Lipopolymer Mediated siRNA Therapy for Cancer: Focus on Acute Myeloid Leukemia

by

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Abstract

Protein silencing by small interfering RNA (siRNA) is a promising treatment strategy for cancer as over-expression of proteins is largely responsible for cancer cells' infinite proliferation, evasion of cell death and multi-drug resistance. However, siRNAs require a carrier as their biological instability, negative charge and large molecular weight prevent cellular delivery. In this thesis, I first provide a review of current non-viral siRNA carrier strategies designed to protect and deliver the siRNA to the cell cytoplasm for RNAi activity and then follow with an over-view of the current state of siRNA development with non-viral carriers specifically in leukemia. One promising cationic polymer for siRNA delivery is high molecular weight polyethylenimine (PEI); however, its toxicity is an obstacle for clinical use. This thesis investigates a library of low-molecular weight (2 kDa) PEI with hydrophobic (lipid) modifications as siRNA carriers. The lipid modification renders this otherwise ineffective low-toxic polymer a safe and effective delivery system for intracellular siRNA delivery and protein silencing. We first explore a lipid modified polymer library in adherent cells lines targeting a model protein target, the house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and several relevant cancer targets; P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and survivin. These initial studies in adherent cells demonstrated that although the exact formulations for efficient silencing depended on the cell line and protein target, silencing with two of the lipid-modified polymers (caprylic and linoleic acid substitutions) were consistently effective, suggesting that these carriers can be applied clinically. Fine-tuning of the siRNA/polymer composition was however critical for silencing particular targets. We then focus our efforts specifically on Acute Myeloid

Leukemia (AML), where siRNA therapy development has lagged behind the cancers that are derived from attachment dependent cells, as evident in the included review of current efforts in AML siRNA therapy. We explored the feasibility of the lipid-modified carriers in AML cell lines. Efficient siRNA delivery and silencing of the model protein target, green fluorescent protein (GFP), was achieved with higher functionality than that of 25 kDa PEI, where again caprylic acid and linoleic acid substitutions stood out as the most desirable polymer substitutions. Further work demonstrated effective silencing of an AML therapeutic target CXCR4, a surface expressed adhesion protein that contributes to leukemic cell survival. The suppression of CXCR4 as well as its ligand, SDF-1 (CXCL12), resulted in a decrease in overall cell survival, which was largely attributed to a decrease in cell proliferation without enhanced effects when silencing the two targets simultaneously. The decrease in cell numbers due to CXCR4/SDF-1 silencing occurred both in the absence and presence of human bone marrow stromal cells (hBMSC), suggesting that the proposed approach would be effective in the presence of the protective bone marrow microenvironment. In more clinically related models, siRNA delivery was achieved in all human AML patient cells tested and CXCR4 silencing was demonstrated in some cases, *ex vivo*. The effects of silencing CXCR4 in an AML subcutaneous *in vivo* tumor model were also explored. Overall, we found that caprylic and linoleic lipid-substituted PEI2 can provide effective siRNA delivery to leukemic cells and can be employed in molecular therapy of leukemia targeting suitable proteins, such as CXCR4, with therapeutic outcomes. We conclude with a discussion on the further development of siRNA carriers with focus on AML therapy, describing potential enhancements that could move the field forward.

Preface

Previous versions of the literature reviews and research presented in this thesis have been published, as described below. All chapters were conceptualized, researched and written under the involvement of H. Uludağ, the supervisory author. Additional acknowledgements are listed at the end of the respective chapters. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "siRNA Based Therapies for Leukemias", No. 687/04/13/D, 01/05/2012. The other research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Novel strategies to overcome drug resistance in leukemia", No. Pro00043783, 21/01/2014 and 26/01/2015. Biosafety approval was obtained under UA file # RES0012356.

Chapter 1 contains the literature review consisting of two parts. **Chapter 1 - Part I**, a review on the delivery of siRNA biomolecules, is included within the manuscript published as H.M. Aliabadi, B. Landry, B, C. Sun, T. Tang, and H. Uludağ, "Supramolecular Assemblies in Functional siRNA Delivery: Where do we Stand?" *Biomaterials*, vol. 33, issue 8, 2546-69. Inclusion of the described paper was limited to the sections I specifically contributed to (Section 1.1 Background on siRNA Carriers was written by Aliabadi with significant insight and contribution by myself and for Section 1.2 A Mechanistic Look At Cellular Delivery Of SiRNA Complexes, I was the primary author.) Figure 1.1 is courtesy of D. Meneskesedag-Erol. Figure 1.2 and Figure 1.4 are courtesy of H.M. Aliabadi. **Chapter 1 - Part II** contains a siRNA leukemia review expected to be published as B. Landry, J. Valencia-Serna, H. Gül-Uludağ, X. Jiang, A.

Janowska-Wieczorek, J. Brandwein, and H. Uludağ, "Progress in RNAi Mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia." As the primary author, I was responsible for the literature review, analysis and manuscript composition. Gül-Uludağ, Jiang, Janowska-Wieczorek, and Brandwein, through their leukemia expertise, ensured accuracy of several ideas covered in the paper. Valencia-Serna provided insight into writing of the manuscript and contributed specifically to the chronic myeloid leukemia (CML) sections of the paper (the major sections that are specific to CML have been removed from the chapter).

Chapter 2 contains portions of three published papers, where the major portion came from a paper published as H.M. Aliabadi, B. Landry, R.K. Bahadur, A. Neamnark, O. Suwantong, and H. Uludağ, "Impact of Lipid Substitution on Assembly and Delivery of siRNA by Cationic Polymers." *Macromolecular Bioscience*, vol. 11, issue 5, 662-72. Although I was not the first author, I was fully and directly involved in the design of the studies, collection of the data, analysis of the data and review of manuscript. Sections of an additional two papers have also been included and were published as H.M. Aliabadi, B. Landry, P. Mahdipoor, and H. Uludağ, "Induction of Apoptosis by Survivin Silencing through siRNA Delivery in a Human Breast Cancer Cell Line." *Molecular Pharmaceutics*, vol. 8, issue 5, 1821-30. H.M. Aliabadi, B. Landry, P. Mahdipoor, C.Y.M. Hsu, and H. Uludağ, "Effective Down-regulation of Breast Cancer Resistance Protein (BCRP) by siRNA Delivery using Lipid-substituted Aliphatic Polymers." *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 81, issue 1, 33-42. Included portions were those that I had direct involvement (design, data collection and data analysis). Other portions of the papers, which I was less involved in, were

minimized, briefly mentioned or removed. Figure 2.5 is courtesy of Aliabadi. Lipid-polymers utilized in these studies were synthesized by Neamnark.

Chapter 3, 4 and 5 are research papers focused on siRNA therapy for acute myeloid leukemia. As the lead author, I designed, performed and analyzed the studies and wrote the manuscript. **Chapter 3** is published as B. Landry, H.M. Aliabadi, A. Samuel, H. Gül-Uludağ, J. Xiiaoyan, O. Kutsch, and H. Uludağ, "Effective Non-viral Delivery of siRNA to Acute Myeloid Leukemia Cells with Lipid-substituted Polyethylenimines." PLoS ONE, vol. 7 issue 8, e44197. Figure 4.1 and Figure 4.2 are courtesy of O. Suwantong. J. Valencia-Serna and B. Sahin provided the data for creation of Figure 3.S3. **Chapter 4** is expected to be published as B. Landry, H. Gül-Uludağ, J. Hongxing, and H. Uludağ, "Targeting CXCR4/SDF-1 Axis by Lipopolymer Complexes of siRNA in Acute Myeloid Leukemia." **Chapter 5** involves AML patient cells and tumor model studies and will be included in a future paper. Samuel was an undergraduate summer student who designed and performed experiments under my guidance. Specific cells used in these studies were provided by A. Janowska-Wieczorek (THP-1, KG-1 and HL-60), O. Kutsch (GFP positive THP-1 cells), H. Jiang (human bone marrow stromal cells), and J. Brandwein and Z. Zak (AML patient cells). Drs. Jiang and Gül-Uludağ afforded leukemia research guidance. Dr. Aliabadi provided assistance in the *in vivo* cancer model through injection of carriers into the tumor, caliper measurements, helped with tumor extractions and general mouse handling. My role in the same *in vivo* cancer model study included design of the experiment, cells/carrier preparation for injection, mouse weighing and monitoring, tumor extractions, general mouse handling, and all post tumor extraction

processing and data analysis. Lipid-polymers utilized in these studies were synthesized by A. Neamnark, R. Bahadur K.C. and J. Fife.

We conclude with **Chapter 6**, Conclusions and Future Directions. This chapter includes portions of the future work sections of the two review papers described above (H.M. Aliabadi, et al. *Biomaterials*, vol. 33, issue 8, 2546-69. and B. Landry et al. In progress.), as well as new content derived from the knowledge gained from the work presented in the thesis.

Acknowledgements

I have had the opportunity to be supported by scholarship funding over the course of my graduate studies. I would like to acknowledge and thank the following institutions for their support: Canadian Institutes of Health Research (CIHR) for the Frederick Banting and Charles Best Canada Graduate Scholarship, University of Alberta for the President's Prize of Distinction, Alberta Cancer Foundation (ACF) for the Graduate Studentship, and Women and Children's Health Research Institute Graduate (WCHRI) for the Graduate Studentship. I have also been supported to present at several conferences and would like to thank the granting programs; Profiling Alberta's Graduate Students Award (University of Alberta), GMN NSERC CREATE Program On Regenerative Medicine and GSA Professional Development Grant (University of Alberta).

Over the course of my program I have had the opportunity to work with many talented researchers within our own lab as well in others across the University of Alberta campus. I would specifically like to thank my lab colleagues (past and present) (in alphabetical order); Dr. Meysam Abbasi, Dr. Rajesh Alphonse, Jeremy Fife, Ross Fitzsimmons, Dr. Hilal Gül-Uludağ, Dr. Charlie Hsu, Dr. Vanessa Incani, Dr. Remant KC, Cezary Kucharski, Xiaoyue Lin, Parvin Mahdipoor, Adam Manfrin, Deniz Meneksedag-Erol, Dr. Hamidreza Montazeri, Nesrine Mostafa, Manoj Parmar, Dr. Laura Rose, Anuja Samuel, Basak Sahin, Juliana Valencia-Serna, Dr. Guilin Wang, and Dr. Sufeng Zhang. The exposure to many researchers from different backgrounds (biological sciences, chemistry, dentistry, engineering, medicine, and pharmacy) where those who have completed their time at University of Alberta have followed many different career directions provided a wide range of experiences. I enjoyed working together with these

talented researchers who made the lab a positive research environment. Specifically I would like to thank Dr. Hamidreza Montazeri whom I enjoyed working on many experimental studies together, Cezary Kucharski for guidance in cell culture studies and Dr. Laura Rose whom I spent most of my PhD years with.

I would also like to thank my previous undergraduate supervisors, Dr. Robert Burrell and his PhD student at the time, Dr. Patricia Nadworny as well as my first summer research supervisor, Dr. Carolina Goano-Diaz. Dr. Goano-Diaz provided me with my first start in research in the summer of 2006. Dr. Robert Burrell and Dr. Patricia Nadworny furthered my interest in research through subsequent research summers.

I would also like to acknowledge the contribution of the co-authors and those listed in the acknowledgment sections in journal publications for all of their help and greatly appreciated contributions. I would specifically like to thank the main contributors; Dr. Hamidreza Montazeri, Dr. Hilal Gül-Uludağ, Juliana Valencia-Serna as well as my supervisor, Dr. Hasan Uludağ. I would also like to thank Anuja Samuel, a summer student who worked with me on the earlier leukemia studies.

I would like to thank my family and friends for their support during my studies; my parents (Lorine Hancock-Landry and Malcolm Landry) as well as my siblings (Nathan (Asami), Adam and Jordan), and my nephews (Naoki and Taiki) and my fiancé (Aaron Lim).

Lastly, I have greatly appreciated the support and mentorship I received from my supervisor Dr. Hasan Uludağ. I am thankful for both the strong academic support he provides and the positive laboratory environment he maintains, which is essential for good and enjoyable research.

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i. Scope

The objective of my thesis project was to explore a new approach for treatment of cancer, with specific focus on Acute Myeloid Leukemia (AML). In most cancers, initial treatment includes broad-spectrum chemotherapy, and in the case of AML, patients may go on to receive additional chemotherapy or a hematopoietic stem cell transplant. However, treatment effectiveness is limited by intolerable toxicities emerging after intensive therapy as a result of non-specific drug actions on healthy tissues and necessary dose increase subsequently due to multi-drug resistance to the drugs. While small molecule drugs are associated with unacceptable side-effects, given their interaction with unintended pathways, small interfering RNAs (siRNAs) may be more suitable for overcoming drug resistance or overexpressed pro-survival proteins as they target the protein at the mRNA level by highly specific base-pairing. The siRNA, however, is highly sensitive to degradation by serum nucleases and its negative charge prevents intracellular uptake on its own, and therefore, efficient carriers are required for intracellular siRNA delivery.

This project explored the feasibility of delivering siRNA to down-regulate proteins that cause aberrant cell growth and/or minimize effective chemotherapy treatments in cancer cells with specific focus on AML. Our main objectives were: 1) development of a polymeric delivery system for siRNA delivery to cancer cells and specifically leukemic cells; 2) characterization of siRNA nanoparticles and cellular uptake; 3) *in vitro* studies for inhibition of leukemic cell growth; and lastly 4) siRNA silencing in *ex vivo* and *in vivo* AML models. The work within this thesis provides

thorough details of current progress of siRNA therapeutics for cancer with specific focus on AML and the studies to address the objectives of the thesis.

I first provide an introduction to supramolecular assemblies used for siRNA delivery in **Chapter 1** with specific focus on siRNA therapy for leukemia. I provide a detailed review of the successes and remaining challenges from the supramolecular carrier entry into the cancer cell to its release of siRNA and finally in achieving a therapeutic result through RNAi activity, in **Chapter 1 – Part I**. A thorough review of siRNA therapy specifically for leukemic cells is then presented in **Chapter 1 – Part II**. Leukemic cells are suspension-growing cells, well known for their challenging properties in respect to gene (polynucleotide) delivery. The review provides the current progress in this challenging field and also addresses the research gaps that remain to be investigated.

Chapter 2 details experiments done on adherent cell lines, two breast cancer cell types as well as a model cell line for Breast Cancer Resistance Protein (BCRP) chemoresistance that occurs in cancer cells. Here, we determined the efficiency of lipid-substituted polymers for siRNA delivery, evaluating the effect of lipid-substitution level, lipid substitution and polymer:siRNA ratios, in the three adherent cell lines. I then examine the ability of the polymer-siRNA complexes to mediate silencing by targeting the house-keeping gene and model target, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an anti-apoptotic and pro-survival protein, survivin, and the cell membrane transporter proteins, P-glycoprotein (P-gp) and BCRP, which cause multi-drug resistance (MDR) by effluxing chemotherapy drugs from the cancer cells. I also discuss the effects that the complexes physiochemical characteristics (zeta-potential and siRNA binding) have on their overall efficiency.

In **Chapter 3**, I build on what we learned and developed for the siRNA therapy in adherent cells and applied our knowledge to siRNA delivery to AML leukemic cells (suspension cells). The extent of siRNA nanoparticle internalization in AML cells was extensively analyzed. Here, I looked at three different AML cell lines, THP-1, KG-1 and HL-60, to investigate reproducibility of the delivery results. The effects of our formulations on cell cytotoxicity as well as their potential ability for siRNA delivery were analyzed. These studies included exploration of various formulations (polymer to siRNA ratios, lipid used for substitutions, lipid substitution levels, and siRNA concentrations) as well as kinetic delivery studies and comparative studies to commercial-available *in vitro* carriers. I then focused on optimizing our system with a GFP reporter gene and was able to better demonstrate effective silencing and improve our silencing efficiency within clinically relevant siRNA concentrations (25-50 nM).

In **Chapter 4**, I further explored our therapeutic goal for siRNA therapy in AML through silencing CXCR4, an adhesion receptor protein as well as its ligand, SDF-1. Here I methodically analyzed the effective response through *in vitro* studies, which included demonstration of effective suppression and resulting therapeutic effects of silencing including decreased proliferation and decreased adhesion to human bone marrow stromal cells (hBMSCs). Silencing with clinically relevant variables, including co-culture with hBMSCs and co-treatment with a chemotherapy drug, was also explored.

After optimization with the reporter gene, GFP, and selection of CXCR4 as an effective target, I pursued silencing in AML patient cell models and animal studies, as presented in **Chapter 5**. Here, I tested siRNA therapy systems in human AML patient cells with our most promising lipid-polymers *ex vivo*. I examined their consistency for

siRNA delivery utilizing a small cohort of human AML patient cells. I additional looked at silencing of CXCR4 and resulting therapeutic effects. I also explored *in vivo* silencing and resulting therapeutic effect (decrease in tumor size) utilizing a subcutaneous tumor xenograft model established with GFP positive AML cells (AML THP-1).

I conclude this thesis with **Chapter 6**, which summarizes our findings and contribution to siRNA therapy for cancer and specifically AML. I delve into research gaps for non-viral siRNA therapy in general, highlighting improvements needed to evaluate siRNA carrier system development and the unknowns in pharmacokinetics on a cellular and intracellular level. The research gaps were probed specifically for siRNA therapy in AML in more detail, discussing the need to improve siRNA therapy efficacy, further understanding of the impediments to efficient siRNA delivery and siRNA effects, methods to improve efficacy and detail potential 'enhanced' AML siRNA treatment strategies. Lastly, I discuss clinically relevant evaluation of siRNA carriers in AML utilizing *ex vivo* human patient cells and bone-marrow mimicking environments as well as suitable in vivo models for progression into pre-clinical work.

1. Supramolecular Assemblies in Functional siRNA Delivery with a Focus on Leukemia Therapy[x]

[X]Versions of sections of this chapter (Part I) was published in:
H.M. Aliabadi, B. Landry, B, C. Sun, T. Tang, and H. Uludağ, "Supramolecular assemblies in functional siRNA delivery: where do we stand?" *Biomaterials*, vol. 33, issue 8, 2546-69.

[X]Versions of sections of this chapter (Part II) to be published as:
B. Landry, J. Valencia-Serna, H. Gül-Uludağ, X. Jiang, A. Janowska-Wieczorek, J. Brandwein, and H. Uludağ, "Progress in RNAi Mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia."*

* A review was published during the preparation of the manuscript: J Guo *et al.* Biomimetic nanoparticles for siRNA delivery in the treatment of leukaemia. (2014) *Biotechnology Advances,* vol. 32, 1396-1409.

PART I. OVERVIEW of SIRNA THERAPY WITH NON-VIRAL CARRIERS

1.1 BACKGROUND ON SIRNA CARRIERS FOR SIRNA THERAPY

Despite the promise of RNA interference (RNAi) and reported success of direct delivery of "naked" siRNA to some tissues [1], administered siRNA has little chance of *in vivo* efficacy if it is not structurally modified or accompanied with an engineered delivery system. The naked siRNA has a poor pharmacokinetics profile. It is almost instantly degraded by RNase A type nucleases [2] that leads to short serum half-life ($t_{1/2}$) on the order of <30 minutes [3]. The rapid siRNA clearance by the kidneys also contributes to its short $t_{1/2}$ (the glomerular molecular weight cut-off of ~60 kDa is larger than the ~14 kDa siRNA) [4]. An additional obstacle for naked siRNA is the negligible cellular internalization; the anionic charge of backbone phosphates (~40/molecule [3]) makes it impossible for siRNA to interact with anionic phospholipid cell membranes. Therefore, many strategies have been evaluated to design siRNA carriers to protect siRNA from *in vivo* degradation, to limit its premature elimination, and to deliver siRNA into target cells for effective silencing. Some of these strategies relied on viruses since the natural abilities of viruses to insert their genome into host cells make them effective delivery agents. Non-viral carriers, the focus of this introductory chapter, aim to mimic viral-like delivery by relying solely on biomolecules to package the nucleic acids. Other siRNA delivery options that have been attempted include viral based deliveries (based on DNA-based expression cassettes designed to express double-stranded short hairpin RNA (shRNA) or microRNA (miRNA) [5-8]) and other physical strategies, such as electroporation, ultrasonic delivery, hydrostatic and 'gene gun', however they will not be reviewed in this chapter.

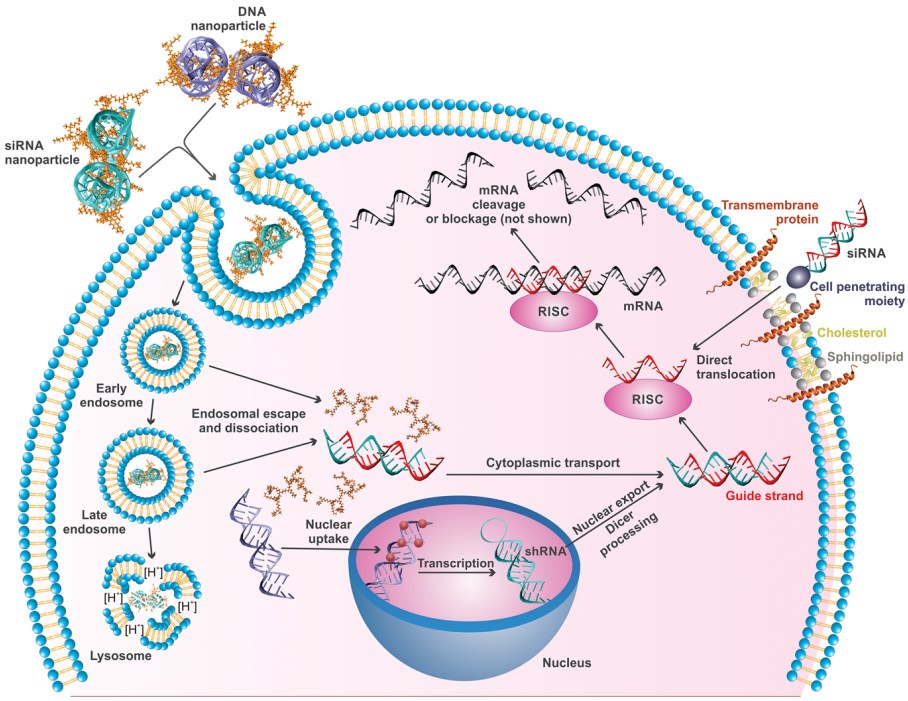

Figure 1.1 Main Processes Involved in Non-viral RNAi Reagent Delivery into a Cell.
Includes siRNA and plasmid DNA encoding for shRNA. The carriers form nanoparticulate complexes with siRNA/DNA that are conducive for passage through cell membrane. Alternatively, chemically modified forms of siRNA can penetrate through cell membrane due to membrane-compatible cell, penetrating moiety and the small size. *Figure courtesy of D. Meneksedag-Erol.*

Carriers that assemble with siRNA to form supramolecular complexes have been engineered for siRNA delivery. Despite significant variations in the design and characteristics of these carriers, the end goal is to overcome the shortcomings of the naked siRNA. Once at the target site, efficient intracellular trafficking and release from the carriers are paramount for effective silencing, **Figure 1.1**. In addition to chemical modification of the siRNA molecule [9, 10], carriers developed for DNA packaging and delivery are being re-designed for siRNA delivery, while new nanotechnology-based strategies are adopted for siRNA delivery. Non-viral carriers offer a more acceptable immunogenicity and safety profiles [11], although clinical validation of this claim remains to be demonstrated. Promising non-viral carriers (**Figure 1.2**) have been reviewed in the next section.

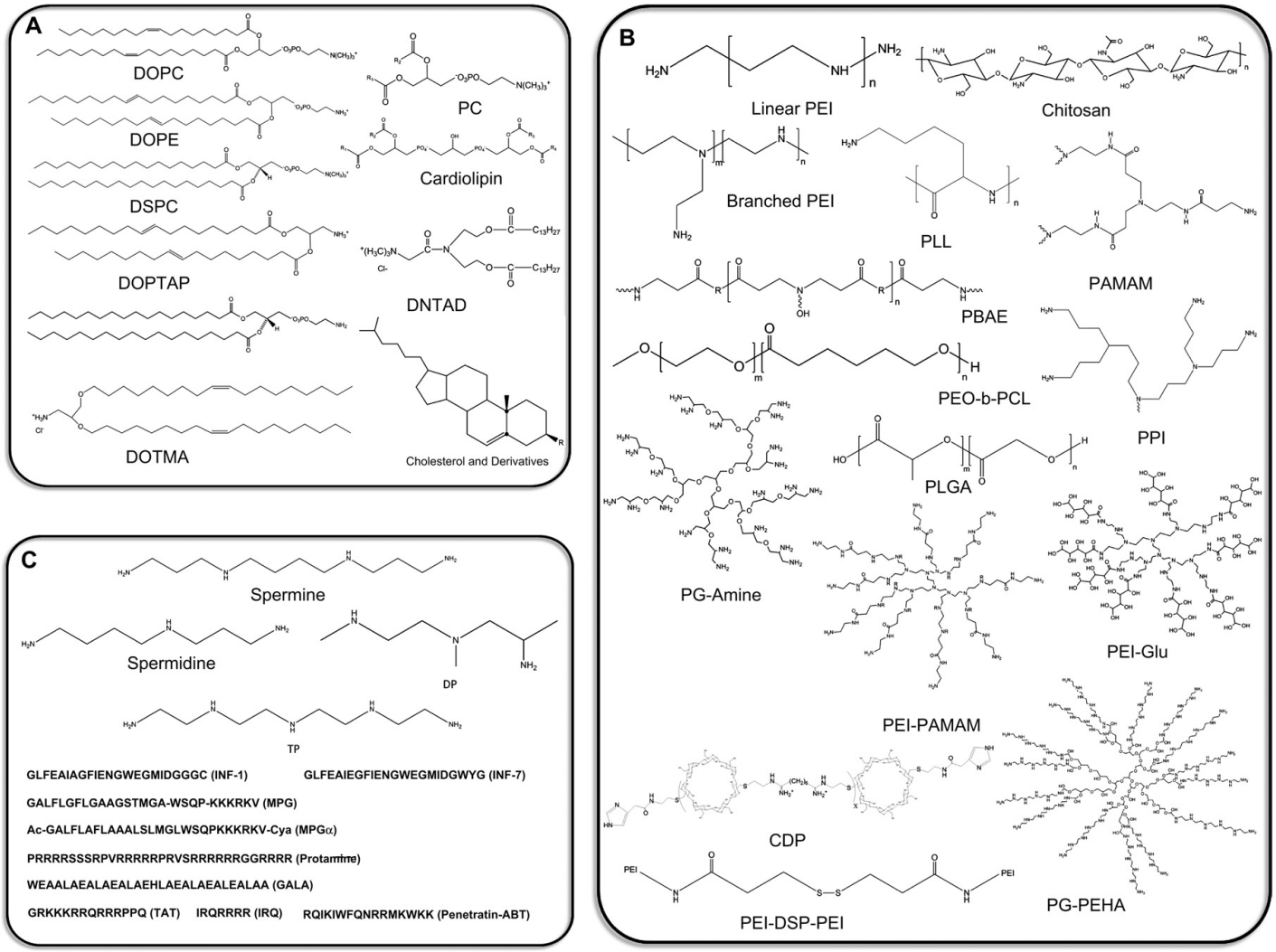

Figure 1.2 Chemical Structure of Several Carriers Used for siRNA Delivery.

(**A**) Cationic Lipids. (**B**) Polymers, (**C**) Peptides and polyamines. The functional carriers constitute a diverse group of molecules that range from cationic lipids to polymeric molecules with repeating cationic and neutral groups. *Figure courtesy of H.M. Aliabadi.*

1.1.1 Liposomes

Highly ordered lipid aggregates at the nano-scale, liposomes are distinguished by an internal aqueous phase and a lipid bilayer envelope, which is reminiscent of naturally occurring phospholipid membrane in cells. Liposomes have been particularly successful for delivery of water-soluble drugs entrapped in the hydrophilic core. "Stealth" liposomes increase the circulation times (longer $t_{1/2}$) and systemic dose (i.e., area under plasma/blood concentration vs. time curve, AUC) of the encapsulated drug, which is a reflection of a decrease in the clearance (CL) and/or volume of distribution (Vd) [12]. Liposomes have been explored extensively for siRNA delivery due to their suitable size (~100 nm), biocompatibility of their components, and especially ease of preparation [13]. For example, neutral 1,2-oleoyl-sn-glycero-3-phosphocholine (DOPC) can encapsulate ~65% of siRNA by simply mixing the solutions of the two components [14]. Dioleoyl phosphatidylethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and phosphatidylcholine (PC) are other neutral lipids employed in preparation of liposomes [15]. Landen *et al.* reported EphA2 (a tyrosine kinase receptor associated with poor clinical outcome in ovarian cancer) down-regulation in a nude mice model using DOPC liposomes [14]. Liposomes formed with DOPC have been also employed for Protease-activated receptor (PAR-1) down-regulation to inhibit melanoma growth and metastasis by decreasing angiogenesis [16] and for adhesion kinase silencing to eradicate ovarian cancer cells [17]. DOPE liposomes have been reported in siRNA delivery for Ubc-13 silencing [18].

1.1.2 Lipoplexes

Cationic lipids complexed with nucleic acids form complexes known as lipoplexes [13]. The main advantage of cationic lipids is the spontaneous interaction with anionic siRNA as well as cell membranes, which lead to higher cell internalization [19]. However, higher toxicity compared to neutral liposomes, shorter serum t1/2 (partly due to uptake by reticuloendothelial system, RES) and higher immunogenicity (due to uptake by macrophages) are among the risks associated with lipoplexes [20]. Use of cationic liposomes has been accordingly confined to *in vitro* systems. Polyethylene glycol (PEG) coating in lipoplexes helps to minimize these risks [20]. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [21] lipoplexes have been successfully used for siRNA delivery against Tumor Necrosis Factor (TNF) by intravenous (IV) injection [22], and against Vascular Endothelial Growth Factor (VEGF) by sub-retinal injection [23] in mouse models. Cardiolipin, a cationic analog of phospholipids found in the cardiac muscle, has been used for siRNA-mediated C-raf silencing in different animal models [24, 25]. A more comprehensive review of lipoplexes in siRNA delivery could be found in [1, 13, 15].

1.1.3 Stable Nucleic Acid Lipid Particles (SNALP)

SNALPs are typically composed of multiple lipids, including neutral, cationic and PEGylated lipids [1] and present a more complicated siRNA formulation. This allows better functionalization of siRNA particles for a variety of purposes, but it may also bring additional complications in the development studies. SNALP formulations of siRNA has been successfully employed for Apolipoprotein B (Apo-B) silencing in cynomolgus monkeys [26] and for polo-like kinase 1 (PLK1) silencing in subcutaneous tumors in

mice (~75% reduction in size) [27]. Recent developments in SNALP-mediated delivery of siRNA [28, 29] indicated excellent potential for their systemic applications.

1.1.4 Cationic Polymers

Supramolecular complexes of siRNA formed with cationic polymers (polyplexes) have evolved into a dominant strategy for siRNA delivery. Self-assembly of complexes results from ionic interaction between the repetitive cationic moieties on polymers and anionic phosphates on siRNA. Depending on the extent of polymer:siRNA ratio, the charges are neutralized to a desirable extent and siRNA is physically protected in the complex against RNase degradation. The main advantage of polymers is their structural flexibility that allows convenient manipulation of the physicochemical characteristics of the delivery system; polymer properties such as molecular weight, charge density, solubility, and hydrophobicity could be engineered at will, as well as addition of desired chemical groups for further functionalization. Both natural and synthetic polymers have been explored for this purpose.

1.1.4.1 Chitosan

A naturally occurring polysaccharide containing repeating glucosamine and N-acetylglucosamine units, chitosan is derived from deacetylation of chitin [11]. PEGylation of chitosan, like other polymers, was effective in enhancing the stability of siRNA complexes and serum t1/2 [30]. Chitosan is biodegradable (readily digested by lysozymes and chitinases *in vivo*; [31]) and is practically non-toxic in mammals (with LD50 of 16 g/kg in rats;[32]). Chitosan/siRNA complexes are characteristically ≤200 nm [33], an appropriate size for *in vivo* delivery. Despite the relative safety and biocompatibility of chitosan, there are only a few *in vivo* studies using chitosan/siRNA

complexes, possibly due to limited efficiency of the polymer for delivering siRNA to its target. Effective siRNA delivery has been reported (against a model target, green fluorescent protein, GFP) in lung epithelial cells after intranasal administration in mice [33]. Intraperitoneal administration of anti-TNF-α siRNA with chitosan showed a ~44% silencing in mice, leading to inhibition of inflammatory response in a collagen-induced arthritis model [34]. Chitosan has been also used as a 'coating' to improve efficiency of other delivery systems. Chitosan-coated polyisohexylcyanoacrylate particles have been reported for *in vivo* delivery of anti-RhoA siRNA to breast cancer xenografts in nude mice, which inhibited tumor growth by >90% [35].

1.1.4.2 Other Natural Polymers

Cyclodextrin, a funnel (or toroid) shaped molecule usually investigated in pharmaceutical delivery formulations, has been used as a component of a cationic polymer to form complexes with siRNA via ionic interactions. Cyclodextrin was proposed not only to protect siRNA from degradation, but also to block immunogenicity of siRNA *in vivo*, even in presence of immune stimulatory sequences in siRNA [36]. Transferrin-targeted cyclodextrin/siRNA complexes were capable of silencing the oncogene EWS-FLI1 in transferrin receptor-expressing Ewing's sarcoma cells [37] and luciferase in Neuro2A-Luc cells [38]. This delivery system was well-tolerated in non-human primates [36]. Atelocollagen (~300 kDa; purified from pepsin-treated Type I collagen; [39]) is another cationic carrier that has been used for siRNA silencing against different tumor targets in mice with considerable success [40].

1.1.4.3 Polyethylenimine (PEI)

Considered by many to be the 'gold standard' in non-viral gene delivery, PEI is a potent carrier due to its exceptional cellular uptake and endosomolytic activity [41]. High MW (25 kDa) PEI has been extensively investigated for siRNA delivery [42]. High charge density of the polymer facilitates strong binding to siRNA and effective protection against enzymatic degradation. However, the toxicity and limited biodegradability of this polymer posed obstacles for its clinical use [43]. Low MW (<2 kDa) PEIs display acceptable toxicity profiles but they do not display efficacious siRNA delivery into cells. It has been hypothesized that PEI and, other cationic polymers, increase cellular uptake of genomic material via creation of transient nanoscale holes in cell membrane, which could enhance material exchange across the cell membrane [44]. The same destabilizing action on membranes has been proposed as the mechanism of cytotoxicity [45]. It is, therefore, not surprising that the polymers more efficient in delivering nucleic acids are also more cytotoxic. Another structural factor affecting the efficiency and toxicity of PEI is the degree of branching in the polymer structure [46]. The branched PEI contains primary, secondary and tertiary amines at an approximate ratio of 1:2:1, whereas the linear polymer is composed of all secondary amines except for the primary amines at terminals [21]. In general, branched PEI was found superior to linear structure in nucleic acid delivery [47]. Despite remarkable potential of this polymer, structural modifications might be required to optimize the efficiency and overcome the limitations that prevented its clinical use.

1.1.4.4 Dendrimers

Highly branched polymers developed in 1980s, dendrimeric molecules from poly(amidoamine) (PAMAM), polypropylenimine (PPI), poly(L-lysine) (PLL), and carbon-silanes [48] have been explored for siRNA delivery. An appropriate concentration of PAMAM was shown to provide the necessary charge density to form stable siRNA complexes [49]. PAMAM polymers are commercially available (Polyfect^{TM} and Superfect^{TM}) for siRNA delivery [50]. A biodegradable arginine ester of PAMAM was effective for siRNA delivery to neurons *in vitro* and *in vivo* (intracranial injection to rabbits) with minimal toxicity [51]. A Luteinising Hormone Releasing Hormone (LHRH)-conjugated PAMAM formulation, capable of restricting its electrostatic charges inside a core, displayed reduced toxicity and effectiveness in tumor targeting [50]. A PEI-related polymer, PPI has been specifically designed for siRNA delivery and functionalized with a PEG and LHRH; growth of human lung A549 xenografts in mice was retarded, while minimizing the liver and kidney concentrations of the carrier/siRNA [52].

1.1.4.5 Other Synthetic Polymers

The linear PLL has a high density of cationic charge suitable for siRNA neutralization. Using PLL/siRNA complexes, a significant silencing of lipoprotein Apo B expression was observed in C57BL/6 mice, without hepatotoxicity and reduction in serum low-density lipoprotein in Apo E-deficient mice (a model of hypercholesterolemia, [53]). Our lab reported ineffective siRNA delivery with the native PLL, suggesting significant variations in the performance of this polymer occurs depending on the context of silencing. P-glycoprotein (P-gp) down-regulation in drug-resistant breast cancer

xenografts (MDA435/LCC6 MDR1) was possible with a PLL-based delivery system, but only after lipid substitution on the polymer. This led to effective tumor growth retardation in NOD-SCID mice after systemic administration of the chemotherapeutic drug DOXIL™ [54]. Several lipids (ranging from C8 to C18) were capable of imparting siRNA delivery capability to the native PLL, although stearic acid substitution functioned better than the others [55].

Poly(beta-amino ester)s (PBAE) are degradable cationic polymers that are synthesized from the conjugate addition of amines to diacrylates [21]. PBAEs have been investigated on their own for DNA delivery, as polycationic coatings on gold nanoparticles or multilayer structures formed with oppositely charged polyelectrolytes. Gold-siRNA nanoparticles coated with PBAEs led to >95% gene silencing, whereas non-coated particles were unable to mediate silencing [56].

Micellar structures from poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) block copolymers have been explored for siRNA delivery after adding polyamine side chains on the PCL block, including spermine (PEO-b-P(CL-g-SP)), tetraethylenepentamine (PEO-b-P(CL-g-TP)), or N,N-dimethyldipropylenetriamine (PEO-b-P(CL-g-DP)). *In vitro* P-gp silencing in MDA435 breast cancer cells has been demonstrated with these micelles [57]. The efficacy was improved after functionalizing the polymer with an integrin αvβ3 targeting peptide (RGD4C) and the cell penetrating peptide TAT [58]. Poly(lactic-co-glycolic acid) (PLGA) microparticles have been reported for antigen-coding DNA delivery in Balb/c mice [59]; siRNA delivery with these particles is in its initial stages [60].

1.1.5 Peptides

Short (<30) amino acid (a.a.) sequences were introduced in 1990s for therapeutic delivery. Peptides are versatile molecules due to considerable variety in the chemical characteristics of the building blocks and are efficient delivery systems that can enhance cellular uptake of siRNA. Basic a.a.s such as arginine and lysine are needed for complex formation with siRNA. Highly charged peptides, however, are impeded by RES, and incorporation of cysteine (and formation of disulphide bonds) in a lysine-rich peptide was reported to improve intracellular delivery due to lower opsonisation [61]. A special class of cationic peptides, known as cell penetrating peptides (CPP; 5-40 a.a. long), have been extensively explored for transferring their cargo across cell membranes. Several CPPs were derived from viral proteins known to be responsible for cell penetrating capability: for example, TAT from HIV-1 [62] and INF-1 and INF-7 from influenza virus [63]. Many mechanisms have been suggested for this efficiency, including signal-activated endocytosis, macropinocytosis, and direct translocation routes (including "inverted micelle" model) [64]. CPPs were used in two approaches for siRNA delivery, one based on covalent binding and one based on electrostatic complexation with the siRNA. The main strategy for covalent linkage between siRNA and CPP is through a disulphide linker (and thioether linkers to a lesser degree), which can degrade in cytosol. Even though this strategy offers a higher siRNA-carrier association, a lower silencing activity may result if the linkage is too stable to prevent siRNA entry into RNA-induced silencing complex (RISC) [65]; however, effective silencing with peptide-conjugated siRNA has been reported [66]. Electrostatically interacting peptides was employed for siRNA delivery

against GAPDH [67]. A CPP peptide known as MPG was also investigated for silencing cyclin B1 in athymic nude mice, with effective inhibition of tumor growth [68].

