purchased from Gibco BRL (Rockville, MD).

2.3.2. Plasmid preparation

pcDNA3-CMV-Luc (pLuc, 7.1 kb) plasmid DNA encoding the firefly luciferase gene under the control of the cytomegalovirus enhancer/promoter was kindly provided by Dr. Leaf Huang at the University of Pittsburgh School of Pharmacy. Cloning and preparation of plasmid DNA were performed by propagating transformed DH5-α E. coli in LB media containing ampicillin (50 µg/mL), followed by isolation and purification with a commercially available plasmid purification kit (Qiagen, Valencia, CA). The concentration and purity of plasmid DNA were assessed by measuring absorbance at 260 and 280 nm (OD_{260}/OD_{280} ~ 1.9) on a Shimadzu UV-160U Spectrophotometer. The size
and integrity of plasmid were confirmed by gel electrophoresis (0.9% agarose gel containing 0.5 μg/mL ethidium bromide) in Tris acetate-EDTA buffer (TAE, pH 8.0).

2.3.3. Cell culture

Two human breast adenocarcinoma cell lines were used in this study. The Sk-Br-3 cells express high levels of HER2, whereas the MDA-MB-231 cells express minimal levels of HER2 [29]. Cells were cultured in DMEM/F-12 (1:1) media supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and 365 μg/mL L-glutamine at 37 °C in a 5% CO₂-humidified incubator.

2.3.4. Synthesis of Herceptin-linked PEI (HerPEI) and IgG-linked PEI (IgG-PEI)

PEI (linear, MW ~ 25 kDa) was dissolved in DMSO to make 1 mL of 100 mg/mL. The PEI solution was added dropwise to 2.43 mg of DSP in 1 mL of DMSO. This reaction was allowed to proceed at room temperature for 2 hr. PEI-DSP was then coupled to trastuzumab by adding 200 μL of PEI-DSP into 800 μL of trastuzumab in PBS (pH 7.4) at ratios of 0.0625 (HerPEI 0.0625), 0.125 (HerPEI 0.125), 0.25 (HerPEI 0.25), 0.5 (HerPEI 0.5), and 1 (HerPEI 1) nmoles of trastuzumab per 100 nmoles PEI. The reaction mixture was incubated for 2 hr at room temperature. To remove reaction by-products and DMSO, the mixture was dialyzed against deionized water. Since trastuzumab is an antibody with an IgG backbone, human IgG conjugated PEI (IgG-PEI) was similarly synthesized (IgG: PEI = 0.25:1) and used as a negative control in
subsequent transfection studies. The concentration of antibody in the HerPEI was measured by BCA protein assays.

2.3.5. Gel retardation assay

The PEI cation:DNA anion ratio is presented as the N/P ratio, the molar ratio of PEI nitrogen to DNA phosphate. Gel retardation assays were used to determine the ability of HerPEI to condense DNA. Three microgram of pcDNA3-CMV-Luc plasmid DNA was mixed with PEI or HerPEI at selected N/P ratios by adding the conjugates into DNA in PBS. The resulting polyplexes were incubated at room temperature for 20 min and then subjected to electrophoresis on 0.9% agarose gel containing 0.5 µg/mL ethidium bromide. A quantity of 0.7 µg of pcDNA3-CMV-Luc plasmid DNA was loaded into each well.

2.3.6. DNase protection assay

The ability of polyplexes to protect DNA from DNase digestion was characterized by DNase protection assays. An aliquot of 30 µL of PEI or HerPEI solutions (in PBS) were added to 30 µL of pcDNA3-CMV-Luc plasmid DNA (0.2 µg/µL) and then allowed to stand for 20 min at room temperature. Each sample was divided into two aliquots of 20 µL and was placed into two separate tubes. Four units of DNase I (4 µL) or 4 µL of PBS (as the control) was added and the samples were incubated at 37 ºC for 15 min. One unit of the DNase I activity was defined as the amount of enzyme required to completely
degrade 1 μg of λ DNA at 37 °C for 10 min. Immediately after incubation, the samples were treated with 10 μL of EDTA (100 mM) at room temperature for 15 min to inactivate the enzyme. Each of the enzyme-inactivated samples was further divided into two aliquots of 15 μL. Five microliters heparin solution (10 μg/μL in double-distilled H₂O) or blank H₂O was added into each tube and the tubes were then incubated at room temperature for 2 hr to allow the complete dissociation of polyplexes by heparin. A qualitative analysis of DNA degradation was performed by gel electrophoreses (0.9% agarose gels containing 0.5 μg/mL ethidium bromide).

2.3.7. Preparation of DNA polyplexes and transfection studies

Polyplexes were prepared in PBS by addition of PEI or conjugated PEI (HerPEI or IgG-PEI) to pcDNA3-CMV-Luc plasmid DNA in 75 μL of PBS to achieve the desired N/P ratios in a final volume of 150 μL. Samples were incubated at room temperature for 20 min prior to transfection studies. For particle size analysis, 10 μg of pcDNA3-CMV-Luc plasmid DNA in 150 μL PBS was complexed with various amounts of PEI or HerPEI in 150 μL PBS and the mean diameter and size distribution of polyplexes were determined by photon correlation spectroscopy using a NICOMP Particle Sizer Model 370. To evaluate the amount of PEI and trastuzumab in polyplexes, polyplexes were incubated with 100 μM PMAA for 30 min at room temperature [29]. The concentration of trastuzumab was measured by BCA protein assay.
For transfection studies, $1.0 \times 10^5$ cells (Sk-Br-3 or MDA-MB-231 cells) per well were seeded on 24-well plates (Falcon) and incubated for 24 hr in DMEM/F-12 (1:1) media supplemented with 10% FBS and antibiotics at 37 °C (~70% confluent). One microgram of pcDNA3-CMV-Luc plasmid DNA per well was used for most transfection studies, unless otherwise indicated. Prior to transfection, the medium was removed and the plates were rinsed with 0.5 mL of PBS. Polypexes or naked DNA was then added to each well and incubated with cells in 0.5 mL serum free media at 37°C for 4 hr. Serum free media were used in most transfection studies, unless otherwise indicated. After incubation, the incubation media were removed and the cells were rinsed with 0.5 mL of PBS followed by the addition of 0.5 mL of fresh medium containing 10% FBS and antibiotics. The cells were incubated for another 24 hr and then lysed and assayed for luciferase activity. All transfection experiments were performed in triplicate.

To assay for luciferase activity, the medium was removed from each well and the cells were washed with 0.5 mL/well of cold PBS. Two hundred microliters of ice-cold lysis buffer (100 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, pH 7.8) was added to each well and incubated on ice for 20 min. The cell lysate was then transferred to 1.7 mL centrifuge tubes and centrifuged at 15,000g for 2 min. The luminescence was measured on a Mini-Lum luminometer (Bioscan, Inc., Washington, DC) immediately after mixing 10 μL of cell lysate with 50 μL of luciferase substrate (Promega). Relative light units were standardized for protein concentration determined by the bicinchoninic acid (BCA) protein assays using bovine serum albumin standards.
2.3.8. Cytotoxicity assay

The cytotoxicity of polyplexes was measured by the MTT assay. The assay determines cell viability based on the mitochondrial conversion of a water-soluble tetrazolium salt (MTT) to the water-insoluble blue formazan product. Cells were seeded in 96-well plates (Falcon) in DMEM/F-12 (1:1) growth media containing 10% FBS at 37 °C in a 5%-CO2-humidified incubator for 24 hr. After incubation, culture media were replaced with 100-µL media containing serial dilutions of polyplexes or free DNA (n = 5 for each concentration). After 4 hr incubation, the media were removed and replaced with 200-µL fresh media for another 24 hr. After 24 hr incubation, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and the cells were incubated at 37°C for another 2 hr. The unreacted MTT and media were then removed by aspiration and 100 µL of DMSO was added to dissolve the blue formazan product. The absorbance at 550 nm in each well was measured by an automated plate reader (Bio-Rad, Hercules, CA). The relative cell viability was calculated as

\[ \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \]

where \( \text{Absorbance}_{\text{sample}} \) is the absorbance for cells incubated in DNA-containing media and \( \text{Absorbance}_{\text{control}} \) for cells incubated in normal culture media.

2.3.9. Statistical analysis

Both Student’s t-test and one way ANOVA with post hoc tests were used in the present study. P values of less than 0.05 were considered significant.
2.4. Results

2.4.1. Preparation of plasmid

pcDNA3-CMV-Luc (pLuc, 7.1 kb), a plasmid DNA encoding the firefly luciferase gene under control of the cytomegalovirus enhancer/promoter, was used as the reporter gene in this chapter. Firefly luciferase is chosen for the following reasons: (1) reporter activity is available immediately after translation since post-translational processing of the protein is not required; (2) this assay is very sensitive due to the high efficiency of its light production and lack of background luminescence in the host cells; and (3) the assay is rapid, requiring only a few seconds per sample. Cloning and preparation of plasmid DNA was performed by propagating transformed E. coli in LB media containing ampicillin, followed by isolation and purification with a commercially available plasmid purification kit. Plasmid DNA concentration was calculated using the absorbance at 260 nm on a Shimadzu UV-160U Spectrophotometer and the purity was calculated by the ratio of absorbance at 260 and 280 nm and the OD260/OD280 of 1.9 was obtained. The size and integrity of plasmid were confirmed by 0.9% agarose gel electrophoresis in Tris acetate-EDTA buffer (TAE, pH 8.0).

2.4.2. Preparation and physicochemical characterization of trastuzumab-conjugated PEI polyplexes

To prepare trastuzumab-conjugated PEI (HerPEI) polyplexes, HerPEIs with various trastuzumab:PEI mol ratios were synthesized using DSP as a cross-linking agent that reacts with primary amines on the PEI and trastuzumab molecules (Figure 2.1). DSP,
a water-insoluble, homobifunctional N-hydroxysuccimide ester (NHS-ester), is a thiol-cleavable, primary amine-reactive reversible cross-linker [30, 31]. Since DSP contains a disulfide bond, bioconjugate synthesized via DSP crosslinking can potentially be cleaved intracellularly where the environment is highly reducing [4]. This might be beneficial to the intracellular trafficking of plasmid DNA and facilitate transgene expression.

Based on the ratio of trastuzumab (in the HerPEI conjugate) and DNA at the N/P ratio selected (N/P = 10), the polyplexes had trastuzumab: DNA molar ratios of 1.7:1, 3.4:1, 6.7:1, 13.4:1 and 27:1, respectively.

The formation of HerPEI polyplexes as a function of trastuzumab content and N/P ratio was determined by gel retardation assay. The N/P ratio of HerPEI/DNA polyplexes reflects the overall positive to negative charge balance of the DNA complex. The results in Figure 2.2 showed that all HerPEI, with trastuzumab/PEI molar ratio ranging from 0.0625 – 1, completely eliminated the mobility of plasmid DNA at N/P > 2, suggesting that HerPEI is capable of forming polyplexes, similar to unconjugated PEI.

Maintaining the stability of plasmid DNA during formulation and delivery processes is an important factor in successful gene delivery. In the current study, the stability of the HerPEI polyplexes was assessed by a DNase protection assay. As shown in Figure 2.3, after incubation with DNase I, much of the plasmid DNA in HerPEI polyplexes remained intact, whereas naked DNA was completely digested. Furthermore, the protection effect was increased as the N/P ratio increased from 5 to 10.
2.4.3. The role of covalent conjugation

To determine whether covalent coupling of trastuzumab to PEI is required for receptor-specific gene transfer, transfection study was conducted using Sk-Br-3 cells comparing the transfection efficiencies of pcDNA3-CMV-Luc plasmid DNA delivered as polyplexes with HerPEI or with a mixture of linear PEI and free trastuzumab (Figure 2.4). The luciferase activity was markedly higher in cells treated with HerPEI polyplexes at all trastuzumab:PEI ratios tested, indicating that covalent conjugation of PEI was necessary for targeted gene delivery.

2.4.4. The role of N/P ratio in transfection

As shown in Figures 2.5 and 2.6, transfection activity increased with an increase in N/P ratio in all polyplex formulations. As N/P ≥ 12.5, however, no further increase in luciferase activity was observed (Figure 2.5). Considering the results shown in Figures 2.5 and 2.6 and the fact that the cytotoxicity of PEI raised with an increasing N/P ratio [32], I concluded that the optimized N/P ratio for HerPEI polyplexes was between 5-10 with the highest luciferase activity observed at N/P ratio of 10. Therefore, subsequent studies evaluating HER2 receptor-targeting effect of HerPEI polyplexes was carried out using polyplexes prepared at an N/P ratio of 5 or 10.
2.4.5. The effect of trastuzumab-to-PEI coupling ratio on transfection

At a fixed N/P ratio of either 5 or 10, HerPEI polyplex formulations showed higher transfection efficiency than non-conjugated PEI polyplexes (Figure 2.6). Nevertheless, luciferase activity exhibited a bell-shaped relationship versus the molar ratio of trastuzumab-to-PEI (Figure 2.6), suggesting the existence of an optimal degree of trastuzumab conjugation for transfection efficiency. Trastuzumab conjugation led to an enhancement of polyplexes transfection up to 20-fold with a maximum occurring at trastuzumab/PEI = 0.25.

2.4.6. The role of HER2 expression level and HER2 on transfection

In order to determine the role of HER2 expression level on the transfection properties of HerPEI polyplexes, two breast cancer cell lines with high and low HER2 expression levels were used in this study, Sk-Br-3 and MDA-MB-231 with HER2 levels of 900 ng/mg protein and 5 ng/mg protein, respectively [33]. As shown in Figure 2.7, while transfection efficiency of HerPEI polyplexes in Sk-Br-3 cells was significantly higher than that of non-conjugated PEI polyplexes and was trastuzumab concentration-dependent, no significant enhancement in transfection was observed for all HerPEI polyplexes evaluated in MDA-MB-231 cells.