1.2 A MECHANISTIC LOOK AT CELLULAR DELIVERY OF SIRNA COMPLEXES

Silencing the target mRNA can be achieved only after supramolecular siRNA complexes reach target cells, interact strongly with cell surfaces, proceed to be internalized and trafficked to appropriate cytoplasmic destination(s) for the siRNA to integrate into RISC complexes without hindrance of the carriers. The ability to navigate each sub-cellular stage contributes to the resulting silencing efficiency and it is critical to understand and optimize each step of this process. Although one is tempted to compare the efficiencies of various supramolecular complexes reported in the literature, it is practically impossible to undertake this task due to extensive variability in experimental parameters, such as the cell type employed, the intrinsic properties of siRNA and target mRNA (e.g., turn-over rate) and dose/duration of treatment. Nevertheless, we attempted to summarize two basic features of supramolecular complexes, namely size and z-potential, as well as the silencing potency (at both protein and mRNA levels) for a select set of studies with different carriers (**Figure 1.3**). The size of complexes did not appear to drastically vary among carriers, where most complexes were typically ~200 nm or less (**Figure 1.3A**). The zeta-potentials of complexes were usually positive (typically 0 to +40 mV; **Figure 1.3B**), but some did exhibit negative zeta-potential. Most studies employed ≥100 nM siRNA in order to achieve effective silencing, a concentration range difficult to translate into preclinical and clinical settings (20-50 nM is preferred), but some carriers

were effective at <100 nM siRNA (**Figure 1.3C**). Not surprisingly, there is no correlation between the extent of silencing and effective siRNA concentration, owing to large numbers of uncontrolled variables among these studies. We recently conducted a similar analysis for silencing a specific target, namely P-glycoprotein (P-gp) involved in multidrug resistance in cancer [69], and a large range of effective siRNA concentrations was also evident with various non-viral carriers for this specific case. It is not immediately clear as to why some carriers are functional at the desirable 20-50 nM range while others require >200 nM siRNA. Defining a minimal effective concentration for each delivery system will clearly identify promising carriers, but this has not been a common practice in the field. In some cases, effective siRNA concentrations were not clearly reported and, more importantly, scrambled siRNA/carrier complexes have been missing as treatment controls, a critical issue since any kind of cellular treatment is bound to give a response. Below, we investigate various steps involved in intracellular transfection pathway.

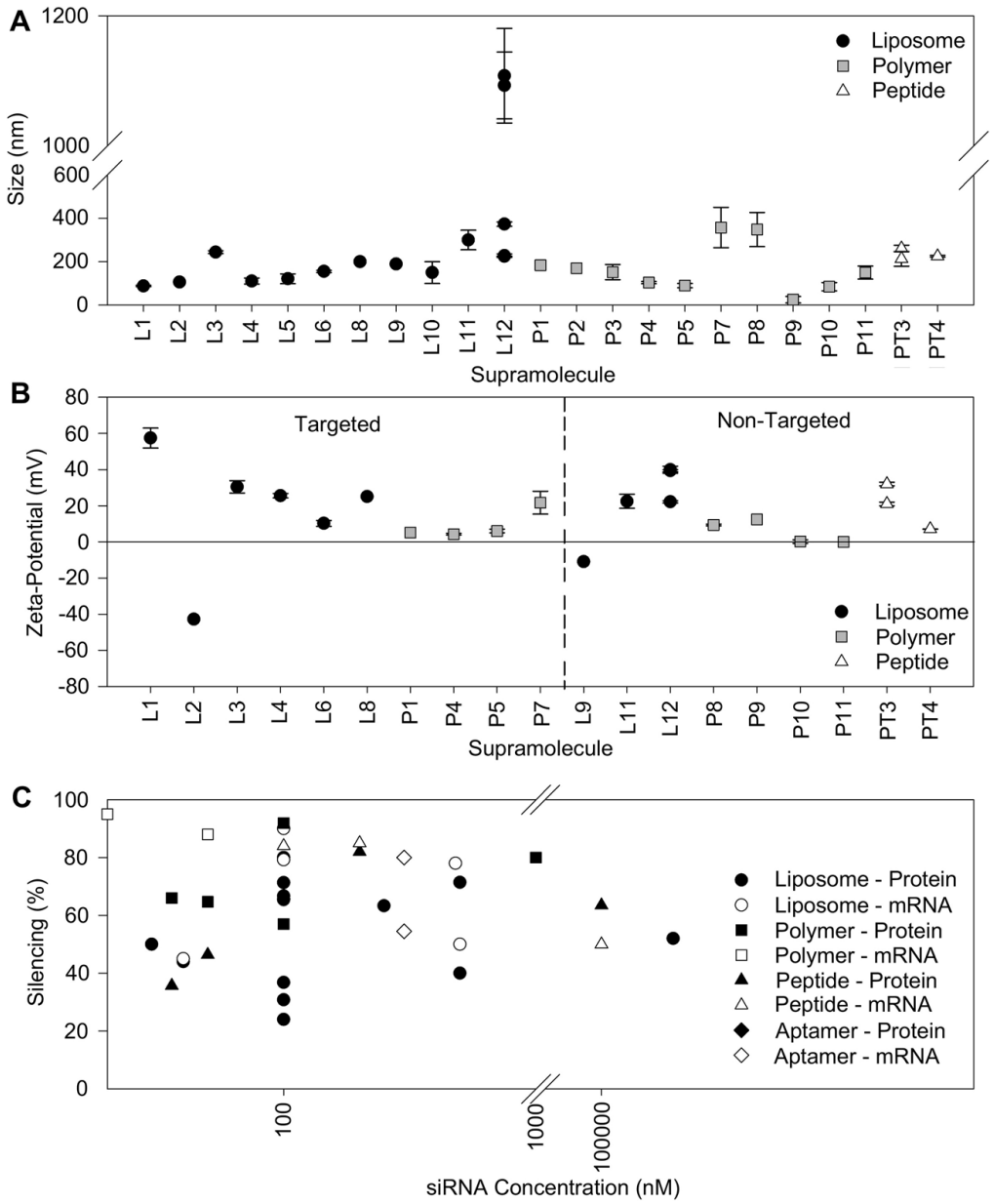

Figure 1.3 A Summary of Select Studies Reporting on the Size, Zeta-Potential and Silencing Efficiency as a Function of siRNA Concentration.

For size (**A**); error bars represent standard deviation or reported range) and for zeta-potential (**B**); error bars indicate standard deviation if reported. The data were chosen from articles reviewed for this manuscript, where siRNA-mediated silencing was reported. Where appropriate, the most suitable carrier was selected among several carriers reported and some values were estimated from the provided graphs and/or calculated from others units and described methods. References: Liposomes (L): L1 [70], L2 [71], L3 [72], L4 [73], L5 [74], L6 [75], L7 [76], L8 [77], L9 [78], L10 [79], L11 [80], L12 [81]; Polymers (P): P1 [82], P2 [83], P3 [84], P4 [85], P5 [86], P6 [87], P7 [88], P8 [89], P9 [90], P10 [91], P11 [92]; Peptides (PT): PT1 [93], PT2 [94], PT3 [95], PT4 [96]; Aptamer (A): A1 [97].

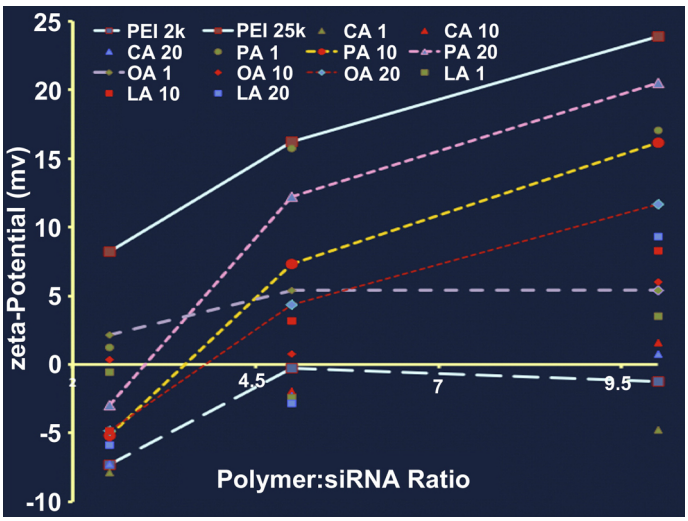

Figure 1.3 Zeta-Potential of Polymer/siRNA Complexes for Native PEI (2 and 25 kDa) and Lipid-Substituted 2 kDa PEIs.

The complexes were formed at polymer:siRNA weight ratios of 2.5:1, 5:1 and 10:1 for zeta-potential measurements. Substituting the PEI2 with lipids increased the zeta-potential of complexes and brought it closer to the zeta-potential of the PEI25 complexes. The substituents on PEI are CA (caprylic acid), PA (palmitic acid), OA (oleic acid) and LA (linoleic acid), each substituted at three different ratios (indicated as 1, 10 and 20). *Figure courtesy of H.M. Aliabadi.*

1.2.1 Cell Surface Binding

Rather than the interactions with individual components, cell surface interactions of the supramolecular complex as a whole are critical for effective entry. Sufficient binding strength is necessary to prevent dissociation of complexes at the cell surface interference from higher concentrations of competing polyelectrolytes [70], keeping in mind that the complex has to dissociate once in the cytoplasm. Charged carriers, such as cationic liposomes, polymers and CPPs, can interact with extracellular matrix components as well as proteoglycans and/or phospholipids at the cell surface (summarized for CPPs in [98]). Rather than the charge of cationic carriers, zeta-potential of the assembled siRNA/carrier complexes dictates the membrane interactions. The nature of charged moieties in a carrier and the carrier:siRNA ratio used for supramolecular assembly are obvious determinants of the zeta-potential; however, other

factors that promote or hinder the supramolecular assembly can affect the zeta-potential. We have seen this when lipid-modified polymers were employed for siRNA delivery (**Figure 1.3**). The siRNA complexes with 2 kDa PEI gave little siRNA delivery across cell membranes and gave an over-all charge close to neutrality; however, upon lipid modification of PEI, the zeta-potential of the complex became positive and siRNA delivery efficiency was significant [89]. Lipid moieties presumably ensured a robust affinity among the components of the assembly under aqueous conditions. Anionic carriers are not the obvious siRNA delivery method but they have been occasionally employed (**Figure 1.3**). Although anionic carriers can demonstrate silencing in some cases, cationic forms are more effective. When polyglycerol-based dendrimers including a cationic dendrimer (+12.4 mV; polyglycerolamine) and anionic dendrimers (-2.2 to -0.614 mV; polyglyceryl pentaethylenehexamine carbamate, PEI-polyamidoamine and PEI-gluconolactone) were utilized, the cationic dendrimer was more effective, demonstrating 50% silencing at carrier concentration over 4-fold less than the lowest anionic dendrimer with mid-range cytotoxicity [90] (note that a thorough optimization of complex charges and siRNA:carrier ratios was missing in that study). A targeting ligand (LHRH peptide) was also required for neutral (+0.11mV; internally cationic but surface neutral) PAMAM (85% quaternized-PAMAM-OH) dendrimer for silencing, but a high siRNA concentration (1000 nM) was needed even in this case [99]. Whereas cationic complexes do not necessarily require targeting ligands (although they were shown to be beneficial as articulated below), anionic ones usually do. Such effect is seen with a liposome-fusion phage protein (DMPGTVLP) system targeting PRDM14, where the liposomal system did not demonstrate silencing unless combined with the phage protein

(40-50% silencing at both mRNA and protein levels) [71]. In case of liposomes formulated with a shortened GALA-peptide (for endosomal release), the anionic assembly (-11 mV) was supportive of silencing, but again at exceedingly high siRNA concentrations *in vitro* (480 nM) and high doses *in vivo* (4 x 4 mg siRNA per kg mouse weight) [78]. A targeting ligand could have been beneficial in this case. The ubiquitous interactions of cationic complexes, however, with soluble anionic species and non-target cells (and resultant uptake) are undesirable. A weak positive charge (<+5 mV) has been suggested as ideal to balance the needed cell surface interactions while minimizing non-specific target carrier binding [82, 91], as long as the propensity for complex aggregation at near neutral charge is addressed.

Hydrophobic moieties in supramolecular complexes should enhance cell membrane affinity non-specifically. Cholesterol has been incorporated into siRNA delivery systems by a variety of means for enhancing interactions with cell membranes. Cholesterol plays a role in many cellular membrane-related events such as membrane fusion, macropinocytosis, caveolin-mediated and lipid raft-mediated endocytosis [79], and cholesterol-containing carriers are expected to improve DNA/RNA transfection through an enhanced interaction with cell membrane [100]. Cholesterol conjugated to siRNA was reported to decrease serum degradation [101], improve siRNA pharmacokinetics and biodistribution, and enhance cellular uptake due to cholesterol interaction with lipoproteins. Cholesterol has been also shown to stabilize the liposomal structure [15] and act as a targeting moiety for liver cells [102]. Aliphatic lipids have been also used to functionalize otherwise non-efficient polymeric carriers (e.g., low MW PEIs) for nucleic acid delivery [103]. We initially speculated that the substituted lipids

could enhance the interaction supramolecular complexes with lipid membranes, but subsequently realized the increased zeta-potential to be also responsible for increased siRNA delivery (**Figure 1.3**). Our experiments have shown the functional silencing with select lipid-modified polymers against P-gp [89], Breast Cancer Resistance Protein [104], and survivin [105], three molecular targets whose expressions are changed in an undesirable manner in tumorigenic cells (further discussed in **Chapter 2**).

Incorporating cell-surface binding ligands into supramolecular complexes is the preferred approach for generating effective and cell-specific binding. Ligands targeting endocytosed receptors, especially in the case of cancers where particular receptors are over-expressed, are preferred for improved internalization (as discussed in [93, 106]). Ligand-mediated binding provides better internalization especially for shielded (e.g., PEGylated) complexes; amphiphilic surfactant and siRNA complexes demonstrated significant reduction in silencing due to substantial decrease in siRNA delivery when PEGylated, but the use of a targeting peptide (bombesin) enabled delivery and silencing at the pre-PEGylation levels [83]. However, targeting ligands could be prone to immunogenicity. Their targets could be low in abundance and display variability from patient to patient [107]. Typical ligands include endogenous molecules (e.g., carbohydrates), synthetic (e.g., phage-displayed derived) and natural proteins/peptides, and antibodies (**Table 1.1**). Positive bias is naturally expected in the disseminated studies with ligand-targeted complexes, where negative outcomes are likely under-reported. Increased cellular delivery by receptor mediated binding is evident even for cationic supramolecular assemblies after incorporation of a ligand, as is the case of cationic DOTMA/DOPE liposomes modified with the K_{16}GACYGLPHKFCG peptide [70] and a

CPP system (CPP-conjugated PLGA with spermidine/siRNA complexes) modified with folate [84]. Multimodal interactions that involve both receptor-mediated and non-specific binding to cell surfaces (e.g., by cationic species and CPPs) can enhance the overall cell association in this way [70, 84]. It is important to note that the beneficial effect of ligands may not be always observed in certain contexts; (i) an RGD/PEG modified branched peptide was found effective at silencing *in vivo* unlike the *in vitro* studies [108], and (ii) a PAMAM-RGD carrier, where improvement in *in vitro* siRNA delivery and silencing was not observed with RGD functionalization, gave enhanced delivery when applied to an *in vitro* spheroid tumor model [109]. One has to be aware of this issue since promising systems could be dismissed under selective testing conditions and their true performance could only be manifested after testing in preclinical (*in vivo*) models [108].

Table 1.1 A Selection of Targeting Ligands Used with Carriers for Creating Functionalized Supramolecular Assemblies.

Category	Ligand	Carrier
Natural proteins and peptides	RGD	Peptide [108], polymer [85, 86, 109]
	TAT	Peptide [110], polymer [85]
	Bombesin	Polymer [83]
	LHRH	Polymer [99, 111]
	Transferrin	Polymer [112]
	Rabies Virus Glycoprotein	Peptide [94]
	Hexapeptide (antagonist G)	Liposome [113]
Synthetic proteins and peptides		Liposome [70-72] polymer [87, 114, 115], fusion protein/peptide [93, 116], aptamer [117]
Endogenous molecules	Folate/folic acid	Liposome [76], polymer [82, 84, 118], aptamer [97]
	Prostaglandin E2	Polymer [119]
	Anisamide	Liposome [73, 74, 120]
	Mannose	Polymer [88]
	Galactose	Liposome [121]
	Hyaluronic acid	Polymer [122]
Antibodies		Liposome [75, 77], peptide [123]

* The compiled list was not meant to be exhaustive, but rather representative of the range of ligands used for facilitating cell surface interactions in siRNA delivery. The classes of specific carriers used for functionalization was provided instead of the chemical nature of the specific carrier.

1.2.2 Cellular Internalization

Intracellular entry of supramolecular complexes may occur by direct transfer through cellular membranes or by energy-dependent membrane buddings known as endocytosis. In the latter case, the specific pathways include clathrin-mediated and clathrin-independent pathways such as caveolae-mediated, clathrin-independent, caveolae-independent, and macropinocytosis. All of these pathways were functional for siRNA internalization depending on the specific siRNA carrier [72, 79-81, 113, 122] and, although not completely understood, each pathway has distinct features and varied intracellular trafficking that then can affect the fate of complexes. Clathrin-mediated pathway follows the traditionally assumed pathway, where the complexes are trafficked from endosomes to lysosomes with a gradual drop in pH and ultimately exposure to degradative conditions. Complexes in caveolae-mediated pathway are directed to caveosomes with a less defined fate, but may escape the drop in pH and degradative conditions that are destructive to siRNAs. Macropinocytosis, also a regulated process, takes up a large amount of liquid by plasma membrane ruffles for intracellular trafficking at a slower speed as compared to other methods [124]. Determining the native and/or optimal endocytosis pathway followed by a supramolecular assembly is a challenging task. A clear consensus on the reliability of endocytosis inhibitors used in mechanistic studies is absent and one needs to optimize the inhibitors for each cell line studied (i.e., to ensure that the effects are not due to non-specific cytotoxicity on the cells) and to further validate the outcomes with additional inhibitors and/or independent methods [125]. Cells might utilize multiple pathways for internalization of the same complexes, displaying rapid adaptation (or compensation) to experimental interventions [126, 127]. Such an

adaptation might be displayed as a function of siRNA dose, where low concentrations of complexes undergo clathrin-/caveolae-mediated endocytosis or macropinocytosis, and internalization becomes non-endosomal at high concentrations as commonly observed for CPPs [98]. A large number of physical characteristics of assembled complexes can affect the internalization method, including size, charge, presence of a ligand and polydispersity [127-130]. As the endocytosis characteristics can change depending on the payload (drugs, DNA or siRNA), we will focus our analysis solely on siRNA studies, which are few in number, but are beginning to provide insight for effective siRNA delivery.

Effective silencing may not result from the major endocytosis pathway employed, but from secondary pathways that may be more conducive for siRNA release into cytoplasm. This could be one reason why intracellular delivery percentages may not correlate with silencing efficiencies. In one study, siRNA formulated with cationic lipoplexes (DharmaFECT1) entered the cells mostly by endocytosis, but silencing was attributed to the siRNA fraction that directly fused with the cell membrane [79]. Liposomal fusion in the case of DNA delivery was found to be undesirable, unlike the siRNA delivery in this case, obviating the efforts previously taken to optimize delivery with plasmid DNAs. Simple alterations in preparative procedures may greatly affect endocytosis pathways and resulting silencing efficacy. When siRNA was formulated with the cationic liposome LipoTrust^{TM}-SR (a mixture of O,O′-ditetradecanoyl-N-(a-trimethyl ammonioacetyl) diethanolamine chloride, DOPE and cholesterol) by vortexing, rather than by spontaneous assembly, decreased size of complexes (possibly due to less aggregation) gave better siRNA accumulation in cytoplasm due to a change in internalization from membrane fusion to clathrin-mediated endocytosis, along with

increased silencing [80]. A contradiction is evident from the latter 2 studies on the optimal pathway for siRNA delivery. In another study, several cholesterol derivatives (amido and carbamate linked hydroxyethylated cationic cholesterol) were used for siRNA delivery to human PC-3 prostate tumor cells. Amido-linked complexes prepared by different methods led to different internalization pathways; the internalization of complexes prepared in water involved faster silencing kinetics via clathrin-mediated uptake and membrane-fusion, whereas complexes prepared in 50 mM NaCl (resulting in larger complexes) gave slower and more effective silencing, and employed clathrin and caveolae-mediated endocytosis. The carbamate-linked complexes, on the other hand, displayed similar high silencing efficiencies under both conditions [81]. These studies highlight the importance of the physical nature (size, shape or elasticity) of the complexes rather than the chemical nature of supramolecular assembly. Aside from the usual variability in the experimental settings (cell type, mRNA target and size/charge of supramolecular assemblies), a 'universally' effective pathway for siRNA entry might be elusive. It is likely that nature of the supramolecular complex (especially the nature of carrier used for assembly) might dictate the appropriate pathway [131]. However, the fact that one can alter or optimize the uptake pathway by adjusting simple preparation variables is encouraging in order to quickly identify the most efficacious pathway for silencing.

The nature of the ligand is expected to affect the endocytosis pathway. In one study, a novel IRQ-peptide grafted cholesterol/phosphatidylcholine liposome was compared to an (arginine)8-grafted liposome (known to undergo macropinocytosis at high concentrations) for siRNA delivery in NIH3T3 fibroblasts [72]. The IRQ-peptide

changed the internalization to caveolae- and clathrin-mediated endocytosis where a portion of the peptide was suggested to interact with caveolae and clathrin receptors. In another study, hexapeptide antagonist G-grafted cationic liposomes were used for siRNA delivery to small cell lung carcinoma (SCLC H69) cells [113]. The hexapeptide directed internalization by clathrin- and caveolae-independent mechanisms with possible small contributions from clathrin-mediated and macropinocytosis; however, no silencing was achieved with either the ligand-modified or unmodified liposomes and lack of caveolae-mediated pathway in the chosen cell line was suggested as a possible reason for this observation. With hyaluronic acid grafted onto hydrophobic amines and spermine (polymer micelle formulation), caveolae-mediated pathway was the major mode of internalization [122]. Although silencing was obtained by this delivery approach, the lack of a control siRNA in silencing studies does not allow a clear assessment of its efficacy. This literature indicates that directing endocytosis along the caveolae-mediated pathway is preferable to avoid late endosome/lysosome degradation. One can envision designing carriers whose supramolecular complexes with siRNA employ this desirable pathway.

1.2.3 Crossing Lipid Membranes for Cytoplasmic Release

The supramolecular complexes have to cross lipid membranes to gain access to cytoplasm for siRNA release. This can be achieved by non-contact mechanisms (such as inducing endosomal swelling) or by direct endosome membrane interactions leading to disruption or fusion. Carriers that exhibit non-physical contact often utilize H+ buffering, a unique mechanism for endosomal escape. Also termed as ‘proton-sponge effect’, this mechanism has been initially recognized in the context of PEI [41]; protonation of PEI amines prevents the endosome from reaching the acidic pH needed for lysosomal

nucleases and causes swelling of PEI/siRNA complexes. The influx of Cl- to balance the H+ influx causes osmotic swelling, eventually bursting the endosome to release the cargo [41]. Such a mechanism might occur with other carriers with similar buffering capacities, for example, with PAMAM-PEG-PLL carrier where PAMAM was intended to increase the buffer capacity for endosomal release, leading to significantly improved silencing [92].

Direct interactions, causing membrane disruption, destabilization or fusion, are the more straightforward approach to penetrate cellular membranes. Membrane interaction with the lipid components of supramolecular complexes are paramount for this purpose and this can occur via a mechanism termed mesomorphic phase behaviour: the cationic lipids form charge-neutral pairs with anionic lipids of cellular membranes, causing a localized change from the usual lamellar structure to a hexagonal phase. The alteration in membrane structure along with carriers' cationic lipid components can allow for siRNA to pass through the membrane. Although details of this mechanism have not been completely elucidated, carriers were designed to promote this phase transition [132, 133]. The cationic-lipid carrier and anionic cell membrane interaction is dependent on the strength of the cationic charge of the carrier. Thus, the ionization constant (Ka) of the lipid headgroups can be optimized to promote the interaction. For endosomal escape, an amino lipid pKa within the range of 6-8 should allow for increased protonation at endosomal pH, thereby increasing membrane interaction and resulting crossing while minimizing interactions at physiological pH, that may lead to increased cytotoxicity or serum protein interactions [134]. Along the same lines, hydrophobicity of complexes has been found to increase silencing through lytic disruption of the cellular membrane. A

diblock copolymer made up of butyl methacylate and propylacrylic acid (which becomes protonated at endosomal pH and significantly elevates hydrophobicity of the carrier) [91] and hydrophobically-modified oligoethylenimine (with hexyl acrylate) [135] demonstrated increased hemolytic activity with increasing hydrophobicity content, which correlated with the siRNA activity. Peptides, such as CPPs and fusogenic peptides, can also mediate transfer across cellular membranes. Various membrane disruption mechanisms was attributed as the mechanism for peptide-mediated delivery, such as pore formation or rearrangement of the lipid bilayer [136]. Hydrophobic peptides, such as arginine [96], have been suggested to promote escape by fusion with endosomal membranes. Peptides are often used in conjunction with other carriers. Such designs include a liposomal siRNA delivery system utilizing the fusogenic peptide (GALA). The fusogenic peptide was introduced into the supramolecular complex because the PEG, intended for 'stealth' properties, also interfered with endosomal escape, thereby almost completely inhibiting silencing activity. The GALA undergoes a conformational change from a random coil structure due to the repulsion of negative charged-glutamic acid at physiological pH to an α-helix at low endosomal pH as the glutamic acid is neutralized, inducing membrane fusion, thereby increasing endosomal escape for subsequent silencing ability (summarized in [78]). How CPPs are incorporated into complexes can influence the functionalities of the CPPs and in some case diminish their effectiveness [137].

1.2.4 Transport within the Cytoplasm

After achieving cytoplasmic entry, the siRNA must be available (dissociated from carrier) in sufficient quantities in order to silence the target mRNA. Competitive binding

with the components of supramolecular complex can lead to desirable disassembly of electrostatically-held complexes. Anionic molecules such as cytoplasmic RNA (mRNA, tRNA, etc.) and glycosaminoglycans are thought to aid siRNA release [70, 138], especially after the intra-complex interactions are weakened during endosomal escape due to interactions with lipid membranes [133]. A decrease in electrostatic binding among carriers and siRNA molecules can also occur during carrier swelling in endosome and changes in overall charge [70]. With a lipid-modified 2 kDa PEI library, the highly bound complexes, although they show efficient uptake, displayed decreased silencing compared to weakly bound complexes [89]. CPPs covalently bound to siRNA are not intended to dissociate, instead the linkage must be located appropriately as to not impede the RNAi mechanism; linkage at the 3' end of the sense strand (passenger strand) of siRNA has been found to be optimal [137]. Rather than relying on supramolecular disassembly with endogenous molecules, it is possible to design biodegradable carriers so that the complexes are disassembled by taking advantage of cleaving agents in the cytoplasm. Disulfide linkages are one such type of labile linkage that are susceptible to reducing environments for siRNA release. Cross-linking low MW PEI using agents such as dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3′-dithiobispropionimidate (DTBP) [139], or 1,3- butanediol (or 1,6-hexanediol) diacrylates [140] has been reported as a strategy to create an efficient carrier with extensive disulphide (-S-S-) and amide (-C(=O)-N(H)-) linkages for degradation. The smaller building blocks will presumably clear in the body on their own without an adverse effect.

Once the siRNA is delivered to cytoplasm, comparing the amount of siRNA within the cell for target mRNA suppression (of similar targets) can provide us with a

sense of carrier efficiency. Only a few studies with supramolecular complexes have provided clues on this aspect; MPGα-mediated (a CPP) siRNA required ~10,000 copies, the cationic liposomal Lipofectamine^{TM} 2000 required ~300 copies and physical methods such as electroporation and microinjection required, respectively, ~400 and <300 copies of siRNA for 50% silencing; large variations in assessed silencing efficiency was evident in these studies (reviewed in [141]). This was indicative that the vast majority of siRNA copies in supramolecular complexes not being available for silencing. What happens to excess siRNA (and associated carrier) is an important issue, as well as elucidating the reasons for sub-optimal release. Determining the number of siRNA copies delivered per complex provides another perspective. A transferrin-targeted cyclodextrin system was suggested to contain ~2000 siRNA copies in a 70 nm nanoparticle [112]. Based on the estimated siRNA copies needed per cell, ~15% release of supramolecular assembled siRNA (300/2000) will be needed for 50% silencing. Timing from cell exposure to cytoplasmic detection is expected to depend on carrier features, among other variables, but delivery typically occurs fairly fast. Delivery within 0.5-6 hours is typical for a range of carriers including a liposomal-targeting peptide system, cationic liposome (Lipofectamine^{TM} 2000), dendrimer (polyglycerolamine), linear PEI, micellar systems (PEO-b-polyester with RGD and/or TAT) and a peptide (arginine) carrier [70, 85, 88, 90, 142-144]. Significant silencing at the mRNA/protein levels occurs in the next 24 to 96 hours, although the duration of silencing is not always reported. Duration of one week is an optimistic estimate, for example ~5 days for a targeted liposome system [73] and 6-7 days for the lipid substituted 2 kDa PEI [104].

Once the siRNA is available in the cytoplasm, RISC (including argonaute 2 and GW182) association is needed to direct the mRNA cleavage. The exact details of this process remain to be elucidated. It is reasonable to assume that intra-cytoplasmic targeting could improve efficiency, as mRNA [145], and possibly RISC components are asymmetrically located within the cytoplasm, leading to greater silencing and/or less siRNA required. However, as it is not known how RISC forms (i.e., which components initiate assembly and how do they form) or how it localizes to proximity of the target mRNA, targeting possibilities in the cytoplasm could include components of RISC, specific cytoplasmic organelles and structures, or the location of target mRNA itself. Targeting P-bodies is one possibility, as it was found that when siRNA was delivered by Lipofectamine^{TM} 2000, the siRNA localized to P-bodies (whose role in RNAi still remains unclear) prior to binding to RISC [143]. Various carriers including liposomes, peptide-targeted liposomes, siRNA/peptide complexes and dendrimers were found to localize to perinuclear region. Additionally, perinuclear localization has been observed to correlate with RNAi activity, suggesting that RISC, at least when activated, is located in this region [70, 96, 110]. If this is in fact the case, targeting microtubules may improve efficiency since they participate in shuttling of cargo between nucleus and cell periphery. An arginine and TAT-peptide delivery systems as well as liposomes (Lipofectamine^{TM} 2000) were found to localize to perinuclear region both in the absence and presence of an mRNA target (e.g., with luciferase, GFP, and endogenous CDK9) [96, 110], suggesting that supramolecular complex targeting to the nuclear periphery is independent of the presence of mRNA [110]. Active delivery to mRNA targets or their general location is another approach to improve silencing; although variability in sub-cellular distribution of

mRNA is noted, the reasons for asymmetrically distribution of mRNA is not well understood [96, 145]. It is not clear how targeting could be achieved apart from the complementary pairing of the siRNA and the target mRNA. However, charge and lipophilicity may play a factor in intracellular localization; in CPPs designed for mitochondrial-penetration, lipophilicity and over-all charge affected their intracellular localization (mitochondria vs. cytoplasmic and nuclear localization [146]. In rare cases, when the siRNA target is in the nucleus, nuclear targeting can be utilized. In one study, siRNA against an essential promoter region of EF1A gene were trafficked to the nucleus by incorporating the nuclear-targeting NLS peptide into CPPs, which achieved highly significant silencing [95]. Finally, the state of cellular physiology has been found to contribute to silencing efficiencies. Loss of RNAi function can occur due to cell stress causing the human argonaute 2 protein being re-located to stress granules, as was seen with the cationic liposome Lipofectamine^{TM} 2000 [147]. Delivery methods should therefore minimize cytotoxicity and stress related factors not only for off target effects on other cells, but to ensure that the RNAi system targeted remains functional. Half-life of the target protein (i.e., its rate of synthesis) is another factor influencing silencing; efficient silencing will occur with proteins produced in low quantity with short half-lives – i.e., a siRNA residence time 3 fold higher than the half-life of the protein target is desirable [148].

1.3 CONCLUSIONS TO NON-VIRAL SIRNA THERAPY

The design and engineering of siRNA carriers gained significant momentum in recent years, as a result of accumulation of predictable and therapeutically promising

molecular targets. Overall efficacy of the developed carriers is difficult to compare among the siRNA formulations as experimental designs are often undetailed (lacking dissemination of siRNA concentrations, and determination of minimal required siRNA-carrier concentrations) and include many uncontrollable variables (such as the cell type and protein targets utilized). In some incidences, what is learned from one carrier can be applied to new carrier designs such as general benefits including charge, nanoparticle size and other beneficial aspects of targeting. However, many factors must be determined and optimized individually for each carrier system. This is evident in the contradictions that arise when determining which endocytosis pathway results in effective RNAi activity depending on the carrier studied. More research is specifically needed in determining the carriers' fate once it enters the cell, from its entry-pathway to the fate of the individual carrier components upon its disassembly somewhere with the cell. Thus, it is likely that carrier systems need to be designed or at least optimized specifically for each individual (cell type, choice of target, etc.) purpose.

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PART II. PROGRESS IN RNAI MEDIATED THERAPY OF LEUKEMIA

1.5 LIMITS OF CURRENT LEUKEMIA THERAPIES AND PROMISE OF RNAI

Leukemic cancers arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, and apoptosis as well as survival of malignant cells. Approximately 350,000 people worldwide are diagnosed with leukemia annually, leading to ~250,000 deaths each year. An overall 5-year relative survival rate of 56.0% (between 2003 and 2009) is estimated for various leukemias combined [149]. The front line therapy in leukemia is chemo (drug) therapy [150, 151], including broad-spectrum cytotoxic agents against fast-proliferating cells and small-molecule inhibitors targeting specific signal transduction pathways, so called molecular therapies [152]. The molecular pathogenesis of some leukemias, such as chronic myeloid leukemia (CML), is relatively clear; aberrant juxtaposition of BCR (breakpoint cluster region protein) and ABL1 (Abelson murine leukemia viral oncogene homolog 1) genes constitutively activates a tyrosine kinase (p210BCR-ABL), whose signalling initially leads to a chronic phase of myeloid cell expansion, while the expanded cells undergo differentiation in peripheral blood. A range of highly specific tyrosine kinase inhibitors (TKIs) has been introduced for clinical use over the last decade and significant improvements in patient survival have been achieved. For acute myeloid leukemia (AML), however, no new drugs have been introduced in recent years and clinical therapy has relied on ‘traditional’ broad-spectrum cytotoxic drugs, where the leukemic cells display a differential sensitivity to drugs. The therapeutic index in this case is relatively small, and significant side effects at efficacious doses typically limit therapy at advanced disease.

Leukemic cells generally respond well to drug therapy at the onset of treatment, but the drugs lose their effectiveness over a period of 6-12 months in a significant fraction of patients. It is now well recognized that the resistance to broad-spectrum drugs is inevitable, but recent evidence also indicated that even the most advanced molecular drugs can lose their efficacy [153]. In CML, development of resistance to current front-line therapy imatinib and failure to reach a complete cytogenetic response occurred in 24% of patients within 18 months [154, 155]. The inherent plasticity of the cells combined with diverse resistance mechanisms allow malignant cells to naturally adapt to drug assault. Additionally, the high relapse rate in leukemia patients has been attributed to existence of a rare population of leukemic stem cells (LSC) capable of evading drug therapies [156, 157]. With better understanding of molecular changes in leukemic transformations, treatments that target tumor-specific changes are expected to lead to more effective therapies as the normal cells transform into malignant cells.

To this end, a highly specific leukemia therapy can be developed by exploiting RNA interference (RNAi) to silence the aberrant protein(s) responsible for the disease [158, 159]. While current small molecular drugs rely on a specific binding mechanism, whether be an active enzyme site or DNA major/minor grooves, RNAi targets a particular mRNA for destruction (or translational blockage) by binding to specific regions in the mRNA. Unlike point mutations that can abolish drug activity, silencing aberrant proteins with RNAi is less prone to resistance development. The mechanism of action for RNAi reagents is similar to previously employed antisense oligonucleotides (AS-ODN) targeting mRNAs (**Table 1.2**), except that RNAi employs endogenous mRNA regulatory machinery to suppress protein production. Furthermore, RNAi can target aberrantly

expressed isoform(s) of the protein (as in BCR-ABL fusion protein), unlike drugs that abolish activity of the target non-specifically (as in both ABL and BCR-ABL proteins). RNAi for leukemia has reached clinical trials in two cases. In the NCT00257647 clinical trial a viral vector, simian virus 40 (SV40), was utilized to deliver siRNA to CML patients against a fusion gene, but there are no published outcomes from the study. The other trial being a non-viral liposomal siRNA tested in one CML patient. A strategy to combine two or more drugs with non-overlapping target resistance profiles could delay the emergence of drug resistance [160]. However, new point mutations could still be expected to induce resistance to drug combinations [161], given the plasticity of LSC. FLT3 inhibitors (midostaurin, AC220 and sorafenib), for example, experience resistance development as a result of secondary FLT3-ITD mutations [162].

Table 1.2 Different Types of Gene Regulators Used for Leukemia Therapy.
While AS-ODNs have reached clinical testing, only one siRNA, and no shRNA or microRNA were tested in clinics for leukemia therapy.

Class of Compounds	Characteristics	Source	Examples in Clinical Leukemia Therapy
Antisense Oligonucleotides	Double-stranded DNA or modified form	Synthetic	GTI-2040 (ribonucleotide reductase), SPC2996 (Bcl-2), LY2181308 (survivin)
Small Interfering RNA	Double-stranded, base-matched RNA	Synthetic	BCR-ABL siRNA
Short hairpin RNA	Double-stranded, base-mismatched RNA	In-situ expressed	Not available
MicroRNA	Double-stranded, base-mismatched RNA	Synthetic or in-situ expressed	Not available

The current review provides a comprehensive summary of RNAi efforts for leukemia therapy. We focus our analysis on myeloid leukemias, specifically AML and to a limited extent CML, where RNAi effort is mostly concentrated (but also provide information on other leukemias as appropriate). RNAi is a therapeutic option for all

leukemias but we want to explore the critical issues in-depth that should be applicable to all leukemias (not just myeloid leukemias). We review the important aspects involved in utilization of RNAi reagents, with a particular focus on siRNA since it is likely to reach clinical testing ahead of other related reagents. Delivery of RNAi agents with non-viral carriers and factors affecting therapeutic efficacy have been emphasized. Where appropriate, experience with other types of RNAi reagents is summarized to generate a better sense of possible future progress. Finally, we provide a perspective on the future of RNAi in leukemic diseases and identify hurdles and solutions to clinical deployment of RNAi technology.

1.6 NON-VIRAL SIRNA DELIVERY FOR LEUKEMIA

The endogenous RNAi mechanism for post-transcriptional gene silencing is triggered by transcription of long pieces of double-stranded RNA (dsRNA) that are subsequently cleaved into smaller (21–23 nucleotides) microRNAs by Dicer [163]. For a pharmacological RNAi intervention, a plasmid encoding for short hairpin RNA (shRNA) or a double-stranded siRNA, to bypass the shRNA transcription and processing steps, have been employed [164, 165]. The use of siRNA is more practical in hard-to-transfect primary cells, and it represents a more physiological mechanism to regulate gene expression as compared to AS-ODN [166], (**Table 1.2**). The siRNA is incorporated into the RNA-induced silencing complex (RISC), where Argonaute proteins cleave the sense strand of siRNA for release from the RISC. The activated RISC, which contains the antisense strand of siRNA, selectively seeks out and cleaves or represses the complementary mRNA [163, 164, 167]. While the activated RISC complex can move on

to cleave additional mRNAs, it also gets diluted during cell division [163], so that repeated siRNA administration may be necessary to achieve a persistent effect. As mentioned with all siRNA therapies in **Chapter 1 - Part I**, the large, hydrophilic and anionic siRNA cannot cross the plasma membrane and an effective carrier is needed to enable internalization and protection from almost immediate degradation by serum nucleases (**Figure 1.1**). Electroporation is a common method to deliver siRNA in culture by creating pores in cell membrane. While helpful to implement RNAi in culture [168-170], such a method cannot be employed *in vivo* [171, 172]. Viral vectors have been alternatively used both in *in vitro* and *in vivo* studies including the clinical trial in CML (NCT00257647) [173-176]. Although viral vectors are a prospective pursuit for leukemia, they present a significant safety risk due to their ability to integrate into a host's genome and/or cause significant immune responses [174, 177], and will not be further addressed in this review. Cationic biomolecules are safer for clinical deployment; they are capable of complexing and condensing anionic siRNA into spherical, stable nanoparticles (NPs) suitable for cellular uptake. Similar delivery systems can be employed for siRNA and AS-ODN since the molecular composition of siRNA is similar to AS-ODN and regulatory microRNAs.