To further confirm that the enhancement in transfection observed in Sk-Br-3 was HER2-mediated, transfection was performed in the presence of 25 µM of free trastuzumab at N/P ratio of 10. In addition, polyplexes of human IgG-linked PEI (IgG-PEI) were used as a control [25]. As shown in Figures 8A and 8B, free trastuzumab was
able to block the transfection of HerPEI polyplexes in Sk-Br-3, but not in low HER2-
expressing MDA-MB-231 cells. In addition, free trastuzumab had little effect on the
transfection activity of PEI or IgG-PEI polyplexes, suggesting that HER2 receptors
mediated the cellular uptake of HerPEI polyplexes in the HER2 overexpressing Sk-Br-3
cells.

Moreover, the luciferase activities were significantly higher in Sk-Br-3 than in
MDA-MB-231 cells for all HerPEI formulations, possibly due to more plasmid DNA
being internalized into HER2 overexpressing cells than HER2-low-expressing cells
(P<0.05). Taken together, our data suggested that the trastuzumab-incorporated PEI
vector could offer a potential strategy for targeted gene delivery, specifically for HER2
overexpressing tumor cells.

2.4.7. The effects of particle size, DNA concentration, and serum in the media on
transfection

Transfection efficiencies and particle sizes of PEI and HerPEI polyplexes at
various N/P ratios were shown in Figure 2.5. At low N/P ratios, polyplexes tend to
aggregate and form larger particles. Higher N/P ratio resulted in smaller particles. The
highest transfection activity was observed at an N/P ratio of 10 (Figure 2.5).

The plasmid dose-response of polyplex transfection was then determined at this
trastuzumab/PEI ratio and at an N/P ratio of 10. As shown in Figure 2.9, increasing
pcDNA3-CMV-Luc plasmid DNA led to a corresponding increase in reporter gene
expression.
To investigate whether serum had any influence on transfection efficiency of HerPEI polyplexes, Sk-Br-3 cells treated with PEI or HerPEI 0.25 polyplexes at N/P ratio of 5 or 10 were incubated in serum free or 10% FBS containing medium (Figure 2.10). Compared with cells incubated in serum free medium, more than 40% of transfection activity was retained at both N/P ratios. The effect of serum was even less significant as N/P ratio increased to 10. These results suggested that HerPEI polyplexes retained their transfection activity in the presence of serum.

2.4.8. Cytotoxicity of PEI-HER polyplexes

The cytotoxicity of polyplexes in SK-BR-3 and MDA-MB-231 cells was evaluated by MTT assay. The results were summarized in Table 2.1. Polyplexes containing PEI alone had some inhibitory effect on the growth of both SK-BR-3 and MDA-MB-231 cells, which was seen in other PEI-based polyplexes [34]. The inhibitory effect increased as the N/P ratio increased in both cell lines. In SK-BR-3 cells, incorporation of Herceptin molecules to polyplex formulations resulted in the increase of cytotoxicity, which is related to the amount of Herceptin used in the formulations. On the contrary, there is no difference among the cytotoxicity of various HerPEI/DNA polyplexes.
2.5. DISCUSSION

Many cancer gene therapy strategies rely on tumor selective expression of the therapeutic gene. Local administration of gene transfer vectors will likely to only result in the transfection of a small fraction of tumor cells. Development of gene transfer vectors for systemic administration is therefore highly desirable. Targeted vectors can potentially increase gene delivery to selective tissues, therefore, are likely to be more effective for systemic gene delivery.

Trastuzumab (Herceptin®) is the first therapeutic antibody to reach clinical use for metastatic breast cancer. Given the prevalence of HER2 overexpression in breast and ovarian cancers, there is good rationale for designing targeted therapeutic agents based on trastuzumab conjugates. In fact, anti-HER2 immunoliposomes have already been investigated as a tumor-targeted drug delivery vehicle [24, 35, 36]. In our design of the HER2-targeted gene transfer vector, a PEI polyplex based system was selected. This was because of the superior DNA binding and inherent endosomolytic activities of this cationic polymer. In addition, a number of tumor targeted vectors based on ligand-conjugated PEI polyplexes have been reported in the literature, including those targeting the transferrin receptor and the folate receptor [34, 37-39]. In this study, a linear PEI was chosen over branched PEI due to its demonstrated relatively high transfection activity in vivo, low polydispersity, and relatively low cytotoxicity [40]. Unlike branched PEI, there is only one primary amine group on each end of linear PEI, which could decrease the chance of intra- and inter-molecular crosslinking during bioconjugate synthesis. In fact, polyplexes of trastuzumab conjugated with branched PEI of same mean MW (25 kDa)
prepared by the same conjugation process as HerPEI and the transfection efficiency of this branched PEI-trastuzumab conjugate showed significantly lower gene transfer activity than those of HerPEI.

A critical parameter of a targeted vector formulation is the antibody to particle ratio, which is related to the density of targeting moiety on the particle surface. At a trastuzumab/PEI ratio of 0.25:100 and N/P ratio of 10, the trastuzumab/DNA ratio is approximately 6.7. If one assumes that the DNA is optimally condensed at this ratio based on its nuclease resistance, an average volume of a condensed DNA molecule of $1.41 \times 10^8 \, \text{Å}^3$ [41], and an average volume of an IgG of $4.49 \times 10^5 \, \text{Å}^3$ [42], the average HerPEI polyplex, which had a diameter of 250 nm would have 475 DNA and 3185 trastuzumab molecules per polyplex. There are more than $1 \times 10^6$ HER2 receptors per Sk-Br-3 cell [33]. The relatively high number of trastuzumab per polyplex evaluated in this study could result in increased receptor binding affinity via multivalent interactions and might sterically block electrostatic interactions between PEI in the polyplex and the cell surface.

For each HerPEI formulation, the transfection activity in Sk-Br-3 was increased as the N/P ratio increased from 5 to 10 (Figures 2.5 and 2.6). However, the transfection activity began to decline at the N/P ratio of $\geq 12.5$ (Figure 2.5). Similar results for PEI polyplexes were also observed by Boussif et al [11] and Oh et al [43]. One possible explanation is that although increased overall positive charge of the polyplexes could result in increased cellular uptake and endosomolytic strength, it could also cause more cytotoxicity [11].
Notably, bell-shaped transfection profiles of HerPEI polyplexes in Sk-Br-3 cell were obtained (Figure 2.6). The higher incorporation ratio of trastuzumab in HerPEI conjugates, the higher targeting capacity of HerPEI polyplexes would be expected. However, the electrostatic properties of the HerPEI polyplexes may be altered by trastuzumab, thereby offsetting the targeting effect. By examining all of the trastuzumab/PEI ratios (ranging from 0.0625 to 1) used, less than 1% of amine per PEI molecule was likely to be affected by the conjugation of trastuzumab. Therefore, the loss of relative charge in PEI due to trastuzumab conjugation was negligible. Furthermore, all HerPEI polyplexes exhibited similar DNA condensation profiles (Figure 2.2), suggesting that factors other than altered electrostatic properties of HerPEI polyplexes might be attributable to the reduced transfection activity. Our cytotoxicity data indicated that, specific for the HER2 overexpressing cells, increased trastuzumab ratio in HerPEI was associated with increased cytotoxicity, which may account for the observed bell-shaped transfection profile.

For a gene transfer vector proven to be successful, in vivo studies are necessary since the extent and duration of gene expression following administration are significantly affected by the biological barriers, including degradation of DNA, extravasation, targeting to specific cell or tissue type(s), internalization, endosomal escape, cytoplasmic stability, nuclear uptake, etc. [44, 45]. Rather than initiating in vivo studies impetuously, some physicochemical or pharmacokinetic properties (e.g., particle size, effect of serum and/or DNase, stability, etc.) of gene transfer vectors can be evaluated in vitro.
Appropriate particle size of polypelexes is a crucial factor for \textit{in vivo} cancer gene therapy. The ideal particle size should be small enough to extravasate the tumor blood vessels, which are relatively permeable and leaky compared to normal blood vessels [46]. Therefore, polypelexes with the size in between the pore size of tumor and normal blood vessels would show higher selectivity towards tumor. The pore size cutoff of tumor blood vessels, depending on tumor types, varies from 380 to 780 nm [46], which could be the upper size limit of PEI polypelexes. Once the polypelexes reach target tumor cells, smaller polypelexes (<100 nm) would be desirable for effective gene delivery via endocytosis. However, studies have shown that \textit{in vivo} transfection efficiency of PEI polypelexes inversely correlated with particle size—i.e., very small particles (<100 nm) exhibited lower transfection efficiencies than larger particles [40, 47, 48]. The results in the previous studies would suggest that lower size limit also exists for efficient \textit{in vivo} gene transfection. Too much DNA condensation by cationic polymer, like PEI, may result in small yet too dense particles, thereby limiting the unpacking of DNA once inside the nucleus. In addition, too small a particle may lead to reduced bioavailability in the target tumors due to nonselective distribution to normal tissues. The exact reasons as to why smaller particles did not show better transfection efficiency \textit{in vivo} remain to be explored.

In summary, adequate particle size of PEI polypelexes not only can achieve higher transfection efficiency but, more importantly, may further enhance the tumor selectivity \textit{in vivo}. In our current study, polypelexes tend to form large particles at low N/P ratio (N/P ≤2) and relatively small particles as N/P ratio is higher than 5 (Figure 2.5). The particle size of HerPEI polypelexes (N/P ratios = 5 to 10) is about 250 nm, which is small enough for extravasation from tumor blood vessels and large enough to prevent the
leakiness from normal blood vessels, therefore, is potentially suitable for \textit{in vivo} gene delivery.

Serum proteins also play important roles in determining a successful gene delivery, which may facilitate the aggregation of cationic vectors, affect the cell-binding step, or even compete for the binding of DNA to cationic polymers or lipids [49, 50]. Cationic gene transfer vectors such as branched PEI are known to be quite sensitive to the presence of serum [10]. In comparison with branched PEI, linear PEI is relatively insensitive to the presence of serum. In the current study, linear PEI-based HerPEI polyplexes maintained more than 50\% of transfection efficiency in 10\% FBS containing medium (Figure 2.9), which could be one of the determinants leading to successful \textit{in vivo} studies in the future.

In this chapter, a novel, non-viral, PEI-based, HER2-targeted gene transfer vector has been developed. The anti-HER2 humanized antibody, trastuzumab (Herceptin\textsuperscript{®}), was used as the targeting moiety. The HerPEI polyplexes were shown to selectively deliver genes to the HER2-overexpressing cancer cells with high resistance to serum. Trastuzumab \textit{per se} has been shown to be both cytotoxic and anti-angiogenic. Therefore, trastuzumab conjugation of PEI may render PEI a much more selective gene delivery vector for anticancer gene therapy. The idea of incorporating a targeting ligand, with intrinsic antitumor activity, into a gene transfer vector may be worthwhile for further exploration.
<table>
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<th>SK-BR-3 N/P=5</th>
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**Table 2.1** Cytotoxicity of various polyplexes in culture tumor cells determined by MTT assay<sup>a</sup>

<sup>a</sup>The result was expressed as [Abs]<sub>sample</sub>/[Abs]<sub>control</sub>×100 and cells incubated with medium alone were used as the Control in the MTT assay. Data are shown as mean ± SD (n=5).

* indicates statistical difference (p < 0.05) from PEI group (analysis of variance)
Figure 2.1 Proposed reaction scheme for conjugating linear PEI with trastuzumab through cross-linking reagent DSP.
Figure 2.2 Agarose gel electrophoresis of HerPEI polyplexes. After incubating HerPEI with plasmid DNA for 20 min at room temperature, HerPEI polyplexes were electrophoresed on 0.9% agarose gels. Gel A, B, C, D, and E are polyplexes formed by HerPEI with HER/PEI mol ratios of 0.0625:1, 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively. Lanes 1-5 represent N/P ratios of 0 (control), 1, 2, 3, and 4, respectively.
Figure 2.3 DNase protection assay. Plasmid DNA alone or complexed with HerPEI at different N/P ratios were exposed to DNase digestion. Lanes 1 and 11 are phage λ DNA digested with HindIII used as a size marker. Lanes 2-4 are pcDNA3-CMV-Luc plasmid DNA alone (N/P=0); Lanes 5-7 and Lanes 8-10 are plasmid DNA condensed with trastuzumab/PEI mol ratio of 0.25:1 at N/P ratios of 5 and 10, respectively. DNase I was added to each sample except for Lanes 2, 5, and 8. After DNase inactivation, except for Lanes 4, 7, and 10, heparin was added to disassemble the polyplexes.
Figure 2.4 Transfection of Sk-Br-3 cells by HerPEI or the mixture of PEI and trastuzumab. Cells were treated with 1 µg of pcDNA3-CMV-Luc plasmid DNA complexed with HerPEI or mixture of trastuzumab and linear PEI (N/P 10). HerPEI were synthesized as described in Materials and Methods. The mixture of PEI and trastuzumab was mixed 30 min and incubated at room temperature prior to forming polyplexes. Same trastuzumab:PEI mol ratio was used for HerPEI and the mixture of PEI and trastuzumab. Results are shown as relative light units (RLU)/mg protein. Each data set represents three replicate assays ± SD.
Figure 2.5 Comparison of transfection efficiencies of PEI and HerPEI in Sk-Br-3 cells. Transfection efficiencies and particle sizes of PEI and HerPEI 0.25 polyplexes shown as a function of N/P ratio ranging from 0 to 15. Results are shown as relative light units (RLU)/mg protein. One µg/well plasmid DNA was used. Data are shown as mean ± SD (n=3). * P<0.05, compared with the transfection efficiency of PEI (trastuzumab/PEI mol ratio = 0) polyplexes.
Figure 2.6 Comparison of transfection efficiencies of PEI and HerPEI in Sk-Br-3 cells. Transfection efficiencies of various HerPEI polyplexes in Sk-Br-3 cells at N/P ratios of 0, 5, and 10. Results are shown as relative light units (RLU)/mg protein. One µg/well plasmid DNA was used. Data are shown as mean ± SD (n=3). * P<0.05, compared with the transfection efficiency of PEI (trastuzumab/PEI mol ratio = 0) polyplexes.
Figure 2.7 Comparison of transfection efficiencies in Sk-Br-3 and MDA-MB-231 cells at N/P ratio of 10. Cells were treated with 1 µg of plasmid DNA complexed with PEI or HerPEI. Results are shown as relative light units (RLU)/mg protein. Each data set represents three replicate assays ± SD. * indicates statistical difference (P < 0.05) using t-test.
Figure 2.8 Effects of free trastuzumab blockade on transfection activity in (A) Sk-Br-3 and (B) MDA-MB-231 cells at N/P ratio of 10. Cells were transfected with 1 µg of plasmid DNA complexed with various PEI-based polyplexes in the absence or presence of 25 µM free trastuzumab in the medium. Results are shown as relative light units (RLU)/mg protein. Each data set represents three replicate assays ± SD. * indicates statistical difference (P < 0.05) using t-test.
Figure 2.9 Comparison of transfection efficiencies in Sk-Br-3 cells. (A) Transfection efficiencies of PEI and HerPEI 0.25 polyplexes with increased amount of plasmid DNA (0.5 µg – 2 µg) at N/P ratio of 10. (B) Transfection efficiencies of PEI and HerPEI 0.25 polyplexes (at N/P ratio of 5 or 10) in serum free medium or 10% FBS containing medium. Results are shown as relative light units (RLU)/mg protein. Data are shown as mean ± SD (n=3).
Figure 2.10 Comparison of transfection efficiencies in Sk-Br-3 cells. Transfection efficiencies of PEI and HerPEI 0.25 polyplexes (at N/P ratio of 5 or 10) in serum free medium or 10% FBS containing medium. Results are shown as relative light units (RLU)/mg protein. Data are shown as mean ± SD (n=3).
LIST OF REFERENCES FOR CHAPTER 2


CHAPTER 3

EFFICIENT DELIVERY OF ANTISENSE OLIGODEOXYRIBONUCLEOTIDE
FORMULATED IN FOLATE RECEPTOR-TARGETED LIPOSOMES

3.1. Abstract

The folate receptor (FR) is a cellular surface marker for numerous solid tumors and myeloid leukemias. The aim of this study was to develop an antisense oligodeoxyribonucleotide (ODN) carrier targeting FR overexpressing cancer cells using folate (FA) as the targeting moiety. G3139, a phosphorothioate antisense oligonucleotide against human bcl2 mRNA, was evaluated in this study. G3139-containing, lipid-based nanoparticles were prepared using an ethanol dilution method. For the targeted formulation, 0.5mol% of folate-PEG-DSPE was incorporated into 3β(N-(N’,N’-dimethylaminoethane)-carbamoyl) cholesterol (DC-Chol)- containing cationic liposomes (DC-Chol/egg PC/PEG-DSPE at 25:65:10, mol/mol) as a targeting ligand. The particle size and surface charge were measured and uptake was assessed by fluorescence microscopy and flow cytometry. ODN-containing formulations were evaluated in FR+
KB cells and Bcl2 down regulation was measured by Western blotting. Cytotoxicity of the formulations was determined by MTT assay. The lipid-based G3139 containing nanoparticles had an average diameter of 80-90nm with high ODN entrapment efficiency (70-80%). The incorporation of folate ligand did not significantly alter the particle size and entrapment efficiency. The formulation was stable in a serum-containing environment. In uptake studies, the folate-targeted formulation exhibited a ligand concentration dependent uptake that was 6-fold more efficient than that of the non-targeted formulation (p<0.05). This uptake can be blocked by an excess amount of free folate, thus indicating an FR dependent mechanism.
3.2. Introduction

Antisense oligodeoxynucleotide (ODN) has been widely used to downregulate gene expression by inhibiting the translation of the mRNA of the target genes through several possible pathways. One major obstacle to the antisense delivery has been the difficulty in introducing them into target cells at concentrations sufficient to bind to the complement mRNA due to the inefficient cellular uptake of naked ODNs [1, 2]. Therefore, liposomal-based antisense delivery, utilizing the cationic properties of the lipids to bind negatively charged DNA or RNA and delivering oligos to the target cells, has been developed and extensively studied in the past decade and soon became one of the most widely used non-viral antisense delivery vehicles [3]. The advantages of liposomes over viral vectors include less immunogenecity, less safety concern, and ease to preparation.

Bcl2 protein is one of the first and the most important mammalian regulators of apoptosis and is overexpressed in more than 85% of lymphomas and other neoplasms. The gene is activated by the t(14;18) chromosomal translocation in B-cell lymphoma [4] and has been implicated in various cancers such as breast, prostate, and lung carcinomas [5]. Bcl2 overexpression also leads to resistance of tumor cells to chemotherapy and the downregulation of bcl2 can reverse chemoresistence [5]. G3139, the first oligonucleotide to demonstrate proof of principle of the antisense effect in human tumors including various leukemias by the documented down-regulation of the Bcl2 protein, was used in this study. G3139 is a phosphorothioate antisense oligonucleotide designed to specifically bind the human bcl2 mRNA, thereby resulting in a decrease in Bcl2 protein translation [6, 7].
The folate receptor (FR) is a 38 kDa membrane glycoprotein that is generally absent in normal tissues and is frequently amplified in human cancers. Folate (FA) conjugated drug carriers, including gene transfer vectors, retain high affinity for the FR. As a low molecular weight ligand for tumor targeting, folate has many advantages such as non-immunogenicity and convenient availability in addition to its high tumor specificity. Folate linked to various viral or non-viral gene transfer vectors can enhance transfection efficiency in various FR-overexpressing tumor cell lines such as KB cells. A variety of gene transfer vectors have been conjugated to folate for folate receptor-mediated tumor cell-specific delivery, including adenoviral particles [8, 9], polyplexes [10-17], lipoplexes [18, 19], and lipopolyplexes [20-24].

In the current study, a FA-conjugated liposomal formulation of G3139 was synthesized and evaluated in FR+ KB cells. An ethanol dilution method was developed to prepare ODN-containing liposomes. The cellular uptake mechanism of the targeted liposomes and antisense activity against Bcl2 were evaluated. Moreover, this study also served as a pilot study for targeted delivery of ODN in leukemia cells.
3.3. Materials and methods

3.3.1. Materials

Egg phosphatidylcholine (egg PC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Poly(ethylene-glycol) (MW ~2000)-distearoyl phosphatidylethanolamine (PEG-DSPE) was purchased from Lipoid (Newark, NJ). Folate-PEG(MW ~3350)-DSPE (folate-PEG-DSPE) was synthesized as described previously [25]. Octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR). Protein assay kits were purchased from Bio-Rad (Hercules, CA). Folic acid, cholesteryl chloroformate, N,N-dimethylenediamine cholesterol (Chol), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), bovine serum albumin (BSA) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Folate-free RPMI 1640 medium and other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA).

3.3.2. Antisense oligodeoxyribonucleotide

All ODNs used in this study were fully phosphorothioated. The antisense G3139 (5’-TCT CCC AGC GTG CGC CAT-3’) [26] is a 18-mer ODN designed to specifically bind the first six codons of the human bcl2 mRNA, thereby resulting in a decrease in Bcl2 protein translation [7]. G3139 was supplied by the National Cancer Institute (Bethesda, MD) and used without further purification. The sequence of mismatch control ODN is 5’-TCT CCC AGC ATG TGC CAT and was purchased from Integrated DNA Technologies (Coralville, Iowa).
3.3.3. Cell culture

KB cells (American Type Culture Collection # CCL17), a folate receptor-positive human oral squamous cell carcinoma, were obtained as a gift from Dr. Philip Low at Purdue University. The cells were maintained in folate-free RPMI 1640 media supplemented with 10% heat-inactivated newborn calf serum (NCS), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a 5% CO₂-humidified incubator.

3.3.4. Synthesis of DC-Chol

DC-Chol (3β(N-(N’,N’-dimethylaminoethane)-carbamoyl) cholesterol), a cationic derivative of cholesterol, was synthesized according to Gao and Huang [27]. Briefly, 2.5g of cholesteryl chloroformate (5.56 mmole) in 5 mL of dry chloroform was added to excess amount of N,N-dimethyethylenediamine in dry chloroform drop by drop and stirred for 2 hr at 0°C. After 2 hr stirring, solvent was removed by evaporation and the residue was purified by recrystallization twice in absolute ethanol at 4°C and dried under vacuum. The reaction yielded 0.8907 g of DC-Chol (white powder, 1.78 mmole, 32%) and the result was confirmed by 1H NMR.

3.3.5. Preparation of liposomes

For non-targeted ODN-containing liposomes, DC-Chol, egg PC, and PEG-DSPE were individually dissolved in 100% ethanol and mixed in a molar ratio of 25/65/10. 0.1
mol% of R18 was added to the formulations for uptake studies. For folate (FA)-targeted formulation, 0.5 mol% of folate-PEG-DSPE (also pre-dissolved in 100% ethanol) was included as a targeting ligand. Lipid mixture was slowly added to ODN-containing citrate buffer (pH 4.0, 20 mM) under vortexing to achieve ethanol concentration of 40% and the ODN-to-Lipid ratio was 0.10 (w/w). The ODN-containing liposome solution was dialyzed against citrate buffer (pH 4.0, 20 mM) for 1 hr and against pH 7.5 Hepes-buffered saline (HBS) overnight to remove ethanol and replace citrate buffer with neutral pH HBS buffer.

ODN-containing DODAP-liposomes (DSPC/Chol/DODAP/PEG-DSPE = 20/45/25/10 molar ratio) were also prepared using same method as non-targeted and FA-targeted DC-Chol liposomes as a reference formulation.

NICOMP Particle Sizer Model 370 was used to measure the size distribution of liposomes. Encapsulation efficiencies of ODN were calculated by the ratio of the final and initial amount of ODN, multiplied by 100. Liposome samples were stored at 4°C and used within 2 weeks unless indicated otherwise.

3.3.6. Cellular uptake of folate (FA)-targeted ODN-containing liposomes

Cellular uptake of R18-labelled liposomes were visualized or quantified by fluorescence microscopy or flow cytometry. For fluorescence microscopy, KB cells (2×10⁴ cells per well) were seeded in 8-well chamber slides 24 hr prior to the studies. ODN formulated in FA-targeted or non-targeted liposomes was added into each well and
cells were incubated at 4°C for 1 hr followed by at 37°C for 2 hr. After the incubation period, cells were rinsed three times with PBS and visualized by fluorescence microscopy.

For flow cytometry, KB cells were suspended and then incubated in ODN-containing liposome formulations in normal culture medium incubated at 4°C for 1 hr followed by 37°C for 2 hr. After the incubation period, cells were washed three times with PBS, resuspended in fresh PBS, and quantified by flow cytometry.

Competition between the liposomes and free folate for cell-surface folate receptor was examined by pre-incubating cells with 100 or 200 µM free folate 30 min prior to adding ODN-containing liposomes. Before fluorescence microscopy or flow cytometry, extended rinsing or washing steps (with PBS) were necessary to remove excess of free folate in the medium.

### 3.3.7. Transfection of cells

FR+ KB Cells (1×10⁶ /well) were plated in 6 well tissue culture plates 24 hr prior to the studies. The whole study was conducted in normal culture medium condition (folate-free RPMI 1640 medium supplemented with 10% NCS and antibiotics). Free ODN or formulated liposome/ODN complex in normal cultural medium were added to each well and were incubated at 37°C for predetermined incubation periods. At the end of incubation period, cells were washed with cold PBS and harvested and lysed for the quantification of Bcl2 protein.
3.3.8. Quantification of Bcl2 protein by Western blotting

For determination of Bcl2 protein level, ODN treated cells were incubated with a lysis buffer containing a protease inhibitor cocktail (CalBiochem, San Diego, CA) on ice for 20 min. The cell lysates were centrifuged for 5 min at 12,000 g at 4°C. The supernatant was collected and stored at -80°C if necessary. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL) and 20 µg for each sample was loaded on a 15% SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline/Tween-20 (TBST) for 2 hr, the membranes were incubated with monoclonal mouse anti-human Bcl2 (Dako, Carpinteria, CA) or polyclonal goat anti-human actin antibody (Santa Cruz, Santa Cruz, CA) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Bioscience, Piscataway, NJ) or rabbit anti-goat IgG (Pierce) for 1 hr at room temperature. Membranes were then developed with Pierce SuperSignal West Dura Extended Duration Substrate (Pierce) and imaged with Kodak X-OMAT film (Kodak, Rochester, NY). In addition to the film, chemiluminescent signals of the protein bands were also directly quantified using the Chemi-Doc phosphor imager system (Bio-Rad) with Bcl2 protein level normalized to the actin level from the same sample.