1.7 FUNCTIONAL CARRIERS FOR RNAI AGENTS

Carriers specifically explored for siRNA delivery in leukemic cells include cationic lipids, oligomers of cationic amino acids and other moieties, cationic polymers and various nano-structured materials (**Figure 1.4 and Table 1.3**). Once the siRNA reaches the leukemic cell, it must gain entry through the cellular membrane, escape the

endosomes (if so entrapped) and effectively release the siRNA into the cytoplasm. The binding and engulfment of siRNA NPs at the plasma membrane adhesion site require effective interactions to overcome the thermodynamics barriers to membrane poration [178]. The lipid composition of the membrane as well as its dynamic nature influences internalization and may contribute to the difference in silencing among different cell types [179, 180]. The highly dynamic lipid rafts [181, 182] may further 'nucleate' interactions with siRNA NPs, leading to different type of affinities along the membrane [178]. Creating cationic NPs capable of interacting with surface proteoglycans has been one approach to enhance siRNA uptake. Cationic single wall carbon nanotubes, for example, were used to silence cell-cycle regulator cyclin A_2 in CML K562 cells [183]; a significant (~70%) reduction of cell numbers was obtained as a result of enhanced apoptosis. When cationic carriers are utilised for delivery, increasing the carrier:siRNA ratio (often referred as the N/P -amine/phosphate- ratio) has been found to improve delivery as a result of increased charge of the complex [184, 185]. The cellular uptake of siRNA (binding and internalization) is generally observed to occur within a few hours for both targeted and untargeted carriers (e.g., ~1 h for liposomes in AML cells [186] and albumin coated CPPs in ATLL cells [187]). Interestingly, a high peak delivery (96%) was achieved with a targeted peptide system at ~2 hr with a rapid decline thereafter [188]. siRNA silencing was not demonstrated with this system and the reason of the rapid decline was not discussed, but could indicate siRNA release (affecting measurable fluorescence levels) or perhaps even exocytosis. siRNA delivery studies, performed with lipid-PEI carrier libraries in CML cells and breast cancer cells emphasized the lower

delivery percentage in CML cells. These results thereby initiated further formulation alterations to achieve more comparable levels of delivery in the CML cells [189].

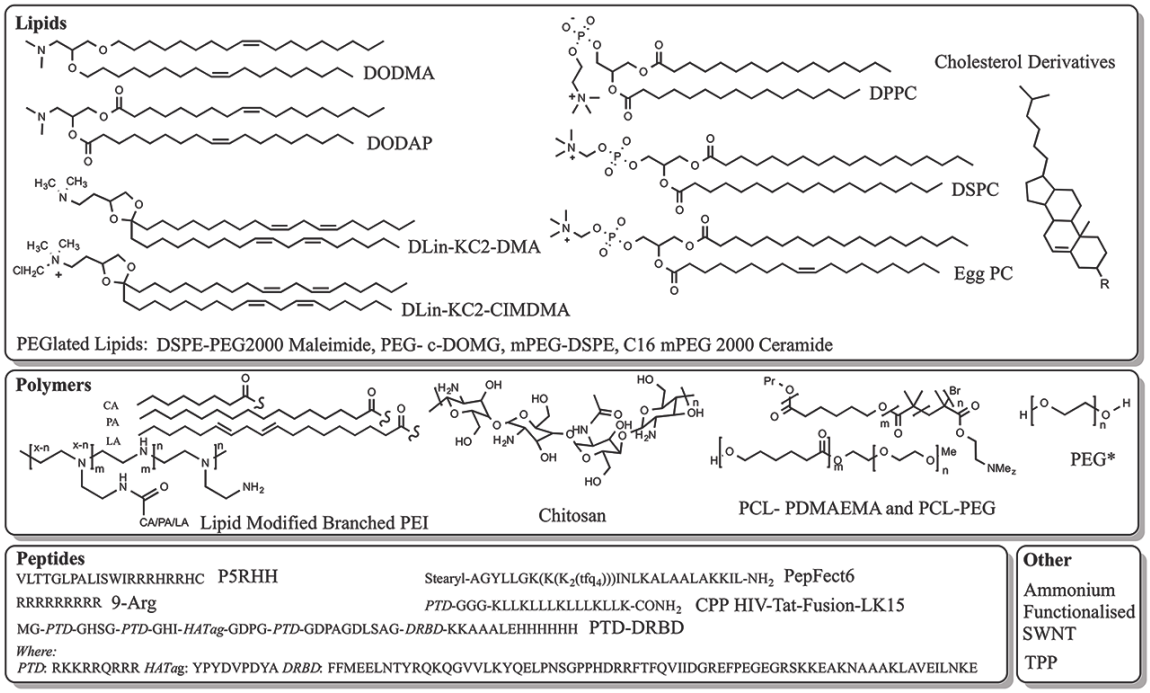

Figure 1.4 Structure of Carrier Components Used for siRNA Delivery in Leukemia.
Chemicals structures are from carriers described and referenced in **Table 1.3**.
CA: Caprylic acid, C16 mPEG 2000 Ceramide: N-palmitoyl-sphingosine- 1-[succinyl(methoxypolyethylene glycol) 2000], DLin-KC2-DMA:1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane , Dlin- KC2-ClMDMA: Alkylated DLin-KC2-DMA, DODAP: 1,2-dioleoyl-3-dimethylammonium-propane, DODMA: 1,2- Dioleyloxy-N,N-dimethyl-3-aminopropane, DPPC: dipalmitoylphosphatidylcholine, DSPC: 1,2-distearoyl-sn-glycero-3-phosphatidylcholine, Egg PC: Egg phosphatidylcholine, SWNT: Single-walled carbon nanotube, mPEG-DSPE: methoxy-polyethylene glycol (MW 2000) distearoyl phosphatidylethanolamine, LA: Linoleic acid, PA: Palmitic acid, PCL: polycaprolactone, PDMAEMA: Poly((dimethylamino)ethylene methacrylate), PEG: polyethylene glycol, PEG-c-DOMG: R-3-[(ω-methoxy poly(ethylene glycol)2000)carbamoyl)]-1,2-dimyristyloxl-propyl-3-amine, PEI: Polyethylenimine, TPP: Tripolyphosphate. *PEG is incorporated into carrier structures

Table 1.3 Non-Viral, Non-Commercial Carriers Developed for siRNA-Based Therapy of Leukemia.

Various carrier formulations were categorized based on the type of leukemia they were tested in.

Ref.	siRNA Carrier	Carrier Design Rationale	siRNA Targets [Cell]	Delivery*		Silencing** (nM)		Therapeutic Effect *** (nM)	
				In Vitro	*In Vivo*	*In Vitro*	*In Vivo*	*In Vitro*	*In Vivo*
Multiple Leukemia Types									
[190]	**Lipid NP** *Cationic lipids (with alkylated DMA) + neutral lipids + PEG*	Alkylated DMA key for improved transfection (increased particle order and stability). Protamine, HA, peptides (PPAA and INF7) enhanced transfection.	KIF11, FLT3 [AML (THP-1, KG-1, Molm13, Mv4-11, HEL), CML (K562), Molm13 *in vivo*.]	✗	✗	✔ (10-500)	✔!	✔ (10-30)	✗
[191, 192]	**Modified siRNA** *TLR9 antagonist CpG*	Not a carrier. Targeted delivery for siRNA. Does not protect against serum nucleases.	STAT3 [AML (MV4-11, patient), MM (KMS-11, patient), TLR9+ hematopoietic cells, human PB blood cells (monocytes, T cells, NK cells, B cells, mDCs, pDCs]^	✔	✔	✔ (500 in MM)	✔	✔!	✔
[193, 194]	**Peptide** *CPP PepFect6*	Characterized and tested amphipathic and arginine-rich CPPs for siRNA silencing. Amphipathic PepFect6 was the most promising CPP. (Comprised of stearyl-TP10 peptide with trifluoromethylquinoline moieties for endosomal escape, effective with serum proteins) Electrostatically formed NP.	Luciferase (reporter gene) [AML (SKNO-1)] HPRT1 [CML (K562) and ALL (Jurkat)] ^	✔ ✗	✗ ✗	✔ (50-200) ✔ (12.5-100)	✗ ✔!	✗ ✗	✗ ✗
[195]	**Fusion Protein** *PTD-DRBD*	DRBD for binding to siRNA, PTD for cellular delivery. Developed for delivery to primary cell and thus also tested in other cell types/animal models. DRBD avidity to siRNA mediated NP formation.	GFP (THP-1 differentiated to macrophages?) ALL (Jurkat)]^	✗	✗	✔ (100-400)	✗	✗	✗
[196]	**Lipid NP** *Transferrin ligand + cationic lipid DODMA)*	Microfluidic formation for controlled mixing parameters during self-assembly.	RRM2 [AML (MV4-11) and CML (K562)]^	✔	✔	✔ (100-500)	✔	✔ (100-1000)	✗
AML									
[197] [184, 198]	**Polymeric Complex** *Caprylic or linoleic acid substituted on 2 kDa PEI*	Lipids for cell interactions. Low MW PEI for decreased toxicity while maintaining beneficial properties of PEI. Electrostatically formed complexes.	GFP (reporter gene), CXCR4, SDF-1, CD44 [THP-1, KG-1, KG-1a, patient]	✔	✗	✔ (25-100)	✗	✔ (25-100)	✗
[199]	**Chitosan NP** *Chitosan*	Chitosan is biocompatible, cationic, and adhesive. Electrostatically formed NP.	VEGF, FLT1 [U937]	✗	✗	✔ Unknown	✗	✔ Unknown	✗
[185]	**Micelle** *Amphiphilic diblock copolymers (4.5 kDa PCL with 15.5 kDa PDMAEMA or 5 kDa PEG.*	PDMAEMA provides charge and buffering for endosomal rupture. PEG provides colloidal stability/ RES protection. Two polymers allow for cell type optimization through cationic charge and resulting toxicity.	Luciferase (reporter gene), RUNX1/ETO [SKNO-1]^	✔	✗	✔ (500)	✗	✗	✗

[186]	**Liposome** *Anti-CD33 Ab + EPC/ chol/ mPEG 2000-DSPE w/wo PEI 25 kDa core*	CD33 for cell targeting; PEI (electrostatical binding to siRNA) increased liposome encapsulation of siRNA however PEI encapsulation did not improve silencing results.	AML1/MTG8 fusion protein [SKNO-1, Kasumi-1]	✔	✗	✔ (600 – 2500)	✗	✔ (30-125)	✗
			CML						
[189]	**Polymeric Complex** *Palmitic acid substituted on 1.2 kDa PEI*	Lipid for enhanced cell interactions. Low MW PEI for decrease toxicity. Electrostatically formed complexes.	GFP (reporter gene), BCR-ABL [K562]	✔	✗	✔ (36-100)	✗	✔ (50-100)	✗
[33]	**Chitosan NP** *Chitosan*	Chitosan formed siRNA complexes (electrostatic) delivery. Tested in multiple cell types (+ CML). Electrostatic formed NP.	BCR-ABL [K562]^	✗	✗	✔ (50)	✗	✗	✗
[174]	**Peptide** *CPP (HIV-Tat (49-57)) + membrane lytic peptide (LK15)*	Peptides for cytoplasm delivery and endosomal escape. Shows studies with shRNA and siRNA. Electrostatically formed NP.	BCR-ABL [K562]	✔	✗	✔ (24-729 Est.)	✗	✔ (1428 - 2142 nM, Est.)	✗
[183, 200]	**Carbon Nanotubes** *Ammonium functionalized SWNT*	SWNT provides high siRNA loading. Ammonium provides positive charge to bind siRNA. Electrostatically formed.	Cyclin A_2	✗	✗	✔ (25)	✗	✔ (25)	✗
[201]	**Liposome** (*Transferrin- PEG2000-DSPE + Chol/ DSPC/ DODAP/ C16 mPEG 2000 Ceramide*	DODAP (+ve at pH 4/neutral at physiological pH) and optimized buffer concentration provides high siRNA encapsulation.	BCR-ABL [K562 and LAMA-84]	✔	✗	✔ (500-2000)	✗	✔ (500-2000)	✗
			ALL and ATLL						
[188]	**Peptide** *Minibody (anti-JL1) conjugated to oligo-9-Arg- peptide CPP*	Specific mini-body against JL1 (specific to leukemic cells and not mature hematopoietic cell). CPP for internalization. Electrostatically formed.	No Target (FITC scrambled siRNA) [CCRF-CEM, Jurkat]	✔	✔	✗	✗	✗	✗
[202]	**NP** *Chitosan + TPP*	Chitosan is biocompatible and adhesive. (Adhesion properties may promote tumor survival). Electrostatically formed NP.	Hsp70 [Jurkat]	✗	✗	✔ (50)	✗	✔ (50)	✗
[187]	**NP** *Albumin coated CPP complex*	Albumin coating for stabilization and prevention of flocculation. Melittin derived P5RHH peptide for endosomal escape. Electrostatically formed NP.	p65 and p100/52 (NfκB) [F8]	✔	✔	✔ (50-200 Est.)	✗	✔ (50-200 Est.)	✗

* Delivery reported if measured (fluorescent siRNA). ** Demonstration of silencing of a target protein if reported. *** Therapeutic effect of silencing a protein target. '✔' indicates study was performed and significant results obtained. '✗' indicates study was not performed or significant results were not obtained. In all cases a '✔' is only indicated when the studies were performed in leukemic cells. In some cases delivery, silencing and/or therapeutic effects were demonstrated in non-leukemic cells, the results of which are not indicated. '!' Indicates study was done in non-leukemic cells but still provides significant benefits to be included in the review. '^' Indicates testing was also done in other cell types. 'Est.' indicates concentration was estimated from data provided. CPP: cell penetrating peptide, C16 mPEG 2000 Ceramide: *N*-palmitoyl-sphingosine- 1-[succinyl(methoxypolyethylene glycol) 2000], DC-Chol: 3b-[N-(N,N -dimethylaminoethane)-carbamoyl] hydrochloride, DSPC:1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine, Chol: Cholesterol, DODAP: 1,2-dioleoyl-3-dimethylammonium-propane, DODMA: Dioleyloxy-N,N-dimethyl-3-aminopropane, Egg PC: Egg phosphatidylcholine, HA: hyaluronic acid, MW: molecular weight, NP: nanoparticle, PEG: polyethylene glycol, PCL: polycaprolactone, PDMAEMA: poly((dimethylamino)ethylene methacrylate), PEI: polyethylenimine, PPAA: poly(propylacrylic acid) peptide, RES: reticuloendothelial system, TPP: tripolyphosphate. Trf: Transferrin.

1.7.1 Cationic Cell Penetrating Peptides in Leukemia siRNA Therapy

Cationic cell penetrating peptides (CPPs) [174, 187, 188, 193, 194] composed of 20-30 amino acids with membrane translocation activity were alternatively employed for siRNA delivery. Their polycationic nature enables them to interact electrostatically with phosphate backbones of nucleic acids, while also allowing them to effectively bind to cell membranes. A Tat-derived CPP (amino acids 49–57 of HIV-1 Tat protein) covalently attached to membrane-active peptide (Tat-LK15) was used to complex electrostatically with nucleic acids and deliver them to K562 cells [174]. The combination of these peptides increased the transfection efficiency by 2-fold compared to Tat peptide alone. Low overall charge (due to charge neutralization) has been found to be an impediment for delivery with peptides. Thus TAT has been alternatively combined with a double stranded RNA-binding domain (DRBD) creating a fusion protein for siRNA delivery where DRBD, due to its high avidity for minor-groove recognition, binds the siRNA and masks the siRNA negative charge resulting in increased overall charge. Delivery with PTD-DRBD (100 to 400 nM siRNA) in GFP-expressing Jurkat T-cells resulted in ~90% reduction of GFP fluorescence (in line with mRNA reduction), while lipofection (Lipofectamine 2000^{TM} and RNAiMAX^{TM}) was generally less effective, with reduced protein levels of 40-50%. Similar results were found when targeting CD4 and CD8 in primary murine T-cells with PTD-DRBD, while no toxicity was found on human umbilical cord vein endothelial cells. About 20% reduction in non-specific target mRNAs was seen when compared to scrambled siRNA [195], which was not surprising considering the high siRNA concentrations used. Amphipathic CPPs (TP10, PepFect6, PF14) as well as arginine-rich CPPs (R9, Tat, hLF and R9-hLF) electrostatically forming

siRNA complexes were physically characterized and analysed for their delivery and silencing ability in SKNO-1 cells [193]. Luciferase reporter silencing was achieved with all peptides, however the amphipathic peptides demonstrated immensely higher silencing ability (60-85% silencing with 50-200 nM siRNA for the best performing CPPs, PepFect6 and Pepfect14), which matched with the cellular localization of the amphipathic CPPs being dispersed within the cytoplasm compared to the cellular membrane localization of the other peptides. The authors highlight physiochemical characteristics including demonstration of siRNA complexation, serum protein resistance and polyanion induced decomplexation (low zeta-potentials) and cellular delivery (not cell association) to be key for efficient CPP carrier systems as demonstrated in the leukemic cell line [193].

1.7.2 Lipidic Carriers in Leukemia siRNA Therapy

Lipidic carriers forming solid NP and core-shell liposomes have also proven effective in AML, CML and ALL cells [186, 190, 196, 201], providing significant *in vitro* silencing as well as therapeutic outcomes in most cases (**Table 1.3**). The lipid components in such NPs was similar to lipids utilized for other cancers [203], with an overall cationic charge (**Figure 1.4**). It was possible to further enhance silencing efficacy in leukemic cells by using modified lipids (DLin-KC2-DMA to DLin-KC2-CIMDMA), as seen in one study with leukemic Molm-13 cells and other AML and CML cell lines [190]. Another targeted and PEGylated liposomal system utilised polyethylenimine (PEI) within its core, which resulted in better siRNA loading efficiency, but did not improve silencing despite PEI's well known ability to escape the endosome and release siRNA within the cytosol [186].

1.7.3 Polymer Carriers in Leukemia siRNA Therapy

Carriers derived from polymers provide ideal control in design and optimization of delivery. PEIs that can serve as non-specific carriers in a range of adherent cells [203] have been derivatized with lipophilic moieties to make them effective in leukemic cells (**Figure 1.4**). The "proton-sponge" feature of PEIs that facilitates endosomal escape of nucleic acids [164] presumably aids in effectively liberating internalized siRNAs in leukemic cells. By modifying the amine groups of low MW (1.2-2.0 kDa) PEI, we designed a range of lipid-substituted PEIs. Our studies with AML cells indicated linoleic acid (C18:2) and caprylic acid (C8) substitution to sustain silencing of a reporter (GFP) and the CXCR4 gene [184]; however, the polymers that were effective in CML cells were different and we found a particular polymer (1.2 kDa PEI) substituted with a relatively high amount of palmitic acid (C16) to be most effective. The ability of this polymer to deliver siRNA intracellularly was high, underpinning its relative efficiency. The oncogene BCR-ABL was effectively silenced in CML (K562) cells, resulting in induced apoptosis of target cells [189]. The liposomal agent Lipofectamine^{TM} 2000 seemed to be equally effective to the polymeric carrier in the K562 model of CML, but this carrier is not recommended for *in vivo* use. Amphiphilic diblock polymers, which form micelles, have been also explored for siRNA delivery [185]. Two diblock copolymers PCL-PDMAEMA and PCL-PEG were utilized in these formulations, so that the components responsible for endosomal escape (PDMAEMA) and protection from reticuloendothelial system (PEG) could be independently optimized. The natural polymer chitosan has also been utilized as an effective carrier due to its perceived biocompatibility [33, 199, 202].

1.7.4 Additional Functionalization of Carriers in Leukemia siRNA Therapy

Additional functionalization of carriers for siRNA delivery was required in some cases [177, 178, 204, 205]. Bioactive peptides for endosomal escape (e.g., P5RHH in albumin-CPP complexes [187], LK15 in a fusion peptide [174] and stearyl-TP10 in CPPs [194]) and other biomolecules for siRNA release (e.g., protamine, HA and peptides PPAA and INF7 [190]) were explored. Interactions with blood can affect the carrier properties and functionality. Cationic CPP-siRNA complexes were found to become negatively charged with decreased particle size when measured in the presence of serum, indicating coating with serum proteins and suggesting that alternative methods of cellular uptake (such as scavenger receptors) occur rather than electrostatic/surface proteoglycans interactions [193]. Stability of CD33 targeting liposomes were tested *in vitro* by incubation in 50% human plasma for up to 10 days and showed a loss of binding of 30-40% after one day with no further significant changes [186].

While successful deployment of different carriers is encouraging, their performances, measured as the effective siRNA concentrations (**Figure 1.5**), are highly variable, with some delivery systems yielding an effective therapy at <50 nM while others requiring ~1000 nM. This analysis inherently assumes the best results (i.e., most effective doses) were obtained with the optimized formulation for each carrier (not necessarily the same N/P ratio, carrier concentration, etc.). The absolute level and turnover rate of target mRNA, as well as characteristics of cell models (e.g., surface proteoglycans, proliferation rate, etc.) could contribute to this variability (and perceived relative efficiency of the delivery system), but little emphasis has been placed on exploring this variability, which will be ultimately critical to understand patient-to-patient

variation in therapeutic responses. For *in vitro* utility, formulations effective in 10-50 nM range will be desirable. Based on analysis in **Figure 1.5**, non-commercial carriers appear to be equally effective as commercial carriers, but the difference might be better revealed in animal models, where the data is limited to-date. Improved performance would be anticipated with newly generated carriers, but our previous analysis [206] did not indicate the new carriers improving in efficacy (i.e., lowering the effective doses of siRNA reagents), leading to proliferation of the type of effective carriers possible but not necessarily leading to carriers with improved efficacy. Towards this goal, more effective therapies may rely on 'leukemia-seeking' carriers in the future.

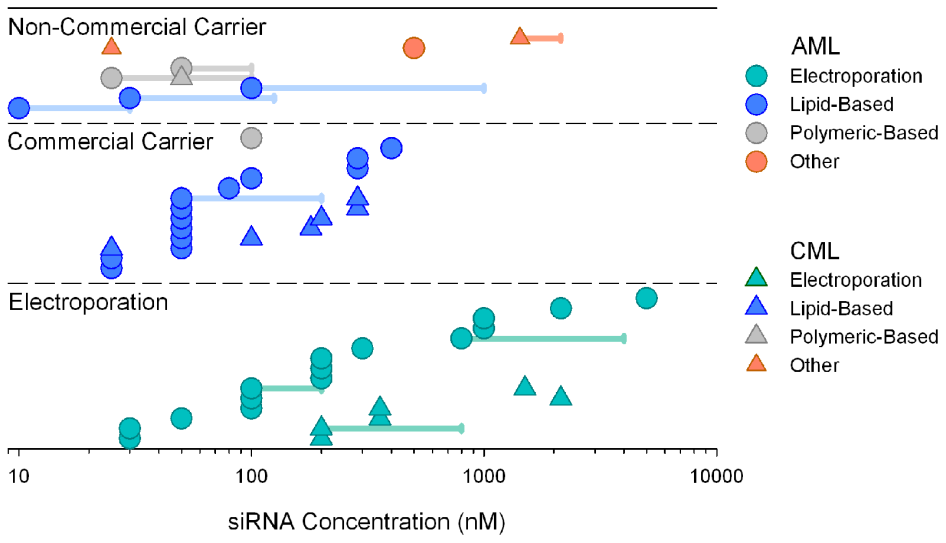

Figure 1.5 Effective *In Vitro* siRNA Dose Ranges for Experimental siRNA Therapies in AML and CML with Non-Viral Carriers.

The 'markers' (circle/triangle) indicate the lowest dosage utilised for siRNA silencing that produced a therapeutic effect on the myeloid leukemia cells whereas the 'lines' indicate any additional dosage range utilised that also provided a therapeutic effect in the reported study. The dose ranges were obtained from *in vitro* studies that demonstrated therapeutic effects and reported both the siRNA concentration and non-viral carrier utilized in **Table 1.6**. Where necessary, siRNA concentrations were estimated by the authors from the reported amounts and volumes used in specific experiments. AML: Electroporation [207-221], Commercial/Lipid-Based [222-234], Commercial/Polymeric-Based [235], Non-Commercial/Lipid-Based [186, 190, 196], Non-Commercial/Polymeric-Based [197, 198], Non-Commercial/Other [191]. CML: Electroporation [168, 170, 236-239], Commercial/Lipid-Based [222, 231, 232, 240-242], Non-Commercial/Polymeric-Based [189], Non-Commercial/Other [174, 183].

1.8 SELECTIVE DELIVERY TO LEUKEMIC CELLS

Most siRNA studies in leukemia focus on down-regulating a target protein to elucidate its function or to develop small molecular drugs against this target, rather than employing siRNA as a therapy. Delivery systems are beginning to be tailored for leukemic cells with a focus on conventional drugs so far, but the information gained will guide the siRNA delivery in the future. Understanding NP uptake in hard-to-transfect non-adherent leukemic cells is important; we noted that CML K562 cells displayed a 15-fold reduction in siRNA uptake using the same lipophilic PEI carriers [189] compared to breast cancer MDA-MB-231 cells. Since the amine content in NPs is the primary determinant of cell interactions [243], less effective uptake by leukemic cells might be due to relatively weak binding of siRNA NPs due to deficient Ca2+-dependent ligands, such as proteoglycans and cadherins [244]. In attachment-dependent cells (i.e., HeLa and mesenchymal stromal cells, MSCs), NPs were found in intracellular compartments, most likely inside endosomes, while in KG1a and Jurkat cells, NPs were located at the cell membrane or periphery [243], suggesting active endocytosis to be limited in leukemic cells. Although weak delivery to leukemic cells can be overcome by increasing the dose, this results in non-specific cytotoxicity [245]. Effective delivery to leukemic cells might need to rely on cell-targeting ligands that not only concentrate siRNA at leukemic cells but also encourage endocytic uptake. While the NP uptake can occur through multiple pathways during endocytosis, therapeutic effect of the payload might not necessarily be equal along all pathways [190].

1.8.1 Employing Ligands Specific for Leukemic Cells

Antibody (Ab) mediated delivery has been used to target surface proteins over-expressed or differentially-expressed on leukemic cells (**Table 1.4**). Other ligands were derived from peptides/proteins, aptamers, saccharides, benzamides, and ODNs with targets including transferrin receptor [196, 201, 246-254], low density lipoprotein [255-257], matrix metalloproteinase receptors (MMP-2/9) [258], toll-like receptor [191], C-type lectin-like molecule-1 (CLL1 receptor) [259, 260], lectins [261], protein tyrosine kinase 7 (PTK7) [245, 262-264], vitamin receptors for biotin [265, 266], folate/folic acid receptor [253, 254, 267-272], alendronate (bone) [272] and sigma receptors [273]. Some of the ligands target 'endocytosing' receptors on cell surface, while others such as CPPs facilitate uptake without necessarily undergoing endocytosis [178, 274]. Combining ligands with different functionalities can further enhance delivery; for example, (i) a JL1-specific Ab with CPPs [188] yielded higher siRNA delivery in JL1-overexpressing ALL cells (~96% $JL1^{high}$-CEM cells vs. ~6% $JL1^{low}$-Jurkat cells) and *in vivo* to CEM cells located in the bone marrow, and (ii) targeting bone marrow with alendronate along with leukemic cells (with folate) improved therapeutic effect *in vivo* [272]. The NPs may follow different pathways than the targeting ligand and optimization of conjugation chemistry and ideal ligand density is needed [249], since 'more' is not always 'better' for affinity and final delivery [270]. Some ligands are very specific for certain leukemias, but others, such as transferrin and folate, function in several types of leukemias, making it possible to develop more generic delivery systems.

Table 1.4 Ligands Used for Derivatizating NPs to Deliver Chemotherapy Drugs, Plasmid DNA, siRNA and microRNA to Leukemic Cells.

siRNA studies are shown in bold. While initial studies were focussed on delivering chemotherapeutic drugs, the developed systems were subsequently adopted for delivery of polynucleotides.

Ligands	Carrier Derivatized	Payload	Leukemia Type	Evaluation (delivery and/or silencing)	Ref.
Ab antiCD3 or Transferrin	PEI	Plasmid	CML (K562), ALL (Jurkat E6-1), Melanoma (H225), Murine Melanoma (B16F10), Neuroblastoma (Neuro2A)	*In vitro*	[275]
Ab antiCD3 or antiCD19	Streptavidin-PEI	Plasmid	ALL (Jurkat Clone E6-1 and J.RT3-T3.5), Lymphoma (Granta 519)	*In vitro*	[276]
Ab antiCD19	Liposome	Norcantharidin	ALL (Nalm-6 Pre-B, patient), CLL (Raji)	*In vitro*	[277]
Ab antiCD20	Lipopolyplex	AS-ODN	CLL (patient, Raji)	*In vitro/vivo*	[278]
Ab antiCD33	PLGA (core) with albumin (shell)	Everlimus, sorafenib	AML (KG1a)	*In vitro*	[279]
Ab antiCD33	**Liposome w/wo PEI core**	**siRNA**	**AML (SKNO-1, Kasumi-1)**	***In vitro***	[186]
Ab antiCD33	Liposome	Cytarabine	HL60	*In vitro/vivo*	[280]
Ab antiCD34	Liposome	DOX	AML (KG-1a)	*In vitro*	[281]
Ab antiCD37, antiCD19, antiCD20 or combination	Liposome	Fingolimod	CLL (patient, Raji, RS11846, Ramos, Daudi)	*In vitro*	[282, 283]
Ab antiCD2, antiCD3, antiCD5, antitransferrin	Liposome	Methotrexate-g-aspartate	ALL (Jurkat, Molt-4, CEM)	*In vitro*	[284]
Ab antiCD74 (milatuzumab)	Liposome	Dexamethasone	CLL (patient, Raji), ALL (697)	*In vitro/vivo*	[285]
Ab antiCD96 or antiCD117 (c-kit)	PEG/Calcium phosphosilicate	Indocyanine green	LSC AML (AML patient cells) or LSC CML (murine 32D-p210-GFP; CML patient)	*In vitro/vivo*	[286]
Ab antiJL1	**CPP**	**siRNA**	**ALL (CCRF-CEM, Jurkat, H9)**	***In vitro/vivo***	[188]
Ab antiJL1	Poly(L-lysine)	Plasmid	ALL (MOLT-4)	*In vitro*	[287]
Ab antiIgM	HPMA copolymer (star or classic)	DOX	Murine BCL (BCL1)	*In vitro/vivo*	[288, 289]
CLL1 peptide	Micelle	DAUN	LSC AML (A549 expressing CLL1; Cd34+ leukemic patient)	*In vitro*	[259]
CLL1	Magnetic NPs	Paclitaxel	CML (K562)	*In vitro/vivo*	[260]
Aptamer against PTK7	Polyplexes	pDNA	ALL (MOLT-4)	*In vitro*	[245]
Aptamer against PTK7	Hairpin DNA /Au	DOX	ALL (CCRF-CEM)	*In vitro*	[262]
Aptamer against PTK7	Single-walled carbon nanotube	DAUN	ALL (MOLT-4)	*In vitro*	[263]
Aptamer against PTK7 or KK1B10	DNA core connector /Photo-cross-linked	Intercalating drug (DOX) and AS-ODN	ALL (CCRF-CEM), CML (K562, DOX R K562),	*In vitro*	[264]
CpG oligodeoxyribonucleotide against TLR9	*	**siRNA**	**AML (MV4-11, patient), MM (KMS-11, patient), TLR9+ hematopoietic cells**	***In vitro/vivo***	[191]
Transferrin	**Liposome**	**siRNA or AS-ODN**	**CML (K562 and LAMA-84)**	***In vitro***	[201]
Transferrin	**Lipid NP**	**siRNA**	**AML (MV4-11) and CML (K562)**	***In vitro/vivo***	[196]
Transferrin	Liposomal with PEI/MiR core	miR	AML (Kasumi-1; OCI-AML3; MV4-11; patient)	*In vitro/vivo*	[246]

Transferrin	Lipopolyplex	AS-ODN	AML (kasumi-1, patient)	*In vitro*	[247]
Transferrin	Lipid/protamine	AS-ODN	AML (MV4-11), CML (K562), CLL (Raji)	*In vitro*	[248]
Transferrin	PEG-Cyclodextrin/ PEG-adamantane/ transferrin-PEG-adamantane	Plasmid	CML (K562)	*In vitro*	[249]
Transferrin	Liposome	AS-ODN	CML (K562)	*In vitro*	[250]
Transferrin	HSA	Sorafenib	CML (K562; imatinib/ dasatinib resistant K562; imatinib refractory patient)	*In vitro*	[251]
Transferrin	Liposome	Rhodamine-PE (label)	ALL (CEM, MOLT-3)	*In vitro*	[252]
LDL	LDL	DiO (label)	CML (K562, KCL22, patient), AML (HL60, AML3), enriched MNCs, prostate (PNT1A, PC3), non-CML patient (quiescent LSC)	*In vitro*	[255]
LDL Peptide	Liposome	DAUN	AML (THP-1 and NB4)	*In vitro/vivo*	[256]
LDL	Liposome	Hygromycin B	Guinea Pig ALL (L_2C)	*In vitro*	[257]
LFA-1 Peptide	PLGA	Empty/ coumarin-6 (label)	ALL (Molt-3, Molt-4), AML (U937, HL-60)	*In vitro*	[290]
Folate	Dextran/Retinoic Acid Micelles	DOX	AML (KG-1)	*In vitro*	[267]
Folate	Liposome	DAUN, DOX	AML (MV4-11, CHO-K1, KG-1, KG-1a), CML (K562), Human Epidermoid Carcinoma (KB), Murine Leukemia (L1210, L1210JF), Folate-Beta transfected cells (CHO-FR-Beta),	*In vitro/vivo*	[268-271]
Folic acid or transferrin	Polylysine	AS-ODN	AML (HL-60)	*In vitro*	[253, 254]
Alendrondate (bone) and folate (CML)	Lipid carrier	Mitoxantrone	CML (K562)	*In vitro/vivo*	[272]
Anisamide against sigma receptors	Lipid-coated nanoscale coordinated polymers	Methotrexate	ALL (Jurkat)	*In vitro*	[273]
Biotin	Single-walled carbon nanotube	Taxoid	Murine Leukemia (L1210FR)	*In vitro*	[265]
Streptavidin + Biotin-G-CSF, antiCD33 Ab or antiCD7 Ab	Liposome	Cytarabine	AML (Kasumi-1 IMS-M2), CML-BC (MEG-01 and K562), ALL (Jurkat, KOPN- 30)	*In vitro/vivo*	[266]
Peptide against MMP-2 and MMP-9 receptors	Liposome	Adriamycin, Rhodamine B (label)	AML (U937), Other (CHO, NRK52E, HT1080)	*In vitro*	[258]
Saccharide against lectins	Liposome	Sarcolysine	AML (HL-60), Lung Adenocarcinoma (ACL)	*In vitro*	[261]

Ab: Antibody, ALL: T-cell acute lymphoblastic leukemia, AML: acute myeloid leukemia, AS-ODN: antisense oligonucleotide, BCL: B cell leukemia, CLL: chronic lymphocytic leukemia, CLL1: C-type lectin-like molecule-1, CPP: Cell-penetrating peptide, DOX R: Doxorubicin resistant, DAUN: Daunorubicin, G-CSF: granulocyte colony-stimulating factor, HSA: Human serum albumin, HA: hyaluronic acid, HPMA: N-(2-hydroxypropyl)methacrylamide, LDL: Low density lipoprotein, LSC: Leukemic Stem Cells, miR: MicroRNA, MM: multiple myeloma, NP: Nanoparticle; PEG: poly(ethylene glycol), PEI: polyethylenimine, PLGA: poly(lactic/glycolic acid), PNA: Triplex-forming peptide nucleic acids, PTK7: protein tyrosine kinase 7, TLR2: toll-like receptor 2 *Not a true NP. Non-NP systems for the table summary were restricted to nucleotide transfection related payloads.

1.8.2 Relying on Targeting to Improve Endocytosis

When untargeted lipid NPs were delivered to leukemic cells displaying low (K562 and HEL cells), medium (Molm13 and THP1 cells) or high (Mv4-11 and KG1 cells) propensity for transfection, the levels of endocytosis-related genes, caveolin 1, caveolin 2 and Rab13, were found to correlate to level of transfection difficulty [190]. Caveolin 1 and 2 expression were also correlated with transfection difficulty in other adherent and difficult-to-transfect cells [190]. The native endocytosis capabilities can be harnessed by employing ligands that induce endocytosis upon receptor binding on the surface of cells. Transferrin is an iron-binding glycoprotein that binds to its receptor in iron-loaded form for endocytosis. The iron requirement increases in rapidly dividing malignant cells and thus transferrin receptors are often over expressed [249]. In early studies, transferrin-PEI conjugates increased transfection (with pDNA) 10-100 fold in CML (K562) cells and transferrin has been successfully employed in NPs carrying siRNA, miR, AS-ODN and plasmids with functional release of the payload and therapeutic outcomes in CML, AML and CLL models [196, 201, 246-250, 254, 275]. While transferrin-conjugated liposomes encapsulating a BCR-ABL siRNA provided effective silencing in CML cells, effects on other proteins and cell viabilities were also observed, likely as a result of high concentrations and repeat treatments [201]. Transferrin-targeting lipid NPs also provided efficient delivery and silencing of R2 subunit of ribonucleotide reductase (RRM2) protein (via siRNA) in both CML (K562) and AML (MV4-11) cells [196]. Transferrin-conjugated liposomal NPs with a PEI/miR-29b core increased uptake and delivery of their payload and resulted in decreased cell and colony numbers in AML cells [246]. The targeted NPs also provided prolonged survival of mice compared with scrambled miR

delivered with the same NPs [246]. Transferrin-lipopolyplexes also provided targeted delivery of an AS-ODN (GTI-2040) against RRM2, where targeted delivery greatly improved mRNA and protein suppression in an AML model (kasumi-1 cells) and patient cells, and sensitized the cells to cytarabine [247]. While providing a strong evidence for the potential of leukemia-specific delivery, transferrin-mediated targeting has highlighted the importance of ligand incorporation method in successful targeting [249], where lysine-mediated attachment of PEG to transferrin provided the least decrease in binding affinity and higher transfection in CML K562 cells [249].

As an alternative to transferrin, folic acid (i.e., folate) that can cause endocytosis upon receptor binding has been incorporated into polylysine for delivery of AS-ODN against c-myb in AML HL-60 cells [253] as well as for chemotherapy drug carrying micelles, liposomes, and lipid carriers [267-272]. An important consideration of folate is its effect on *in vivo* clearance; folate-functionalized liposomes gave faster clearance possibly due to folate receptor-beta expression on phagocytic cells of the reticuloendothelial system [270]. This is not unique to folate and others ligands, such as all-trans retinoic acid [271] and CD33-targeting antibodies [266] also affected the *in vivo* pharmacokinetics of the delivery systems.

1.8.3 Antibody-Mediated Targeting

Targeting with Abs is especially attractive due to its wide applicability. One can envision incorporating Abs directly into carriers, or using a secondary Ab to target cells already labeled with a primary Ab [266, 284]. Early efforts have identified functional Abs against CD2, CD3 and CD5 in ALL cells, [275, 284], although transferrin-mediated uptake was found to be superior to Ab-targeting in one study [284]. Representative

formulations recently explored for leukemia include; (i) a CD33-seeking liposome which showed improved delivery and silencing in AML cells (CD33 has little expression in hematopoietic stem cells and non-myeloid cells [280]), albeit the siRNA concentrations were high and an improvement in efficacy was needed [186]; (ii) a CD3-seeking polyplex was functional in Jurkat T-cells (CD3+/CD19-), while a CD19-seeking polyplex was functional in Granta B-cells (CD3-/CD19+) for plasmid delivery [276], with good selectivity in a heterogeneous cell population. However, only ~11% of Jurkat cells and ~2% for Granta cells were transfected, indicating difficulties in transfecting non-adherent cells once again, and; (iii) a CD20-seeking lipopolyplex was used to suppress Bcl-2 in CLL with AS-ODN G3139, which suffered from low delivery and immune stimulation when delivered naked, providing reduced immunostimulatory effects and improved Bcl-2 silencing in CLL cells [278]. The complications related to Fc domain-related systemic clearance by macrophages might be circumvented with Fab' fragments of Abs [280].

Ab-mediated NP targeting might not always lead to enhanced internalization. In the case of doxorubicin-loaded liposomes attached to an anti-CD34 mAb, the IC50 of the delivery system was 8-fold higher than non-targeted system in CD34+ AML (KG-1a) without any evidence of increased internalization [281]. This was attributed to local release in the vicinity of cells and rapid transport of doxorubicin through cell membrane. This might be limiting for siRNA therapeutics since locally released siRNA cannot enter cells on their own. If it is the NP that limits internalization (e.g., a particular type of liposome), other types of NPs, such as poly(lactic/glycolic acid) NPs that demonstrated high internalization even without targeting, could be more useful [291]. Alternatively, modified siRNAs capable of entering cells on their own might be required. Chemically-

modified siRNAs (e.g., with palmitic acid [292], cholesterol [293-295], CPPs [296] and oligodeoxyribonucleotides [191]) have been described that traverse the cell membrane on their own or via specific receptors. Only the latter agent was explored in leukemia; a TLR9 agonist CpG-oligodeoxyribonucleotide (with STAT3 or Bcl-XL siRNA) yielded effective silencing in normal TLR9+ hematopoietic cells, KMS-11 multiple myeloma and MV4-11 AML cells, and delivery in multiple myeloma and AML patient cells [191]. *In vivo* intratumoral delivery to MV4-11 xenografts gave delivery to ~76% of tumor cells (100 μg siRNA) and effective silencing of STAT3 and Bcl-XL (>60%).