3.3.9. Cytotoxicity

To determine the effect of antisense ODN treatment on cell growth, KB cells pre-seeded in 96-well plates were treated at 37°C for 24 or 48 hr and the cytotoxicity was
evaluated by MTT assay as described in Section 2.3.8. Briefly, KB cells were seeded in 96-well plates in folate-free RPMI1640 growth media containing 10% FBS at 37 °C in a 5%-CO₂-humidified incubator 24 hr prior to the study. The culture medium was then replaced with 200 µL medium (with 10% FBS and antibiotics) containing G3139 alone, FA-targeted or non-targeted G3139-containing liposomes, or FA-targeted or non-targeted empty liposomes (without G3139). In a separate study, serial dilutions of daunorubicin (0-100 µM) were also added in order to evaluate the effect of combined treatment of antisense therapy and chemotherapy on KB cells. Cells were incubated with ODN-containing liposomal formulations and daunorubicin for 24 or 48 hr and then subject to MTT assay as described previously.

3.3.10. Statistical analysis

The results were represented as the mean ± standard deviation (SD) of 3 or 4 repeat studies unless otherwise indicated. Means and standard deviations were calculated using standard methods. Student’s t-test or one way ANOVA with post hoc tests was used in the present study. P values < 0.05 were considered statistically significant.
3.4. Results

3.4.1. Preparation and physicochemical characterization of ODN-containing FA-targeted or non-targeted liposomes

In the current study, we adapted and modified an ethanol dilution method developed by Maurer et al [28] to encapsulate ODN into liposomes. The mean diameter and particle size distribution were determined by photon correlation spectroscopy using a NICOMP Particle Sizer Model 370. The particle size of non-targeted or FA-targeted liposomes without ODN is around 60 nm. For ODN-containing formulations, after dialysis, non-targeted and FA-targeted ODN-containing liposomes have particle size distribution of 84.3±10.4 and 82.9±9.4 nm, respectively. The incorporation of FA-PEG-DSPE as the target ligand did not increase the particle size distribution of liposomes, possibly due to the small molar percentage of FA ligand in the formulation (0.5 mol%).

Maintaining the stability of ODN-containing liposomes during the formulation and delivery processes is an important factor for successful antisense delivery. In the current study, the stability of liposomes was evaluated by monitoring the colloidal stability of liposomes and the amount of ODN remaining in the formulation up to 8 weeks. The colloidal stability evaluated by the particle size change up to 8 weeks is shown in Fig. 3.1. No significant particle size change was observed for both formulations during this period.

The encapsulation of ODN was measured at OD 260nm. The lipid concentration (0.8 mg/mL after dialysis) used in the current study had minimal absorbance at this wavelength. Encapsulation efficiencies of ODN in the formulations were about 70-80%
to the initial G3139 amount with no significant difference between non-targeted (72.3±3.2%) and FA-targeted liposomes (77.0±3.3%) (Figure 3.2). ODN-containing DODAP-liposomes (DSPC/Chol/DODAP/PEG-DSPE = 20/45/25/10 molar ratio) were also prepared using the same method as a reference formulation since this formulation has been reported as a high-ODN-encapsulation efficiency formulation [29]. In the current study, DC-Chol-based liposomes achieved higher encapsulation than DODAP liposomes (41.0±2.3%) (p<0.05).

Liposome samples were stored at 4°C and used within 2 weeks, during which time no significant leakage (<1%) of the entrapped ODN was detected by spectrophotometry (OD 260nm). After 8-week storage, more than 90% of loaded-ODN remained in the formulations.

Overall, small, homogeneous, stable, and high ODN-encapsulated liposomal nanoparticles were formed by the method we used in the current study.

### 3.4.2. Uptake of FA-targeted liposomal ODNs by FR+ KB cells

The uptake of R18-labelled ODN-containing liposomes was analyzed in KB cells by fluorescence microscopy and flow cytometry to compare the difference in the uptake of various liposomal ODN formulations. In the absence of liposomes, ODN uptake was minimal (Figure 3.3(A)). FA-targeted ODN containing formulation resulted in a significantly higher cytoplasmic accumulation than non-targeted liposomal formulations (Figure 3.3). The uptake is FA-ligand-concentration dependent as visualized by fluorescence microscopy (Figure 3.4) and the results were confirmed by flow cytometry.
Furthermore, free folate blocked the uptake of FA-targeted liposomal ODN but not non-targeted liposomes suggested that the intracellular delivery of FA-liposomal ODN was mediated by FR-mediated pathway. Results from the uptake studies clearly demonstrated improvement in the uptake of ODN by KB cells following administering FA-targeted formulations.

### 3.4.3. Bcl2 downregulation effect of FA-targeted G3139-containing liposomes

The Bcl2 downregulation effect by G3139 was evaluated by the Bcl2 protein level of G3139 treated KB cells. All transfection experiments were done in 10% serum containing culture medium. KB cells were incubated with G3139-containing liposomes either for 4 hr followed by another 44 hr incubation time period (Figure 3.6(A)) or continuously for 48 hr with liposomes (Figure 3.6(B)). The result showed that 4 hr incubation is not enough for Bcl2 downregulation to occur. While increasing the incubation period to 48 hr, FA-targeted G3139-containing liposomes demonstrated a significant decrease of Bcl2 protein expression as compared to mismatch ODN and free G3139. Empty liposomes alone have no effect in Bcl2 downregulation (data not shown). Moreover, the Bcl2 downregulation effect by FA-targeted G3139-containing liposome is more prominent than commercial liposome formulation (Lipofectamine). In general, FA-targeted G3139-containing liposomes can efficiently downregulate Bcl2 protein in FR+ KB cells. However, further optimizations and adjustment are needed to show more dominant targeting effect. Since the main purpose of this study was to serve as a pilot
study for development of a targeted ODN-delivery system in leukemia cells, which FR may not be a good target, we therefore did not attempt to optimize the formulation.

3.4.4. FA-targeted delivery of G3139 sensitizes cells to chemotherapeutic agents

It has been shown that Bcl2 overexpression leads to resistance of tumor cells to chemotherapy and the downregulation of Bcl2 may reverse chemoresistance and therefore sensitize cells to chemotherapy [30]. Daunorubicin is a widely used chemotherapeutic drug that displays significant antitumor activity against a broad range of tumors. Therefore, studies were conducted to evaluate whether targeted delivery of G3139 can efficiently sensitize KB cells to daunorubicin treatment. An MTT colorimetric assay was used to evaluate the cytotoxicity of G3139-related liposomal formulations and the cytotoxicity of daunorubicin in the presence or absence of various G3139 containing formulations.

For the effect of combined treatment of targeted antisense therapy and chemotherapy, the study was conducted using G3139-containing formulations (0.2 or 5 µM G3139) and incubated for 24 or 48 hr. Table 3.1 showed that the treatment of KB cells with 5 µM of FA-targeted G3139-liposomes significantly increased the capability of daunorubicin to induce cytotoxicity. In all formulations, 0.2 µM G3139 had only minimal effect on the KB cell growth after 24 or 48 hr treatment. As G3139 concentration increase to 5 µM, FA-targeted G3139-containing formulation decreased the IC50 of daunorubicin from 2.71 µM to 0.47 µM, compared with 1.83 µM by free G3139 and 1.21 µM by the non-targeted G3139 formulation, respectively after 24 hr treatment.
(p<0.05). Similar results were observed for 48 hr treatment groups, as the IC$_{50}$ of
daunorubicin decreased from 1.81 µM to 0.12 µM, compared with 0.81 µM by free
G3139 and 0.54 µM by the non-targeted G3139 formulation, respectively (p<0.05). Both
non-targeted and FA-targeted G3139-containing liposomes had a moderated inhibitory
effect on cell growth and the cytotoxicity of the liposomal formulations without G3139 is
minimal (Figure 3.7), indicating that the liposomal formulation itself at this concentration
level is not toxic for the cells and that the biological effect is due to the reaction between
bcl2 mRNA and G3139.

In conclusion, our data suggests that FA-targeted G3139-containing liposomes
increase the uptake of ODNs, and, consequently, these ODN-containing formulations
increase the cytotoxicity of daunorubicin in K562 cells.
3.5. Discussion

Overexpression of FR in tumor cells makes it a prominent candidate for receptor-mediated drug/gene/antisense delivery. Low expression in normal cells can decrease toxicity and increase tissue specificity. The folate, a stable and safe small molecule with low immunogenicity, can bind to FR with high binding affinity, which is an important factor for receptor-mediated delivery systems. The conjugation chemistry of folate is relatively simple and the modification of folate via γ-carboxyl group does not decrease its binding affinity [31]. Folate-conjugated polymers and liposomes have been evaluated for tumor cell selective delivery of therapeutic agents including plasmid DNA via the FR [22, 32, 33].

The objective of this study was to investigate the use of FA-targeted liposomes for the delivery of encapsulated ODN in FR+ tumor cells. More importantly, we would like to develop a method that is more widely suitable for applications such as other DNA-based therapeutics and other ligands for targeted gene delivery or antisense delivery.

Liposomes were composed of DC-Chol, egg PC, and PEG-DSPE. DC-Chol was employed as an ionizable cationic lipid as it has an apparent pKₐ of 7.8 [34]. The encapsulation of ODNs relies on electrostatic interaction between ODN and DC-Chol at acidic pH values (pH 4). As the external pH is adjusted to neutral pH (pH 7.5-7.6), it would result in liposomes with neutral-to-slightly-positive surface charge. At pH 4, >99% of DC-Chol was protonated. Assuming one positive charge per DC-Chol molecule and one negative charge per nucleotide, we chose the total lipid-to-ODN ratio to be 0.10 in order to make “near-neutral” liposomes. As confirmed by Zeta potential analysis, the
The ζ potential for G3139-containing liposome is $1.02 \pm 0.17$ mV. Neutral charge liposomal nanoparticles are more favored than cationic liposomes since cationic liposomes have several problems when administered intravenously, such as rapid elimination from circulation and poor local accumulation in tumors [35-37].

For an antisense delivery system to prove successful, in vivo studies are necessary since the extent and duration of antisense effect following administration are significantly affected by biological barriers, including degradation of DNA, extravasation, targeting to specific cell or tissue type(s), internalization, endosomal escape, cytoplasmic stability, nuclear uptake, etc. [38, 39]. Liposomes have been used to enhance the intracellular delivery of ODNs in vitro; however, this type of carrier may have unfavorable pharmacokinetic properties for most in vivo applications, possibly due to rapid uptake into tissues of the mononuclear phagocytic system [36, 40]. In the current study, polyethylene glycol (PEG) was grafted onto the liposome surface in order to (a) decrease the uptake of liposomes into mononuclear phagocytic system, which consequently increases circulation time and enhances localization of liposomes in disease tissues [41-44], and (b), more importantly, serve as a steric barrier lipid to minimize aggregation and fusion between particles during the formulation process and storage period.

After enhanced intracellular delivery of ODN via the FA-targeted liposomes was demonstrated, their therapeutic application was then evaluated using anti-Bcl2 ODN (G3139) as a model. G3139 is designed to target the coding region of bcl2 mRNA, which consequently inhibits Bcl2 protein synthesis. FA-targeted G3139-containing liposomes exhibited more prominent downregulation effect of Bcl2 protein after 48 hr
Chemotherapy induced tumor cell death can be blocked by the Bcl2 protein in various tumors and leukemias [45-47]. The chemosensitization study showed that, after 48 hr incubation, FA-targeted G3139-containing liposomes significantly enhanced the chemosensitization effect of G3139 by decreasing the IC$_{50}$ of daunorubicin from 1.81 µM to 0.12 µM (15-fold increase) while free G3139 treatment only caused 2.2-fold increase. No difference between empty liposomes and the untreated group was observed, indicating that the sensitization effect was due to the reaction between bcl2 mRNA and G3139. The enhanced uptake observed in fluorescence microscopic studies suggests that the superior chemosensitization effect by FA-targeted G3139-containing liposomes was possibly due to the enhanced delivery of G3139 into KB cells.