Finally, Ab-mediated targeting holds great potential for specific delivery to LSC since they are usually refractory to current drugs. Numerous LSC surface protein targets for monoclonal Ab therapy have also been highlighted (CD25, CD32, CD44, CD47, CD96, and CD123, CLL1) [297, 298] and one could foresee their use in NP targeting as well. Using calcium phosphosilicate NPs, a photoactivatable drug (indocyanine green) was delivered to AML and CML LSC by using CD96 or CD117 Abs, respectively, which dramatically improved the efficacy [286]. C-type lectin like molecule-1 (CLL1) was additionally employed, as CLL1 is expressed on AML LSCs and CD38+ progenitor cells but not on CD34+/CD38- hematopoietic stem cells [259, 299]. A ligand for CLL1 was also utilized on magnetic NPs to take advantage of receptor-mediated endocytosis in CML K562 cells [260].

1.8.4 Aptamers for Targeting

Aptamers, synthetic ODNs or peptides with engineered binding affinities and specificities, is another ligand type that attracted recent attention. Anionic aptamers can be electrostatically attached to cationic NPs. An aptamer (sgc-8c), which recognizes

protein tyrosine kinase 7 (PTK7) present on ALL cells, was utilized for targeting PEI/plasmid polyplexes and carrying a luciferase reporter plasmid to MOLT-4 cells [245], hairpin DNA-Au NPs delivering doxorubicin to CCRF-CEM cells [262], and daunorubicin loaded single-walled carbon nanotubes to MOLT-4 cells [263]. Additionally, PTK7 as well as KK1B10 (for directing to doxorubicin resistant K562 cells) provided targeting for an aptamer-DNA NPs delivering doxorubicin (intercalated with DNA) and antisense oligonucleotides [264].

1.8.5 Targeting Adhesion Receptors

There is usually a low level of expression of receptors for attachment proteins in leukemic cells; K562 cells displays only fibronectin receptors (VLA-5) on cell surfaces, but not vitronectin (avb3), collagen (VLA-2) or hyaluronan (CD44) receptors [300], but they could be induced to express CD44 upon differentiation into myeloid lineage [301]. Unlike K562 cells, AML cells SHI-1, THP-1 and NB4 cells [302] express significant levels of CD44, which is involved in mobilization of leukemic cells [303]. Although others have explored CD44 for various malignancies by utilizing its endogenous ligand hyaluronic acid (HA) [304], few have focused on leukemic disease. A HA-coated chitosan-triphosphate NP was investigated for delivery to high CD44-expressing macrophages (murine RAW 264.7) and low CD44-expressing K562 cells [305]. Although targeted-NPs were not compared to non-targeted NPs, plasmid transfection efficiency was in proportion to CD44 levels in target cells. Using dual targeting with mannose and HA, beneficial effect of HA was independently shown in macrophages (RAW 264.7) as well as in AML (THP-1) cells [306]. The highly relevant CXCR4, involved in homing to bone marrow microenvironment and survival pathways, was not

targeted in leukemic models, but pursued in other systems. A cationic peptide (T22) targeting CXCR4 provided enhanced intracellular delivery to self-assembling NPs in CXCR4+ cells including HeLa and metastatic colorectal cancer model cells (SW1417) [307]. In another study, CXCR4 Ab-mediated targeting of liposomes carrying lipocalin-2 siRNA were delivered to CXCR4+ breast cancer cells; CXCR4 Ab was utilized as an additive therapy to lipocalin-2 siRNA, not for demonstrating CXCR4 mediated endocytosis [308]. As CXCR4 and CD44 can serve as therapeutic targets for inhibitors [309] as well as siRNA [197, 198] targeting siRNA-bearing NPs specifically to these proteins should improve both potency and specificity of the therapy.

1.9 SIRNA DELIVERY IN LEUKEMIA AND RELATED MODELS

Relatively few studies have explored siRNA therapy in animal models of leukemia. The studies included subcutaneous and systemic xenograft models and related disorder models that involved siRNA delivery to systemic blood cells (**Table 1.5**). Experimental studies with intratumoral delivery may act as a bridge to systemic studies by providing basic information on cellular uptake, doses for effective silencing and siRNA clearance [191]. As leukemic cells mostly exist in blood and bone marrow, it is not surprising that IV injection of NPs (**Table 1.5**) has effectively delivered siRNA to leukemic or circulating cells where significant delivery was achieved with and without specific targeting. An increased delivery to subcutaneous AML (MV4-11) xenografts was achieved after IV injection of transferrin-targeted lipid NPs [196], thereby demonstrating improved efficacy with specific targeting. Peptide-mediated delivery (anti-JL1) demonstrated delivery of fluorescence-labeled siRNA to 7.3% of the CEM leukemic cells

in the bone marrow (which comprised of 3.3% of the total bone marrow cells) after direct injection into the mouse bone marrow with minimal delivery to other bone marrow cells after 2 h post-injection [188]. Dosage regimes varied widely among the *in vivo* studies (**Figure 1.6**), ranging from a single treatment (end-point 24 h later) to 5-weeks of siRNA treatment every 48 h, while the total siRNA dose ranged from ~0.5 to ~30 mg/kg (first 10 days). The CpG-conjugated system utilized a large quantity of siRNA; 400 μg over 4 days for intratumoral injection [191] and 600 μg over 6 days for systemic delivery [192], presumably due to rapid extracellular degradation by nucleases. *In vivo* Jet-PEI delivery also utilized a large quantity of siRNA (~900 μg over 5-weeks) [310]. Such high siRNA amounts may sometimes be needed for silencing high levels of reporter (luciferase) activity. In the lowest reported dose (0.1 mg/kg), it was unclear if the carrier used in the *in vitro* studies was also used in the *in vivo* studies, and efficacy was not compared to scrambled siRNA, making it difficult to assess the results [311, 312].

In the first non-viral clinical siRNA study, BCR-ABL siRNA liposomes were used to treat a BCR-ABL positive CML patient by IV (10-30 μg/kg) and intratumorally (300 μg) at CML nodules; some evidence of silencing was noted after the first IV treatment but not afterwards [313]. The dosage used for the first human trial was relatively low and it was based on the assumption of (i) siRNAs similarity to AS-ODNs for biodistribution, (ii) reasonable half-life of modified siRNAs, (iii) recommended dosing of an AS-ODN (G3139) being 2-4 mg/kg [314] and (iv) siRNA bioactivity being 100-1000 fold higher than AS-ODNs [313]. It is likely that a higher dosage of BCR-ABL siRNA may be required for a significant effect. To determine possible clinical siRNA dosages for future studies, we can compare AS-ODN preclinical and clinical studies

previously done. Clinical AS-ODN studies include LY2181308 AS-ODN study targeting survivin using multiple dosages of 750 mg (7.5-15.0 mg/kg in 50-100 kg patient) with clinical benefits in AML patients [315], AEG35156 AS-ODN targeting XIAP with effective dosages used being 110-350 mg/m^2 (2.8-9.5 mg/kg estimated based on the human adult km factor of 37 [316]) in AML [317], and AS-ODN Cenersen^{TM} AS-ODN study targeting p53 with multiple dosages of 2.4 mg/kg with clinical efficacy seen in in AML patients [318]. Pre-clinical mouse model dosages of AS-ODN models include single or multiple dosages of the AS-ODN LY2181308 ranging between 5-50 mg/kg [319], the AS-ODN AEG35156 ranging between 1-25 mg/kg and the AS-ODN G3139 dosages ranging between 5-7 mg/kg [320, 321]. The pre-clinical models (displayed in **Figure 1.6** and **Table 1.5**) are comparable to the low end of the pre-clinical AS-ODN studies described. However, carrier toxicities may limit the siRNA dosage that can be applied. Due to the higher specific activities of siRNAs as compared to AS-ODNs, a more consistent and effective therapeutic response should be achievable at lower doses.

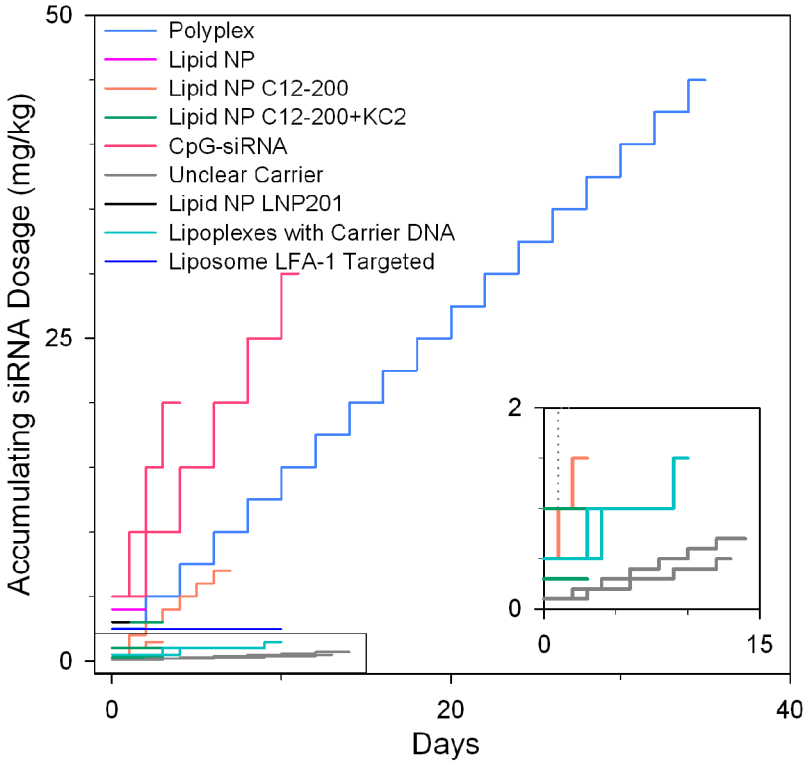

Figure 1.6 Dosages Used for Non-Viral siRNA Therapies in Preclinical Models.
The data were obtained from *in vivo* studies reported in **Table 1.5**. Accumulating dosages of the delivered siRNA (mg siRNA/kg b.w.) over time until the end of the associated study is displayed in a step-wise graph, where each injection can be visualized (vertical line). Dosages were estimated by assuming 20-g mouse weight when the study reported only siRNA amount (μg) for injection for different delivery systems. Day 0 was taken as the first treatment of siRNA. Note that the range of administered dose varied between ~0.5 and ~30 mg/kg in the first 10 days of administration. The insert is an expansion of the lower left corner of the graph and each line corresponds to a different study with the type of delivery system indicated in the legend. Polyplex [310], Lipid NP [190], Lipid NP C12-200 [322, 323], Lipid NP C12-200 +KC2 [324], CpG-siRNA [191, 192], Unclear Carrier [311, 312], Lipid NP201 [325], Lipoplexes with Carrier DNA [326, 327].

Table 1.5 Studies Involving siRNA Administration in Animal Models of Leukemia and Related Disorders.

Type of NP	Mouse Model / Treatment Goal	Injection Route	siRNA Dosage /Frequency	Silencing [target]	Therapeutic Effect	Ref.
Lipid NP	Healthy athymic nude mice / Leukemia	IV	80 µg* (4 mg/kg) /Once x 2 days	mRNA: 45% spleen [KIF11], 37% bone [KIF11], 89% liver [AHSA1]	Y	[190]
Polyplex *In vivo-JetPEI™*	IV (MLL-AF4 SEM-luciferase) Leukemia in NOD/SCID mice^ / Leukemia	IV	50 µg (2.5 mg/kg*) /48h x 5 weeks	Protein: 62.2% (week 4) 47.0% (week 5) [luciferase]	NS	[310]
Lipid NP *C12-200*	Healthy nude mice (COX-7)^ / Inflammatory monocytes	IV	10 /20 µg* (0.5/1 mg/kg) /24h x 3-7 days Other frequencies also used	mRNA: 45.5%; Protein: 36.4% (Splenic Ly-6C^{high} Monocytes) [CCR2] mRNA: 92.6%, 73.1%, 93.8% Protein: 75.8%, 66.7%, 89.5% (Splenic Ly-6Cl_{ow} monocytes, CD11c+ dendritic cells, F4/80+ macrophages resp.) [CD45]	Y	[322, 323]
Lipid NP *KC2 and C12-200*	Rodent myeloid cells and Non-human primates (male cynomolgus monkeys)/Myeloid Cells	IV	0.3/1/3 mg/kg /once x 4 days	Significant silencing in monocyte/ macrophage lineage in liver/ blood/spleen/bone marrow/ peritoneal cavity [various targets]^	Y	[324]
Lipid NP *LNP201*	Healthy Crl:CD-1/ICR mice/*in vivo*	IV	3 mg/kg /once x 1 day	NSC [Ssb]	NS	[325]
Lipoplexes (*with carrier DNA found to enhance siRNA delivery)*	Collagen-induced arthritis in DBA/1 mice / Myeloid Cells in Chronic Inflammatory Disorders	IV	10 µg (0.5 mg/kg) /72h x 4 days 10 µg (0.5 mg/kg) /96 +120hrs x 10 days	Protein: 90% (93.8% activated form) [TAK1] Protein: 58.75% [cPLA_2α]	Y Y	[326, 327]
Modified siRNA *CpG-siRNA*	SC (MV4-11) in NSG mice^ IV (Cbfb-MYH11/Mpl+ AML) Leukemia in C57BL/6 mice and naïve mice/Leukemia	IT IV	100 µg (5 mg/kg) /24h x 4 days 5 mg/kg /48h x 11 days	mRNA:52% [STAT3] Protein: 61% [STAT3], 65% [BCL-X_L] mRNA: 61.9% [STAT3] Protein: 80.0% [activated STAT3]	Y Y	[191] [192]
Unclear Carrier	SC (THP-1) tumors in athymic BALB/c nude mice/Leukemia	IT/IP	2 µg* (0.1 mg/kg) /48 or 72h x 13-14 days	NSC	NSC	[311] [312]
Lipid NP *Transferrin Targeted*	SC (MV4-11) in NOD-SCID mice/ Leukemia	IV	(2.5 mg/kg) /72h (unknown number of days in treatment)	mRNA: 82% [RRM2]	NS	[196]
Liposome Ab antiLFA-1 targeted	HIV-seronegative PBMCs in NOD/SCID/IL2rγ^{null} and BLT mice / HIV	IV	50 µg (*2.5 mg/kg)/ Up to 10 days with one treatment	Protein: ~50-70% [CD4] in liver, spleen and blood mRNA: >60% Day 3 and ~50% Day 10 in PB CD14+ monocytes	Y	[328]
Peptide *CPP PepFect6*	C57Bl/6JOlaHsd mice / (Also NMR1 mice luciferase silencing in the liver model , not discussed)	IV	0.25-1 mg/kg followed by 1 mg/kg dosage 24h later. Silencing measured at 24h and 72h	HPRT1 silencing in main organs		[194]
Peptide *Minibody (anti-JL1 targeted)*	BM (CCRF-CEM) in NOD-SCID mice 24h prior to complex injection / Leukemia	BM	1 nmole/mouse / 2h	No specific siRNA utilised	NS	[188]
Albumin coated CPP complex	Mice with ATLL tumors / Leukemia	IV	1 mg/kg	No specific siRNA utilised		[187]

BM: Bone marrow injection, IP: Intraperitoneal, IT: Intratumoral, IV: Intravenous, SC: Subcutaneous, Y: Yes, NS: Not Shown NSC: No appropriate control utilized (scrambled siRNA) for assay. *Estimated based on 20 g mouse. ^Other mice models also used for some assays. Other results are also demonstrated in many of the studies, results shown here are those that relate to leukemic type cells also reported. Days of treatment are counted from first injection to end-point date (last day of analysis for siRNA suppression). Silencing percent are calculated relative to scrambled siRNA to ensure comparability between studies

1.9.1 Biodistribution and Pharmacokinetics

Biodistribution of various NPs was relatively similar, where the highest delivery was almost always seen at spleen and liver after IV administration [192, 194, 196, 322, 324, 326, 327], and significant silencing was observed in relevant cells and locations (circulation and bone marrow). The exception was albumin-coated CPP complexes which were shown to locate to the ATLL tumor periphery (Cy5.5 labeled siRNA) and noted to minimally locate to the liver and spleen after IV injection, where the authors suggest that albumin coating protected the complex from opsonisation [187]. As another example, IV delivery of siRNA resulted in uptake in c-Kit+/GFP+ leukemic cells and myeloid immune cells within 3 h [192]. The highest siRNA delivery was in leukemic and myeloid immune cells in spleen and liver (30-70%), but significant delivery was also seen in bone marrow and lymph nodes. In naïve mice, IV CpG-siRNA provided minimal delivery to myeloid progenitor cells and no delivery to hematopoietic cells, limiting possible side-effects.

The systemic half-life of lipid NPs (C12-200) in nude mice was only 8.1 min [322]. The liver and spleen retention (in red pulp) was relatively constant starting immediately after injection whereas bone marrow accumulation was detected after 30-60 min [322]. After IV administration of lipid NPs, the CD11b+F4/80+ cells (monocytes and macrophages) had high uptake in circulation and spleen, and significant delivery was seen in inflamed ankle joints (arthritis model) and lymph nodes, and minimal delivery to CD3+ T-lymphocytes and B220+ B-lymphocytes. High uptake was seen in monocytes, dendritic cells and macrophages, and especially splenic Ly-6Chigh monocytes [326]. In a pharmacokinetic study of transferrin-NPs, the plasma half-life was 10.2 h, whereas free

siRNA had a plasma half-life of only 2.9 h [196], clearly reiterating the requirement of a carrier. A lipoplex system designed for delivery to myeloid cells involved in chronic inflammatory disorders displayed a high delivery (5-25%) to CD11b+ and CD11c+ cells in circulation/spleen/liver on day 1 and 2 after IV injection (0.5 mg/kg), and low but significant delivery to draining lymph nodes and joints with significant decrease of Cy3-siRNA detection in all areas after 2 days [326]. It was not known whether the decrease reflected actual degradation of siRNA or loss of label. Additionally, low uptake was noted in CD146+ endothelial cells located in the spleen (3%) and liver (10%). Another lipid NP formulation gave higher levels of siRNA in liver and kidney and lower levels in the duodenum [325]. A CPP peptide (PepFect6) was monitored for silencing in main organs (kidney, brain, lung, spleen, liver and heart) with the strongest silencing seen in the liver, kidney and lung [194]. Biochemical markers of kidney and liver functions were unchanged with no indication of acute toxicity, suggesting a lack of toxic effect by the CPP treatment. Liposomes with LFA-1 targeting (a ligand relevant for leukemia) demonstrated delivery to human T cells, B cells and monocytes but not to murine derived CD45+ cells or brain cells with effective silencing of CCR5 (co-receptor for macrophage-tropic strains of HIV) in CD14+ monocytes (2.5 mg/kg) [328].

1.9.2 Silencing Efficiency

Significant silencing ranging from 37 to 93% for mRNA and 36 to 80% for protein was reported where leukemic cells typically reside (circulation, bone marrow and spleen). However, silencing efficiency did not seem to relate to any specific variable, such as siRNA dosage or administration schedule, owing to vast number of differences among the studies. Lipid NPs designed for delivery to leukemic cells demonstrated

successful KIF11 silencing in healthy blood cells in the spleen (45%) and bone marrow (37%), and separately AHSA1 silencing in liver (89%) [190]. Lipid NPs (C12-200 or KC2) demonstrated silencing in monocyte/macrophage lineage cells in the liver, blood, spleen, bone marrow and peritoneal cavity [322-324]. Effective silencing with similar NPs was also demonstrated for the first time in myeloid cells of non-human primates in blood, bone marrow, peritoneal cavity, liver and spleen [324]. Silencing was maintained with repeated siRNA treatments of Jet-PEI^{TM} polyplexes (every 48 h for 5 weeks); *in vivo* suppression of luciferase in leukemic cells was evident at 2 weeks after siRNA treatment and showed significant silencing up to 5 weeks [310]. A single injection of a lipid NP formulation (KC2) with CD45 siRNA (2 mg/kg) provided long-term silencing in GFP- peritoneal lavage cells (macrophages) for up to 3 weeks [324]. In another demonstration of long-term silencing, LFA-1 targeted liposomes achieved silencing of CCR5 that lasted for at least 10 days after a single IV injections of siRNA (2.5 mg/kg) [328]. Several studies confirmed the RNAi activity by RACE for the cleavage of target mRNAs [191, 322, 324].

To probe silencing in circulating monocytes and leukocytes that may relocate after uptake of NPs, mice were injected IV with lipid NPs (KC2) followed by isolation of monocytes/macrophages for *in vitro* culture; maximum silencing was seen at 15 min for blood cells, 60 min for splenic cells and 120 min for peritoneal macrophages and no silencing for bone marrow cells [324]. With lipid NPs (C12-200), silencing was seen in blood cells sampled after 5 min of NP injection followed by 3 days of *in vitro* incubation. Silencing in the peritoneal macrophages was confirmed to be a result of NPs localizing to peritoneal cavity. IV delivery in non-human primates of C12-200 (1 mg/kg) or KC2 (3

mg/kg) NPs followed by blood collection and *in vitro* culture of the cells demonstrated delivery to blood cells within 1 hour of injection as well as effective silencing.

1.10 THERAPEUTIC TARGETS EXPLORED FOR RNAI IN LEUKEMIA

Many studies adopted RNAi for elucidating suitable targets for leukemia therapy without necessarily focusing on clinically translatable siRNA therapeutics. Through these studies, we aim to accentuate many potential targets with therapeutic potential on myeloid leukemias. These targets have been summarized and categorized based on their perceived mechanisms of action in **Table 1.6**. Electroporation has dominated siRNA delivery in these studies (52% of listed studies), followed by commercial carriers (33%) while non-commercial carriers were employed to a lesser extent (15%). We review the myeloid leukemia studies, with emphasis on the AML studies due to chapter length constraints, with a focus on desired outcomes of siRNA therapy.

1.10.1 Effects on Leukemic Cell Survival

Silencing of chosen targets regardless of category has typically resulted in decreased survival in the form of decreased proliferation/viability or increased apoptosis, or increased differentiation, as described for individual studies in **Table 1.6**. Several studies utilize RNAi screens to determine potential targets. Screens are advantageous as they allow comparison among large numbers of targets and may make it possible to 'personalize' the therapy. A large-scale siRNA screen of tyrosine kinases for survival of AML cells highlighted many possible targets (EPHA4, JAK1, JAK3, KIT, LTK, LYN, PTK2 [FAK], PTK2B, PTK6, PTK9 and SRC) [218]. The same authors also highlighted tyrosine kinase targets in patient cells, demonstrating the feasibility of patient specific

leukemia targets. Decreased cell survival was found in 10 of 30 leukemia patients with kinase siRNAs in one study [219]. An shRNA screen of chromatin regulators highlighted protein bromodomain-containing 4 (Brd4) epigenetic pathway as a potential target [329] in AML. shRNA screens in conjunction with a complementary screen (e.g., proteomic or small-molecule screening) determined Syk [330] and GSK-3α [331] as potential targets again for AML. In CML, the BCR-ABL kinase has been the main target (**Table 1.6**) and several studies unequivocally demonstrated increased apoptosis as a result of specific BCR-ABL silencing [172, 236].

1.10.2 Sensitizing Leukemic Cells to Chemotherapy

Silencing targets to increase sensitivity of leukemic cells to drug therapy have proven beneficial and primary targets found to increase sensitivity to drugs (e.g., azacitidine, daunorubicin, bortezomib and arsenic trioxide) were anti-apoptotic proteins such as Mcl-1, Bcl-2, Bcl-210, Bcl-XL, C-FLIP$_L$, and survivin [209, 212, 223, 225, 226, 229, 332-335] in AML. Additionally, cell-cycle checkpoint proteins had the highest synergistic effects in a genome wide-shRNA/cytarabine and a kinase siRNA/cytarabine screen including CHEK1, HGS, and WEE1 proteins [336, 337]. Cell-cycle checkpoint proteins can prevent cells from committing to apoptosis and their silencing could open the door to increased induction of apoptosis (preferentially in leukemic cells over normal cells). Specifically, WEE1, acting as an intra-S-phase checkpoint, prevents cytarabine induced S-phase arrest and was suggested as a promising target for siRNA to sensitize several AML cell lines (TF-1, THP-1, HEL and MDS-L) [336]. Additionally, several proteins involved in other cellular mechanisms have been found to contribute to drug sensitivity. Suppression of NPM1, a molecular chaperone and a well known AML site of

mutation, caused inhibition of cell cycle progression and colony growth, increased differentiation and increased chemosensitivity to All-Trans-Retinoic Acid and cytarabine in mutant-NPM1 expressing AML cells [210]. Signalling proteins in the MEK/ERK pathway (MEK1 [338], Mnk1/2 [339] and 4E-BP1 [216]), and PI3K/Akt pathway (Akt [340] and OPN [341]) also increased drug sensitivity. In one study, cytarabine was found to activate Mnk and MEK/ERK signalling and thus Mnk siRNA and cytarabine co-treatment enhanced suppression of leukemic colony formation [339]. siRNA suppression of TESC, a pH regulation protein up-regulated during sorafenib treatment, was found to increase sorafenib sensitivity [342]. Increased FOXO1 suppression was found to correlate with increased efflux-pump P-glycoprotein (MDR1) expression and silencing of FOXO1 restored doxorubicin sensitivity [343]. Interestingly, FLT3 mutation also suppresses P-gp expression and thus FOXO1 is a potential target for FLT3-negative cells. Suppression of adhesion proteins including CXCR4 [198], whose silencing enhanced cytarabine sensitivity in free and BMSC-attached THP-1 cells and FAK [214], which increased daunorubicin sensitivity in free KG-1 cells but not as much in fibronectin-attached cells, also increased drug sensitivity. Other drug-sensitizing targets included S100A8 involved in autophagy [333] and transcription factor related proteins HO-1, GSK3β and NF-κB subunit p65 [213, 344].

1.10.3 Effects on Mobility and Homing

In addition to direct effects on cell proliferation and survival, [198, 222, 345-348], suppressing adhesion proteins can diminish homing of cells to protective bone marrow niche. Suppression of CXCR4 [198], CD44 [197], ITGB3 (and pathway members) [349], ITGA6 [345], EVI1 [345], and ITGB4 [345] decreased AML adhesion to BMSCs (or

extracellular matrix coatings such as fibronectin). The CD82 adhesion molecule, overexpressed in AML LSC population (CD34+CD38-), was silenced with shRNA/siRNA in CD34+CD38- or EOL-1(R) cells, leading to decreased adhesion to fibronectin (by up-regulation of MMP-9), increased migration, and decreased engraftment in NOD/SCID mice [347]. Additionally, IGFBP7, a tumor suppressor in solid tumors, was found to be involved in leukemic cell adhesion to endothelial cells, migration, as well as invasion [346]. siRNA silencing of NRP-1 (a VEGF receptor) decreased chemotaxis [348]. Silencing of MMPs and activators (e.g., MMP-2, MT1-MMP and TIMP-2) decreased mobility towards SDF-1 [233]. A FAK siRNA also decreased the migration ability in FAK+ AML cells [214]. Ultimately, decreased adhesion and/or mobility towards bone environment are expected to retain the malignant cells in circulation, allowing better response to therapy.

1.10.4 Eliminating LSCs

LSCs reside in bone marrow and their interactions with bone marrow stroma provide extrinsic factors favouring long-term survival and protection against drugs. As with systemic leukemic cells, reducing LSC survival and minimizing resistance to drugs is desirable to prevent the residual disease, in addition to enhancing LSC mobilization to peripheral circulation. Treating LSCs specifically is challenging, as they constitute a relatively minor fraction among the leukemic population. NPs delivered to LSCs combined with a cargo that targets LSC-specific proteins (whose suppression would not affect normal hematopoietic cells) would be ideal. The specific protein signatures of LSC have been recently highlighted. Expression of proteins involved in apoptosis, cell cycle, expression, proliferation, and signaling (as well as activation) is different in LSCs from

AML and CD34+ populations, for example PU.1 (SP1), P27, Mcl-1, HIF1α, cMET, P53, Yap, and phosphorylated-Stat 1/5/6 [350]. Other targets include CD32, CD25, WT1 (transcription factor) and HCK (kinase) which are highly expressed in quiescent and chemotherapy-resistant LSCs and suppression of which does not negatively effect normal hematopoietic cells [351]. The protein Mcl-1 was particularly up-regulated in FLT3-ITD AML LSCs, where suppression of Mcl-1 (shRNA) increased apoptosis and suppression of STAT5 (siRNA) down-regulated Mcl-1 expression [352]. Additionally, multi-drug resistance transporter proteins P-glycoprotein, MRP, and LRP were found to be overexpressed in AML LSC population [353]. In fact, increased P-glycoprotein expression has been shown to be a distinctive feature of LSC derived from AML patients [353, 354], as well as LSC associated with CML [355]. This drug transporter appears to protect the LSC particularly from chemotherapy and it might be highly relevant to eradicate the residual AML disease. The adhesion molecule CD82, mentioned above, is also over-expressed in AML LSCs, serving as a potential target. Additionally, an *in vivo* shRNA screening with a LSC model (MLL-AF9 oncogene expressing granulocyte-monocyte progenitor cells) determined the potential of Itgb3 as a therapeutic target, whose suppression decreased homing, induced differentiation, and suppressed LSC gene-expression signatures [349]. The adhesion protein, CD44 [356], was targeted with Abs to eliminate LSCs. siRNA-mediated silencing of CD44 with a therapeutic response was recently demonstrated in primitive KG-1a cells (CD34+/CD38-; a LSC model), more differentiated KG-1 cells and CD34+ patient cells [197]. Other LSC surface antigens targeted with Abs include CD33, CD44, CD47, CD123, and WT1 [297, 298] and these antigens could be readily targeted with siRNAs.

Table 1.6 siRNA Targets Shown to be Beneficial in AML and CML Models.

AML studies are shown as blue background and CML studies shown with green background in the table. The targets were segregated based on the role of the protein target.

Ref.	Target (Role)	Rationale & Related Outcomes	siRNA Carrier [effective *in vitro* conc.]	siRNA Silencing Outcomes
Fusion Gene				
[357]	AML/MTG8 (Transcription Factor)	AML/MTG8 fusion gene found in AML. (Also studied MLL/AF4 found in ALL)	Electroporation (unknown)	Reduced clonogenicity, induction of replicative senescence, (also decreased TERT expression and increased telomere shortening)
[186]	AML/MTG8 (Transcription Factor)	Design of CD33 targeted PEGylated liposome for siRNA targeting AML/MTG8 fusion gene	Liposome (30-125 nM) [siRNA for silencing demonstration was 600-2500 nM]	Decreased leukemic clonogenicity of CD33 positive AML cells
[227]	MLL/AF9	Fusion gene in infant AML. To determine new targets and understanding of pathway.	Dreamfect (50 nM) [every 72 h]	Decreased cell size under certain *in vitro* variables (no effect on proliferation, cell cycle distribution, apoptosis) in THP-1
[358]	BCR-ABL	Compare efficiency of cell killing by Imatinib to that of silencing of BCR-ABL with siRNA	Oligofectamine (unknown)	Reduction of mRNA and protein were found with apoptosis levels 2.5x higher than controls. Apoptosis rate of anti-BCR-ABL siRNA treated cells was at the same level as cells treated with Imatinib or ~5 times more than control cells.
[238]	BCR-ABL	Demonstrate therapeutic effect of BCR-ABL down-regulation by siRNA delivery	Electroporation (0.5 µg/ 100 µl - 357 nM, Est.)	Reduction of viable cells by 75%. No proliferation inhibition in primary CML cells.
[236]	BCR-ABL	Inhibit BCR-ABL expression and evaluate sensitization to imatinib	Electroporation (200-800 nM)	Decreased cell viability and sensitization of imatinib-resistant K562 cells to imatinib.
[172]	BCR-ABL	Study anti-leukemic properties of BCR-ABL by RNAi	Electroporation (1 µg per 5 x 10^5 cells)	60% reduction of *BCR-ABL* mRNA expression. Slight increase of apoptosis. 2-fold increase of DNA fragmentation. Caspase-7 and -9 activated. Cells unable to actively divide for at least 2 weeks after silencing.
[174]	BCR-ABL	To assess efficacy of Tat-LK15 peptide in delivering siRNA to target BCR-ABL	Tat-LK15 peptide: fusion of HIV-Tat-derived peptide to cationic peptide LK15 (20 to 30 µg siRNA in 1 mL – 1428 - 2142 nM, Est.)	Expression of p210 BCR-ABL was reduced for all concentrations. Cytotoxicity due to siRNA nanoparticles ranging from 0% (10 µg) to 30% (30 µg). No silencing detected after 48 h.
[189]	BCR-ABL	Demonstrate efficacy in down-regulating BCR-ABL	Lipid-modified polymer (50 – 100 nM)	Increase in apoptotic cells.
[241]	BCR-ABL	Compare effects of two pathways of BCR-ABL suppression (siRNA for inhibition of protein synthesis and Glivec for inhibition of already synthesized protein).	Lipofectamine (180 nM, 3 times every 2 d)	Reduction of tyrosine kinase activity (57%) and cell proliferation capacity (50%).

[201]	BCR-ABL	Encapsulate BCR-ABL siRNA with Transferrin-liposomes and assess efficacy	Transferrin receptor-targeted liposomes [200 – 2000 nM) for demonstration of silencing]	*BCR-ABL* mRNA down-regulation.
[232]	BCR-ABL and WT1	Anti-leukemic additive effect of co-silencing of BCR-ABL and WT1.	TransMessenger (0.8 µg siRNA in 24 well plates - 200 µl of final vol. according to manufacturer. 286 nM, Est.)	Additive effect in the inhibition of cell growth and in the increase of apoptosis in comparison with transfection of either siRNA alone. WT1 siRNA on its own also induced apoptosis and decreased proliferation.
[359]	BCR-ABL and GFI1B	Anti-leukemic additive effect of co-silencing of BCR-ABL and GFI1B	DOTAP, liposomal transfection (Unclear)	Additive effect in the inhibition of cell growth and in the increase of apoptosis in comparison with transfection of either siRNA alone.
		Cell Cycle		
[360]	SGOL1	SGOL1 is a centromeric protein overexpressed in leukemia's including AML.	Electroporation (unknown)	Decreased proliferation; mitotic arrest, intrinsic apoptosis.
[337]	WEE1, CHEK1, PKMYT, ATR (Drug Sensitizing)	siRNA kinase/cytarabine screen to determine chemosensitizing targets to use with cytarabine	Cationic lipid-based (unknown)	Increased cytarabine efficacy.
[183, 200]	Cyclin A_2	Deliver cyclin A_2 siRNA with SWNTs and evaluation of cyclin A_2 role upon doxorubicin treatment.	Ammonium functionalized single wall carbon nanotubes (25 nM)	Silencing cyclin A_2 without doxorubicin caused increased growth inhibition and apoptosis. Silencing with doxorubicin elucidated a pro-apoptotic role of cyclin A_2.
		Apoptosis-Related Mechanisms		
[234]	MCL-1	MCL as an siRNA target for AML	Lipofectamine 2000 (50 nM)	Decreased proliferation and cell survival in HL60 AML cells.
[226]	MCL-1 (BMSC Adhesion)	Survival effects of adhesion interactions with BMSCs. (Induced CD44 expression upregulated MCL-1)	Lipofectamine RNAiMax (50 nM)	Increased apoptosis.
[332]	MCL-1 (Drug Sensitizing)	MCL-1 is over expressed in FLT3-ITD cell lines	Electroporation (unknown)	Increased chemosensitivity in FLT3-ITD+ AML.
[333]	MCL-1 (Drug Sensitizing)	Involvement in arsenic trioxide effect in AML	Unknown	Increased arsenic trioxide-induced mitochondrial apoptosis (chemosensitivity).
[212]	BCL2 (Drug Sensitizing)	Determining CDDO mechanisms in AML	Electroporation (100-200 nM)	Decreased cell proliferation and increased apoptosis with co-treatment of CDDO (but not without) in CDDO resistant cells.
[225]	BCL2 (Drug Sensitizing)	Involvement in curcumin action in daunorubicin insensitive CD34+ AML	Lipofectamine 2000 (50 nM)	Increased chemosensitivity of daunorubicin in CD34+ AML.
[209]	BCL2L10 (Drug Sensitizing)	BCL210 over-expression in azacitidine resistant cells	Electroporation (50 nM)	Sensitized cells to azacitidine.
[334]	BCL-X_L, BCL2, MCL-1 (Drug Sensitizing)	BCL-X_L, BCL-2, MCL-1 as sensitizing targets for 5-Azacytidine	Buffer-transfection reagent (unknown)	BCL-X_L and MCL-1 reduced viability in SET-2. TF-1, HEL, THP-1, OCI-AML3 and ML-2. BCL-2 had less effect on cell viability. BCL-X_L and MCL-1 increased sensitivity to 5-Azacytidine.
[335]	C-$FLIP_L$ (Drug Sensitizing)	Higher expression of C-FLIP (drug resistance role) correlated with decreased patient survival	Electroporation (1.5 µg/1-2.5 x 10^6 cells)	Increased apoptosis, sensitization to rTRAIL induced apoptosis.

[223]	HSP27 (Stress Response)	Role of HSP27	Oligofectamine (25 nM)	Increased VP-16 mediated apoptosis but not CD95/Fas mediated apoptosis.
[229]	Survivin (Drug Sensitizing)	Survivin as a siRNA target for AML	Lipofectamine 2000 (80 nM)	Decreased proliferation and increased apoptosis in HL-60. Increased sensitivity to etoposide.
[361]	GCS or MDR1	Relation of GCS to regulation of P-gp expression and function activity in drug retention	Lipofectamine 2000™ (unknown)	Silencing of GCS can affect MDR1 expression and inhibit P-gp efflux. Silencing of GCS or MDR1 sensitized drug-resistant cells to chemotherapy and increased drug retention.
[242]	MCL1	Antileukemic effects of MCL1 silencing and synergistic effects with Imatinib in CML	Lipofectin (200 nM)	Decreased proliferation and synergistic effect with Imatinib
		Cell Homing and Mobility		
[198]	CXCR4 (SDF-1 Receptor), SDF-1 (Drug Sensitizing)	Demonstration and effect of CXCR4 and SDF-1 silencing in AML	CA-PEI 2 kDa (25-50 nM) [THP-1 cells]	Decreased proliferation, decreased BMSC adhesion.
[222]	SDF-1	Role of SDF-1 in survival and proliferation in AML.	HiPerFect (25 nM)	Decreased proliferation. (Study included CML K562 cells)
[197]	CD44	Demonstration and effect of CD44 silencing in AML cells	LA-PEI 2 kDa (50-100 nM)	Increased apoptosis, decreased adhesion to hyaluronic acid coating and BMSC.
[348]	NRP-1 (VEGF Receptor)	Involvement in AML	Lipofectamine 2000 (unknown)	Decreased VEGF mediated proliferation and chemotaxis
[362]	CDC25A (Cell Cycle)	Effects on cell adhesion and proliferation	Electroporation (8 pmol per 6 x 10^6 cells)	Decreased adhesion dependent increase in proliferation.
[213]	GSK3β (Transcription Factor Related), NF-κB subunit p65 (Transcription Factor Related, Drug Sensitizing)	Resistance due to adhesion molecules/integrin and morphogen Wnt soluble factors in AML	Electroporation (200 nM	Restored chemosensitivity (daunorubicin).
[346]	IGFBP7 (Tumor Suppressor)	To investigate the role of the known solid tumor suppressor (IGFBP7), in childhood AML	Lipofectamine 2000 (unknown)	Decreased adhesion, migration, invasion, proliferation. Role in BM microenvironment interaction was apparent.
[214]	FAK (Tyrosine Kinase, Drug Sensitizing)	FAK involvement in AML	Electroporation (200 nM)	Decreased migration, increased chemosensitivity (daunorubicin), did not improve fibronectin adhesion provided resistance.
[233]	MMP-2, MT1-MMP, TIMP-2	Role in AML extramedullary infiltration	Lipofectamine 2000 (400 nM Est.)	Decreased invasion.
[222]	SDF-1	* Same reference as above (AML cells)	HiPerFect (25 nM)	Decreased proliferation.
		Transcription Factor Related Mechanisms		
[235]	Gli1 (Transcription Factor)	Effects of aberrant expression and inhibition of Gli	Jet-PEI (100 nM)	Decreased proliferation and decreased survival.
[207]	HO-1 (Enzyme), Nrf2 (Transcription Factor), c-FLIP (Anti-Apoptosis)	Involvement in NF-κB and TNF-induced apoptosis in AML	Electroporation (30 nM)	Susceptible to TNF-induced cell death (HO-1, Nrf2), Susceptible to TNF but not with NF-κB inhibitor BAY 11-7082 (c-FLIP).

[208]	NF-κB (Transcription Factor), HO-1 (Enzyme)	Inhibition of highly expressed NF-κB did not cause apoptosis due to HO-1	Electroporation (30 nM)	Increased apoptosis after targeting both HO-1 and NF-κB in AML cells but not in CD34+ non-malignant cells.
[230]	hnRNP K (Docking Protein)	Role of hnRNP K in drug induced suppression and apoptosis induction	DharmaFECT-4 (100 nM)	Induced apoptosis.
[344]	HO-1 (Enzyme) (Drug Sensitizing)	Determination of HO-1 regulation in AML by Bach1 (transcription regulator) and Nrf2 (activator)	Electroporation (unknown)	Reduced cell survival with and without cytarabine.
[363]	NF-κB subunit p65, IKK subunits [IKK1, IKK2, NEMO]	Understanding the role of NF-κB activation in AML	Electroporation (unknown)	Increased apoptosis.
[364]	NF-κB subunit p65 (Transcription Factor)	NF-κB is continuously activated in P39 MDS/AML cells	Electroporation (unknown)	Increased apoptosis.
[191]	STAT3 (Transcription Factor)	Development of targeted STAT3 (role in cancers) siRNA delivery in TLR9+ hematopoietic cells.	TLR9 antagonist CpG-siRNA (500 nM)	*In vivo*, decreased tumor growth. The delivery system is immunostimulatory and can contribute to overall anti-cancer effects. *In vitro*, Immunostimulatory properties are enhanced by STAT3 silencing in DC cells.
[192]	STAT3 (Transcription Factor)	To determine the immunostimulatory ability of STAT3 silencing and TLR9 activating system.	TLR9 antagonist CpG-siRNA	*In vivo*: STAT3 siRNA and CpG (TLR9) cause immune response against AML cells
[224]	WT1 (Transcription Factor)	Involvement in miR-15a and miR-16-1 tumor suppressors	HiPerFect (50 nM)	Decreased proliferation.
[232]	WT1 (Transcription Factor)	WT1 is overexpressed in leukemia *Also listed above in CML - Fusion Gene Category.	TransMessenger (800 ng siRNA in 24-well plate (200 µl according to manufacturer. 286 nM, Est.))	Decreased proliferation, increased apoptosis in AML/CML (not in naïve CD34+ cells). Increased anti-survival effects when WT1 and BCR-ABL were targeted in K562 cells.
[231]	GFI1B (Transcription Factor)	Evaluation of GFI1B expression in some types of leukemias	TransMessenger (800 ng siRNA in 24-well plate (200 µl according to manufacturer. 286 nM, Est.))	*Both CML and AML: GFI1B* overexpressed in only certain leukemias. Silencing induces reduction in proliferation and increase in apoptosis unlike healthy cells.
[231]	GFI1B (Transcription Factor)	Same reference as above.	TransMessenger (800 ng siRNA in 24-well plate (200 µl according to manufacturer. 286 nM, Est.))	As above. Silencing induces reduction in proliferation and increase in apoptosis unlike healthy cells.
[169]	STAT5A	Effects of STAT5A siRNA knockdown on cell growth and apoptosis induction	HiPerFect (unknown)	~75% suppression of *STAT5A* mRNA. Resistant K562 cells became ~4 times more sensitive to Imatinib. An increase in caspase-3 activation was seen.
[240]	STAT3, STAT5A/B	Detect gene expression profile of JAK/STAT pathways members	HiPerFect (100 nM)	Induced apoptosis
Tyrosine Kinase Signalling				

[365]	Axl (FLT3 Related)	Determine role of Axl in FLT3 signalling in AML	Electroporation (unknown)	Inhibited cell growth, arrested cell-cycle, induced apoptosis and differentiation in FLT3-ITD+ AML.
[366]	CSFIR	Identification of tyrosine-phosphorylated proteins in AML M7 (AMKL)	Electroporation (unknown)	Decreased proliferation and increased apoptosis in AML M7 MKPL1 cells but not in CML K562 cells. (C-KIT siRNA did not decrease proliferation).
[218]	EPHA4, JAK1/3, KIT, LTK, LYN, PTK2 [FAK], PTK2B PTK6/9, SRC	siRNA screen of tyrosine kinases in AML cells	Electroporation (1000 nM)	Decreased viability.
[219]	EPHA4, JAK1/3, FLT1, FYN, PDGFRα/β, PTK2B.	siRNA screen of tyrosine kinases in leukemic patient cells	Electroporation (1000 nM)	Decreased viability. Also targets: ALL: K-RAS, CSF1R, N-RAS, ROR1. CMLL: JAK2, EPHA5. CNL: JAK2, EPHA4, LYN, LMTK3
[215]	FAK (Adhesion Protein)	Over expression in AML stem cells; Potential Target	Electroporation (200 nM)	Decreased survival.
[367]	JAK2	To determine kinases that cause STAT5 phosphorylation in AML	Electroporation (unknown)	Decreased proliferation and viability in AML (HEL) but not in CML (K562). Decreased phosphorylation of STAT1/3/5 and Erk1/2. JAK1, JAK3, TYK2 had no effect.
[368]	JAK3	To identify activated tyrosine kinases in AMKL cells without FLT3 and KIT mutations	Electroporation (unknown)	Decreased proliferation, inhibition of STAT5 tyrosine phosphorylation, increased apoptosis in AMKL. JAK2 and TYK2 had no effect.
[217]	FES, FER (FLT3 Mutation Related)	Investigation of FES and FER in AML in relation to FLT3 mutation	Electroporation (0.4-0.8 nmol in 0.2-0.5 ml; 800-4000 nM estimate)	Decreased proliferation (FER) and decreased survival (FES) in FLT3-ITD+ AML but not in non-mutated cells.
[220]	Lyn	Lyn is highly activated. PP2 (SRK inhibitor) caused decreased proliferation and increased apoptosis	Electroporation (3 µg/100µl for $2x10^6$cells, 2143 nM est.)	Decreased leukemic colony formation, linked to mTOR pathway.
[369]	Lyn (FLT3 Mutation Related)	Lyn and FLT-ITD interactions in AML	Electroporation (3 µg)	Decreased proliferation in FLT3-IDT+ 32D cells. Decreased STAT5 phosphorylation.
[370]	Ubc9	To identify target proteins of C/EBPαp30	Electroporation (500 ng)	Prevents differentiation block caused by C/EBPαp30 (co-transfected) when CD34+/ U937 cells go through granulocytic differentiation.
[199]	FLT1, VEGF	Development of Chitosan NP for siRNA silencing in U937 cells.	Chitosan NP	Decreased proliferation (Both VEGF and FLT1)
[312]	FLT3	FLT3 over-expressed/mutated	sc-29528, Santa Cruz (unknown) (^)	Arrested in G0/G1 phase, decreased proliferation *in vivo* and *in vitro*, increased apoptosis.
[371]	FLT3	FLT3 over-expressed/mutated in AML. Developing multiple methods for inhibiting FLT3	Electroporation (1 µg/1 x 10^7 cells)	Decreased proliferation, increased apoptosis, and increased sensitivity to MLN518 (a FLT3 inhibitor).
[190]	FLT3	Effective siRNA carriers for leukemic cells	Lipid Nanoparticles (10-30 nM) [Silencing demonstration was effective from 10-500 nM]	Decreases proliferation

[228]	KIT	siRNA and shRNA studies target c-kit (over-expressed / mutation)	Lipofectamine 2000 (50-200 nM)	Effects were not studied for siRNA transfections. (shRNA studies)
[237]	Lyn	Study effects of Lyn ablation in CML blast crisis cells	Nucleofection (0.5 µg siRNA in 100 µl, 357 nM, Est.)	Lymphoid CML blasts underwent induction of apoptosis.
PI3K/Akt and MEK/ERK Signalling Pathway				
[372]	ILK (PI3K/Akt)	Investigation of ILK and FLT3 as targets (inhibitors used for FLT3 suppression)	Accell modified siRNA (unknown)	Decrease leukemic colony formation.
[373]	ILK (PI3K/Akt)	ILK role. Possible benefit in targeting both ILK and FLT-3	Electroporation (50 µg per 5 x 10^6 cells)	Decreased colony formation, increased cell death.
[340]	Akt (PI3K/Akt, Drug Sensitizing)	Cell surface sialylation patterns and multidrug resistance	Unknown	Reversed multidrug resistance/increased sensitivity to adriamycin, paclitaxel, vincristine
[216]	4E-BP1 (MEK/ERK), MCL-1 (Anti-Apoptotic) (Drug Sensitizing)	AZD6244 causes apoptosis and suppresses 4E-BP1 and MCL-1 in HL-60 cells but not in EOL-1 and MOLM13 cells	Electroporation (300 nM)	Decreased MCL-1 expression and increased apoptosis with AZD6244 (4E-BP1). Increased apoptosis with/out AZD6244 (MCL-1).
[338]	MEK1 (MEK/ERK, Drug Sensitizing)	Study of 5-AzadC (DNA methyltransferase inhibitor) and AZD6244 (MEK inhibitor) in AML	Electroporation (unknown)	Decreased viability with 5-Aza-2'-deoxycytidine co-treatment but not without.
[339]	Mnk1/2 (MEK/ERK, Drug Sensitizing)	Involvement in cytarabine mechanism of action	Unknown	Decreased leukemic colony formation with cytarabine treatment but not without.
[341]	OPN (PI3K/Akt/ Ser585)	Investigated Ser585-survival pathway. OPN is a secreted protein.	Unknown (50-150 nM)	Increased cell death and decreased survival in AML blasts and leukemic stem and progenitor cells.
[221]	COT1 (Drug Sensitizing)	COT1 increases effect of silibinin/1,25-dihydroxyvitamin D3 combinations	Electroporation (5000 nM)	Increase G1 arrest and differentiation caused by Silibinin/ 1, 25-dihydroxyvitamin D3 combinations.
Other Mechanisms of Action				
[374]	NOTCH1, NOTCH2 (NOTCH Pathway)	Effects of NOTCH targeting in leukemia	Electroporation (no therapeutic effect in AML) [Silencing demonstration was performed at 40 nM]	AML: Did not effect proliferation (THP1 and TMD7). Signalling was affected. T-ALL: decreased proliferation and increased apoptosis (DND-41 and KOPT-K1)
[342]	TESC (Cell pH Regulation, Tyrosine Kinase Inhibitor Resistance)	TESC is upregulated during sorafenib treatment and may be involved in resistance to tyrosine kinase inhibitors	Electroporation (unknown)	Decreased proliferation, decreased intracellular pH, increased apoptosis in MOLM-13 and MV4-11
[375]	S100A8 (Autophagy, Drug sensitizing)	S100A8 role in autophagy, cell survival and chemoresistance in AML	Lipofectamine RNAiMAX (unknown)	Increased chemosensitivity, increased arsenic trioxide induced apoptosis, decreased autophagy.
[376]	CIP2A (Oncoprotein)	Determine role of CIP2A in AML as it is involved in cancers.	Electroporation (unknown)	Decreased proliferation, decreased clonogenic activity, increased differentiation.
[343]	FOXO1 (Multidrug Resistance, Drug Sensitizing)	FOXO1 expression correlates with P-gp expression. FLT3 also suppresses FOXO1 and also results in decreased P-gp expression.	Electroporation (unknown)	Inhibited P-gp expression, restored doxorubicin sensitivity.

[210]	NPM1 (Molecular Chaperone) (Drug Sensitizing)	Common mutation	Electroporation (100 nM)	Chemosensitizes (ATRA and cytarabine), decreased cells in S-phase, induced differentiation, increased apoptosis (NPM1 mutant+ AML).
[211]	EZH2 (epigenetic regulator)	Effect of EZH2 on AML cells	Electroporation (100 nM)	Co-treatment with LBH589 (inhibitor) decreased colony formation (HL-60 and U937) and increased differentiation (U937).
[196]	RRM2 (R2 subunit of ribonucleotide reductase)	Design of carrier by microfluidic formation for controlled mixing parameters during self-assembly.	Lipid NP with Transferrin Ligand (100-1000 nM ^)	Decreased cell viability.
[377]	Rho, Rac, Cdc42 (Rho family GTPases) (CBL Mutation)	Understanding AML CBL mutations. CBL+ cells required FLT3, CBL, Akt, STAT5 and Rho, Rac and Cdc42.	Electroporation (unknown)	Decreased proliferation (CBL + AML).
[378]	c-CBL (CBL mutation)	Identification and study of c-CBL and CBL-b mutations	Electroporation (unknown)	Decreased cell proliferation.
[168]	Syk and Axl	Identify downstream effectors of Lyn involved in resistance to nilotinib	Nucleofection (200 nM)	Silencing Lyn's downstream effectors Syk and Axl restored capacity of nilotinib to inhibit cell proliferation.
[170]	PRAME	Investigate function of PRAME in CML progression by RNAi in K562 cells	Nucleofection (1500 nM)	70% knockdown of PRAME mRNA. Significant inhibition of cell proliferation and decrease of clonogenic growth. 60% of apoptotic cells in comparison with
[239]	PPP2R5C (protein phosphatase)	Effect of PPP2R5C down-regulation in imatinib-sensitive and –resistance CML cells	Nucleofection (3 µg/100µl, 2140 nM, Est.)	Inhibition of the proliferation of CML cells. Rendered imatinib-resistant cells more sensitive to TKIs.

CDDO: synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; Casitas B lineage lymphoma: CBL, Est.: estimated, ^*in vivo* study

1.11 CONCLUSIONS ON SIRNA THERAPY IN LEUKEMIA

For siRNA therapy to find a place in clinical management of leukemia, considerable progress in siRNA carrier development is required. Recent work has begun to determine the barriers to siRNA therapy in hard-to-transfect leukemic cells. A better understanding of the mechanisms that block efficient silencing, through investigation of uptake and intracellular trafficking of the siRNA carriers in leukemic cells (especially in patient samples) are needed, since most of intracellular mechanistic studies have employed attachment-dependent cells due to convenience of analysis. Benefit of better understanding of the siRNA therapy impediments is clear; knowing that the endocytosis rates are low in leukemic cells and may impede endocytosis of a carrier allows for design

of carriers utilizing an endocytosis inducing ligand. Furthermore, tailoring siRNA carriers to leukemic cells, likely primarily through the use of targeting ligands, should greatly improve their efficacy and is especially required for targeting LSC populations, which exist in lower numbers. Current non-viral carriers under development should be pursued further, the routine physiochemical studies and silencing demonstrated in the initial publications are not followed by more detailed studies in clinical samples, and no further studies are published with the developed carrier, indicating a lack of commitment to most carriers.

Functional targets need to be identified that are specific for LSCs and its progeny. The siRNA therapy that has shown the greatest progress in leukemia has been developed for CML; this can largely be attributed to the effective and broad-range occurring target BCR-ABL. The discovery of highly effective and broad-range targets, or effective co-targets, in other leukemia types will rapidly progress siRNA therapy for the respective leukemia; a task already underway as demonstrated by the vast number of electroporation siRNA studies performed in context of determining new targets and better understanding of potential targets for all molecular therapies. Identifying a "magic" target, however, might be difficult in myeloid leukemia due to clonal heterogeneity in the disease, where a heterogeneous population of sub-clones are capable of expanding under favourable conditions [379]. With better characterization of clonal heterogenetic at the genetic level, it might be possible (and necessary) to deliver cocktails if siRNA to target different sub-clones simultaneously at the onset of therapy, and adjust the composition of such a cocktail in case of relapse [380].

Despite these uncertainties, early experience with siRNA-based therapeutic approach has been promising and new, more-effective and less-toxic approaches are expected to emerge for the control of leukemia. The speed at which new therapeutic agents (i.e., siRNAs) are identified is exceptionally fast as compared to development process needed to identify and assess conventional drugs (i.e., small organic molecular entities). This bodes well for a cure of the leukemic disease in the near future.

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2. siRNA Silencing in Adherent Cells[x]

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2.1 INTRODUCTION TO SIRNA SILENCING IN ADHERENT CELLS

Post-transcriptional gene silencing mediated by RNA molecules is currently explored as a unique and promising therapeutic strategy. RNA interference is an evolutionary conserved gene silencing mechanism triggered by small interfering RNAs (siRNAs), which mediate sequence- specific mRNA degradation [1]. In the cytoplasm, siRNAs are incorporated into the RNA-induced silencing complex (RISC) protein complex that contains the Argonaute 2 endonuclease [2]. Only antisense, or guide, strand of the siRNA duplex is retained inside the RISC. Subsequently, the activated RISC uses the guide strand to bind to the complementary region on the target mRNA, followed by cleavage (also called 'slicing') of the complementary mRNA at a discrete position between bases 10 and 11 with respect to the 5' end of the guide strand [3]. The cleavage fragments are then further degraded by cellular RNases [4]. Delivering siRNA against intracellular targets in an effective way, however, has been challenging. The rapid degradation of siRNAs in the extracellular environment with RNase A type nucleases combined with the poor cellular uptake of anionic siRNA has made it a practically incompetent silencing agent on its own.

Advanced materials are needed for therapeutic delivery of siRNA molecules and cationic polymers are attractive for this purpose since they can be tailored to neutralize the anionic charge of nucleic acids and are not hampered by the safety concerns associated with viral carriers. The electrostatic interactions between the anionic phosphates in siRNA and cationic moieties in polymers can assemble the siRNA molecules into nanoparticles suitable for cellular uptake. High-molecular-weight polyethylenimines (PEIs) are one class of polymers that have been shown to be effective

siRNA delivery agents [5-7]. While the unprotonated amines of PEI create an opportunity for endosomal escape due to the ‘‘proton sponge effect’’ [8], the high density of positive charges facilitates strong binding to siRNA, which in turn creates a strong protection effect against enzymatic degradation. However, even though high molecular weight PEI has been used extensively in vivo [9], and even commercialized [10], the toxicity of high molecular weight PEIs has been a hurdle for clinical use [11-16]. Lower molecular weight PEIs present acceptable toxicity profiles but, unfortunately, the small polymers do not display efficacious siRNA delivery into cells. A promising approach to improve nucleic acid delivery into cells is to incorporate hydrophobic moieties onto the polymer amines, since hydrophobic substituents are expected to increase polymer interactions with lipophilic cell membranes and facilitate the uptake of the cargo. Such a beneficial effect of lipid substitution has been established in the context of plasmid DNA delivery for several cationic carriers, where enhanced gene expressions were typically obtained when plasmid DNA was delivered with lipid-substituted polymers [17]. However, whether lipid substitution on polymers are also beneficial for siRNA delivery remains to be investigated. A cholesterol-substituted 1.8 kDa PEI was recently shown to be suitable for siRNA delivery [18], but the role of the lipid substituent on siRNA delivery could not be assessed, owing to lack of comparative studies with native (i.e., unmodified) polymers. No other lipids apart from the cholesterol were investigated and it is not known if other lipids are functional for siRNA delivery.

In order to evaluate the efficiency of the carriers for siRNA therapy both their ability to deliver the siRNA to the cells as well as siRNA-mediated action must be assessed. For siRNA delivery, a fluorescent label attached to siRNA is typically utilised,

followed by assessment by flow cytometry and/or fluorescent microscopy. To determine siRNA silencing, a model target or house-keeping gene such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), can first be chosen. Selection of a therapeutic target can then be chosen, with typical choices for cancer therapy being anti-apoptotic proteins and other proteins that promote cancer cell survival. One such potential target is survivin, which is best known by its anti-apoptotic function but also has other pro-survival supporting roles including cytoprotection and cell-cycle regulation [19]. Survivin is found to be upregulated in many cancers and is associated with their overall enhancement of cancer cell survival (evasion of apoptosis) and linked to resistance to chemotherapy [19]. Another target option are proteins that can improve the effects of current drug treatments by either synergistic effects or preventing resistance to a given drug. By reversing drug resistance, a patient could continue treatment with the given drug that was previously effective. Potential targets involved in chemotherapy resistance are P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP), which are both found to be over-expressed (as a result of drug treatment or naturally prior to treatment) in cancer cells and contribute to multi-drug resistance (MDR) [20, 21]. P-gp and BCRP are cellular membrane transporters capable of effluxing the drugs from cells. P-gp induced drug resistance can occur as result of any drug treatment and is a broad-spectrum multidrug efflux pump [20]. However, not all cancer cells express P-gp. BCRP has been found to be specifically involved in resistance to drugs such as mitoxantrone (which are not as effectively effluxed by P-gp). P-gp is known (or suspected in some cases) to efflux doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, and homoharringtonine, while BCRP is known (or suspected) to

efflux doxorubicin, daunorubicin, mitoxantrone, topotecan, and SN-38. Many mutations can occur that alter substrate specificity of the transporters; for example a single amino-acid mutation in the BCRP gene resulted in the BCRP protein being able to efflux doxorubicin and the model efflux reporter, rhodamine [20, 22].

The present study systematically investigated siRNA delivery systems based on lipid substitution on cationic polymers, with the purpose of (i) identifying advanced materials for siRNA delivery and (ii) better understanding of substituent effects on siRNA complex properties, cellular delivery and targeted gene silencing. Here, we report characterization of a library of non-toxic low molecular weight 2 kDa PEI (PEI2) synthesized with hydrophobic modifications, including caprylic acid (CA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic acid (OA), and linoleic acid (LA). We tested the carriers' ability in three different attachment-dependent cell lines and with four different protein targets: GAPDH, P-gp, BCRP and survivin.

2.2 METHODS

2.2.1 Materials.

The 2 kDa PEI (PEI2; M_n, 1.8 kDa; M_W, 2 kDa), 25 kDa PEI (PEI25; M_n, 10 kDa; M_W, 25 kDa), anhydrous dimethyl sulfoxide (DMSO), caproyl chloride (C8; >99%), palmitoyl chloride (C16; 98%), octanoyl chloride (C18:1 9Z, 12Z; 99%), linoleyl chloride (C18:2 9Z,12Z; 99%), Hanks' balanced salt solution (HBSS with phenol red), trypsin/EDTA, heparin, EDTA, ethidium bromide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) were obtained from SIGMA (St. Louis, MO).

Clear HBSS (phenol red free) was prepared in house. Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX^{TM}-l, Dulbecco's modified Eagle's medium (DMEM; low glucose), ultrapure agarose, penicillin (10000 U/mL) and streptomycin (10 mg/mL) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from PAA Laboratories (Etobicoke, Ontario). The scrambled siRNAs were AllStars Negative siRNA Fluorescein (catalog number: 1027290) and AllStars Negative Control siRNA (catalog number: 1027281), both from Qiagen (Huntsville, AL, USA) as well as negative control and negative control Fluorescein from Gene Pharma Co. LTD (Shanghai, China). Silencer GAPDH siRNA was from Ambion; Streetsville Ontario. P-gp specific siRNA was from Qiagen; Huntsville, AL, USA. The human survivin siRNA (catalog number 29499) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Silencer siRNAs versus ATP-binding cassette sub-family G member 2 (ABCG2; Breast Cancer Resistance Protein gene) were purchased from Ambion (catalog numbers: s18056, s18057, and s18058). The KDalert GAPDH Assay Kit was from Ambion. Fluorescein isothiocyanate (FITC) labeled P-gp antibody was from BD Pharmingen; Franklyn Lakes, NJ, USA. The anti-human survivin-fluorescein monoclonal antibody (catalog number: IC6472F) and FlowTACS Apoptosis Detection Kit (catalog number: 4817_60-K) were provided by R&D Systems Inc. (Minneapolis, MN, USA). The Phycoerythrin-labeled monoclonal anti-human BCRP antibody (catalog number: FAB995P) was purchased from R&D Systems Inc. (Minneapolis, MN).

2.2.2 Cell Lines

The P-gp transfected human MDA-MB-435 cells were kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC), the M. D. Anderson human

metastatic breast cancer 231 (MDA-MB-231) cells were a generous gift from Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, AB). Wild-type and BCRP-transfected Madin–Darby Canine Kidney (MDCK) cells were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute). The preparation and characterization of the BCRP-expressing cell line was previously reported [26], where an IRES promoter was used to derive co-expression of BCRP and the reporter Green Fluorescent Protein (GFP). MDA-MB-435 cells were cultured in RPMI 1640 medium, the MDA-MB-231 cells with DMEM medium and MDCK cells in high glucose DMEM medium with L-glutamine substituted with GlutaMAX^{TM}-l on a molar equivalent basis, all with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) in 37°C and 5% CO_2. Cell culture was considered confluent when a monolayer of cells covered more than 80% of the flask surface. To propagate the cells, a monolayer was washed with Hank's balanced salt solution (HBSS), and subsequently incubated with 0.05% trypsin/ethylenediaminetetraacetate (EDTA) for 5-10 min and room temperature or at 37°C. The suspended cells were centrifuged at 600 rpm for 4 min, and were re-suspended in the medium after removal of the supernatant. The suspended cells were either sub-cultured at 10% of the original count or seeded in multiwell plates for testing.

2.2.3 Synthesis of Lipid-Substituted Polymers

The process of lipid-substituted polymers synthesis has been described elsewhere [23, 24]. Briefly, a 50% 2 kDa PEI solution (in water) was purified by freeze-drying, and substitution was performed by N-acylation of PEI with commercially available lipid chlorides. Acid chlorides were typically added to 100 mg of PEI in anhydrous dimethyl sulfoxide (DMSO). The lipid:PEI ratios were systemically varied between 0.012 to 0.2.

The mixture was allowed to react for 24 h at room temperature under argon, after which excess ethyl ether was added to precipitate and wash the polymers. The substituted polymers were dried under vacuum at ambient temperature overnight. Polymers were analyzed by ^{1}H NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shifts of lipids (δ ~0.8 ppm; -CH_3) and PEI (δ ~2.5-2.8 ppm; NH-CH_2-CH_2-NH-) were integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on polymers (**Table 2.1**). The polymers used in this study were designated as either PEI2-XXYY or PEI2-XXZ.Z, where XX refers to the lipid substituted, YY refers to lipid:ratios used during synthesis where 0.066 for XX=1, 0.1 for XX=10 and 0.2 for XX=20 and Z.Z to the level of substitution (e.g., PEI-CA6.9 refers to CA substitution at 6.9 lipids/PEI2). Alternative naming is also shown in **Table 2.1**.

2.2.4 Cytotoxicity Evaluation by MTT Assay

The cytotoxicity of the polymers was evaluated in human MDA-MB-435 MDR cells using an MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in 48-well flat-bottomed plates. Confluent cell cultures were trypsinized, seeded in 48 well plates with 0.2 mL medium in each well, and allowed to reach ~80% confluence (1–2 d). Polymer/siRNA complexes were prepared using the scrambled siRNA at the ratio of 8:1 and were added to the wells to give final polymer concentrations of 1.25, 2.5, 5, and 10 μL/mL in triplicate. Cells were incubated for 24 h in their normal maintenance conditions and then 40 μL of MTT solution (5 mg/mL in HBSS) was added to each well. After 2 h of incubation in 37°C, the medium was removed, and 500 μL of DMSO was added to each well to dissolve the crystals formed. The optical density of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments; Winooski,

VT, USA) with cell-less medium as a blank. The absorbance of polymer- treated cells was compared to untreated cells (as 100% viability) and the % cell viability was calculated for each concentration of polymers. Cytotoxicity evaluation by MTT were also performed for MDA-MB-231 cells and the BCRP-positive MDCK cells and are reported in the original papers [25, 26].

2.2.5 Cellular Uptake of siRNA

Confluent cell cultures were trypsinized, re-suspended as described before and seeded in 48 well plates (0.35 mL in each well) at ~50% confluence. After 24h, 200 μL fresh medium was added to each well, followed by the addition of polymer/siRNA complexes. The complexes were prepared in sterile tubes using both 5-carboxy-fluorescein (FAM)-labeled scrambled siRNA and non-labeled scrambled siRNA (as a negative control) with polymer:siRNA ratios of 2:1 and 8:1 (corresponding 36 nM siRNA and 1 and 4 μg/mL polymer in culture medium). The prepared complexes were added to wells in triplicates and were incubated in 37°C for 24 h. After the incubation period, cells were washed with HBSS (x3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells and the siRNA uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect cell-associated fluorescence. The percentage of cells showing FAM fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined. Calibration was performed by gating with the negative control (i.e., "No Treatment") group such that the autofluorescent cell population represented 1-2% of the total cell population.

2.2.6 siRNA Protein Suppression

2.2.6.1 GAPDH Knockdown in MDA-MB-435 MDR Cells

Confluent cell cultures were trypsinized and seeded in 96 well plates (100 mL in each well) at ~50% confluence in medium containing 10% FBS. The polymer/siRNA complexes were prepared at different ratios of polymer:siRNA in sterile tubes using the Silencer GAPDH siRNA and a manufacturer-supplied negative control siRNA with polymer:siRNA ratios of 2:1, 4:1, and 8:1 (corresponding to 71 nM siRNA and 2, 4, and 8 μg/mL polymer in cell culture medium), and were added to the wells in triplicates. The plates were incubated at 37°C for 72 h, after which they were transferred to microcentrifuge tubes and were centrifuged at 1000 rpm for 4 min. The pellets were washed with HBSS and the GAPDH enzyme expression was measured by the KDalert GAPDH Assay Kit. Briefly, the cells were lyzed with 200 μL of lysis buffer and were incubated for 20 min at 4°C. After the incubation time, 90 μL of the KDalert Master Mix reagent was added to 10°C of the lyzed samples and the fluorescence of the samples were measured using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA) with λ_{ex}=536 and λ_{em}=604 nm.

2.2.6.2 P-gp Knockdown in MDA-MB-435 MDR Cells

Confluent cell cultures were trypsinized and seeded in 24 well plates (200 μL in each well) at ~50% confluence in medium containing 10% FBS. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and P-gp specific siRNA with polymer:siRNA ratios of 2:1 and 8:1 (corresponding to 36 nM siRNA and 1 and 4 μg/mL polymer in cell culture medium), and were added to the wells in triplicates. The plates were incubated in 37°C for 48 h, after

which the medium was removed from the wells, and 100 μL fresh medium was added to each well. Fluorescein isothiocyanate (FITC) labeled P-gp antibody was added to each well (10 μL), and plates were incubated at room temperature for 45 min. The cells were then washed with HBSS (x3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells and the P-gp down-regulation was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect the fluorescence. The percentage of cells showing FITC fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined.

2.2.6.3 Survivin Knockdown in MDA-MB-231 Cells

Confluent cell cultures were trypsinized and seeded in 24-well plates (500 μL in each well) at ~50% confluence. After 24 h, the medium was removed and 200 μL of fresh medium was added to each well. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and survivin siRNA with polymer:siRNA weight ratio of 2:1 (corresponding 54 nM siRNA and 1 μg/mL polymer in cell culture medium), and were added to the wells in triplicate. Plates were then incubated in 37°C for 72 h, after which the medium was removed, and cells were trypsinized, fixed with 3.7% formaldehyde solution, and transferred to tubes. Cells were then washed with HBSS and permeabilized for intracellular staining with a 0.1% solution of Triton X100 in HBSS. Permeabilized cells were exposed to fluorescein-conjugated antisurvivin monoclonal antibody for 45 min, and then were washed with the same permeabilizing solution twice before being resuspended in HBSS for flow cytometry assay. The percentage of cells showing FAM-fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined by

fluorescence measurement in FL1 channel. Calibration was performed by gating with the negative control such that the autofluorescent cell population represented 1-2% of the total cell population. Effect on viability was measured by MTT assay, as described above except performed 3 days after siRNA treatment (data not shown).

2.2.6.4 BCRP Knockdown in BCRP-positive MDCK Cells

Confluent cell cultures were trypsinized and seeded in 24 well plates (500 μL in each well) at ~50% confluency (~$5x10^5$ cells). After 24 h, the medium was removed and 200 μL of fresh medium was added to each well. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and a cocktail of the three different BCRP-specific siRNAs with polymer:siRNA ratios of 2:1 and 8:1 (corresponding to a total of 36 nM siRNA with 12 nM of each BCRP-specific siRNA, with 1 and 4 μg/mL polymer in cell culture medium, respectively) and were added to the wells in triplicates. The plates were incubated in 37°C for 48 h, after which the medium was removed and cells were washed with HBSS and trypsinized, and transferred to separate tubes for each well. Cells were then centrifuged at 1200 g for 4 min to remove the supernatant and were then washed (x3) with PBS supplemented with 0.5% Bovine Serum Albumin (BSA). Cells were then re-suspended in 50 μL of the same PBS/BSA buffer, and 4 μL of the Phycoerythrin-labeled anti-human BCRP antibody was added to each tube. Tubes were incubated at 2–8°C for 45 min, were washed (x2) with PBS/BSSA buffer, and then were fixed with 3.7% formaldehyde solution. The BCRP down-regulation was quantified by the flow cytometer using the FL1 channel to detect the fluorescence of GFP and FL2 channel for the antibody label. The percentage of cells positive for the label and the mean fluorescence in the total cell population were

determined. Analysis was performed by calibrating gating to the negative control (i.e., ‘‘No Treatment’’ group) such that the autofluorescent cell population represented 1–2% of the total cell population.

2.3 RESULTS

2.3.1 Lipid Substituted Polymer Library

Three series of lipid substitutions (with lipid:PEI2 amine mole ratios of 0.066, 0.1 and 0.2) were performed on PEI2 with caprylic acid (CA), palmitic acid (PA), oleic acid (OA), and linoleic acid (LA) based on a method described elsewhere (**Table 2.1**) [23]. There was a general increase in lipid substitution as the lipid:PEI ratio was increased during the synthesis (determined by ^{1}H NMR spectroscopy) and the highest number of lipids substituted was achieved with CA at lipid:PEI amine ratio of 0.2 (6.9 CAs/PEI). All polymers remained water-soluble. Physiochemical assessment of the lipid-polymer and formed siRNA complexes such as size, zeta-potential and siRNA binding and release has been performed and described below [27]. Briefly, after complexation of polymers with siRNA, the particle sizes ranged from 300 to 600 nm. The siRNA complexes formed with the native PEI2 showed negative zeta-potential indicating weak assembly of the polymer with siRNA in solution. Whereas PEI25 complexes showed positive zeta-potential for all ratios studied, indicating stronger affinity of the higher MW polymer to siRNA. For all lipid-substituted polymers, a continuous increase in the zeta-potential was observed with increasing polymer:siRNA weight ratio, and all polymers showed positive zeta-potential at the ratio of 10:1 (except PEI-CA1, which is consistent with the lower

binding affinity of CA substituted polymers) [27]. Given the need to protect siRNA from serum nucleases, the siRNA integrity after complex incubation in serum was measured (data not shown). All lipid-substituted polymers showed complete protection against degradation except for native (substituted) PEI2 (with ~68% intact siRNA remaining) and naked siRNA was readily degraded (<5% intact siRNA remaining) (not shown) [27].

Table 2.1 Lipid-Substituted PEI 2kDa Library

Polymer	Alternative Name[A]	Substituted Lipid	Lipid:PEI Ratio[B]	Lipid / PEI[C]	Methylene / PEI[D]
PEI-CA1	PEI-CA1.1	Caprylic Acid	0.066	1.1	8.8
PEI-CA10	PEI-CA2.4		0.1	2.4	19.0
PEI-CA20	PEI-CA6.9		0.2	6.9	56.8
PEI-PA1	PEI-PA0.6	Palmitic Acid	0.066	0.6	9.5
PEI-PA10	PEI-PA0.8		0.1	0.8	12.6
PEI-PA20	PEI-PA1.1		0.2	1.1	18.0
PEI-OA1	PEI-OA1.0	Oleic Acid	0.066	1.0	18.1
PEI-OA10	PEI-OA1.7		0.1	1.7	30.0
PEI-OA20	PEI-OA2.5		0.2	2.5	44.1
PEI-LA1	PEI-LA1.0	Linoleic Acid	0.066	1.0	17.3
PEI-LA10	PEI-LA1.8		0.1	1.8	33.2
PEI-LA20	PEI-LA3.2		0.2	3.2	57.7

A) Alternative names are due to different naming style depending on publication that the data is associated with; **B**) Molar ratios used for synthesis; **C**) Extent of lipid substitution per PEI calculated from 1H NMR analysis; **D**) Extent of methylene substitution per PEI, calculated based on the extent of substitution (from 1H NMR) and number of methylene groups in each lipid.

2.3.2 Cytotoxicity in MDA-MB-435 Cells

Cytotoxicity has been a major concern for polymeric systems for siRNA delivery. The low-molecular-weight PEI2 is known to be relatively biocompatible, but lipid substitution may impact its cellular interactions and alter its toxicity. In vitro toxicity of the lipid-substituted polymers was accordingly assessed with the MTT assay after forming polymer complexes with a scrambled siRNA. Human melanoma MDA-MB-435 cells, stably transfected with P-glycoprotein (P-gp) and serving as a model for MDR, were used for this purpose. **Figure 2.1** summarizes the cell viability after 24 h exposure to polymer/siRNA complexes. While PEI2 complexes showed almost no toxic effect

(even at 10 μg/mL), the PEI25 complexes were significantly toxic at 5 μg/mL and higher concentrations. Lipid substitution on PEI2 increased the toxicity of the complexes, especially for CA- and OA-substituted PEI2. However, the observed toxicity of the complexes with lipid-substituted polymers was significantly lower than the PEI25 complexes. Cytotoxicity evaluation by MTT was also performed for MDA-MB-231 cells and BCRP-positive MDCK cells, as reported in the original papers [25, 26], with similar results.

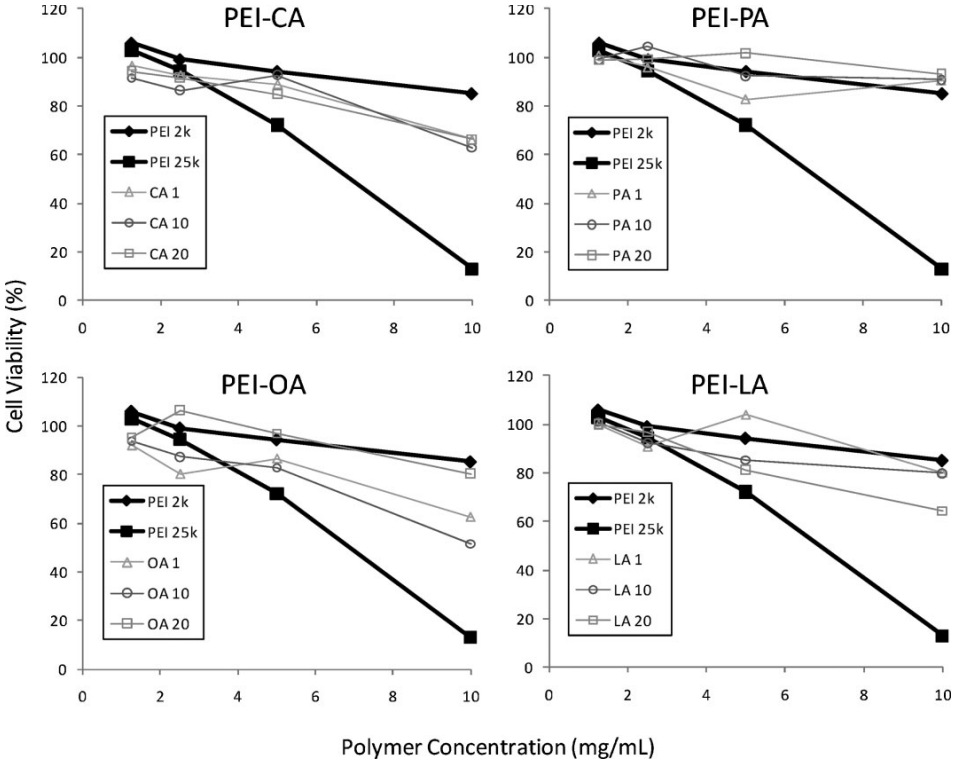

Figure 2.1 The Viability of the P-gp+ MDA-MB-435 Cells after Treatment with Polymer/siRNA Complexes.

Cells were exposed to complexes for 24hrs. While PEI25 was obviously toxic to the cells at concentrations above 2.5 μg/mL, the toxicity profiles of the lipid substituted polymers were similar to the relatively non-toxic PEI2, with OA- and CA-substituted polymers showing most toxicity among the lipid-substituted polymers.