In summary, the ethanol dilution method provides a suitable platform to prepare ODN-containing liposomes. The encapsulation procedure is not limited to a particular combination of polyelectrolyte or lipid composition and can be applied to formulations containing polyelectrolyte with oppositely charged liposomes. The small particle size, overall near-neutral charge, and, more importantly, high encapsulation efficiency of ODNs at optimized conditions are important characteristics for the development of DNA-based therapeutics delivery systems. In the next two chapters, a similar method will be applied to other antisense delivery systems including ODNs and small interfering RNAs (siRNAs) with a high molecular weight ligand (transferrin) to evaluate whether this method is suitable for more comprehensive applications.
<table>
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<tr>
<th>Formulations / IC₅₀ (µM)</th>
<th>IC₅₀ (24hr)</th>
<th>IC₅₀ (48hr)</th>
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<tr>
<td>untreated</td>
<td>2.71 ± 0.09</td>
<td>1.81 ± 0.05</td>
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<td>Free G3139 (0.2 µM)</td>
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<td>1.79 ± 0.04</td>
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<td>1.82 ± 0.03</td>
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<td>FA-targeted G3139 (0.2 µM)</td>
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<td>1.49 ± 0.04</td>
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<tr>
<td>Free G3139 (5 µM)</td>
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</tr>
<tr>
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</tr>
<tr>
<td>FA-targeted G3139 (5 µM)</td>
<td>0.47 ± 0.05*</td>
<td>0.12 ± 0.02*</td>
</tr>
</tbody>
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Table 3.1 FA-targeted delivery of G3139 sensitizes KB cells to chemotherapeutic agent (daunorubicin) treatment.a.

aCells were treated with 0.2 or 5 µM of free G3139, non-targeted or FA-targeted G3139-containing liposomes for 24 or 48 hr. b the data represent the mean of three separate experiments and the unit of IC₅₀ of daunorubicin is µM. * indicate statistical difference between this group and all other groups (p<0.05).
Figure 3.1 Colloidal stability studies of liposomal formulations. Particle size distribution of (○) non-targeted ODN-containing liposomes, or (●) FA-targeted ODN-containing liposomes up to 8 weeks.
Figure 3.2 ODN encapsulation efficiency of different liposomal formulations.
Figure 3.3 Fluorescence microscopic examination of cellular uptake of ODN-containing liposome formulations. KB cells \(2 \times 10^4/\text{well}\) were seeded in 8-well chamber slides 24 hr prior to uptake studies. ODN (0.22 \(\mu\text{M}\)) formulated in liposomes with or without a folate ligand were added into each well and cells were incubated at 4°C for 1 hr followed by at 37°C for 2 hr. After the incubation period, cells were washed three times with PBS, re-suspended in fresh PBS, and viewed by either fluorescence microscopy (left panels) or phase contrast (right panels). Cellular uptake of R18-labelled ODN-containing formulations was examined under a Nikon fluorescence microscope at 400x magnification. A: untreated (no liposomes); B and C: non-targeted ODN-containing liposomes; D and E: FA-targeted ODN-containing liposomes; A, B, and D: in the absence of free folate; C and E: in the presence of free folate (100\(\mu\text{M}\)).
**Figure 3.4** Fluorescence microscopic examination of cellular uptake of Folate-targeted ODN-containing liposome formulations. KB cells (2*10⁴/well) were seeded in 8-well chamber slides 24 hr prior to uptake studies. ODNs (0.09 or 0.22 µM) formulated in FA-targeted liposomes were added into each well and cells were incubated for 1 hr at 4⁰C followed by 2 hr at 37⁰C. After the incubation period, cells were washed three times with PBS, re-suspended in fresh PBS, and viewed by either fluorescence microscopy (left panels) or phase contrast (right panels). Cellular uptake of R18-labelled ODN-containing formulations was examined under a Nikon fluorescence microscope at 400x magnification. A: untreated (no liposomes); B and C: FA-targeted ODN(0.09 µM)-containing liposomes; D and E: FA-targeted ODN(0.22 µM)-containing liposomes; A, B, and D: in the absence of free folate; C and E: in the presence of free folate (100µM).
Figure 3.5 Cellular uptake of Folate-targeted or non-targeted ODN-containing R18-labeled liposomal formulations in the absence or presence of free folate measured by flow cytometry. (A) a representative histogram of comparison of fluorescence intensity in different ODN-containing formulations. a: untreated control; b: 1µM ODN complexed with non-targeted liposomes; c: 1µM ODN complexed with non-targeted liposomes with preincubation of 200µM free folate; d: 1µM ODN complexed with FA-targeted liposomes; e: 1µM ODN complexed with FA-targeted liposomes with pre-incubation of 200µM free folate. (B) KB cells treated with ODN(0.16-1.6µM)-containing non-targeted or FA-targeted liposomal formulations in the absence or presence of 200µM of free folate.
Figure 3.6 Western analysis of Bcl2 expression in KB cells following targeted delivery of bcl2 antisense ODN. KB cells were treated with ODN-containing formulations as described in this figure. KB cells were incubated with formulations for 4 hr, and then change to fresh medium for additional 44 hr (A) or for continuous 48 hr (B).
Figure 3.7 Cytotoxicity of non-targeted or FA-targeted liposomal formulations in KB cells determined by MTT assay after 48 hr incubation. (□) non-targeted empty liposomes; (△) FA-targeted empty liposomes; (■) non-targeted G3139-containing liposomes; and (▲) FA-targeted G3139-containing liposomes. Data are shown as mean ± SD (n=5).
LIST OF REFERENCES FOR CHAPTER 3


CHAPTER 4

EFFICIENT DELIVERY OF A BCL2-SPECIFIC ANTISENSE OLIGODEOXYRIBONUCLEOTIDE (G3139) VIA TRANSFERRIN RECEPTOR TARGETED LIPOSOMES

4.1. Abstract

A novel transferrin receptor (TfR) targeted liposomal formulation was synthesized and evaluated for the delivery of a phosphorothioate antisense oligodeoxyribonucleotide (ODN) against Bcl2 (G3139) in K562 leukemia cells. Liposomes composed of DC-Chol/egg PC/PEG-DSPE (25:73.5:1.5, mole/mole) were loaded with G3139 with high efficiency (70-80%). To prepare Tf-targeted liposomes, transferrin was first coupled to Mal-PEG-DSPE and then incorporated by post insertion. The liposomes had mean diameter in the 100 – 150 nm range and exhibited colloidal stability when incubated with serum-containing media. Liposome uptake was evaluated in TfR positive K562 cells and was found to be dependent on Tf ligand density on the liposomes and more efficient than that of the non-targeted control formulation. Uptake of Tf-targeted liposomal ODN by K562 cells could be blocked by excess free transferrin.
Incubation of K562 cells with G3139 antisense oligonucleotides in Tf-targeted liposomes resulted in 2-fold and 10-fold greater Bcl2 protein downregulation than cells treated with non-targeted liposomes and with free G3139, respectively (p<0.05). Treatment of these cells with 2 µM G3139 in Tf-targeted liposomes resulted in >80% reduction in Bcl2 expression. In addition, treatment of K562 cells with Tf-targeted liposomal G3139 increased their sensitivity to chemotherapy agent daunorubicin, as determined by an MTT cytotoxicity assay. In conclusion, Tf-conjugated liposomes are effective delivery vehicles for G3139 antisense oligos in TfR positive K562 cells and warrant further investigation as a potential therapeutic agent.
4.2. Introduction

Antisense oligodeoxyribonucleotides (ODNs) have shown substantial promises as therapeutic agents in the treatment of cancer and leukemia [1-3]. ODNs have relatively rapid plasma clearance and are given clinically via prolonged intravenous infusion. Novel formulation strategies for ODNs can potentially increase the systemic circulation time of ODNs and achieve targeted delivery to tumor cells via conjugation to a targeting moiety, which may lead to improvement in their therapeutic efficacy [4, 5].

G3139 is a phosphorothioate antisense ODN specific for Bcl2, which is a regulator of cellular apoptosis that is frequently overexpressed in tumor cells [6]. Bcl2 overexpression is associated with increased tumor cell resistance to chemotherapy and its downregulation has been shown to reverse chemoresistance [7]. Initial clinical evaluation of G3139 in conjunction with chemotherapy has led to promising results in leukemia patients [6, 8]. This study is aimed at evaluating a novel formulation of G3139 formulation, based on transferrin (Tf) conjugated liposomes, for targeted delivery to leukemia cells.

The transferrin receptor (TfR) is a 180 kDa dimeric transmembrane glycoprotein overexpressed in cancer and leukemia cells. Transferrin (Tf), a 80 kDa glycoprotein, is the ligand for TfR and is internalized by receptor mediated endocytosis [9-12]. Transferrin conjugated polymers [13, 14] and liposomes [15-21] have been evaluated for tumor cell selective delivery of therapeutic agents including plasmid DNA via the TfR. In this chapter, in order to demonstrate the targeting ability of the formulation, Tf was incorporated as the targeting moiety to bind to TfR, which were overexpressed in several
leukemia cell lines. The incorporation method, however, does not limit to transferrin and is applicable to other protein ligands or antibodies. A Polyethylene glycol (PEG)–lipid derivative was also introduced into the liposomal formulations to enhance the stability as well as to increase circulation lifetime of the nucleic-acid-containing formulations in vivo.

In the current study, a Tf-conjugated liposomal formulation of G3139 was synthesized and evaluated in human leukemia K562 cells. The cellular uptake mechanism of the Tf-targeted liposomes and antisense activity against Bcl2 were evaluated.
4.3. Materials and methods

4.3.1. Materials

Methoxy-polyethylene glycol (M.W. 2,000)- distearoyl phosphatidylethanolamine (DSPE-PEG) was purchased from Lipoid (Newark, NJ). Egg phosphatidylcholine (egg PC) and DSPE-PEG-maleimide (DSPE-PEG-Mal) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DC-Chol was synthesized as described in Section 3.3.4. Octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR). Holo-transferrin (Tf), 2-Iminothiolane (Traut’s reagent), 5,5’-dithio-bis-(2-nitrobenzoic acid) (Ellman’s reagent), bovine serum albumin (BSA) protein standards, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All tissue culture media and supplies were purchased from Life Technologies (Rockville, MD).

4.3.2. Antisense oligodeoxyribonucleotide

All ODNs used in this study were fully phosphorothioated. The antisense G3139 (5’-TCT CCC AGC GTG CGC CAT-3’) [22] is a 18-mer ODN designed to specifically bind the first six codons of the human Bcl-2 mRNA, thereby resulting in a decrease in Bcl2 protein translation [6]. G3139 was supplied by the National Cancer Institute (Bethesda, MD) and used without further purification. The sequence of mismatch control ODN is 5’-TCT CCC AGC ATG TGC CAT and was purchased from Integrated DNA Technologies (Coralville, Iowa).
4.3.3. Cell culture

K562, a human myelogenous leukemia cell line obtained from the American Type Culture Collection (ATCC) (Manassas, VA), was used in this study. The cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂.

4.3.4. Preparation of transferrin (Tf)-targeted G3139-containing liposomes

The process of preparation of Tf-targeted G3139-containing liposome is summarized in Figure 4.1. For non-targeted G3139-containing liposomes, DC-Chol, Egg PC, and PEG-DSPE were individually dissolved in 100% ethanol and mixed in a molar ratio of 25/73.5/1.5; 0.1 mol% of fluorescent probe R18 was added to the formulations for cellular uptake studies. The lipid mixture was slowly added to G3139 dissolved in a citrate buffer (pH 4.0, 20 mM) under vortexing to achieve a final ethanol concentration of 40% and an ODN-to-lipid ratio of 0.10 (w/w). The preparation was then dialyzed against citrate buffer (pH 4.0, 20 mM) for 1 hour and then against pH 7.5 HEPES-buffered saline (HBS) overnight, using a MWCO 10,000 Dalton membrane, to remove ethanol and free ODNs.

A post-insertion method was adopted to incorporate Tf ligand into the G3139-loaded liposomes. Holo-Tf (diferric transferrin) was reacted with 5x Traut’s reagent to synthesize holo-Tf-SH. Free sulphhydryl content in holo-Tf-SH was measured by Ellman’s reagent. The holo-Tf-SH was then reacted to micelles of Mal-PEG-DSPE at a
molar ratio of 1:10, and then incubated with G3139-containing liposomes at different ligand to liposome ratios for 1 hr at 37°C. To prepare liposomes containing different amounts of Tf, Tf-PEG-DSPE-to-liposomal lipid ratios used were 1:400 (0.25 mol%), 1:200 (5 mol%), 1:100 (1 mol%), and 1:50 (2 mol%). Non-targeted control liposomes were prepared by coupling bovine serum albumin (BSA) to the liposomes by the same method.

Liposome size was analyzed on a NICOMP Particle Sizer Model 370. Encapsulation efficiencies of ODN were calculated based on the ratio of the initial and final amount of ODN after dialysis, multiplied by 100.

4.3.5. Cellular uptake of Tf-targeted G3139-containing liposomes

Uptake of Tf-targeted and non-targeted control G3139 loaded liposomes fluorescently labeled with R18 was evaluated in K562 cells. For uptake studies, 5×10^5 cells were incubated with G3139(1 μM)-containing liposomes in RPMI1640 medium for 4 hr at 37°C. Receptor blocking studies were performed by pre-incubating the cells with 125 μM holo-transferrin 30 min prior to adding ODN-containing liposomes. The cells were then washed three times with pH 7.4 phosphate-buffered saline (PBS) and re-suspended in fresh PBS for analysis by fluorescence microscopy or flow cytometry.
4.3.6. Transfection studies

K562 Cells (10×10^6/well) were plated in 6 well tissue culture plates with 1.2mL/well of fresh RPMI1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics prior to the studies. Three hundred µL/well of free or liposomal G3139 in was added to each well and the cells were incubated for 4 hours at 37°C. The cells were then transferred to T25 flasks, diluted with fresh culture media, incubated for a further 24, 48, 72, or 96 hr, and then analyzed for bcl2 mRNA expression levels and Bcl2 protein expression levels.

4.3.7. Quantification of Bcl-2 RNA by real-time RT-PCR

The effect of G3139, delivered by Tf-targeted liposomes, on bcl2 mRNA expression was evaluated using real-time RT-PCR, as previously described [23]. Briefly, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) by measure OD at 260 nm (PerkinElmer, Boston, MA). For cDNA synthesis, 2 µg of total mRNA from each sample was mixed with 1.5 µL of 20 µM random hexamer primer (Perkin Elmer, Boston MA) and heated to 70°C for 2 min followed by cooling on ice for 5 min. Seventeen µL of master mixture containing M-murine leukemia virus reverse transcriptase (Invitrogen), 5x reaction buffer (Invitrogen), 100 mM dithiothreitol, 10mM of each dNTP, and RNAsin (Promega, Madison, WI) was added into each sample and the samples were then incubated in a thermal cycler (Applied Biosystem, Foster City, CA) at 42°C for 60 min following by 94°C for 5 min. The resulting cDNA was amplified by real time PCR (ABI Prism 7700 Sequence Detection System (Applied Biosystem)). The
following oligonucleotides primers designed by the Primer Express program (Applied Biosystems) were used: Bcl2, forward primer CCCTGTGGATGACTGAGTACCTG; reverse primer CCAGCCTCCGTTATCCTGG; probe ACCGGCACCTGCACACCTGGA. Each cDNA sample was used as a template in two separate PCR amplification reactions: (a) a set of primers and a FAM/TAMRA dual labeled probe for Bcl2 transcripts, and (b) primers and a VIC-labeled probe for a housekeeping gene ABL. Bcl2 mRNA was normalized to ABL mRNA levels.