2.3.3 Delivery to Adherent Cells

The uptake of polymer/siRNA complexes at 2 different polymer:siRNA ratios (2:1 and 8:1, **Figure 2.2, Figure 2.3** and **Figure 2.4**) was tested in three different adherent cell lines. For all cell lines tested (MDA-MB-435 cells, MDA-MB-231 and MDCK cells), PEI2 yielded minimal siRNA delivery into the cells as expected. PEI25 was among the most effective polymers at both ratios for MDA-MB-435 cells (**Figure 2.2A**), but its relative strength was not as dominant in MDA-MB-231 and MDCK cells. For MDA-MB-435 cells, the complex uptake at 8:1 ratio was higher than the 2:1 ratio and, for most lipid-substituted polymers, >90% of the cells were positive for the siRNA after 24 h of incubation (**Figure 2.2B**). In MDA-MB-231 cells, **Figure 2.3**, the ratio of 8:1 was again significantly more effective in siRNA delivery (compared to the 2:1 ratio) for LA- and OA-substituted polymers (based on the mean fluorescence of the cells), such a significant difference was not evident for other polymers (PA and CA-substitutions). In the MDA-MB-231 cell line, LA- substituted polymers provided the highest cellular uptake, while other polymers gave much lower siRNA delivery, in contrast to the delivery result in MDA-MB-435 cells where delivery efficiency varied more on a per lipid-polymer basis without a certain lipid substitution being noticeably better. Similarly to the mean fluorescence results in MDA-MB-231 cells, a higher percentages of siRNA uptake occurred with most of the lipid-substituted polymers where a maximum of ~96% of cells with siRNA delivery was achieved with LA1 (PEI-LA1.0), **Figure 2.3B**. Lastly, in MDCK cells, Canine kidney cells, yet again, the polymer:siRNA ratio of 8:1 was generally more effective in siRNA delivery to the cells as compared to the 2:1 ratio, indicating that at least for delivery, a high ratio is more efficient independent of the cell

line (**Figure 2.4**). Among the lipid-substituted polymers, LA- and CA-substituted polymers showed the highest siRNA delivery, and OA-substituted polymers had the lowest efficacy. For the effective polymers, the highest level of substitution (3.2 LA/PEI2 and 6.9 CA/PEI2) showed the highest delivery efficacy. Once again, polymer:siRNA ratio of 8:1 showed higher percentages of cells with siRNA, and lipid-substituted polymers gave a maximum of ~80% siRNA-positive cells, **Figure 2.7B**.

When the siRNA delivery was correlated to the extent of lipid substitution, a different pattern was observed for individual polymers at the two ratios employed and the three different cell lines. For MDA-MB-435 cells, MDA-MB-231 and MDCK cells at the 2:1 ratio, all polymers showed a higher efficacy in siRNA delivery with higher lipid substitution (all r^2 values >0.88 except for one which was r^2=0.60 (MDCK; PEI2-OA) **Figure 2.5**. However, at the 8:1 ratio these trends were not as clear. For MDA-MB-435 and MDA-MB-231 cells at the 8:1 ratio, this trend was observed only for CA- and OA-substituted PEI2 (r^2> 0.68), **Figure 2.5A-B**. Lastly, for MDCK cells at the 8:1 ratio, all polymers (CA, LA and PA) except for PEI2-OA showed a positive correlation, although not as strongly as the 2:1 ratio. Therefore, while the lipid substituent clearly helped the cellular uptake of siRNA complexes, a direct relationship between the extent of lipid substitution and the siRNA delivery was dependent on the polymer:siRNA ratio used to form the complexes. Once the siRNA delivery reaches saturation levels, such as uptake at the 8:1 ratio, the effect by the lipid substitutions was no longer clearly observed.

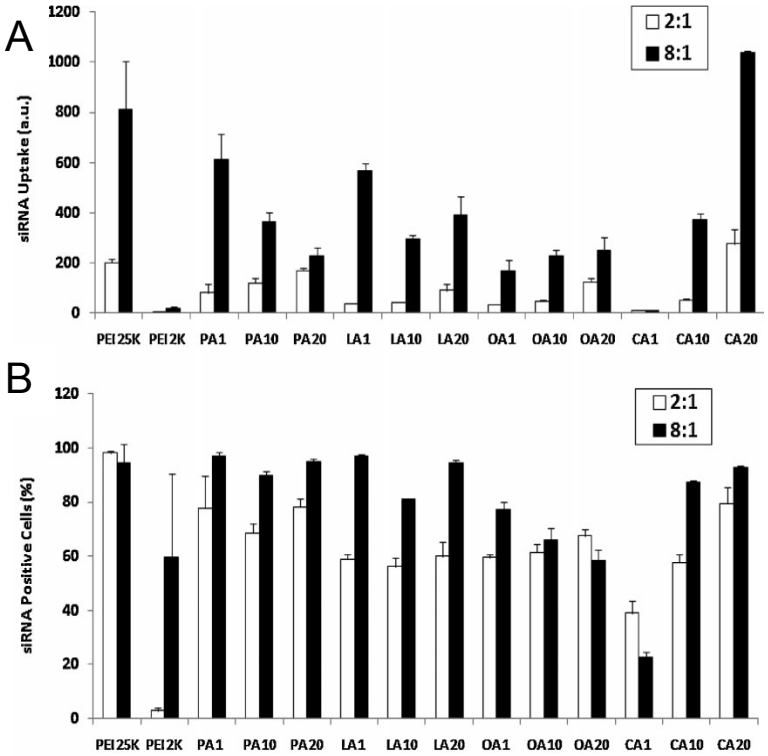

Figure 2.2 Cellular Uptake of Polymer/siRNA Complexes in MDA-MB-435 Cells.

(**A**) The mean fluorescence of the MDA-MB-435 MDR cells after 24 h exposure to complexes formed with FAM-labeled siRNA at weight/ weight polymer: siRNA ratios of 2:1 and 8:1. (**B**) The percentage of cells positive for FAM-siRNA after 24h exposure to siRNA complexes.

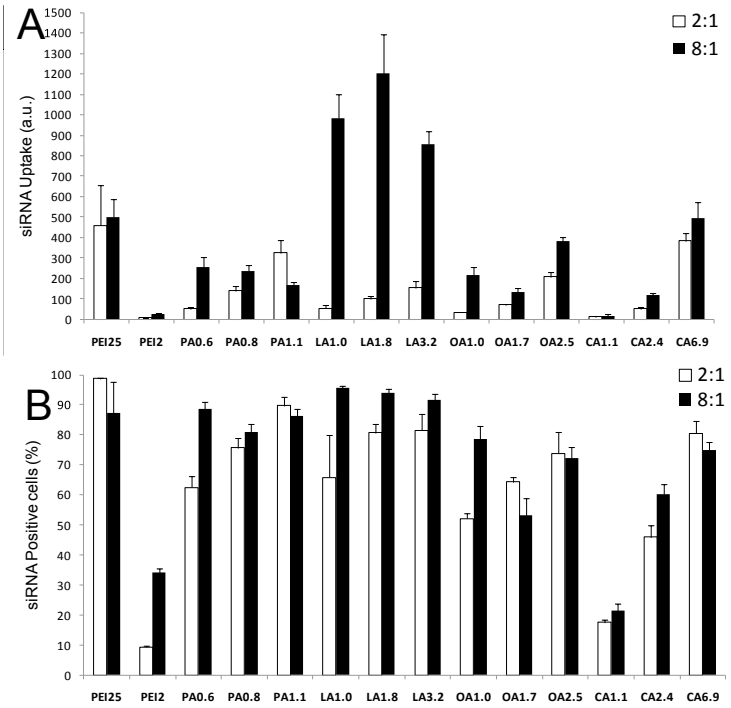

Figure 2.3 Cellular Uptake of Polymer/siRNA Complexes in MDA-MB-231 Cells.

(**A**) The mean fluorescence of the MDA-MB231 cells after 24 h exposure to complexes formed with FITC-labeled scrambled siRNA at polymer:siRNA ratios of 2:1 and 8:1 (weight/weight). (**B**) The percentage of cells positive for FITC-siRNA after 24 h exposure to siRNA complexes. Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of LA-substituted polymers at ratio of 8:1). In general, siRNA uptake was more significant with the polymer:siRNA ratio of 8:1.

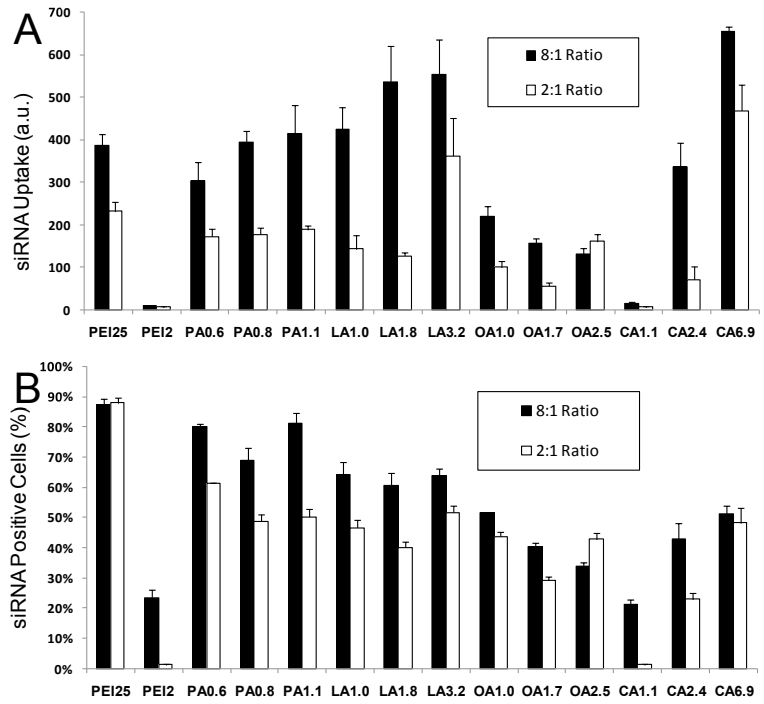

Figure 2.4 Cellular Uptake of Polymer/siRNA Complexes in BCRP+ MDCK Cells.

(**A**) The mean fluorescence of the wild-type MDCK cells after 24 h exposure to complexes formed with FITC-labeled siRNA at polymer:siRNA ratios of 2:1 and 8:1 (weight/weight). (**B**) The percentage of cells positive for FITC-siRNA after 24 h exposure to siRNA complexes. Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of LA-substituted polymers and PEI2- CA6.9). In general, siRNA uptake was more significant with the polymer:siRNA ratio of 8:1.

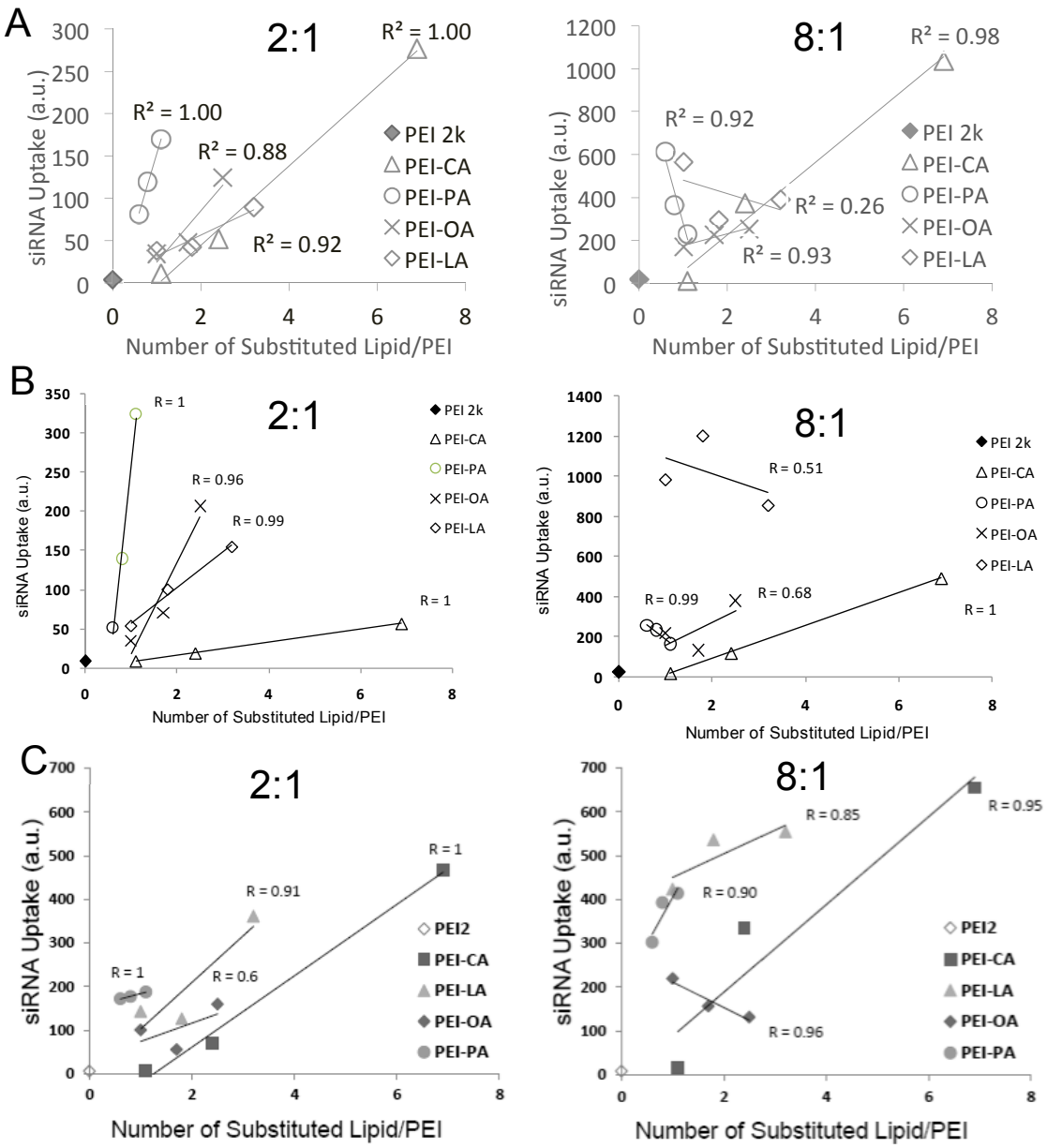

Figure 2.5 Correlation Between Polymer Substitution Level and siRNA Cellular Uptake of the Polymer/siRNA Complexes.

Values were based on number of lipids per PEI chain and cellular uptake calculated based on mean fluorescence). (**A**) MDA-MB-435 cells: Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of CA20). There was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties; such a correlation was only observed for CA- and OA-substituted polymers at 8:1. (**B**) MDA-MB-231 cells: There was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties. (**C**) MDCK cells: Again there was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties; such a correlation was not observed for OA-substituted polymers at 8:1. *Figure courtesy of H.M. Aliabadi.*

2.3.4 siRNA Silencing in Adherent Cells

The functional performance of the lipid-substituted PEIs was evaluated based on down-regulation of GAPDH and P-gp in MDA-MB-435 MDR cells, survivin in MDA-MB-231 cells and BCRP in BCRP expressing MDCK cells. All the down-regulation experiments were performed in presence of 10% fetal bovine serum (FBS). While GAPDH is a house-keeping enzyme commonly used as a prototypical target for silencing [28, 29], P-gp and BCRP are drug transporter whose up-regulation has been linked to resistance to chemotherapy in cancers [20, 21, 30-33]. Survivin is a member of the IAP family with multiple functions including inhibition of cell death and cell cycle regulation [19]. For the silencing experiments, the complexes were prepared at the polymer:siRNA ratios of 2:1, 4:1, and 8:1.

For MDA-MB-435 cells, GAPDH silencing at the ratio of 2:1 was minimal, and was only observed for PEI25 and LA20 (PEI2-LA3.2) (12–16%), **Figure 2.6A**. The silencing effect was more significant at the 8:1 ratio, especially for all LA-substituted polymers that gave 23–32% silencing. The toxic effect of PEI25 was significant at this ratio (note the low level of GAPDH recovered), whereas PEI2-based polymers did not result in significant toxicities under equivalent conditions (i.e., polymer concentration of 8 μg/mL). Among other lipid-substituted polymers, only PEI-CA20 (PEI-CA6.9) showed significant GAPDH down-regulation (~17%) at this ratio. The down-regulation of GAPDH was higher at the 4:1 ratio as compared to the other two ratios, with almost all polymers showing some effect. The PEI2-LA20 (PEI-LA3.2) showed the maximum effect at ~66%, higher than the down-regulation achieved with PEI25 (~55%). **Figure 2.6B** shows the correlation between the substitution level and GAPDH down-regulation

for individual polymers. An increase in GAPDH down-regulation was observed with increasing substitution levels for both LA- and CA-substituted polymers, consistent with the trends observed for the siRNA delivery results. In PA- substituted polymers, however, a reverse trend was observed: the highest down-regulation was observed for PA1 (PA0.6), similar to the siRNA uptake pattern seen in PA-substituted polymers.

Again performing the silencing in MDA-MB-435 cells, the down-regulation of P-gp is summarized in **Figure 2.7A** (showing ratio of 8). Correlations of P-gp knockdown to siRNA uptake studies and P-gp knockdown to number of substituted lipids per PEI are shown in **Figure 2.7A and Figure 2.7B**, respectively. The complexes at the ratio of 8:1 generally showed a more effective down-regulation as compared to the 2:1 ratio (consistent with GAPDH results for these ratios; note that 4:1 ratio was not attempted for P-gp). At the polymer:siRNA ratio of 8:1, the PEI-LA1 (PEI-LA1.0) was the most effective (~67%), which was higher than the P-gp down-regulation achieved with the PEI25 (~61.2%). At this ratio (8:1), only PEI-PA20 (PEI-PA1.1) showed some effect (~15.4%) among the PA-substituted polymers, while OA-substituted polymers and Lipofectamine 2000 (~3.9%) were ineffective. Correlations were also performed of P-gp knockdown versus siRNA uptake (**Figure 2.7B**) or number of substituted lipid/PEI (**Figure 2.7C)**. Increasing P-gp suppression with increasing siRNA uptake and number of substitution on carriers was most evident at the 2:1 ratio (as compared to the 8:1 ratio). Not all polymers showed this trend, however PEI-CA20 (PEI-CA6.9) consistently demonstrated the positive correlation. Similar to the correlations seen between siRNA delivery and lipid substitution (as discussed above), the correlation of siRNA silencing

with lipid substitution was more apparent at the 2:1 ratio, as saturation of siRNA delivery was reached at 8:1 ratio but not yet at 2:1 ratio.

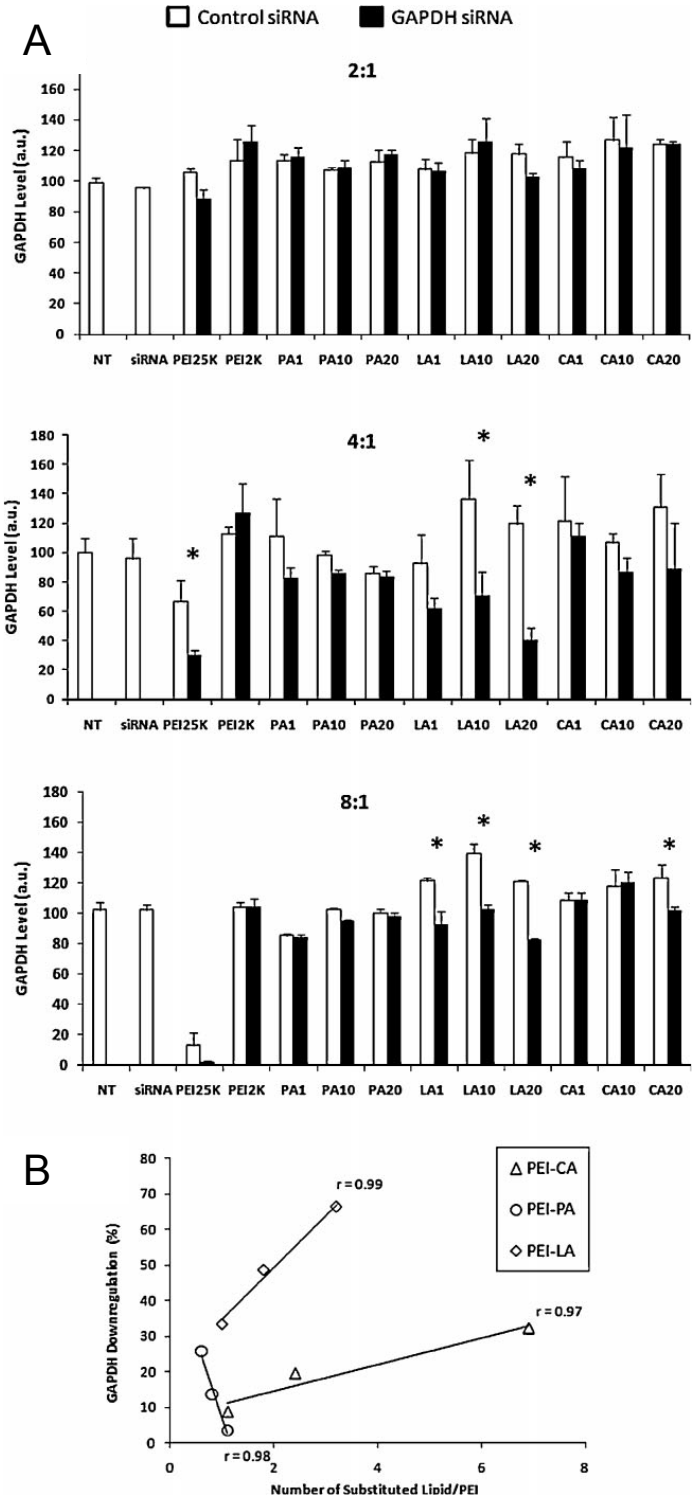

Figure 2.6 Down-Regulation of GAPDH Expression by Polymer/ siRNA Complexes.

(**A**) The GAPDH levels in MDA-MB-435 MDR cells after 72 h exposure to polymer/siRNA complexes at weight/ weight polymer:siRNA ratios of 2:1 (top panel), 4:1 (middle panel), and 8:1 (bottom panel). The white bars represent GAPDH levels for scrambled siRNA treated cells, whereas the black bars represents cells treated with GAPDH specific Silencer siRNA. NT (No Treatment) refers to cells treated with buffer alone. Asterisks represent significant down-regulation compared to scrambled siRNA treated cells (t-student's test; p 0.05). (**B**) Correlation between the level of GAPDH down-regulation (represented by the level of GAPDH expression as a percentage of scrambled siRNA treated cells) for weight/weight polymer:siRNA ratio of 4:1 and the extent of lipid substitution based on the number of lipids per PEI. Correlations with 2:1 and 8:1 ratios were not shown due to relatively weaker GAPDH down-regulation. While none of the complexes were effective in down-regulating GAPDH expression at 2:1 ratio, significant down-regulation was observed with some of the lipid-substituted polymers at higher ratios, most notably with LA20 at ratio of 4:1. Naive PEI2 was ineffective in all polymer:siRNA ratios and a positive correlation was observed between the GAPDH down-regulation and the substitution level for more effective CA- and LA-substituted polymers.

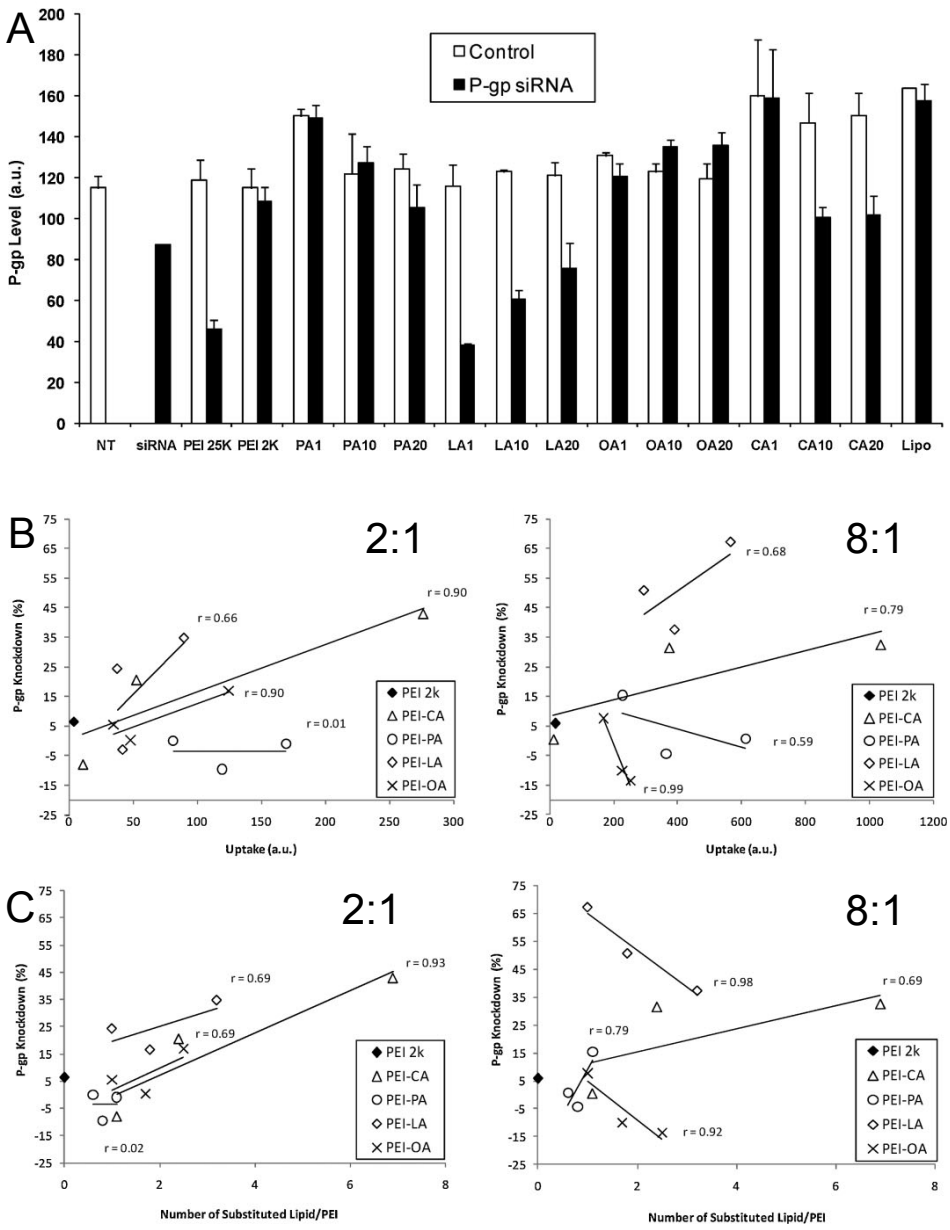

Figure 2.7 Down-Regulation of P-gp Expression by Polymer/siRNA Complexes.
(**A**) The level of P-gp expression in MDA-MD-435 MDR cells after 48 h exposure to complexes. Prepared with ratio of 8:1. The white bars represent P-gp levels for scrambled siRNA treated cells, whereas the black bars represents cells treated with P-gp specific siRNA. NT (No Treatment) refers to cells treated with buffer alone. (**B**) The correlation between the extent of P-gp down-regulation and the cellular uptake of the polymer/siRNA complexes (data from Figure 2.2). (**C**) The correlation between the extent of P-gp down-regulation and the extent of lipid substitution based on the number of lipids per PEI for the weight/weight polymer:siRNA ratio. Highest level of P-gp down-regulation was achieved with CA-substituted polymers at the polymer:siRNA ratio of 2:1, and with LA-substituted polymers at 8:1. At ratio of 2:1, an increase in down-regulation was observed with an increase in uptake, and the extent of lipid substitution. At ratio of 8:1 such a correlation was not observed for all of lipid-substituted polymers.

The effect of siRNA delivery on survivin protein down-regulation in MDA-MB-231 cells was evaluated after 72 h of treatment with the polymer/siRNA complexes. Based on pre-selection of the most effective polymer for silencing by measuring resulting viabilities by MTT assay (not shown), it was determined that PEI-CA6.9 (PEI-CA20) and PEI-LA3.2 (PEI-LA20) at polymer: siRNA ratio of 2:1 and a siRNA concentration of 56 nM were the most effective formulations. **Figure 2.8A** summarizes the mean survivin levels analyzed by flow cytometry after treatment with siRNA complexes of PEI-LA3.2 (PEI-LA20) and PEI-CA6.9 (PEI-CA20). While both LA- and CA-modified polymers showed a decrease in survivin levels in comparison to the cells treated with scrambled siRNA, PEI2-CA6.9 (PEI2-CA20) gave the most significant down-regulation in survivin levels (~82% vs. 25-40% for PEI2-LA polymers). The unmodified PEIs were ineffective in survivin silencing (not shown). **Figure 2.8B** represents the percentage of survivin-positive cells from the same experiment, which again confirmed the superior effect of PEI2-CA6.9 to silence survivin expression.

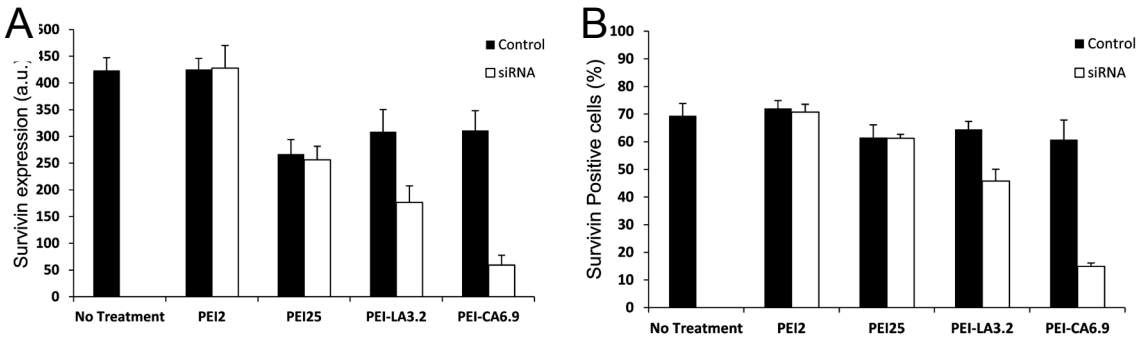

Figure 2.8 Down-Regulation of Survivin Expression by Polymer/siRNA Complexes.
(**A**) The survivin levels in MDA-MB-231 cells after 72 h exposure to siRNA complexes prepared with unmodified PEIs, PEI-LA3.2 and PEI-CA6.9, at a polymer:siRNA ratio of 2:1. The black bars represent survivin levels for scrambled siRNA treated cells, whereas the white bars represent cells treated with survivin specific siRNA. NT (No Treatment) refers to cells treated with buffer alone. (**B**) The level of survivin expression presented as percentage of survivin-positive cells after 72 h exposure to the same complexes. Survivin down-regulation was observed for both polymers; however, the level of down-regulation was more significant for PEI-CA6.9 polymer.

The feasibility of down-regulating BCRP levels, in the BCRP expressing MDCK cells, was evaluated after 48 h of treatment with polymer/siRNA complexes prepared at 8:1 ratio. Since the cells were prepared by an IRES plasmid expressing both GFP and BCRP, a strong GFP fluorescence was observed with the BCRP-positive cells in flow cytometry. **Figure. 2.9A and B** show the BCRP and GFP protein levels, respectively, after the treatment of BCRP-positive cells with siRNA complexes of different polymers. We observed that BCRP-specific siRNA delivery also caused a parallel down-regulation of GFP levels for select polymers. Similar to siRNA delivery results, PEI2 had minimal effect on the BCRP and GFP levels, while PEI25 was effective in down-regulating both GFP and BCRP protein levels. Among the lipid-substituted polymers, PEI-LA polymers and PEI-CA6.9 (PEI-CA20) were the most efficient carriers for down-regulating the protein levels (at 8:1 ratio). The lipid-polymers were also evaluated at the 2:1 ratio Similar results were also obtained based on the analysis of BCRP-positive cell population obtained after siRNA treatment (not shown): the LA-substituted polymers gave the most effective BCRP down-regulation, and PA- and OA-substituted polymers were least effective. The extent of BCRP and GFP down-regulations obtained is summarized in **Figure 2.9C** (calculated as a percentage of BCRP/GFP levels from specific siRNA delivery with respect to non-specific siRNA delivery). Clearly the down-regulation of BCRP and GFP are very similar. Increasing the lipid substitution level had a significant impact on improving siRNA efficacy for CA- and PA-substituted polymers, as only the highest CA- and PA-substituted polymers were effective in silencing BCRP expression. The LA-substituted polymers were all effective, but a reverse trend between the substitution level and down-regulation was obtained, declining from 77.8% to 61.7%

with increasing substitution levels. All OA-substituted polymers were ineffective in BCRP down-regulation. The LA-substituted polymers (i.e., the most successful polymers) were also evaluated at the polymer:siRNA ratio of 2:1 as well. Only PEI-LA3.2 (PEI-LA20) showed a small but significant down-regulation of BCRP/GFP, and other polymers (including PEI25) showed no significant BCRP/GFP down-regulation (not shown).

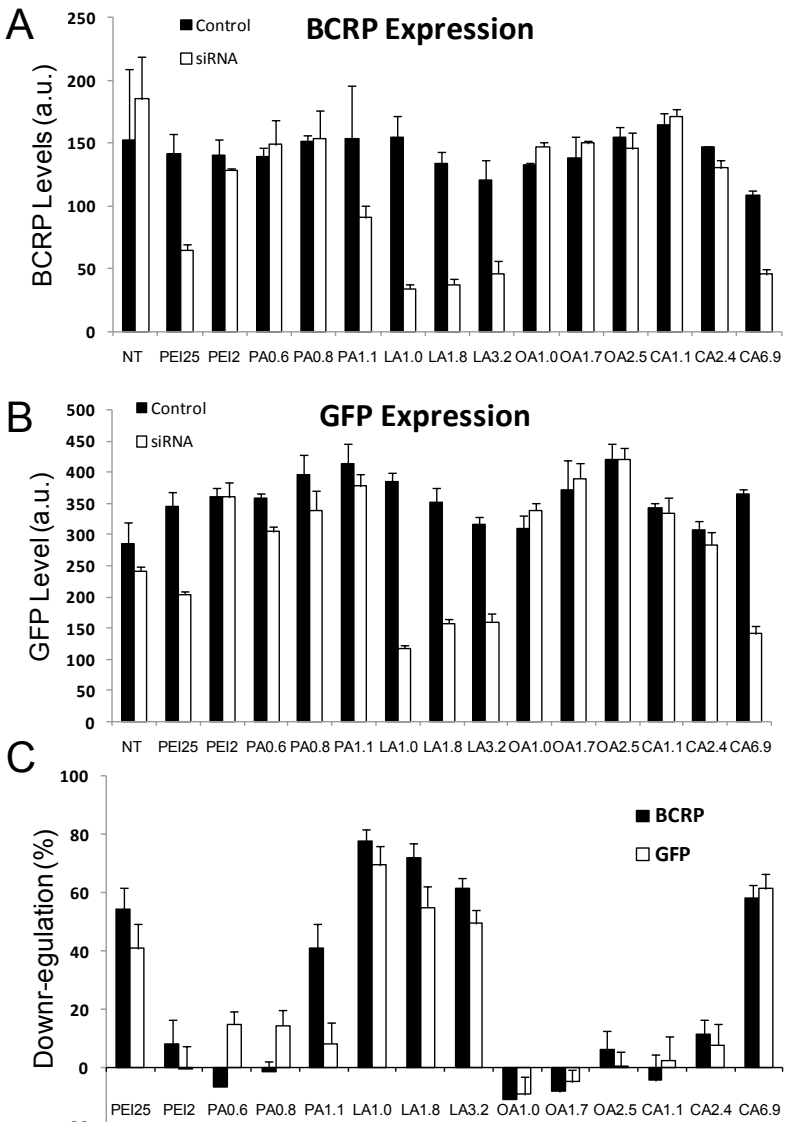

Figure 2.9 Down-regulation of BCRP and GFP expression by Polymer/siRNA Complexes.
The BCRP (**A**) and GFP (**B**) levels in BCRP-transfected cells after 48 h exposure to polymer/siRNA complexes at polymer:siRNA ratio 8:1. The black bars represent BCRP levels for scrambled siRNA-treated cells, whereas the white bars represent cells treated with BCRP-specific siRNA cocktail. NT (No Treatment) refers to cells treated with buffer alone. (**C**) The level of BCRP and GFP down-regulation in BCRP-transfected MDCK cells after 48 h exposure to complexes. The level of down- regulation was calculated as a percentage of protein levels in cells treated with scrambled siRNA complexes (calculated based on A and B).

2.4 DISCUSSION

Lipid-modified polymers clearly improved the efficacy (delivery and silencing) of polymer-siRNA complexes as compared to native PEI in this study. The addition of the lipids increases the hydrophobicity of the polymers, which results in a decrease in the binding affinity of the polymer to the siRNA. The lipid substitution were also found to result in an overall increase in zeta-potential compared to native PEI2 making the net charge of the complexes closer to the higher cationic charge seen with PEI25 (data not shown) [27]. This increase was indicative of better interaction among polymer molecules involved in each particle and better assembly with siRNA molecules.

Increasing the substitution level did have a positive effect on the cellular uptake of the complexes. This correlation is especially obvious at the ratio of 2:1 for each substituted lipid in each cell line, however it was less apparent at the 8:1 ratio (**Figure 2.5**). This is likely due to excessive polymer and/or complex exposure to the cells, which could saturate the cellular uptake mechanism. (The zeta-potential did not seem to be the driving force for the cellular uptake, as we did not observe a strong correlation between the zeta-potential of the complexes and the corresponding cellular uptake (not shown)). Interestingly, there was a strong difference in effective siRNA delivery with the lipid polymers depending on the cell line. In the MDA-MB-231 breast cancer cells, PEI-LA regardless of substitution levels displayed markedly higher delivery than the rest of the lipid polymers. However, in MDA-MB-435 breast cancer cells and MDCK canine kidney cells the differences in the polymer delivery abilities were not so distinctive.

The better performing (most efficacious) polymers, when considering siRNA silencing instead of siRNA delivery, was more consistent among the three cell lines with

PEI-LA and PEI-CA demonstrating the best silencing ability in all cases. However, when considering the specific formulations of the lipid polymer (the polymer to siRNA ratio and lipid substitution), there were still variances among the cell lines. Down-regulation of survivin in MDA-MB-231 cells was clearly the most effective with PEI-CA20 (PEI-CA6.9) at a ratio of 2:1. Silencing GAPDH was best with PEI-LA10 (PEI-LA1.8) and PEI-LA20 (PEI-LA3.2) at 4:1 and 8:1 but not 2:1 and silencing P-gp was best with PEI-LA1 (PEI-LA1.0) and PEI-LA10 (PEI-LA1.8) at 2:1 and 8:1 (however P-gp silencing revealed more formulations that were almost as efficient as those listed). Lastly silencing BCRP was clearly the best with all three PEI-LAs and PEI-CA20 (PEI-CA6.9) at 8:1 ratio but not 2:1.

The higher zeta-potential at the ratio of 8:1, which seems to be an advantage in increasing the cellular uptake (data not shown; [27]), could have became an obstacle since the stronger binding may prevent siRNA availability in free form to reach their site of action. Considering the GAPDH and P-gp silencing in MDA-MB-435 cells and BCRP silencing in MDCK cells, this could explain the higher silencing efficiency achieved at the ratio of 8:1 compared to ratio of 2:1 (because of a higher uptake), and for P-gp silencing the higher efficacy at the ratio of 4:1 compared to 8:1 and 2:1 ratios (because of more free siRNA available after the uptake), which made the 4:1 the optimal ratio for siRNA silencing. The MDA-MB-231 cells displayed the best siRNA silencing of survivin at a 2:1 ratio instead, which seems to not follow the above explanation. However, resulting cell viabilities after survivin silencing revealed a significant decreased in cell viability in many of the formulations, with both control siRNA and survivin siRNA, (not

shown; [27]) so there maybe further factors involved with these cells which impact the most optimal formulation of lipid-polymer.

Overall, high levels of silencing were achievable with the best lipid-polymer formulation. For GAPDH and P-gp silencing in MDA-MB-435 cells, the most significant silencing was achieved with LA20 (LA3.2) at ratio 4:1 (66% decrease in GAPDH protein levels) and with LA1 (LA1.0) at 8:1 (67% decrease in P-gp protein levels). For survivin suppression in the breast cancer cell line MDA-MB-231, CA20 (CA6.9) (2:1 ratio) demonstrated the most significant silencing (~82%). BCRP suppression in a BCRP expressing model cell line (MDCK-BCRP) with LA1 (8:1 ratio) demonstrated the most significant silencing (~80%). Overall, it appears that optimization with PEI-LA and PEI-CA was needed to determine the best formulations.

Since the BCRP positive MDCK cells were made utilizing an IRES promoter, which provided co-expression of BCRP and the reporter protein GFP, we were able to suppress both by the BCRP siRNA utilized. This implies that encoded mRNA were rapidly degraded after silencing with the BCRP siRNA, so that the GFP protein could not be produced from its portion of the mRNA strand. Another interesting aspect of dual silencing the proteins is the ease at which one can monitor silencing while measuring therapeutic effects. Dual-silencing of the target protein and a reporter protein has foreseeable benefits both in complex *in vitro* therapeutic effect studies as well as a multitude of applications *in vivo*. It is important to also note that the siRNA silencing described in this chapter has been shown to result in therapeutic effects, the necessary end-point to demonstrate an effective therapy. Silencing survivin resulted in decreased

cell viability, as well as evidence of apoptosis, determined by apoptosis assay and visualized by DNA fragmentation (not shown) [25]. Increase in cytotoxic effect of the anticancer drugs doxorubicin, paclitaxel, and mitoxantrone was also observed after pre-silencing of survivin (not shown) [25]. The positive effect of silencing BCRP, a drug transporter, was clearly evident with treatment with mitoxantrone after BCRP silencing (not shown) [26]. Collectively, these results indicate the functional effects of the specific silencings pursued in this study.

2.5 CONCLUSION

In conclusion, lipid substitution on low-molecular-weight PEIs was shown to lead to functional materials for siRNA delivery and effective gene knockdown with minimal cytotoxicity. The lipid substitution leads to better assembly of siRNA complexes, and higher intracellular delivery of therapeutic siRNA molecules. The gene knockdown efficiency was ultimately dependent on the nature of the substituted lipid, the level of substitutions, and the relative ratio of polymer to siRNA, which had to be tailored and optimized for therapeutic purposes. Although the exact formulations for efficient silencing depended on the cell line and protein target, silencing with two lipid-polymers (CA and LA) modified low molecular weight PEIs was consistently obtained, suggesting that these carriers can be clinically applied in the future. Fine-tuning the siRNA/polymer composition was critical for silencing particular targets.

2.6 ACKNOWLEDGEMENTS

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3. Effective Non-Viral Delivery of siRNA to Acute Myeloid Leukemia Cells with Lipid-Substituted Polyethylenimines[x]

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3.1 INTRODUCTION

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with an estimated >13,000 new cases yearly and a mortality rate of ~10,000 in the US alone [1]. Development of novel AML therapies is urgently needed due to poor prognosis of the disease with a five-year survival rate of 30% for younger adults and ~15% for elderly patients [2]. Only in childhood AML, ~60% of patients can be cured of AML with very intensive chemotherapy [3]. The chemotherapy remains the front-line treatment, but alternative therapeutic approaches are required due to high relapse rates and limited treatment options for patients that cannot bear the toxic side-effects of chemotherapy [4]. Chemotherapy also leads to long-term undesired consequences; ~66% of survivors have either a chronic or late-effect due to cancer treatment and ~33% of these effects are considered major, serious or life threatening [1]. With better understanding of molecular changes in malignant transformations, treatments that target tumor-specific changes will lead to more effective therapies as the normal cells transform into malignant cells. Towards this end, a highly specific leukemia therapy can be developed by exploiting the RNA interference (RNAi) to silence the aberrant protein(s) responsible for the disease [5, 6].

There are two main approaches for RNAi, using either a plasmid encoding for short hairpin RNA (shRNA) or delivering small interfering RNA (siRNA) where the shRNA transcription and processing steps can be omitted [7]. The use of siRNA is a more practical approach bypassing the need to express the shRNA at sufficient quantities in hard-to-transfect primary cells. In cytosol, the siRNA duplexes assemble into a pre-RISC (RNA-induced silencing complex) containing specific proteins, including argonaute

proteins (AGO1, 3 or 4) [8, 9]. The siRNA duplexes become unwound in AGOs, where the guiding strand directs the mature-RISC to target desired mRNA based on complementary base pairing [8]. Endonucleolytic cleavage and/or translational repression of the mRNA [8, 9] subsequently silences the desired protein target. Delivery systems, however, are an absolute necessity for effective use of siRNA since the molecules are highly sensitive to serum nucleases and their large (~13 kDa) and anionic nature (due to its phosphodiesterase backbone) prevents siRNA to traverse cellular membranes. Cationic biomolecules capable of binding and neutralizing the anionic charges of siRNA and packaging the siRNA into nano-sized complexes can serve as effective siRNA carriers [10]. The utility of cationic carries for siRNA therapy in AML has been explored as early as 2003, where Raf-1 and Bcl-2 proteins were suppressed in AML cells by using the synthetic carrier Oligofectamine^{TM}. However, the resulting apoptotic response required 400 nM siRNA [5], a concentration too high for practical applications. It was evident that a more efficient delivery system was required to advance siRNA therapy for AML. Recent RNAi delivery attempts in leukemia cells have employed a variety of commercial carriers, which included; (i) Lipofectamine^{TM} 2000 in chronic myeloid leukemia (CML) K562 cells, and AML cells (KG-1/HL-60/U937/primary) [11-16], (ii) RNAiMAX^{TM} in K562 [17], (iii) HiPerFect^{TM} in K562 and T-ALL (Jurkat) cells [18, 19], (iv) DOTAP in BCR-ABL positive CML cells (2Dp210-modified/patient samples) [20], (v) Lipofectin in myeloid neoplasm cells (HMC-1) [21] (vi) and Oligofectamine^{TM} in T-cell lymphoblastic leukemia cells (CCRF-CEM) [22]. Other carriers used for siRNA delivery were cell penetrating peptides (Tat–LK15 peptide in K562 cells) [16], CADY peptide in THP-1 cells [23], and functionalized carbon nanotubes in K562 cells [24].

Many of the above studies focused on discovery of possible targets for silencing and/or mechanisms of drug action, without pursuing siRNA delivery as a therapy. A systematic analysis of carrier features responsible for effective siRNA delivery was not conducted, which is critical for design of more effective siRNA carriers suitable for clinical use.

We previously reported on siRNA delivery by amphiphilic cationic polymers with lipid substituents to anchorage-dependent malignant cells [25]. The polymers provided the necessary cationic charge for siRNA binding whereas the lipid component provided the hydrophobicity for improved interactions with cellular membranes. The polymeric component was derived from polyethylenimine (PEI), whose prototypical member, 25 kDa branched PEI (PEI25), is widely used as an effective transfection agent [26-28]. Since the cytotoxicity of PEI25 has been a major impediment for its therapeutic use, we employed a smaller PEI (2 kDa; PEI2) as the polymer backbone since it displays minimal cytotoxicity [29-34]. Although PEI2 displays effective binding to nucleic acids in buffers, the resultant complexes were ineffective for nucleic acid delivery into cells. Lipid substitution on PEI2 enhanced the assembly of nucleic acids into nano-particles, improved the cellular uptake and, depending on structural features of lipid substituents, enabled silencing of selected molecular targets in breast cancer cells [25, 35]. Leukemic cells, on the other hand, are structurally different from anchorage-dependent cells, with minimal surface area and endocytic activity, and are known to be difficult to transfect (as discussed in [36]).

This study explored the utility of lipid-substituted polymers for siRNA delivery to leukemic cells. It was our aim to determine the relative effectiveness of these carriers for siRNA delivery and to elucidate carrier features critical for delivery. We focused on

AML subgroup of leukemia and employed 3 well-characterized cell models (THP-1, KG-1 and HL-60 cells). The PEI25 was employed as a reference reagent, given its prominent use for siRNA delivery to anchorage-dependent cells. A systematic approach was employed to investigating the role of lipid substitution as well as the nature of substituted lipid on siRNA binding, toxicity, and siRNA delivery and silencing. The results showed that (i) PEI25 was not effective in siRNA delivery to leukemic cells unlike the anchorage-dependent cells, and (ii) lipid substitution improved the siRNA delivery of cationic polymers, and (iii) effective silencing could be obtained at clinically acceptable siRNA doses (20-50 nM). These results provide encouraging data to pursue the described carriers for siRNA-based molecular therapy of leukemia.

3.2 MATERIALS AND METHODS

3.2.1 Materials

PEI25 (M_n: 10 kDa, M_w: 25 kDa) and PEI2 (M_n: 1.8 kDa, M_w: 2 kDa), anhydrous dimethyl sulfoxide (DMSO), myristoyl chloride (C14; 97%), palmitoyl chloride (C16; 98%), octanoyl chloride (C18:1 9Z; 99%), linoleoyl acid (C18:2 9Z, 12Z; 99%), 3-(4,5-demethyl-2-thiazoylyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), *N*-[1-(2,3-dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate (DOTAP), trypan blue solution (0.4%), and heparin sodium from porcine intestinal mucosa were purchased from Sigma-Aldrich (St. Louis, MO). Stearoyl chloride (C18; >98.5%) was obtained from FLUKA. Clear filtered HBSS (phenol red free) was prepared in-house. Unlabeled negative control siRNA, 5'-carboxyfluorescein (FAM)-labelled negative control siRNA, GFP siRNA (GFP-22) and CXCR4 siRNA (HSC.RNAI.N001008540.12.1) were

purchased from Ambion (Austin, TX), Shanghai GenePharma Co., Ltd (Shanghai, China) and Qiagen (Toronto, ON) and IDT (Coralville, IA), respectively. Hanks Balanced Salt Solution (HBSS), Dulbecco's modified Eagle medium (DMEM; low glucose with L-glutamine; 11885), and RPMI Medium 1640 with L-glutamine (11835), opti-MEM® I reduced serum medium (31985), penicillin (10000 U/mL), and streptomycin (10 mg/mL) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS; A15-751) was purchased from PAA Laboratories Inc. (Etobicoke, ON). RNAi-mate was obtained from Shanghai GenePharma Co., Ltd, Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX Reagent from Invitrogen, Metafectamine Pro from Biontex (San Diego, California) and FuGENE® HD from Roche (Laval, QC) and HiPerFect Transfection Reagent from Qiagen (Mississauga, Ontario).

3.2.2 Cell Models and Culture

The cell lines THP-1, KG-1 and HL-60 cells used as the AML models were obtained from the American Type Culture Collection (Manassas, VA). THP-1 and KG-1 cell were maintained in RPMI medium and HL-60 cells were maintained in DMEM Low Glucose medium, all containing 10% FBS (heat inactivated at 56 °C for 30 min) and 1% penicillin/streptomycin under normal conditions (37 °C, 5% CO_2 under humidified atmosphere). The cells were maintained at concentrations between $0.1x10^5$ and $4x10^5$ cells/ml (monitored by hemocytometer cell counts) and by weekly passage by dilution after removing the spent medium with centrifugation at 600 rpm (72g) for 5 min. To obtain Green Fluorescent Protein expressing THP-1 cells, a retroviral vector expressing enhanced GFP (EGFP) was generated by cloning EGFP into pMSCVpuro (Invitrogen). The murine stem cell virus-based vector was chosen as it provides relatively stable long-

term expression of the transgene and is less prone to transcriptional shutdown in THP-1 cells than other retroviral vector systems tested. To generate retroviral particles, pMSCV-EGFP was transfected into 293T cells with Fugene HD. Gag/pol were provided in trans and VSV-G was utilized as viral coat protein. Retroviral supernatants were harvested 24 h post transfection and used to transduce THP-1 cells. The cells were then selected using puromycin and further enriched for EGFP expression using fluorescence activated cell sorting. The resulting GFP-expressing THP-1 cells were cultured as above.

3.2.3 Synthesis of Lipid-Substituted Polymers

The PEI2 polymers substituted with lipids (caprylic acid; CA, palmitic acid; PA, oleic acid; OA, linoleic acid; LA; stearic acid; StA, myristic acid; MA) were prepared in house, where the synthesis and characterization have been previously described [37, 38]. Briefly, a 2 kDa PEI solution (50% in water) was first purified by freeze-drying. Commercially available lipid chlorides (CA, PA, OA, LA, StA and MA) were then substituted by N-acylation of PEI onto the amine groups by addition of the lipid chlorides to 100 mg of PEI in DMS0 for 24 h at ambient temperature under argon. To produce a range of substitution levels for each lipid, four different feed ratios were utilized (lipid:polymer = 0.012, 0.066, 0.1 and 0.2) and the polymers were precipitated and washed with excess ethyl ether. The lipid-substituted polymers were dried under vacuum at ambient temperature over night. The substitution was analysed by ^{1}H-NMR (Bruker 300 MHz; Billerica, MA) in D_2O). The characteristic proton shifts of lipids (δ~0.8 ppm; -C$\underline{H}_3$) and PEI (δ~2.5-2.8 ppm; NH-C$\underline{H}_2$-C$\underline{H}_2$-NH-) were integrated and normalised to the number of protons in each peak in order to calculate the lipid substitution levels. **Table 3.S1** summarizes the employed feed ratios as well as the final level of lipid substitutions

obtained. The numbers of lipid methylenes substituted in each polymer were calculated by multiplying the level of lipid substitution (from ^{1}H-NMR) with the number of methylenes in each lipid. Percent lipid substitution was calculated by dividing the number of lipid substituted with the number of amines available in each PEI2 (14).

3.2.4 siRNA/Polymer Complex Formation

Polymer/siRNA complexes were formed by first adding a desired amount of siRNA (0.37 μg; 25 nM final siRNA well concentration) to 150 mM NaCl solution. The polymers (PEI25, PEI2 and lipid-substituted PEI2s; all dissolved in ddH_2O) were then added to the siRNA solutions at desired polymer:siRNA ratios (8:1, 4:1 and 2:1 w/w, corresponding to 63.2:1, 31.6:1, 15.8:1 N/P ratios), bringing the volume to 30 μL. After mixing, the complexes were incubated at room temperature for 30 min before addition (triplicate; 10 μL/well) to the cells (note that 30 minutes of complex incubation was within the optimal range for siRNA delivery; **Figure 3.S1)**. For electron microscopy imaging, complexes were prepared in the same manner, except the 150 mM NaCl solution was replaced with ultra pure water to prevent NaCl crystal formation. After 30 min of incubation, 5 μL of complex solution was transferred to a 3 mm Formvar film coated grid. The grid was allowed to dry (~20 min) and complexes were imaged by a Philips/FEI (Morgagni) Transmission Electron Microscope with CCD camera (TEM-CCD).

3.2.5 Cytotoxicity

The extent of the polymer/siRNA complex cytotoxicity was determined by the MTT assay. The complexes were prepared at 8:1 polymer:siRNA w/w ratio as described above. The cells were seeded in 24-well plates with 0.5 mL medium per well and allowed

to acclimatize for 24 h. The complexes were then added in triplicate for final concentrations of 1.25, 2.5, 5 and 10 μg/mL and incubated for 24 h under normal maintenance conditions. The MTT solution (40 μL, 4 mg/mL in HBSS) was then added to each well and the cells were incubated for 2 h. The plates were centrifuged, the medium removed, and 200 μL of DMSO was added to each well to dissolve the MTT crystals formed. The optical density of the solutions (570 nm) was measured by an ELx800 Universal Microplate Reader (BioTek Instruments; Winooski, VT, USA). Background was determined with medium only wells and subtracted from the obtained optical densities. The percentage of cell viability was calculated as follows: 100% x (absorbance of polymer treated cells/absorbance of untreated cells).

3.2.6 Analysis of siRNA Binding to Polymers

Gel electrophoresis was performed for assessment of siRNA binding efficiency of polymers, as well as for dissociation of siRNA/polymer complexes with heparin. For the binding studies, 3 mL of 0.1 mg/mL control siRNA (in ddH_2O) was incubated with various concentrations of polymers (in ddH_20) in 25 μL of 150 mM NaCl for 20 min to form complexes. Loading dye (4 μL, 6x, 40% sucrose with bromophenol blue/xylene cyanole) was added to samples and the samples were run on a 0.8% agarose gel containing 1 μg/mL ethidium bromide (130 V for 20 min). The gels were visualized under UV illumination and bands corresponding to free siRNA were quantified by spot densitometer. siRNA alone was run as a reference control (i.e., 0% binding). Percent binding (% Binding) was calculated as: 100% × [(control siRNA − free siRNA) ÷ control siRNA]. Percent binding was plotted as a function of polymer concentration and

concentration required for 50% binding of siRNA (BC_{50}) was estimated based on sigmoidal curve fits.

3.2.7 siRNA Delivery to Leukemia Cells

Effectiveness of carriers for siRNA delivery was determined by measuring the percentage of cells positive for siRNA and mean fluorescence of cells after delivery of FAM-labelled control (scrambled) siRNA (CsiRNA-F). To account for cellular auto-fluorescence due to complex exposure, a non-labelled scrambled (control) siRNA (CsiRNA) was utilized as a control for each siRNA-polymer complex prepared. In cases where the results from CsiRNA are not shown, the autofluorescence was found to be insignificant. THP-1, KG-1 or HL-60 cells were seeded in 24-well plates (0.35 mL fresh medium/well) and allowed to acclimatize for 24 h in normal maintenance conditions. The siRNA-polymer complexes were prepared as in Section 2.4 and 10 µl of complex solution was slowly added to each well containing the cells (0.35 mL medium/well in triplicate). A 30 min of complex formation between the siRNA and the polymers was found to give the optimal uptake (**Figure 3.S1**), so that complexes were exposed to the cells after this incubation time. At indicated time points (see figure legends), the cells were transferred to eppendorf tubes and centrifuged (1200 rpm; 100 rcf). Cells were washed with clear HBSS, resuspended in 100 µl of clear HBSS and then fixed with 3.7% formalin in HBSS. The siRNA delivery to the cells was assessed by flow cytometry (Cell Lab Quanta^{TM} SC; Beckman Couter) using the FL-1 detection channel, fluorescence plate reader at λ_{EX} of 485 nm and λ_{EM} of 527 nm (Fluoroskan Ascent, Thermo Labsystems), or by epifluorescence microscopy (FSX100; Olympus) as elaborated in the figures.

Competitive inhibition studies were performed by incubating the cells with free lipids (LA, OA, StA; 0-100 μM) followed by treatment of the cells with FAM-labelled siRNA/polymer complexes. Effect of serum on complex delivery was also determined; the percentage of FBS in medium was varied between 0 and 50% prior to complex treatment and cell uptake was determined by flow cytometry as described above.

For internalization studies, siRNA delivery was performed as described above with the following modifications. Delivery was performed at both 4 and 37°C from 1 to 24 h and subsequently split into trypan blue treated and non-treated groups. For the 4 °C groups, cells were placed at 4 °C, 20 min prior to addition of complexes and immediately put on ice in subsequent steps. At each time point, cells were transferred to 1.5 ml tubes, centrifuged and the medium was removed. Each group was split into a trypan blue and a without trypan blue group. 100 μl of 0.4% trypan blue in HBSS (or HBSS) was added to each tube (containing 100 μl medium) and cells were resuspended and incubated for 5 min. They were then fixed with 3.7% formalin and washed twice with 1 ml HBSS (to remove trypan blue) prior to flow cytometry.

A comparison between lipid-substituted polymers and commercial reagents (RNA-mate, Lipofectamine^{TM} 2000, RNAiMAX^{TM}, Metafectamine, DOTAP, Fugene HD and HiPerFect) was performed by delivering siRNA complexes (24 h) prepared with CsiRNA-F. Complexes were prepared as closely as possible to the manufactures directions while maintaining a consistency necessary for comparison. The incubation time with the cells, medium volume and type and serum concentrations were all standardized. As most vendors suggest the use of OPTI-MEM for complex preparation, the complexes were prepared with OPTI-MEM as the buffer. siRNA (0.37 μg) and

desired reagents was added to 150 µL OPTI-MEM solutions separately. The reagent amounts were 2.94 µL (1 mg/ml) for PEI25 and PEI2-LA20, 5.88 µL for RNAi-mate, 4.00 µL for Lipofectamine™ 2000, 7.50 µL for RNAiMAX™ (pre-diluted 1:4 in OPTI-MEM), 1.84 µL for Metafectamine, 4.00 µL for Fugene HD, 2.21 µl for DOTAP (1 mg/mL), and 4.5 µL for HiPerFect (pre-diluted 1:4 in OPTI-MEM). The amount of the reagents was halved for low concentration experiment. The siRNA and reagent solutions were then vortexed, except Metafectamine that was mixed by pipetting once. The siRNA-reagent solutions was then mixed by gently vortexing except for DOTAP which was mixed by pipetting and Metafectamine which was not mixed. PEI25, PEI2-LA20 and RNAi-mate complexes were incubated for 30 min, Lipofectamine™ 2000, RNAiMAX™ and Metafectamine were incubated for 20 minutes, Fugene HD and DOTAP were incubated for 15 min and HiPerFect was incubated for 10 min, prior to drop-wise addition (100 µL) to cells in 200 µL of RPMI medium. The commercial reagents were ranked (from 1 to 9; 1 being the best and 9 being the worst) according to siRNA uptake results from flow cytometry, based on percentage of cell population positive for siRNA and mean siRNA fluorescence/cell. If reagents had comparable fluorescence levels (due to overlapping SDs), their ranks were averaged and each was given the same mean value. The ranking was then averaged over the three cell lines to provide an overall performance ranking.

3.2.8 GFP Silencing in THP-1 Cells

GFP-expressing THP-1 cells were used as a model for silencing studies. siRNA complex formation and delivery to the cells was performed as described in Section 2.4 and 2.7, utilizing GFP specific siRNA (GFP-siRNA) and scrambled siRNA (CsiRNA).

For the time course studies, cells were treated with desired siRNAs continuously during the experimental duration; cells were subcultured every 3 days to prevent over-growth. Subculturing was performed by dilution (x10) into fresh medium after resuspension. All groups were subcultured with the same ratio regardless of cell concentration to ensure that the concentration of any remaining complexes stayed constant. For studies including the commercial reagents, selected reagent preparation was performed with OPTI-MEM as described in Section 2.7 for the commercial reagent delivery comparison study, keeping the same reagent:siRNA ratios (high ratios) and at siRNA concentration of 50 nM. GFP silencing was assessed by flow cytometry after cell fixation (as described in Section 2.7) using the FL-1 detection channel. Percent decrease in mean fluorescence was calculated as follows: 100 - {[Mean FL1 of cells treated with GFP-siRNA/polymer complexes] / [Mean FL1 of cells treated with CsiRNA/polymer complexes] x 100%}. Percent decrease in GFP-positive cells was calculated as follows: [% of GFP-negative cells of GFPsiRNA/polymer treated cells] - [% of GFP-negative cells of CsiRNA/polymer treated cells]. Gating was performed as shown in **Figure 3.11A**.

For studies where GFP silencing was followed at the mRNA level, total RNA was extracted from treated THP-1 cells in 12-well plates (biological duplicates) with the RNeasy Mini Kit (Qiagen). The extracted RNA was then quantified by spectrophotometry (GE Nanovue). cDNA was synthesised following Invitrogen's protocol, briefly adding 2 μL master mix 1 (0.5μL Oligo$(dT)_{12\text{-}18}$ Primer, 0.5 μL random primers and 1 μL (10mM) dNTP's per sample) to 10 μL of RNA (2500 ng) and then heated at 65°C for 5 min. 7 μL of Master Mix 2 (4 μL 5x Synthesis Buffer, 2 μL DTT (0.1M) and 1 μL RNAout RNase inhibitor (1.8 U/μL)) was then added and the samples

heated at 37 °C for 2 min. 1 μL of M-MLV RT enzyme was then added per sample and they were heated at 25 °C for 10 min, 37°C for 50 min and 70°C for 15 min. Real-time PCR was performed on a ABI 7500 HT with human beta actin (Forward: 5'-CCA CCC CAC TTC TCT CTA AGG A-3' Reverse: 5'-AAT TTA CAC GAA AGC AAT GCT ATC A-3') as the endogenous house keeping gene and the specific GFP primers (Forward: 5'-GGG CAC AAG CTG GAG TAC AAC-3', Reverse: 5'-CAC CTT GAT GCC GTT CTT CTG -3'). 7.5 μL of master mix containing 5 μL of 2X SYBR Green master mix (MAF Centre, U. of Alberta) and 2.5 μL primer (3.2 μM; per sample) was added to each well. Then, 2.5 μL of template of each sample was added in triplicate. A template concentration (9.76 ng/μL) was determined optimal based on a standard curve. To ensure that the efficiencies of the human beta actin and GFP primers were approximately equal, to validate use of the $2^{-\Delta\Delta CT}$ method, ΔCT vs. cDNA dilution was plotted and the slope was verified to be approximately zero. Analysis was performed by $2^{-\Delta\Delta CT}$ method [39] using the no-treatment group as the calibrator. Finally, the change in mRNA levels (in percent form) was calculated as follows: [% mRNA rel. NT of cells treated with CsiRNA/polymer complexes] – [% mRNA rel. NT of cells treated with GFPsiRNA/polymer complexes]. Standard deviation was calculated from the biological replicates.

3.2.9 CXCR4 Silencing in THP-1 Cells

THP-1 cells were treated with CXCR4 siRNA or control siRNA by using the polymer complexes (4:1) as described above. At day 2 and day 3, cells were stained with 4 μL of PE-labeled mouse anti-human CXCR4 (CD184) or PE-labeled mouse IgG

isotype control (BD Pharmingen) antibody in 90 μL of medium (after centrifugation and resuspension) for 45 min at room temperature. They were subsequently re-suspended in HBSS and fixed in 3.7% formalin and immediately analysed by flow cytometry (FL2 channel). As in GFP analysis, changes in mean CXCR4 levels (based on Ab fluorescence levels) and the CXCR4-positive cell population were calculated. The cell population stained with non-specific antibody was used for flow cytometry calibration (i.e., 1% CXCR4-positive population).

3.2.10 Statistical Analysis

Results are displayed as the mean ± standard deviation (SD) of triplicate samples. For binding and dissociation studies, variations between the group means were analyzed as described in figures. To determine linearity, linear regression was performed; r^2 coefficient and P values (to test for significant slope) were reported. Statistical analysis was performed with GraphPad InStat v3.06 (GraphPad Software, San Diego, CA USA).

3.3 RESULTS AND DISCUSSION

Lipid substitution to PEI2 was explored as a means to improve siRNA delivery to AML cells, THP-1, KG-1 and HL-60. The substituted lipids included CA, MA, PA, StA, OA and LA (in the order of increasing carbon chain length form C8 to C18) at a range of substitution levels (**Table 3.S1**) [37, 38]. There was a general increase in lipid substitution as the lipid:PEI feed ratio was increased during the synthesis (determined by ^{1}H-NMR). The highest number of lipids substituted was achieved with CA at lipid:PEI amine ratio of 0.2 (6.9 CAs/PEI). All polymers remained water soluble, except PEI2-

StA20 that had the highest number of lipid methylenes substituted per PEI2 chain (89.0) and it was excluded from the study.

3.3.1 Polymer Binding to siRNA

It is imperative for the polymers to bind and neutralize the anionic charge of siRNA to form a siRNA complex. The siRNA binding ability polymers was determined by the semi-quantitative EMSA using CsiRNA. The fraction of unbounded siRNA (i.e., free siRNA capable of moving into the gel) was determined in this assay, which was used to calculate the amount of siRNA participating in complex formation. This method is similar to quantitative dye binding assay based on SYBR Green [25], but actually measures complex formation directly rather than binding of a fluorescent probe to free sites on siRNA. As expected, increasing the polymer:siRNA ratio during complex formation resulted in an increase in siRNA binding for all polymers (**Figure 3.1**). The binding curves typically followed a sigmoidal curve for most polymers, except a few linear curves obtained for some polymers (e.g., PEI2-LA20 in **Figure 3.1D**). The linear curves were usually the case for polymers with lower capacity for siRNA binding. The PEI25 and PEI2 typically yielded the most binding at a given polymer:siRNA ratio as compared to lipid substituted equivalents, indicating a lowering of binding efficiency after lipid substitutions. Based on the generated curve fits, BC_{50} values were determined as a relative measure of the siRNA binding efficiency. The PEI2 and PEI25 had the lowest BC_{50} values among the polymers (0.07 and 0.09, respectively), and all lipid-substituted polymers displayed a BC_{50} value higher than the native PEIs (**Figure 3.2**). For some lipids (PA and OA), a general trend of increasing BC_{50} with increasing lipid substitution was clearly evident, but not all lipids (CA and StA) gave such a clear trend.

A more general relationship between the degree of lipid substitution and BC_{50} values was explored based on the correlation coefficient between BC_{50} and the extent of lipid substitution for all polymers. The obtained linear regression coefficient (r^2~0.2024; dashed line in **Figure 3.2A**) indicated a relatively weak but a significant correlation ($p<0.05$) between the two variables. Since each type of lipid contained a differing number of lipid carbons, we also explored a correlation between the BC_{50} and the extent of lipid Cs substituted (see **Table 3.S1** for exact values of lipid Cs). The regression coefficient obtained was relatively higher (r^2~0.2828; dashed line in **Figure 3.2B**), again indicating a significant correlation ($p<0.01$) between these two variables.

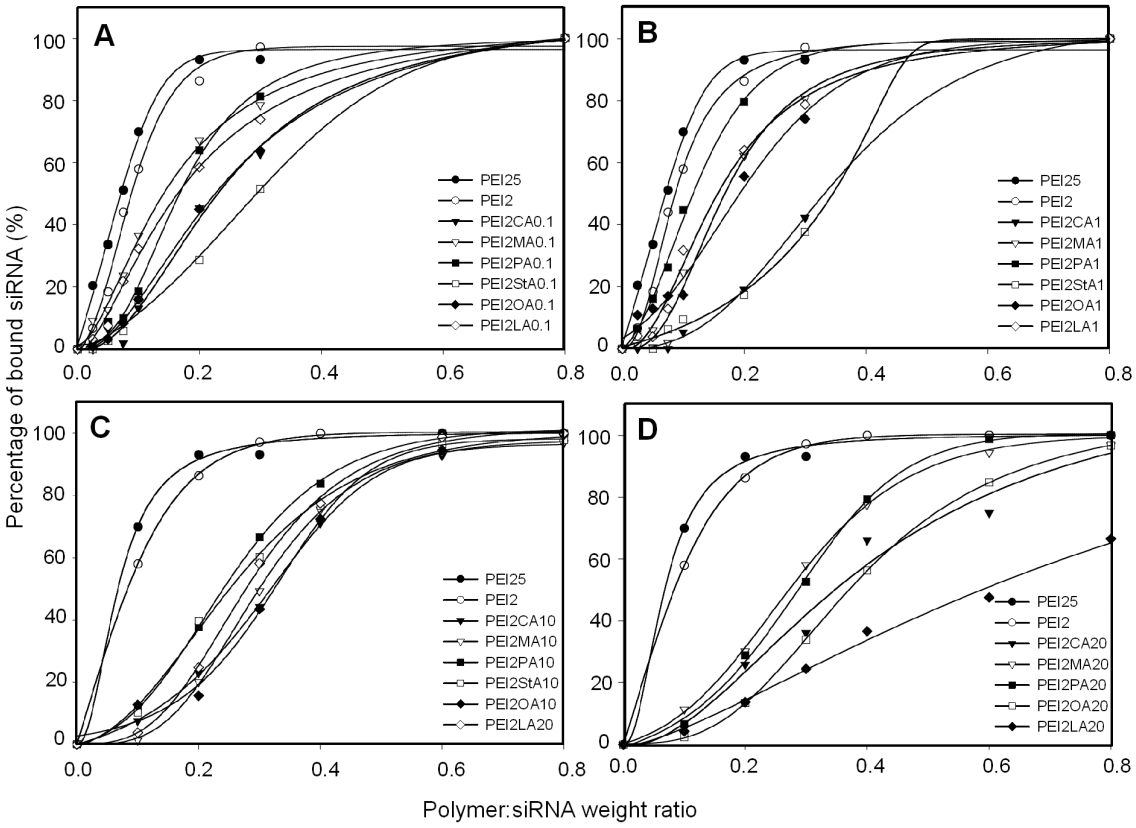


Figure 3.1 Binding of lipid-substituted polymers to siRNA.
Binding of lipid-substituted polymers to siRNA. Percentage of siRNA bound as a function of polymer:siRNA weight ratio in EMSA analysis. Polymers obtained from lipid:polymer feed ratios of 0.012, 0.066, 0.1 and 0.2 are shown in A, B, C and D, respectively. *Figure courtesy of O. Suwantong.*

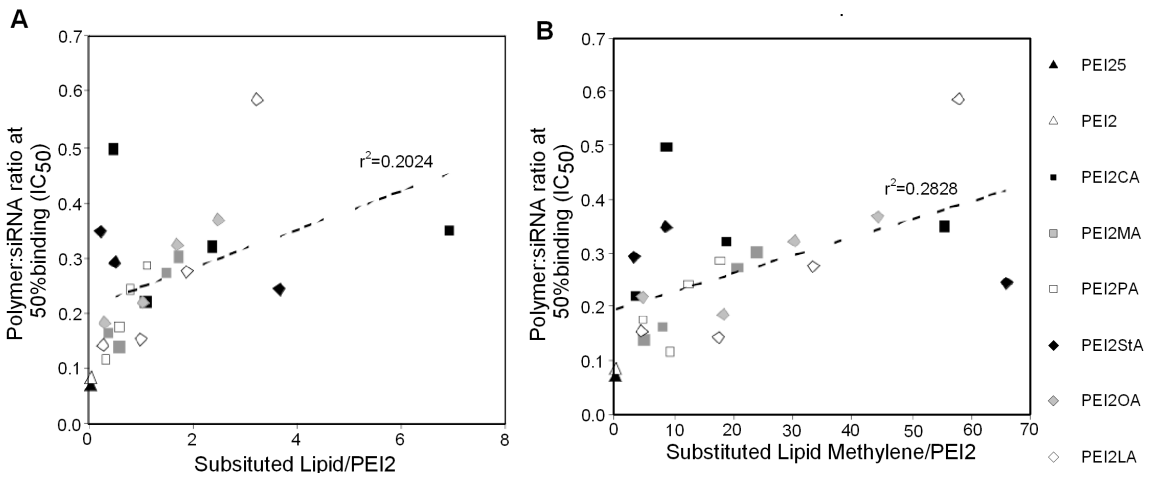


Figure 3.2 Correlations Between Polymer Binding Affinity IC50 and Extent of Lipid Substitutions.

IC50 is shown as a function of number of lipids substituted (**A**) or number of lipid methylenes substituted (**B**). As lipid substitution is increased, the binding affinity (given by IC50) decreased. Statistical analysis was determined by Student's t-test ($p<0.05$). *Figure courtesy of O. Suwantong.*

TEM imaging for the complexes with native PEIs (PEI25 and PEI2) and representative CA, PA, OA and LA substituted PEI2 are summarized in **Figure 3.3**. Distinct complexes were observed in most cases, but some polymers (PEI2 and PEI2-PA) gave aggregated particles where smaller spherical particles appeared to fuse together. Fusing of particles in TEM images have been seen in other studies as well [40], which was likely due to drying during the sample preparation. Most complexes appeared relatively homogenous (similar contrast throughout the complex) with the exception of PEI2-OA, where spherical particles appeared to be multiphasic. The size of individual complexes were typically <100 nm, with PEI2-OA particles being notably larger (>200 nm). Directly comparable images of TEM complexes, such as PEI25/siRNA or PEI2/siRNA complexes, are not available in the literature; however, TEM imaging of PEI25/plasmid DNA complexes were reported to be larger than our PEI25/siRNA

complexes [41], consistent with larger size of plasmid DNAs used to assemble the particles.

In order to minimize the scale of further experiments, the lowest substitution for each polymer was excluded from the experiments as they are expected to be behave the least different from the unmodified PEI2. Additionally, MA- and StA-substituted polymers were excluded, as these substitutes did not appear to be unique in the extent of substitutions and the siRNA binding studies.

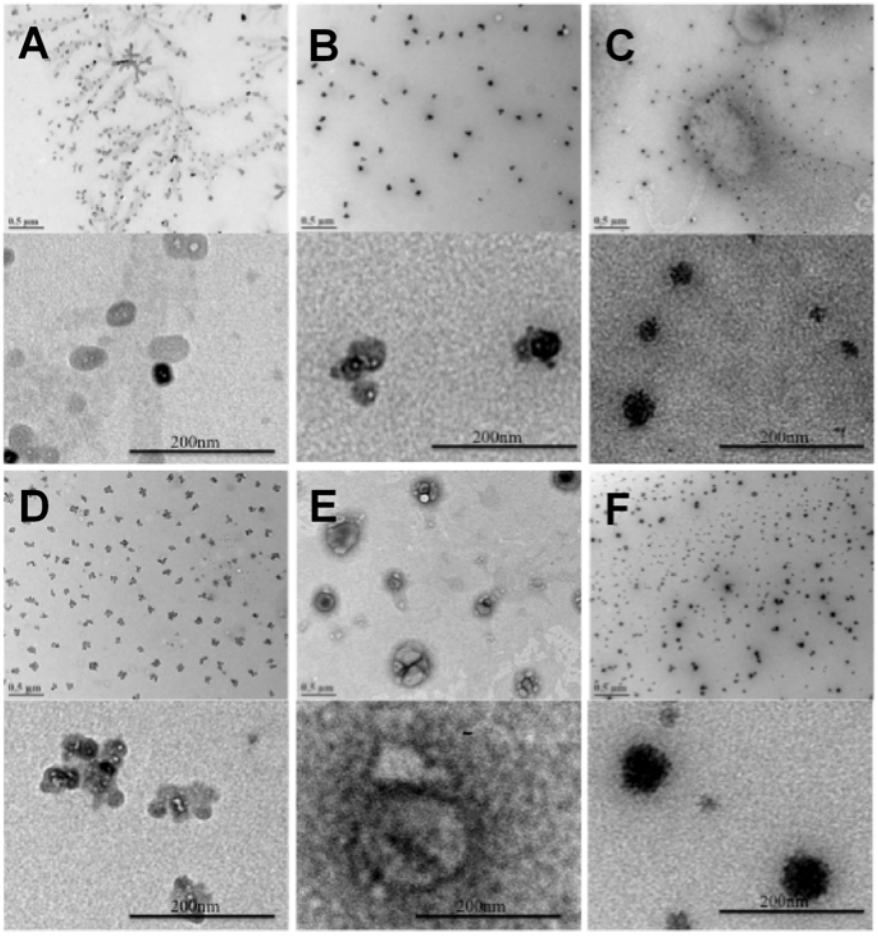


Figure 3.3 Morphology of Polymer/siRNA Complexes Imaged by TEM.
(A) PEI25 complexes, (**B**) PEI2 complexes, (**C**) PEI2-CA20 complexes, (**D**) PEI2-PA20 complexes, (**E**) PEI2-OA20 complexes, (**F**) PEI2-LA20 complexes. All complexes were prepared at an 8:1 polymer:siRNA weight ratio. Scale bar in the high magnification images indicates 200 nm.

3.3.2 Cytotoxicity of Polymeric Carriers

It is well established that the high MW PEI25 generally displays high cytotoxicity in contact with cells, whereas low MW PEIs display minimal cytotoxic effects [29-34]. High MW polymers were suggested to be more effective in creating membrane invaginations and/or pores, which is desirable for siRNA delivery, but this also causes more harm on the cells by disrupting membrane integrity [42]. While lipid-substitution is intended to increase membrane affinity of polymers, our lipid-modified polymers are expected to expose the cells to lipid concentrations of 1-10 μM, assuming a polymer concentration of ~6 mg/mL in contact with the cells (practical concentration used for siRNA delivery) and average lipid substitutions of ~3 lipids/PEI2. The lipids are naturally occurring molecules and palmitic, oleic and linoleic acids are present in plasma membranes. Additionally, the toxicity of lipids on leukemia cell lines have been investigated; tolerable concentration of lipids in Jurkat (human T leukemia) and Raji (human B leukemia) cell lines depended on the specific lipids, but all concerned lipids were tolerable at ~50 μM [43], a value much higher than lipid concentrations to be exposed to cells with our carriers.

The cytotoxicity of the complexes on AML cells is summarized in **Figure 3.4**. As expected, PEI25 displayed an obvious, concentration-dependent toxicity in all cells. The PEI2 displayed minimal toxicity that was evident only in HL-60 cells (see **Table 3.S2** for detailed analysis of cytotoxicity trends). For lipid-substituted PEI2, a concentration dependent loss of viability was evident for some cell-polymer pairs, but other polymers did not display toxicity in the investigated concentration range. However, cytotoxicity of the PEI2 and PEI2-lipids did vary among the cell lines. For THP-1 cells, minimal toxicity

was observed with CA and PA substitutions, while OA and LA substituted PEI2 gave a significant decrease in cell viability. In KG-1 cells, minimal, if any, decrease in cell viability was seen with CA, PA and OA substitutions, while a slight increase in cytotoxicity was seen with LA substituted PEI2. For HL-60 cells, PEI2 substitutions with CA and PA displayed no changes in cytotoxicity, but OA and LA substitutions displayed a small negative effect on cell viability. Taken together, LA was the only lipid substituent that clearly increased the cytotoxicity of the polymers in all three cell lines. This was presumably due to better interaction of this type of polymer with these AML cells.