4.3.8. Quantification of Bcl-2 protein by Western blotting

The detail process of quantification of Bcl-2 protein by western blot was described in Section 3.3.8

4.3.9. Cytotoxicity assay

The cytotoxicity of daunorubicin, alone and in combination with G3139-containing liposomes, was evaluated in K562 cells by an MTS cell proliferation assay (Promega). Briefly, the cells were transferred in 80 µL per well of media to 96-well plates and incubated with empty liposomes, free G3139, or G3139-containing liposomes (0, 0.5, or 4 µM G3139) in 10µL (n=5). Serial dilutions of daunorubicin (0 to 100 µM) in 10µL were then added and the cells were incubated at 37°C for 4 hr. Another 100 µL of fresh medium was then added to each well and the cells were incubated for an additional 48 hr. At this point, 20 µL of CellTiter 96® AQueous One Solution (Promega)
was added to each well and the cells were incubated at 37°C for another 4 hr. Absorbance was measured at 492 nm on an automated plate reader (Molecular Devices, Sunnyvale, CA). Chemosensitization ratio was calculated by the IC₅₀ of daunorubicin plus pretreatment with free G3139 or G3139-loaded liposomes divided by the IC₅₀ of daunorubicin alone.

4.3.10. Statistical analysis

Data points were represented as the mean ± standard deviation (SD) of 3 or 4 repetitions unless otherwise indicated. Experimental groups were compared using Student’s t-test and one way ANOVA with post hoc tests. P value of 0.05 was used as a cutoff for statistical significance.
4.4. Results

4.1. Preparation and physicochemical characterization of G3139-containing liposomes

Figure 4.1 illustrated the overall process of preparation of Tf-targeted G3139 containing liposomes. Ethanol dilution method modified from Maurer et al [24] was used to encapsulate G3139 into liposomes and a post-insertion method was applied to prepare Tf-targeted G3139-containing liposomes. Holo-Tf (diferric transferrin) was used in the current study due to its high binding affinity to TfR, compared to apo-Tf [11]. The particle size distribution of liposomal formulations was measured by NICOMP Particle Sizer Model 370 (Table 2). The particle size of empty liposomes (without G3139) was around 80 nm. The particle size increased to 114.2 ± 6.2 nm after the encapsulation of G3139. The incorporation of Tf ligands only slightly increased the particle size to 126-156 nm, depending on the ratio of Tf-PEG micelles-to-liposomes (Table 4.2). The encapsulation efficiency of G3139 in the formulations was about 70-80%. Overall, small, homogeneous, and high G3139-encapsulated liposomal nanoparticles were formed by the method we developed in the current study.

The colloidal stability of G3139 loaded liposomes was evaluated by monitoring change in the size distribution of the liposomes and the amount of G3139 remaining in the formulation during storage. The initial particle size distribution of empty liposome (no G3139), non-targeted, and Tf-targeted (5 mol% Tf-PEG-DSPE) G3139-containing liposomes was 70.7±8.3, 115.7±7.9, and 131.5±8.5 nm, respectively and no significant
changes were observed for 8 weeks at 4°C (Figure 4.2). In addition, greater than 90% of loaded-ODN remained in the formulations following the storage.

4.4.2. Uptake of Tf-targeted liposomal G3139 by K562 cells

The uptake of R18-labelled G3139-containing liposomes was analyzed in K562 cells by fluorescence microscopy. BSA-liposomes were similarly synthesized as Tf-liposomes and used as a non-targeted control in these studies. In the absence of liposomes, ODN uptake was minimal. As shown in Figure 4.3, Tf-targeted G3139 containing liposomes were efficiently internalized by the cells and the level of cellular uptake was significantly higher than that of non-targeted control liposomes (Figure 4.3) after 4 hr incubation. Liposomal G3139 without surface modification with proteins exhibited a similar uptake level to BSA-conjugated liposomes. Furthermore, free transferrin blocked the uptake of Tf-targeted liposomal G3139 but not BSA-conjugated liposomes suggesting that the cellular uptake of Tf-liposomes was mediated by transferrin receptor. Results from the uptake studies demonstrated the improvement in the uptake of G3139 by K562 cells following conjugation with transferrin.

4.4.3. Downregulation of Bcl2 by Tf-targeted G3139-containing liposomes.

The bcl2 downregulation effect of G3139 in liposome formulations was evaluated by both Bcl2 protein and bcl2 mRNA levels of treated K562 cells. Unless indicated otherwise, all transfection experiments were performed in 10% serum containing culture medium. Real-time RT-PCR analysis demonstrated a significant decrease of bcl2 mRNA
level following treatment by G3139 containing liposomes (Figure 4.4). In contrast, no bcl2 downregulation was observed following treatment with a mismatch ODN, indicating that antisense effect was sequence specific. Empty liposomes alone also had no effect in bcl2 mRNA down-regulation (data not shown). The downregulation effect persisted for ~72 hr. The enhancement of downregulation due to Tf-targeting was also most profound at 72 hr, with maximum effect of 80% reduction in bcl2 transcript. The difference in bcl2 mRNA reduction between the Tf-targeted formulation (80%) than the non-targeted formulation (70%) and free G3139 (50%) treated cells were statistically significant (p< 0.05), as shown in Figure 4.4.

At protein level, the Bcl2 effect was maintained for ~ 96 hr in cells treated with liposomal formulations of G3139 compared to 48-72 hr in free G3139 (Figure 4.5). Densitometry analysis revealed that Bcl2 levels were decreased by up to 70% by G3139 in Tf-targeted liposomes, compared to 8% by free G3139 and 40% by G3139 in non-targeted liposomes, respectively (Figure 4.6). The Bcl2 downregulation effect was Tf-concentration dependent, as shown in Figure 4.7.

The effect of serum on transfection efficiency was also investigated. After treatment with 1 µM G3139 for 48 hr in 10%, 25%, or 50% serum-containing medium, cells were lysed and Bcl-2 mRNA level was quantified by real-time RT-PCR. As shown in Figure 4.8, both Tf-targeted and non-targeted G3139-containing liposomal formulations studied were resistant to the presence of serum, which might be an important factor that contributes to the future success of in vivo ODN delivery.
4.4.4. Tf-targeted delivery of G3139 sensitizes leukemia cells to chemotherapeutic agents

Previous studies showed that Bcl2 overexpression led to tumor cell resistance to chemotherapy and that downregulation of bcl2 may reverse chemoresistence and sensitize tumor cells to chemotherapy [25]. Daunorubicin is a widely used chemotherapeutic drug that displays significant activity against a broad range of tumors and leukemias. Therefore, we evaluated whether Tf-targeted delivery of G3139 could sensitize K562 cells to daunorubicin using an MTS-based colorimetric cellular proliferation assay. As shown in Table 4.2, 4 µM of Tf-targeted G3139-liposomes decreased the IC\textsubscript{50} of daunorubicin from 1.8 µM to 0.18 µM, comparing to 0.67 µM by free G3139 and 0.32 µM by non-targeted G3139 formulation, respectively (p<0.05). In addition, the cytotoxicity of the liposomal formulations without G3139 was minimal.

It has been shown that Bcl2 overexpression leads to resistance of tumor cells to chemotherapy and the downregulation of bcl-2 may reverse chemoresistence and therefore sensitize cells to chemotherapy [25]. Daunorubicin, a widely used chemotherapeutic drug, displays significant antitumor activity against a broad range of tumors. Therefore, studies were conducted to evaluate whether targeted delivery of G3139 can efficiently sensitize K562 cells to daunorubicin treatment. An MTT-based colorimetric assay was used to evaluate the cytotoxicity of daunorubicin in the presence or absence of various G3139 containing formulations. Cell viability was also assessed to evaluate whether transferrin and plain liposomes were toxic to the cells. G3139 at a concentration of 0.5 µM had minimal effect on the cytotoxicity of daunorubicin in K562 cells (data not shown). Table 1 showed that the treatment of K562 cells with 4 µM of Tf-
targeted G3139-liposomes increased the capability of daunorubicin to induce cytotoxicity. Tf-targeted G3139-containing formulation decreased the IC₅₀ of daunorubicin from 1.8 µM to 0.18 µM, comparing to 0.67 µM by free G3139 and 0.32 µM by non-targeted G3139 formulation, respectively (p<0.05). In addition, the cytotoxicity of the liposomal formulations without G3139 is minimal. The growth of K562 cells exposed to the non-targeted or targeted empty liposomes was indistinguishable from that of untreated cells (Table 4.2), indicating that the liposomal formulation itself at this concentration level is not toxic for the cells and that the biological effect is due to the reaction between bcl2 mRNA and G3139. In conclusion, our data suggest that Tf-targeted liposomes increase the uptake of ODNs, and, consequently, these ODN-containing formulations increase the cytotoxicity of daunorubicin in K562 cells.
4.5. Discussion

The objective of this study was to investigate the use of Tf-targeted liposomes for the delivery of G3139 to TfR-positive leukemia cells and other tumor cells. We showed here the liposomes prepared with a cationic cholesterol derivative (DC-Chol) enhanced the efficiency of G3139, a bcl2 antisense oligonucleotide, in K562 cells. In addition, the incorporation of transferrin as a targeting ligand further improved ODN delivery efficiency.

The lipid composition used in this study was DC-Chol/egg PC/PEG-DSPE. DC-Chol is a weakly basic cationic lipid containing a tertiary amine with a pKₐ close to neutral pH [26]. The encapsulation of ODNs in the liposomes is facilitated by electrostatic interaction between ODN and DC-Chol at pH 4, where >99% of DC-Chol was protonated. When pH was subsequently adjusted to pH7.5, DC-Chol is only partially ionized. This results in a reduction in liposome surface charge, as shown by its low zeta potential of 1 mV. PEG-DSPE in the liposome formulation serves to reduce plasma protein binding. Therefore, the liposome formulation was designed to extend the in vivo circulation time and reduce non-specific tissue distribution [27-29]. In fact, in a pilot study, G3139-formulated in the liposomes exhibited a much greater AUC and longer half life as compared to free G3139 (data not shown).

Chemotherapy-induced tumor cell death can be blocked by the Bcl2 protein in various tumors and leukemias [30-32]. The chemosensitization study showed that the Tf-targeted G3139 liposomal formulation enhanced the chemosensitization effect of
G3139 by decreasing the IC$_{50}$ of daunorubicin from 1.77 $\mu$M to 0.18 $\mu$M (9.8 -fold increase). In contrast, pretreatment with free G3139 only caused 2.7-fold increase, which was similar to the results observed by Kim et al [33]. No difference between the empty liposome and untreated groups indicated that the sensitization effect was due to the reaction between bcl2 mRNA and G3139. The enhanced uptake observed in fluorescence microscopic studies suggested that the superior chemosensitization effect by Tf-targeted G3139-containing liposomes was possibly due to the increased delivery of G3139 into K562 cells. Overall, this suggests that it might be possible to combine Tf-targeted G3139 liposomes with chemotherapy for treatment of leukemias.

Last but not least, the approach that we described to selectively introduce antisense oligonucleotides into K562 cells might be applied to other cell types that overexpress TfR or other surface receptors such as epidermal growth factor (EGF) receptors and folate receptors. In addition, the system could also be adapted to the delivery of other nucleotides including small interference RNA (siRNA) since ODN and siRNA share a similar molecular weight range and have similar charge properties.

In summary, a Tf-targeted liposomal formulation of antisense ODN G3139 was evaluated K562 leukemia cells. This formulation exhibited excellent characteristics in terms of particle size, loading efficiency, colloidal stability, and vehicle toxicity. Furthermore, this formulation was very efficient in antisense delivery, showing excellent bcl2 downregulation efficiency and TfR specificity. Further preclinical studies are warranted to evaluate the therapeutic potential of this novel formulation.
Table 4.1 Particle size distribution of various liposomal formulations.\textsuperscript{a}

\textsuperscript{a} the data represent the mean of three separate experiments.

\textsuperscript{b} Except for “empty liposomes” group, all other formulations contain G3139.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Particle size (nm)</th>
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<tbody>
<tr>
<td>Empty liposomes</td>
<td>79.7 ± 4.1</td>
</tr>
<tr>
<td>non-targeted liposomes</td>
<td>114.2 ± 6.2</td>
</tr>
<tr>
<td>Tf-targeted liposomes (0.25 mol% Tf-PEG-DSPE)</td>
<td>126.8 ± 10.3</td>
</tr>
<tr>
<td>Tf-targeted liposomes (0.5 mol% Tf-PEG-DSPE)</td>
<td>134.2 ± 7.7</td>
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<tr>
<td>Tf-targeted liposomes (1.0 mol% Tf-PEG-DSPE)</td>
<td>143.4 ± 3.1</td>
</tr>
<tr>
<td>Tf-targeted liposomes (2.0 mol% Tf-PEG-DSPE)</td>
<td>156.0 ± 6.8</td>
</tr>
<tr>
<td>Formulations</td>
<td>IC$_{50}$ (µM) of daunorubicin$^b$</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>control (PBS only)</td>
<td>1.77 ± 0.15</td>
</tr>
<tr>
<td>Non-targeted vector alone</td>
<td>1.60 ± 0.10</td>
</tr>
<tr>
<td>Tf-targeted vector alone</td>
<td>1.93 ± 0.15</td>
</tr>
<tr>
<td>Free G3139</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td>non-targeted G3139-liposomes</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Tf-targeted G3139-liposomes</td>
<td>0.18 ± 0.03$^*$</td>
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**Table 4.2** Tf-targeted delivery of G3139 sensitizes K562 cells to chemotherapeutic agent (daunorubicin) treatment$^a$.

$^a$Cells were treated with 4 µM of free G3139, G3139 liposomes, or empty liposomes.  
$^b$the data represent the mean of three separate experiments.  $^*$indicate statistical difference between this group and all other groups (p<0.05).
Figure 4.1 Preparation of Tf-targeted G3139-containing liposomes.
Figure 4.2 Colloidal stability of liposomal formulations. Particle size distribution of (▲) empty liposomes, (○) non-targeted G3139-containing liposomes, or (●) Tf-targeted G3139-containing liposomes up to 8 weeks. (n=3).
Figure 4.3 Cellular uptake of Tf-targeted G3139-containing liposomes in K562 cells. ODN formulated in liposomes with or without Tf or BSA ligand were added into each well. Four hours following the incubation at 37°C, the cells were washed three times with PBS. Cells were then re-suspended in fresh PBS for fluorescence microscopy. Cellular uptake of R18-labelled ODN-containing formulations was examined under a Nikon fluorescence microscope with at 400x magnification. A: no liposomes; B and C: G3139-containing non-targeted liposomes; D and E: BSA-coated G3139-containing liposomes; F and G: Tf-targeted G3139-containing liposomes. A, B, D, and F: in the absence of free holo-transferrin. C, E and G: in the presence of free holo-transferrin (125µM).
Figure 4.4 Kinetic of bcl2 mRNA downregulation by G3139 in various formulations. K562 cells were treated with free mismatch ODN (Δ), free G3139 (▲), non-targeted G3139-containing liposomes (○), or Tf-targeted G3139-containing liposomes (●). ODN concentration was 1 μM in all groups except for untreated group (■). Cells were analyzed at 24, 48, 72, or 96 hr by real-time RT-PCR to quantify the bcl2 mRNA levels. (n=3)
Figure 4.5  Kinetic of Bcl2 protein down-regulation effect by G3139. K562 cells were treated with free G3139, non-targeted G3139-containing liposomes, or Tf-targeted G3139-containing liposomes. G3139 concentration was 1 µM in all groups except for untreated group. Bcl2 protein level was determined by Western blot. A representative Western blot of Bcl2 protein expression (A), its corresponding densitometry data (B).
Figure 4.6 G3139 decreases Bcl2 protein and mRNA expression in K562 cells. A representative Western blot of Bcl2 protein expression (A), its corresponding densitometry data (B), and results of real-time RT-PCR analysis(C) at 48 hr following treatment with G3139-containing formulations. (n=3).
Figure 4.7 Effect of G3139 concentration on bcl2 downregulation. A representative Western blot of Bcl2 protein expression (A), its corresponding densitometry data (B), and results of real-time RT-PCR analysis (C) at 48 hr following treatment with G3139-containing formulations. (n=3)
Figure 4.8 Effect of serum concentration on transfection efficiency of G3139 liposomes. K562 cells were treated with 1 μM G3139 for 48 hr and the bcl2 mRNA levels were quantified by real-time RT-PCR. (△) untreated, (▲) free G3139, (○) non-targeted G3139-containing liposomes, and (●) Tf-targeted G3139-containing liposomes. (n=3)
LIST OF REFERENCES FOR CHAPTER 4


CHAPTER 5

ENHANCEMENT OF TRANSFERRIN RECEPTOR-TARGETED DELIVERY
OF siRNA BY DESFERRIOXAMINE

5.1. Abstract

Iron deficiency can upregulate transferrin receptor (TfR) expression at mRNA and protein levels. This study is aimed at evaluating the effect of iron chelator desferrioxamine (DFO) on TfR-targeted siRNA delivery in leukemia cells. K562 cells, which are TfR positive, were cultured in medium containing 0-20 µM DFO for one week prior to siRNA treatment. Cell viability was assessed by MTT assay. siRNA against luciferase gene was incorporated into cationic liposome formulations composed of DC-Chol/egg PC/PEG-DSPE (25:73.5:1.5, mole/mole) by an ethanol dilution method. For TfR-targeted formulations, holo-Tf was incorporated into siRNA-containing liposomes as a targeting ligand. Uptake of Tf-FITC was analyzed by fluorescence microscopy and flow cytometry. For luciferase expressing cells, plasmid DNA containing a luciferase gene under CMV promoter was complexed with polyethylenimine and transfected into DFO-treated or non-treated cells for 1 hr. For gene silencing studies, siRNA-containing liposomes were incubated with the transiently transfected cells for an additional 24hr. Transfection efficiency and gene silencing were evaluated by luciferase assay. Uptake of
Tf-FITC increased significantly in K562 cells pretreated with 20 µM DFO, as shown by flow cytometry and fluorescence microscopy. This uptake process can be blocked by excess free transferrin, indicating a TfR-dependent uptake mechanism. In contrast, treatment with a lower concentration (2 µM) DFO produced no significant effect. siRNA transfection studies showed that TfR-targeted siRNA liposomes can downregulate luciferase activity by 66.3%, as compared to 48% by non-targeted formulation and 36.2% by Lipofectamine®, respectively (p<0.05). In 20 µM DFO -pretreated cells, TfR-targeted siRNA formulations are 2-5-fold more efficient than that in non-DFO-pretreated cells (p<0.05). No DFO effect was observed in cells treated with non-targeted formulations under the same conditions. In summary, our data suggest that DFO pre-treatment increased the uptake of TfR-targeted siRNA in K562 cells. Tf-targeted siRNA formulation, combined with DFO pre-treatment, was a highly efficient delivery vehicle for siRNA for leukemias that express the TfR.
5.2. Introduction

Since RNA interference (RNAi) was first discovered in *C. elegans* in 1998 [1], it has rapidly become one of the hottest topics in DNA-based therapy due to its high efficiency and selectivity. Synthetic small interfering RNAs (siRNAs), which bypass the process of DICER cleavage, were extensively studied in the past few years [2]. siRNAs are currently being investigated in many gene-related diseases, e.g., cancers, acute liver failure, and some infectious disease such as HIV, influenza viruses, Hepatitis B and C viruses, Herpesviruses, etc [3-5]. In cancer therapy, RNAi can be applied to silence specific oncogenes such as BCR-ABL [6, 7], AML1/ETO [8, 9], and RAS [10] or to reduce multi-drug resistance by inhibiting MDR-1 [11]. Compared with other gene-ablation techniques such as AS-ODNs, siRNAs exhibited superior efficacy, possibly due to their high specificity to the target mRNAs and higher resistance to ribonucleases [12]. Efficient and selective delivery and rational siRNA design may lead to the success of RNAi therapy.

Desferrioxamine (DFO) is a FDA approved iron chelator for the treatment of secondary Fe overload [13, 14] that also exhibited anti-tumor activity, possibly by depleting iron in the tumor or by causing selective oxidative stress in tumors [15-17]. It has been proposed that, in response to hypoxic conditions, expression of genes involved in angiogenesis, erythropoises, and iron metabolism are upregulated [16, 18]. Transferrin (Tf) and transferrin receptor (TfR) are among the key components in iron metabolism. Therefore, in the current study, K562 cells were pretreated with various concentrations of DFO and their uptake of Tf-FITC was studied.
TfR is a 180 kDa dimeric transmembrane glycoprotein overexpressed in cancer and leukemia cells. Transferrin, a 80 kDa glycoprotein, is the ligand for TfR and is internalized by receptor mediated endocytosis [19, 20]. Tf-targeted formulations have been evaluated extensively for tumor cell selective delivery of therapeutic agents including plasmid DNA via the TfR [21-31]. In the current study, a Tf-conjugated siRNA-containing liposomal formulation was developed and evaluated in human leukemia K562 cells. K562 cells were also pretreated with DFO to investigate whether the enhancing effect was TfR-related.
5.3. Materials and methods

5.3.1. Materials

1,2-Distearoyl-sn-Glycero-3-phosphoethanolamine-N-[(Polyethylene Glycol)] (DSPE-PEG) was purchased from Lipoid (Newark, NJ). Egg PC and DSPE-PEG-Maleimide (DSPE-PEG-Mal) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). DC-Chol was synthesized as described in Section 3.3.4. Holo-transferrin, Fluorescein Isothiocyanate (FITC), desferrioxamine (DFO), bovine serum albumin (BSA) protein standards, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All tissue culture media and supplies were purchased from Invitrogen.

5.3.2. Plasmid and siRNAs

Luciferase Plasmid was prepared as described in Section 2.3.2. Luciferase siRNA (Luc siRNA) was purchased from Dharmacon (Chicago, IL) with target sequence 5’-GCC ATT CTA TCC TCT AGA GGA TG-3’.

5.3.3. Cell culture

HELA, a human epithelial adenocarcinoma cell line, was used in a pilot transfection study due to its easy-to-be-transfected property. HELA cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂.
K562, a human myelogenous leukemia cell line obtained from the American Type Culture Collection (ATCC) (Manassas, VA), was used in this study. The cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine at 37°C in a humidified atmosphere containing 5% CO₂.

In order to study the effect of DFO pretreatment, fresh K562 cells were separated into three flasks and were cultured in normal culture medium, normal culture medium plus 2 µM DFO, or normal culture medium plus 20 µM DFO for 1 week. Cell viability was checked every two days by trypan blue assay.

5.3.4. Preparation of siRNA-containing liposomes

For non-targeted siRNA-containing liposomes, DC-Chol, egg PC, and PEG-DSPE were individually dissolved in 100% ethanol and mixed in a molar ratio of 25/74/1. siRNA in small volume was slowly added to lipid ethanol solution under vortexing. The siRNA-lipid solution was then diluted with HBS (pH 7.5) to achieve a final ethanol concentration of 10%.

For Tf-targeted siRNA-containing liposomes, the post-insertion method described in Chapter 4 was applied to incorporate Tf into siRNA-containing nanoparticles. This method provides a simple yet efficient way to introduce a large molecule like transferrin to pre-made, nucleic acid or drug encapsulated liposomes. Briefly, holo-Tf was first modified to holo-Tf-SH and then coupled to Mal-PEG-DSPE preformed-micelles and then transferred the surface of pre-made, siRNA-containing liposomes for 1 hr at 37°C.
fixed the ratio of Holo-Tf-SH: Mal-PEG-DSPE at 1:10 (mol/mol) since more than 95% of Tf was included in Tf-PEG micelles in this condition.

NICOMP Particle Sizer Model 370 was used to measure the size distribution of liposomes.

5.3.5. Synthesis of Tf-FITC

To synthesize of Tf-FITC, holo-transferrin was dissolved in 50 mM pH 8.5 Tris buffer and FITC was dissolved in DMSO. FITC was added to the Tf solution at an FITC-to-Tf ratio of 1.5:1 (mol/mol ratio) and incubated at room temperature for 2 hr, after which, FITC-Tf was passed through a PD-10 column with pH 7.4 PBS as eluent to remove free FITC. Transferrin concentration was measured at OD 280 nm and at OD 595nm with protein assay reagent (Bio-Rad). FITC concentration was measured at OD 495 nm. The concentrations were calculated based on the corresponding protein or FITC standard curves. Purified Tf-FITC was sterilized by filtration with 0.22 µm syringe membrane and stored at 4°C. After PD-10 column, the total recovery of Tf-FITC was 87% of initial amount and was ready to use for uptake studies. BSA-FITC was also synthesized using the same method.

5.3.6. Cellular uptake of Tf-FITC by non-treated or DFO-pretreated K562 cells

An uptake study was conducted to evaluate the effect of DFO pretreatment on the uptake of transferrin. Tf-FITC was synthesized and used in this study. BSA-FITC was
applied as a negative control assuming the cellular uptake of transferrin was mainly through a TfR-mediated pathway. For uptake studies, $5 \times 10^5$ cells were used. Uptake of Tf-FITC into pretreated or non-pretreated K562 cells was assessed after incubating cells with Tf-FITC (0, 0.5, or 2 µM) in normal culture medium for 2 hr at 37 °C. Competition between Tf-FITC and free transferrin for cell-surface receptor was examined by pre-incubating cells with 125 µM holo-transferrin 30 min prior to adding Tf-FITC. After the incubation period, cells were washed three times with pH 7.4 phosphate-buffered saline (PBS) and then re-suspended in fresh PBS for fluorescence microscopy or flow cytometry. DAPI staining was also applied to some samples in order to stain for the nucleus.

5.3.7. Preparation of DNA polyplexes and transfection studies

DNA/PEI Polyplexes were prepared in PBS by addition of branched PEI (M. W. 25,000 Dalton) to pcDNA3-CMV-Luc plasmid DNA in 75 µL of PBS to achieve the desired N/P ratios in a final volume of 150 µL. Samples were incubated at room temperature for 15 min prior to transfection studies.

For transfection studies, $1.0 \times 10^6$ K562 cells per well were transferred to 24-well plates prior to the transfection studies. Serum free culture medium was used in the incubation period. One microgram of pcDNA3-CMV-Luc plasmid DNA per well was used for most transfection studies, unless otherwise indicated. Prior to transfection, the medium was removed and the plates were rinsed with 0.5 mL of PBS. Polyplexes were then added to each well and incubated with cells in 0.5 mL serum free media at 37°C for
4 hr. Thirty min after adding DNA/PEI polyplexes, siRNA-containing formulations were added to each sample. Serum free medium as used during the 4 hr incubation period. After 4 hr incubation, the media were removed and the cells were rinsed with 0.5 mL of PBS followed by the addition of 0.5 mL of fresh medium containing 10% FBS and antibiotics. The cells were incubated for another 24 hr and then lysed and assayed for luciferase activity. All transfection experiments were performed in triplicate.

To assay for the luciferase activity, the medium was removed from each well and the cells were washed with 0.5 mL/well of cold PBS. One hundred microliters of ice-cold lysis buffer (100 mM Tris-Cl, 2 mM EDTA, 1% Triton X-100, pH 7.8) was added to each well and incubated on ice for 20 min. The cell lysate was then transferred to 1.7 mL centrifuge tubes and centrifuged at 15,000g for 2 min. The detailed steps for luciferase assay were described in Section 2.3.7.

5.3.8. Statistical analysis

The results were represented as the mean ± standard deviation (SD) of 3 or 4 repeat studies. Means and standard deviations were calculated using standard methods. Both Student’s t-test and one way ANOVA with post hoc tests were used in the present study. P values < 0.05 were considered statistically significant.
5.4. Results and discussion

5.4.1. Preparation and physicochemical characterization of siRNA-containing liposomes

In this chapter, we modified the ethanol dilution method due to the stability consideration of siRNAs. The particle size distribution of siRNA liposomes measured by NICOMP Particle Sizer Model 370 was similar to G3139-containing liposomes. The particle size of empty liposomes (without siRNA) was around 90 nm, which was not totally unexpected considering the similar size and similar charge properties of AS-ODN and siRNA. The particle size increased to 125.8 ± 10.2 nm after the encapsulation of siRNA. The incorporation of Tf ligands only slightly increased the particle size to about 140 nm, as we had observed in Chapter 4. Overall, small, and homogeneous, siRNA-encapsulated liposomal nanoparticles were formed by the method we used in the current study.

There are many commercialized, read-to-use siRNA on the market. Luciferase siRNA was chosen for the same reason that I chose luciferase plasmid DNA: (a) reporter activity is available immediately after translation since the post-translational process of the protein is not required; (b) this is a very sensitive assay due to the high efficiency of its light production and no background luminescence in the host cells; and (c) the assay is rapid, requiring only a few seconds per sample.
5.4.2. Cell viability of K562 cells after DFO pretreatment

After 1-week pretreatment with DFO, cell viability was measured by trypan blue assay (Figure 5.1). Compared to the non-pretreatment group, the cell number decreased to 55% in the 20-µM DFO group. 2-µM DFO treatment only slightly decreased cell viability to 86%. This is possibly due to the anti-tumor activity of DFO. Although DFO was approved by FDA as an iron-chelator, it has been shown to have anti-tumor activity, especially in those tumors that overexpress transferrin receptors [16]. TfR tended to be upregulated in rapidly proliferating tumor cells and those cells were shown to have an increased requirement for iron [32]. Therefore, it is reasonable to speculate that DFO as an iron-chelator may cause the death of TfR-overexpressing cells due to inefficient influx of iron.

5.4.3. Uptake of Tf-FITC by K562 cells

Uptake studies were performed to evaluate the effect of DFO pretreatment of K562 cells on transferrin cellular uptake. BSA-FITC were similarly synthesized as Tf-FITC and used as a non-targeted control in these studies. The uptake of Tf-FITC was analyzed by fluorescence microscopy and by flow cytometry. After incubation of K562 cells for 2 hr with Tf-FITC, cells pretreated with 20-µM DFO had a significantly higher cytoplasmic accumulation of Tf-FITC than did non-DFO-treated K562 cells (Figure 5.2). In the absence of Tf-FITC, fluorescence intensity was minimal (Figure 5.3 and Figure 5.4). Although 2-µM DFO pretreatment is less toxic (Figure 5.1), uptake studies confirmed that this concentration was not high enough to induce the upregulation of TfR.
and therefore, did not enhance the transferrin uptake in K562 cells (Figure 5.4). No difference was observed between non-pretreatment or 2-µM DFO pretreated K562 cells (Figure 5.4). Furthermore, free transferrin blocked the uptake of Tf-FITC but not BSA-FITC suggested that the intracellular delivery of Tf-liposomal ODN was mediated by transferrin receptor-mediated pathway. Results from the uptake studies demonstrated the improvement in the uptake of Tf-FITC in K562 cells by pretreated cells with 20-µM DFO.

5.4.4. Transfection of siRNA-containing liposomes

Before we tried the transfection of Luc siRNA-containing liposomes in K562 cells, a pilot study using HELA cells was conducted since HELA cells are known to be very easy to transfect. As seen in Figure 5.5, Luc siRNA-containing liposome formulations that I developed in this chapter were pretty efficient and exhibited significantly higher inhibition effect than did Lipofectamine in HELA cells. At 0.25 µg Luc siRNA-containing liposomes per sample, >99% luciferase activity induced by luciferase plasmid DNA was inhibited.

Compared to HELA cells, leukemia cells are much more difficult to transfect. Therefore, a higher amount of siRNA was needed to achieve an inhibitory effect. In the current study, 1 µg siRNA per sample was used. The transfection study (Figure 5.6) showed that TfR-targeted siRNA liposomes can downregulate luciferase activity by 66.3%, as compared to 48% by the non-targeted formulation and 33.2% by Lipofectamine®, respectively (p<0.05) in K562 cells. Next, the effect of DFO-
pretreatment or the transfection of Tf-related siRNA-containing liposomes was evaluated. Similar to the uptake study, there was no difference in transfection results between non-treated or 2-µM DFO pretreated cells. In 20-µM DFO pretreated cells, TfR-targeted siRNA formulations are 2-5-fold more efficient than observed in no-DFO-pretreated cells (p<0.05). The effect of DFO pretreatment was transferrin-concentration dependent. Combining the results of the transfection studies and uptake studies suggested that the enhancement effect by DFO pretreatment was possibly due to the upregulation of TfR.

In summary, I developed a targeting strategy for siRNA that was potentially applicable in treating leukemias overexpressing transferrin receptors. The novel Tf-targeted siRNA carrier developed in this study was shown to generate nanometer-size particles with siRNAs and to effectively deliver siRNA to target cells. The inhibition effect by siRNA-containing liposomes was remarkable in HELA cells. In TfR overexpressing K562 leukemia cells, Tf-targeted siRNA liposome formulations exhibited higher inhibition than non-targeted formulation as well as one commercial liposomal product. The delivery of siRNA to cells via a receptor-mediated pathway provides the prospect of more selective targeting of these reagents in association with increased intracellular concentrations in target cells. More future studies directed toward optimization of the formulation and in vivo studies are needed for this formulation to work clinically.
Figure 5.1 Cell viability of K562 cells after one-week DFO treatment (0, 2, or 20 μM).
Figure 5.2 Cellular uptake of Tf-FITC (0.5 µM) in K562-related cell lines after 2 hr incubation. Left panel: K562 cells; right panel: K562 pretreated with 20 µM DFO. Cellular uptake was examined under a Nikon fluorescence microscope with at 1000x magnification. (A) and (D): fluorescence microscopy; (B) and (E): DAPI staining for nucleus; (C) and (F): overlapping images from fluorescence microscopy and DAPI staining.
Figure 5.3 Cellular uptake of FITC-Tf (0.5 μM) in K562-related cells after 2 hr incubation. Cell uptake was measured by flow cytometry. (a) 20 μM DFO-pretreated K562 cells; (b) K562 cells; and (c) K562 cells without Tf-FITC.
Figure 5.4 Cellular uptake of Tf-FITC or BSA-FITC in DFO-pretreated (2 or 20 μM) or non-pretreated K562 cells measured by flow cytometry. Cells were treated with (A) 0.5 μM of Tf-FITC or BSA-FITC, or (B) 2 μM of Tf-FITC or BSA-FITC in the absence or presence of free Tf (125 μM). (n=3)
Figure 5.5 Transfection of HELA cells with DNA/PEI complex and siRNA-containing formulations as indicated in each figure. Cells were treated with 1, 0.5, or 0.25 µg of plasmid DNA complexed with PEI at N/P ratio of 10. Cells were first incubated with DNA/PEI complex for 30 min and then siRNA-containing formulations were added into each sample. Results are shown as relative light units (RLU)/mg protein. (n=3)
Transfection of K562 cells with DNA/PEI complex and siRNA-containing formulations as indicated in each figure. Cells were treated with 1 µg of plasmid DNA complexed with PEI (N/P 10). Cells were first incubated with DNA/PEI complex for 30 min and then siRNA (1 µg)-containing formulations were added into each sample. Results are shown as relative light units (RLU)/mg protein. (n=3)
Figure 5.7 Transfection of pretreated or non-pretreated K562 cells with DNA/PEI complex and siRNA-containing formulations with different amount of transferrin in liposome formulations. Cells were treated with 1 µg of plasmid DNA complexed with PEI (N/P 10). Cells were first incubated with DNA/PEI complex for 30 min and then siRNA(1 µg)-containing formulations were added into each sample. Results are shown as relative light units (RLU)/mg protein. (n=3)


CHAPTER 6

SUMMARY AND PERSPECTIVES

The numerous formulations developed to target specific receptors and the exciting preliminary results in cell culture reflect a high level of enthusiasm in the gene delivery community for this tumor targeting strategy. The findings indicated that besides receptor-mediated tumor cell uptake, formulation characteristics that provide reduced non-specific cellular uptake, efficient endosomal escape, increased plasma stability, and prolonged systemic circulation are critical for the specificity and efficiency of gene transfer by receptor targeted vectors. Optimization of these properties is, therefore, urgently needed. Furthermore, key determinants of in vivo gene transfect vector performance include its pharmacokinetic property and stability in circulation, and its ability to localize in and distribute within the target tumors. Novel strategies, such as covalent vector stabilization and PEGylation of the vector, might prove critical for the in vivo performance of receptor-targeted vectors. In addition, the effects of tumor endothelial permeability and the rate of extravascular diffusion within the tumor may have a profound impact on the ability of gene transfer vectors to reach its target tumor
cells. Continued efforts in optimization of receptor-mediated gene transfer vectors will likely lead to the development of a tumor-specific vehicle for therapeutic gene delivery and promote the advancement of clinical translation of cancer gene therapy.

The specific aims of this dissertation were to develop receptor-mediated non-viral delivery agents for DNA-based therapeutics and evaluate their biological activity in vitro.

In Chapter 2, HER2-specific antibody (trastuzumab) conjugated PEI was shown to selectively deliver luciferase plasmids to the HER2-overexpressing cancer cells with high resistance to serum, which may render PEI a much more selective gene delivery vector for anticancer gene therapy.

Chapter 3 was mainly focused on developing a method to formulate AS-ODN or siRNAs into liposomes. FA was chosen since FA-PEG-DSPE is a ready-to-use FA derivative that can be easily incorporated into liposome composition. DC-Chol was chosen as the cationic lipid due to its unique charge property in different pH. Overall surface charge of prepared liposomes is only slightly positive. Combining with the incorporation of PEG-derivatives, this formulation can avoid some problems occurred on cationic ODN-liposomal formulations, such as rapid circulation elimination. Small, homogenous liposomes with high ODN encapsulation efficiency were produced by the method developed in this chapter. Uptake studies of FA-targeted or non-targeted ODN-containing formulations demonstrated that the successful incorporation of FA-ligand into liposomes led to higher cellular uptake of liposome-associated ODNs in FR+ tumor cells. The low cytotoxicity of liposomes without ODNs is also an important factor for formulation development.
The aim of Chapter 4 was to develop a targeted ODN-containing liposome formulation that can efficiently and specifically delivery ODNs to leukemias. G3139, a potent Bcl2 AS-ODN currently in Phase 3 clinical trial of various leukemias, is the model ODN chosen in this chapter. Transferrin receptors were overexpressed in many tumor and leukemia cells, especially those rapidly proliferating tumor cells. The method can also be applied to other leukemia-specific receptors or other AS-ODNs in the future. Similar to FA-targeted ODN-containing liposomes prepared in Chapter 3, we got stable, small, homogenous, serum-resistant Tf-targeted G3139-containing liposomes with 70-80% ODN encapsulation efficiency, which is higher than many current ODN-liposomal formulations. Downregulation of bcl2 mRNA and Bcl2 protein by Tf-targeted G3139 liposomes was markedly higher than free G3139 and non-targeted formulations. The results confirmed that the G3139 maintained its activity after the enhanced cellular uptake by Tf-targeted liposomes.

In Chapter 5, we applied similar idea to siRNA delivery. DFO was used to upregulate TfR in K562 leukemia cells, which can also further confirm the enhanced delivery was due to Tf-targeting effect. The results showed that the preparation method can be applied to siRNA. With the aid from DFO-pretreatment, Tf-targeted liposomal Luc siRNA exhibited higher luciferase downregulation effect in K562 cells, which is an encouraging result for Tf-targeted DNA-based therapeutics delivery.

In this dissertation, we attempted to develop a reliable and scalable method to produce stable, efficacious, and cell-type specific DNA-related liposomal formulations. Some purposes were achieved; however, we should realize that we are still far from
applying this preparation method and/or targeted formulation to clinical use. More optimization of the formulations is needed. The detailed of cell internalization process is still yet to be clarified. More specific targets to leukemia cells are preferred. In vivo studies are also required for the pharmacokinetics and pharmacodynamic of the targeted formulations.
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