Since a major concern of polymeric carriers is the dose-dependent cytotoxicity [44], as obviously manifested with the PEI25, it is notable that our carriers did not display definitive dose-response curves in investigated cell lines. Relatively low cytotoxicity is the likely reason for the lack of clear dose-response curve. We previously noted that lipid substitution generally increased the toxicity of PEI2 on anchorage dependent bone marrow stromal cells [38] and breast cancer cell line MDA-435 [25]. To further explore this issue with leukemic cells, a correlation between the lipid substitution and the resulting cytotoxicity was explored as in the binding studies (**Table 3.S3**). Very few obvious correlations occurred in this analysis; the strongest trends were seen at the highest polymer concentrations of 10 μg/ml where high r^2 and significant slopes (p values) were observed for CA- and PA-substitutions in KG-1 cell lines. Again, relatively low cytotoxicity in the working range did not allow for a strong correlation and toxicity at higher concentrations was not explored since this is not the practical range for siRNA delivery.

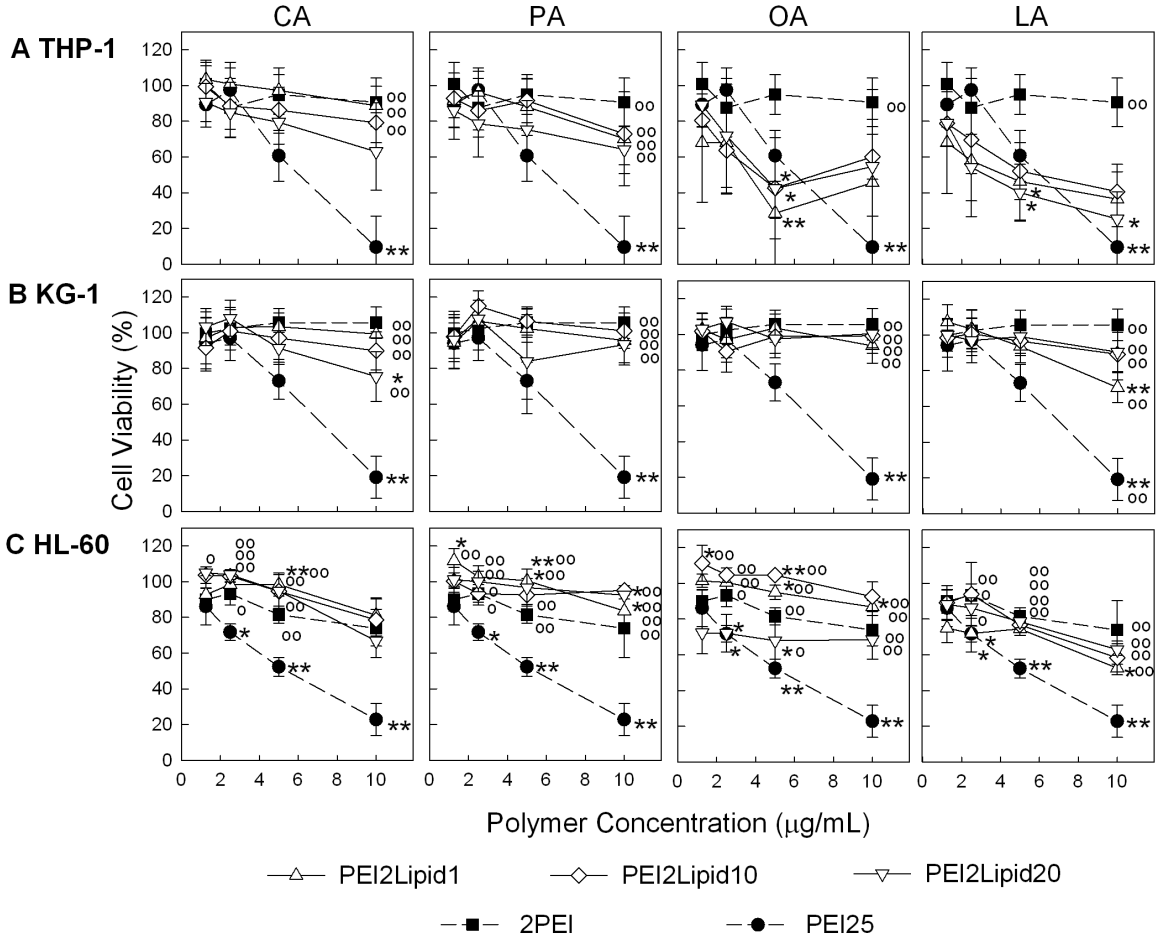


Figure 3.4 Cytotoxicity of Complexes in Leukemia Cells
Complex cytotoxicity in THP-1, KG-1 and HL-60 cells (top, middle and bottom panel, respectively) where cell viability values are expressed relative to no-treatment control. Viability was measured 24 h after incubation of complexes with cell lines. PEI25 displays a linear relationship with increased cytotoxicity in line with increasing concentration. Effect of lipid-substituted polymers on cell viability was similar to that of unmodified PEI2. * : $p<0.05$, ** : $p<0.01$, as compared to PEI2 and ^{o} : $p<0.05$, ^{oo} : $p<0.01$ as compared to PEI25, using one-way ANOVA tests with Dunnett post test.

3.3.3 siRNA Delivery with PA-substituted PEI2 to THP-1 Cells

Initial siRNA delivery studies were performed with THP-1 cells and by using PEI2-PA as a prototypical carrier. Two concentrations of siRNA complexes (corresponding to 36 and 72 nM siRNA and, 4 and 8 μg/mL polymer) were used in this study, as well as an unlabeled siRNA (CsiRNA) as a control to account for the possibility

of autofluorescence displayed with certain carriers [45]. The percentage of cells with siRNA uptake and the mean siRNA fluorescence in cells are summarized in **Figure 3.5A**. Exposure of the cells to CsiRNA did not indicate significant autofluorescence at the low concentration, but a significant autofluorescence was evident at the high polymer concentration (8 μg/mL) with PEI2-PA. The PEI2-PA was successful in delivering siRNA to majority of the cells (>50%) at both doses and also demonstrated higher delivery than PEI25, which gave a lower percentage of siRNA-positive cells (<16%) that decreased at higher complex dose. This decrease was likely due to high toxicity of PEI25 at the 8 μg/mL used in this experiment. The low effectiveness of PEI25 was unlike most siRNA delivery studies reported in the literature that typically employed anchorage-dependent cells, such as human breast cancer cells [25, 35, 46], mouse albino neuroblastoma cells [27], human ovary cells [28], human prostate carcinoma cells [46], human cervical cancer cells [26], and mouse glioblastoma cells [47]. The ineffectiveness of PEI25 in haematopoietic cell lines was previously noted for delivery of plasmid DNA [48], which found PEI2 to be superior to PEI25. Unlike the study on the plasmid DNA delivery, the PEI2 was not effective in our hands for siRNA delivery.

To further compare the relative efficiency of PEI25, PEI2 and PEI2-PA, siRNA delivery was explored as a function of polymer:siRNA ratio (**Figure 3.5B**) and seeded cell density (**Figure 3.5C**). As before, PEI2 was not effective under all investigated conditions. While PEI25 was more effective at lower ratio (4:1), PEI2-PA was more effective at higher ratios (8:1 and 12:1), indicating the polymer:siRNA ratio to be critical for uptake. As the cell concentration was increased, the percentage of siRNA-associated cells remained the same, but the mean fluorescence/cell was decreased (**Figure 3.5C**),

indicating less siRNA uptake/cell at higher cell concentrations. Finally, confocal microscopic analysis of the siRNA uptake confirmed the quantitative results obtained. Distinct cell-associated complexes were clearly seen with PEI2-PA, but not with PEI25 and PEI2 (**Figure 3.5D**). It was therefore clear that the lipid substitution on PEI2 (PA in this case) mediated improved delivery of siRNA to the leukemic cells.

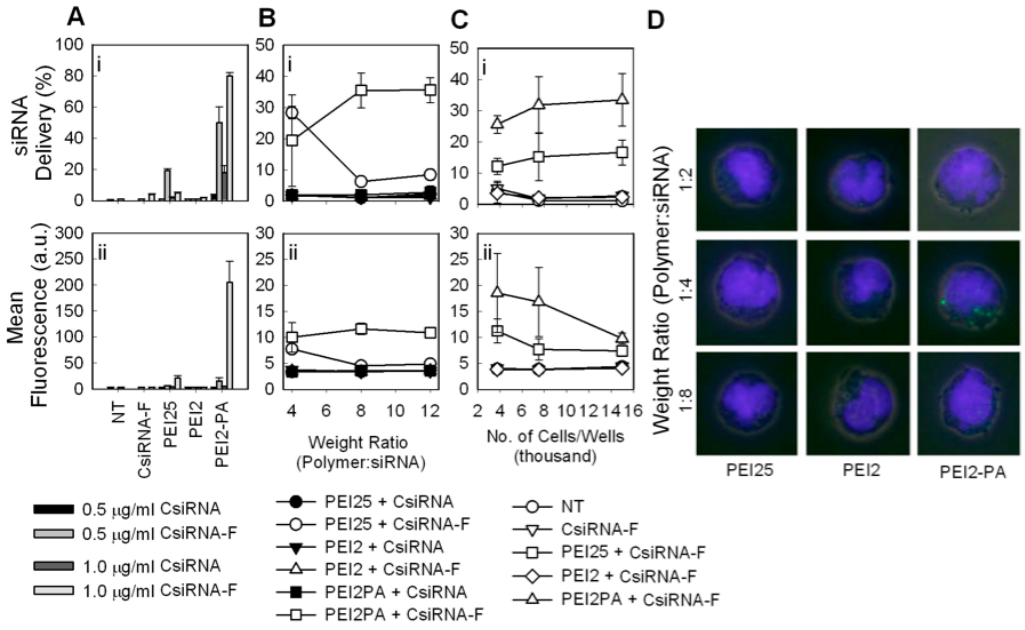
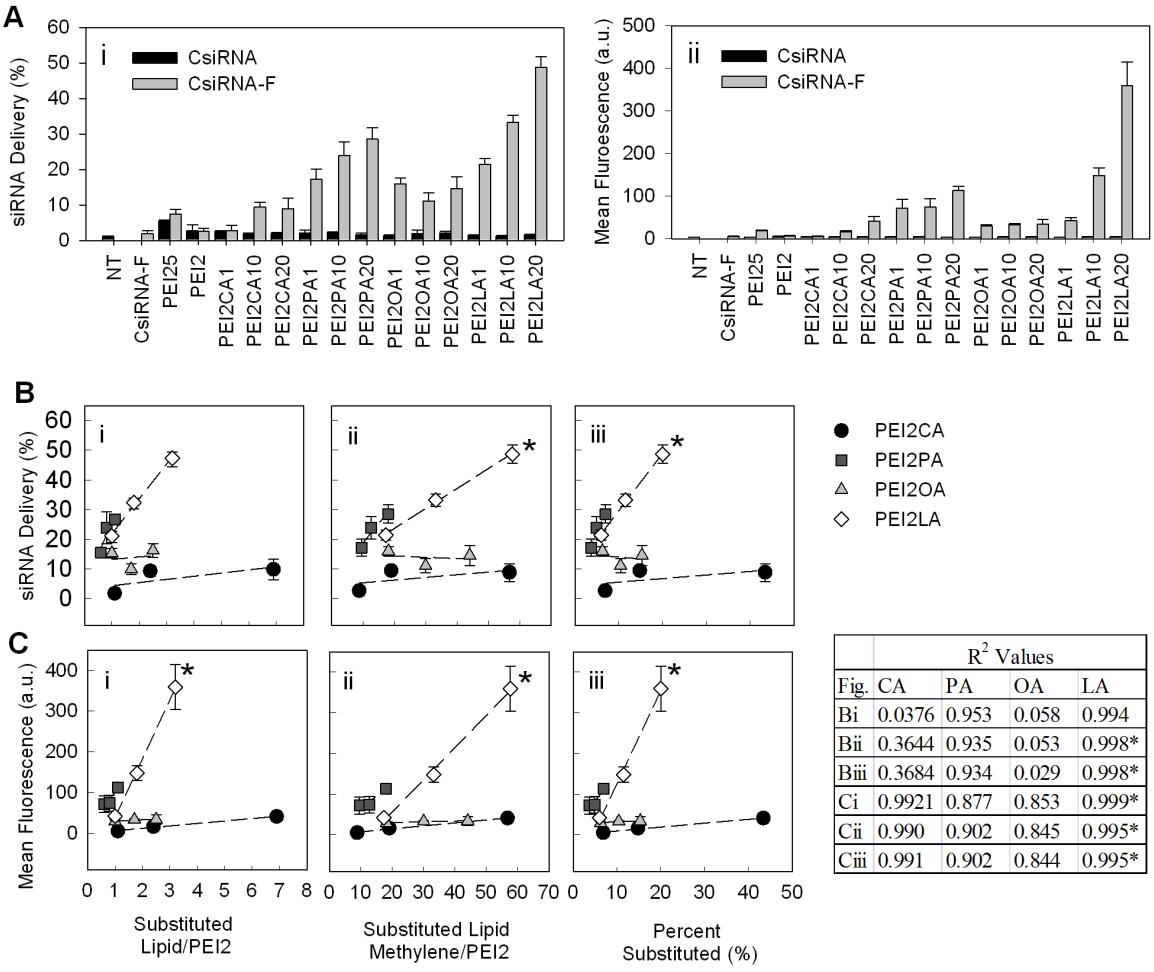


Figure 3.5 siRNA Delivery to THP-1 Cells.
Complex formulation studies were performed by (**A**) varying complex dose (0.5 and 1.0 μg/mL siRNA). (**B**) varying polymer:siRNA weight ratio. (**C**) varying initial cell number (0.35 μg/mL siRNA). The results are summarized as (i) percentage of siRNA-associated cells in cell population, and (ii) mean fluorescence of cells due to complex association. (**D**) confocal microscope images of individual cells (0.5 μg/mL siRNA). Hoechst stained nucleus in blue and FAM-labelled siRNA-polymer complexes in green. Polymer:siRNA ratios were 2:1, 4:1 and 8:1 from top to bottom panel.

3.3.4 Effect of Lipid Substitution on siRNA Delivery

To develop a broader understanding of the role lipid substitution on siRNA delivery, PEI2 substituted with CA, PA, OA and LA were used to evaluate siRNA delivery to THP-1 cells (**Figure 3.6**). The siRNA delivery varied significantly among the

polymers, whether it was assessed by the siRNA-positive cell population (**Figure 3.6Ai**) or the mean siRNA level per cell (**Figure 3.6Aii**). Based on these two parameters, siRNA delivery was correlated to the number of lipids substituted/PEI2 (**Figure 3.6Bi** and **3.6Ci**), the number of lipid methylenes/PEI2 (**Figure 3.6Bii** and **3.6Cii**) and percentage of lipid substitution (**Figure 3.6Biii** and **3.6Ciii**). As shown in the table in **Figure 3.6**, strong correlations (see r^2 values listed in the figures) were observed with PA and LA substitution in all cases. These positive correlations were indicative of lipid substitutions to be directly responsible for intracellular siRNA delivery. Among the polymers, PEI2-LA polymers appeared to be most effective, based on the strong correlations between siRNA delivery and LA substitutions, as well as absolute levels of siRNA delivery per cell. It was also clear that the enhanced delivery was dependent on the individual lipid, as the explored correlations failed if all lipid-substituted polymers were considered together. This was unlike the case with the binding studies where the correlation was valid with all lipids, indicating a similar role of lipids on the siRNA binding, but significantly different roles in delivering the siRNA to the cells.



	R^2 Values			
Fig.	CA	PA	OA	LA
Bi	0.0376	0.953	0.058	0.994
Bii	0.3644	0.935	0.053	0.998*
Biii	0.3684	0.934	0.029	0.998*
Ci	0.9921	0.877	0.853	0.999*
Cii	0.990	0.902	0.845	0.995*
Ciii	0.991	0.902	0.844	0.995*

Figure 3.6 Effect of Lipid Substitution on siRNA Delivery to THP-1 Cells.
Polymer:siRNA ratio was 8:1 and siRNA concentration was 25 nM (0.35 µg/mL). (**A**) siRNA delivery percentage (percentage of cells with complexes, i) and mean fluorescence (mean fluorescence of cells due to fluorescence labelled siRNA-polymer complexes, ii). (**B**) Correlations between siRNA delivery percentage and lipid substitution. (**C**) Correlations between mean fluorescence and lipid substitution. Very strong positive correlations (r^2 values) are seen with PA and LA regardless how the lipid substitution is expressed (no of lipid per PEI2, no of lipid methyl carbons per PEI2 or percentage of PEI2 amines substituted) and with both siRNA delivery and mean fluorescence. Strong correlation (r^2 value) is seen with CA when considering mean fluorescence. * indicates where the slope is statistically significant.

To determine the extent at which the complexes are internalized as opposed to remaining surface-bound, a time course of complex uptake was performed (**Figure 3.7**). The polymer that provided the highest siRNA delivery, PEI2LA, was used for this purpose in THP-1 cells. Trypan blue staining was employed to differentiate between

surface bound and internalized complexes, since it quenches the fluorescence of surface-bound complexes [49], as well as incubation at 4 °C since it prevents active complex internalization. A gradual increase in cell-associated siRNA was evident in the first 24 hours for the cells incubated at 37 °C (**Figure 3.7A**), where ~80% of the cells became siRNA-positive (**Figure 3.7Aii**). For cells incubated at 4 °C, no further increase in cell-associated siRNA was evident after the initial binding at 1 hour (**Figure 3.7Ai**). With trypan blue quenching, a continuous increase in siRNA uptake was evident at 37 °C, but not at 4 °C (**Figure 3.7Bi**). This was consistent with abolished active uptake at the latter temperature. Whereas the proportion of siRNA-positive cells gradually increased to ~42% at 37 °C, this value remained <7% for 4 °C incubated cells during the 24-hour study period (**Figure 3.7Bii**). We note that the fluorescence levels obtained by trypan blue treatment was greatly diminished, consistent with results reported by an independent group [49]. As trypan blue coats the surface of the cells, it is likely that it decreased the excitation of the internalized fluorescent complexes within the cells.

Although the beneficial effect of lipid modification of carriers in cellular delivery of nucleic acids is established [50], the mechanism(s) by which they due so remains ill-defined. It has been suggested that the lipid modifications may elicit specific biological responses in interacting with cellular membrane, facilitating uptake and intracellular transport [50]. From a physical perspective, membrane phospholipids, consisting of various combinations of lipids, significantly contribute to the membrane stability, permeability and fluidity. Saturated lipids such as CA (C8) and PA (C16) are linear and allow tighter membrane packing leading to decreased fluidity and permeability, whereas unsaturated lipids (one double bond in OA (C18:1) and two double bonds in LA (C18:2))

introduces non-linear chains and disorder (and fluidity) into membranes [51]. Therefore, it is not surprising that the LA-substituted complexes will increase the membrane fluidity the most and display the highest uptake. The composition of lipids in the cellular membrane is another possible cause of variations in delivery. In an analysis of lipid composition in AML cells, the weight percentage of PA, OA and LA were 20.8±1.2%, 15.9±2.2% and 7.0±1.0% respectively (values equivalent to healthy controls [52]). Similar percentages were also reported in Jurkat (T-lymphocyte), Raji (B-lymphocyte) K562 cells, and foetal calf serum [43, 53]. LA content seems to be significantly less than the PA and OA contents. After incubation in LA-supplemented medium, the LA content of cellular membranes can be increased extensively (~20 times) [53]. It is conceivable that lipids that are present at lower concentrations originally (i.e., LA) would be taken up and incorporated in the cellular membrane to a greater degree. Thus, in order to investigate the effect of specific lipid uptake into the cellular membrane, cells were incubated with the free LA (0-100 μM) for 24 h hours followed by siRNA uptake for 24 h. The exposure of cells to LA prior to adding the complexes did not effect siRNA delivery percentages, regardless of using polymer/siRNA complexes at 2:1 or 8:1 polymer:siRNA (**Figure 3.S2A)**. Additionally, incubation of LA, OA and StA (0-50 μM) with simultaneous siRNA complex (PEI2-LA, PEI2 and PEI25) treatment did not influence the extent of siRNA delivery (**Figure 3.S2B**). These results suggested that free lipids did not affect the uptake of the complexes, so that specific uptake was likely not the reason for increased uptake of LA-containing complexes.

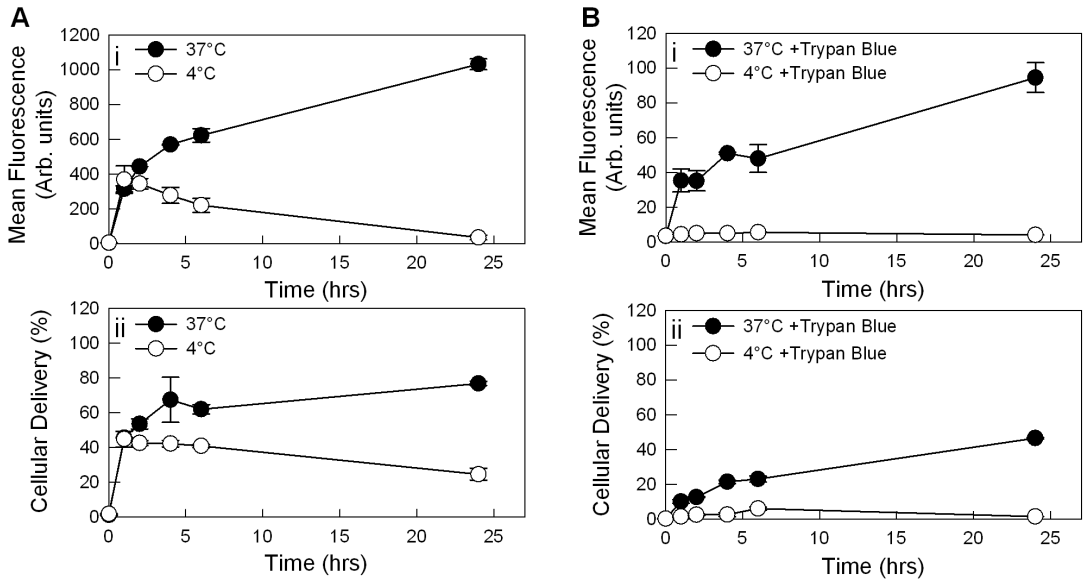


Figure 3.7 Effect of Temperature (4 and 37 °C) and Trypan Blue Treatment on siRNA Delivery to THP-1 cells.

PEI2-LA (2.1 LA/PEI2) was used in this study with polymer:siRNA ratio of 8:1 and final siRNA concentration of 25 nM (0.35 μg/mL). (**A**) siRNA delivery based on mean FAM fluorescence (i) and FAM-siRNA positive cell population (ii) with untreated cells (**B**) siRNA delivery based on mean FAM fluorescence (i) and FAM-siRNA positive cell population (ii) with cells treated with trypan blue.

3.3.5 siRNA Delivery to Other AML cells

Two other AML cell lines, the acute myelogenous leukemia (M1) KG-1 cells and the acute promyelocytic leukemia (M2) HL-60 cells, were next tested for siRNA delivery with the modified PEI2s, along with the THP-1 cells (**Figure 3.8**). Of the cell lines studied, KG-1 (M1) is the least differentiated (myeloblast), HL-60 (M2) is in the early stages of differentiation (promyeloblast) and THP-1 (M5) is the most differentiated (monocyte). As classification is dependent on differentiation stage, the cells vary in expression of the differentiation markers CD11b and CD14 (monocytic markers) and CD33, CD13, CD65s, CD15/15s (myeloid markers), as summarized in [54]. The siRNA delivery was investigated at polymer:siRNA ratios of 2:1, 4:1 and 8:1. Various lipid

substitutions were successful for siRNA delivery to THP-1 cells, given by the large increases in siRNA delivery after lipid substitution on PEI2 (**Figure 3.8A**).

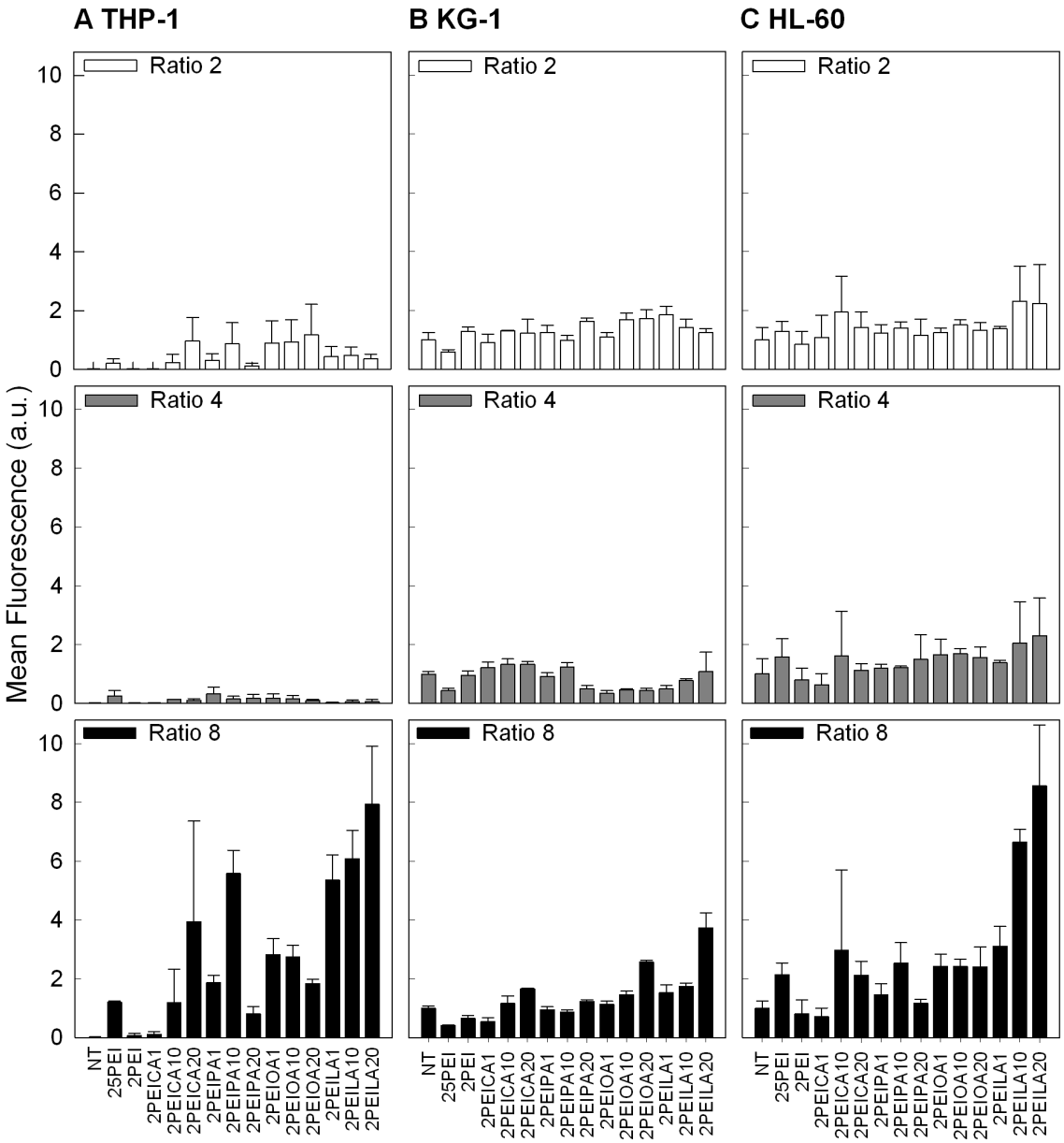


Figure 3.8 siRNA Delivery to THP-1, KG-1 and HL-60 Cells at Various Polymer:siRNA Weight Ratios.

(A) THP-1 (**B**) KG-1, and (**C**) HL-60 cells. Fluorescence intensity refers to the mean fluorescence of the cell population. Non-labeled control siRNAs did not show any significant fluorescence (autofluorescence) and were removed for figure clarity. siRNA concentration was 25 nM (0.35 μg/mL) and polymer:siRNA weight ratios were 2:1 (top panel), 4:1 (middle panel) and 8:1 (bottom panel).

In the KG-1 and HL-60 cells, LA-substituted PEI2 was again the most effective (**Figure 3.8B** and **3.8C**). Of the three different polymer:siRNA ratios tested, the 8:1 ratio consistently gave the best delivery. This was attributed to higher cationic charge of the complexes formed at higher polymer:siRNA ratios [24], which should facilitate better binding to anionic cell surfaces. The PEI25 and PEI2 did not appear to be an effective delivery agent for the KG-1 and HL-60 cells either, confirming our previous observation with THP-1 cells. It was also evident that the cells displayed differing propensity to uptake polymer/siRNA complexes; whereas THP-1 appeared to be most readily display siRNA uptake, KG-1 cells displayed the least uptake. This was expected since KG-1 being the least differentiated phenotype (leukemic progenitor cell) with minimal endocytic activity and smaller size (12-16 mm) and THP-1 being the most differentiated with larger size (15-20 mm) [55]. It will be important to further explore the molecular basis of this observation, since it might be an indicative of patient-to-patient variations in siRNA delivery.

Time-dependent siRNA delivery was then assessed by utilising only the polymers with the highest substitution levels at the best performing ratio (8:1) and exposing the complexes to cells for a period of up to 48 hours (**Figure 3.9**). The highest siRNA delivery was obtained at the earliest time point (6 h). As time increased, the percentage of cells associated with complexes and the mean fluorescence/cell was decreased. The mean fluorescence levels declined at a faster rate than the percentage of siRNA-positive cells. The decline was most likely a result of rapid cell proliferation, as the doubling-rate of THP-1 cells is ~26 h and KG-1 and HL-60 cells being slightly longer. In such a case, a rapid drop in the levels of siRNA concentrations is expected, whereas the percentage of

siRNA-positive cells might not change drastically. The latter will be seen for cells where the siRNA amounts were lower (i.e., at the detection threshold). As the variation of mean fluorescence (given by standard deviations in **Figure 3.9**) did not increase with time, it is reasonable to assume that the splitting of complexes between the dividing cells were fairly even with internalized siRNA not particularly restricted to mother or daughter cells. Even with the prolonged time of analysis, both PEI25 and PEI2 did not yield a significant siRNA delivery, based on the mean siRNA fluorescence associated with the cell population (**Figure 3.9ii**). Collectively, these results indicated that the uptake of the siRNA complexes was relatively rapid (<6 h) and prolonged incubation with complexes did not yield a 'net' accumulation of siRNA inside cells.

As the interaction with serum proteins can affect delivery of siRNA complexes, we explored the effect of serum on siRNA delivery, utilizing the best performing polymer PEI2-LA (**Figure 3.10**). At low serum percentage of 2.5%, PEI25 displayed comparable delivery percentage to PEI2-LA, although the siRNA delivery/cell remained low as usual for this polymer. However, as the serum concentration was increased, the delivery ability of PEI25 decreased to a greater degree than the PEI2-LA in all cell lines. In THP-1 cells, the effect of serum on PEI2-LA delivery was evident even at low concentration (i.e., from 2.5 to 10%), whereas delivery was largely unaffected up to 25% serum in the KG-1 and HL-60 cells. Although, the polymer-serum interactions were expected to produce similar results in all cell lines as the serum percentage and polymer remain the same, properties that promote of uptake, can be dependent on the cell type. The ability to deliver siRNA in serum is clearly important especially for leukemia cells, however similar uptake studies in the presence of serum have not been reported in literature for leukemic cells. Clearly

PEI25 was affected by the serum proteins to a much greater extent than the lipid-modified PEI2. We previously found that lipid substituted PEIs afforded better protection against degradation in the presence of serum [40]. This observation along with the uptake results suggest that the lipid modification decreased interaction of complexed nucleic acids with serum proteins, so that their delivery ability is less affected by high serum concentrations.

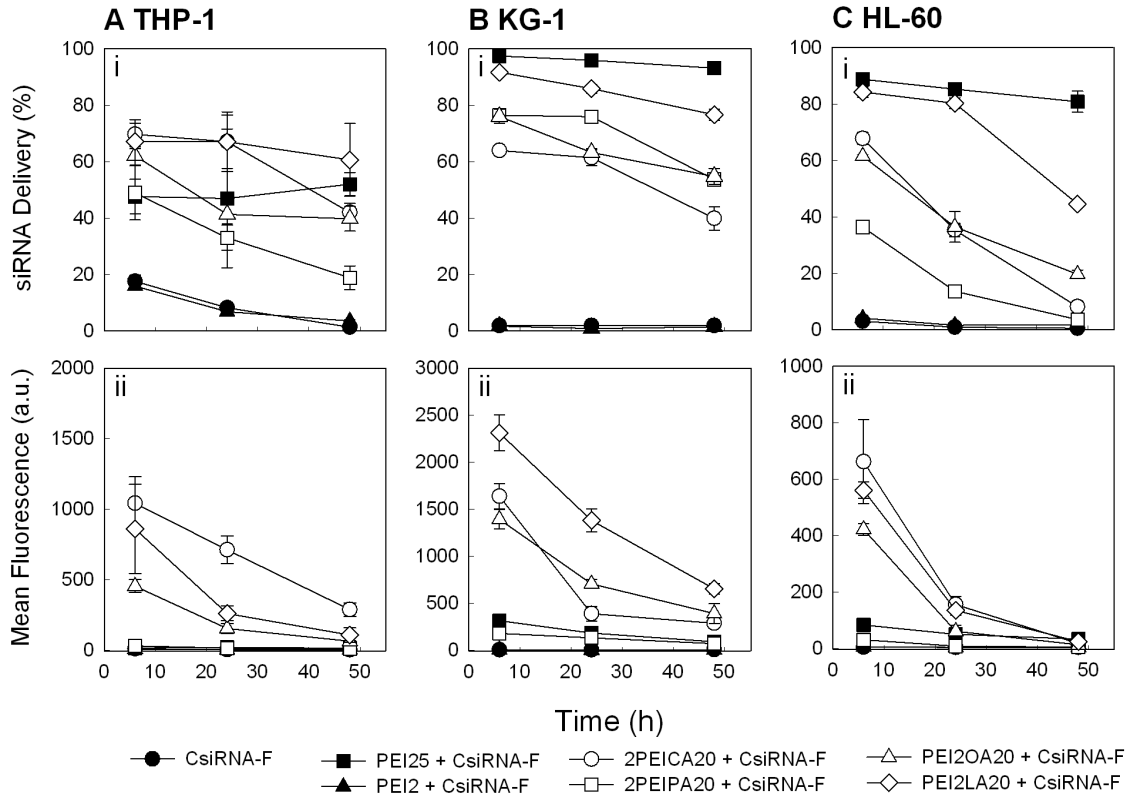


Figure 3.9 siRNA Delivery to THP-1, KG-1 and HL-60 cells at Various Time Points.
(**A**) THP-1 (**B**) KG-1, and (**C**) HL-60 cells. Non-labeled control siRNAs did not show any significant fluorescence (autofluorescence) and were removed for figure clarity. siRNA concentration was 25 nM (35 µg/mL) and polymer:siRNA weight ratio was 8:1. The results are summarized as percentage of siRNA positive cells (top panel), mean fluorescence per cell (bottom panel).

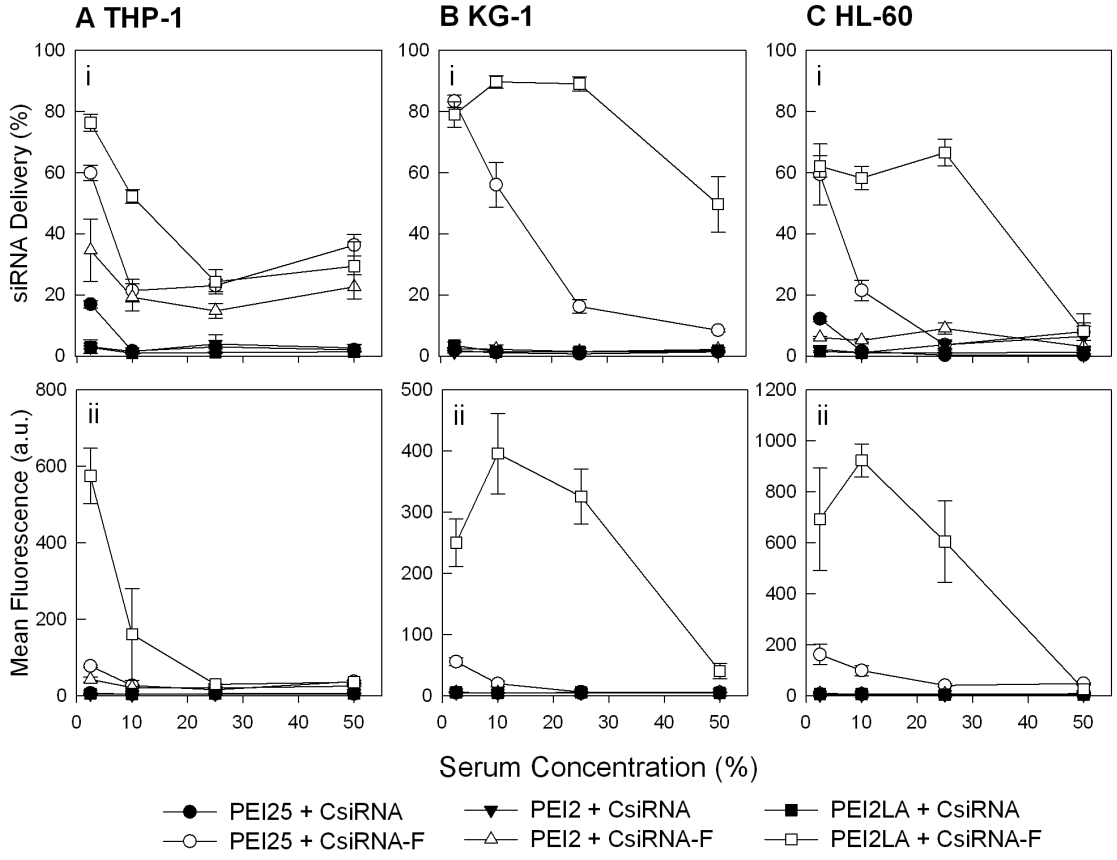


Figure 3.10 Effect of Serum on siRNA/Polymer Complex Delivery to THP-1, KG-1 and HL-60 cells.

(**A**) THP-1 (**B**) KG-1, and (**C**) HL-60 cells. siRNA concentration was 25 nM (35 µg/mL) and polymer:siRNA weight ratio was 8:1. The results are summarized as percentage of siRNA positive cells (top panel) and mean fluorescence per cell (bottom panel).

3.3.6 Comparison of siRNA Delivery with Commercial Reagents

The best performing polymeric carrier, PEI2-LA, was next compared to several commercial reagents for siRNA delivery to AML cells. The commercial reagents were chosen based on their previous use for siRNA delivery to leukemic cells (see Introduction). All of the commercial reagents were cationic liposomes, since no polymeric carrier was utilized in leukemic cells previously. The siRNA delivery was investigated at the polymer:siRNA ratios of 8:1 and 4:1, but the results from the 8:1 ratio

is shown only since the results from the 4:1 ratio also gave equivalent outcomes. The results were summarized as either the percentage of siRNA-positive cells (**Figure 3.11A**), or the extent of siRNA delivery per cell (**Figure 3.11B**). The siRNA delivery by the chosen reagents varied significantly, depending on the cell line. Fugene HD, PEI25, Metafectamine and PEI2-LA were the carriers with the highest delivery percentages (>80%; **Figure 3.11A**), whereas Lipofectamine^{TM} 2000, RNAiMAX, DOTAP and HiPerFect displayed variable results depending on the cell line. Based on the mean fluorescence levels, Metafectamine, Lipofectamine^{TM} 2000 and PEI2-LA were the top three carriers (**Figure 3.11B**), but RNAi-mate and DOTAP did not demonstrate significant delivery at all. The polymeric PEI2-LA was among the top three carriers when ranked in both the mean siRNA fluorescence (**Figure 3.11C**, **top**) and the percentage of siRNA-positive cell population (**Figure 3.11C**, **bottom**). The ranking was not consistent with the two parameters assessed, indicating that the individual carriers behaved differently in the extent of modification and the mean siRNA delivered into each cell type. We noted a significant variation in siRNA delivery among the three cell types for these different carriers, as noted in **Figure 3.9** as well.

There has not been any comparison of the efficiency of commercial reagents for siRNA delivery to leukemic cells, but it is clear that large differences in efficiency existed among these reagents. Lipofectamine^{TM} 2000, Metafectamine and Fugene HD seemed to be sufficiently effective when one wishes to employ an 'off-the-shelf' siRNA delivery system. In performing this analysis, we attempted to follow the manufacturer's recommendations for optimal reagent:siRNA ratio, and employed a single complexation buffer for all reagents. It is likely that the efficiency for some reagents may be improved

with further optimization of complexation conditions, however such an effort was not spent in this study due to extensive numbers of variables that can be optimized. Our main goal was to identify a few obviously effective commercial reagents and to compare our polymeric carriers to these reagents in silencing studies (below).

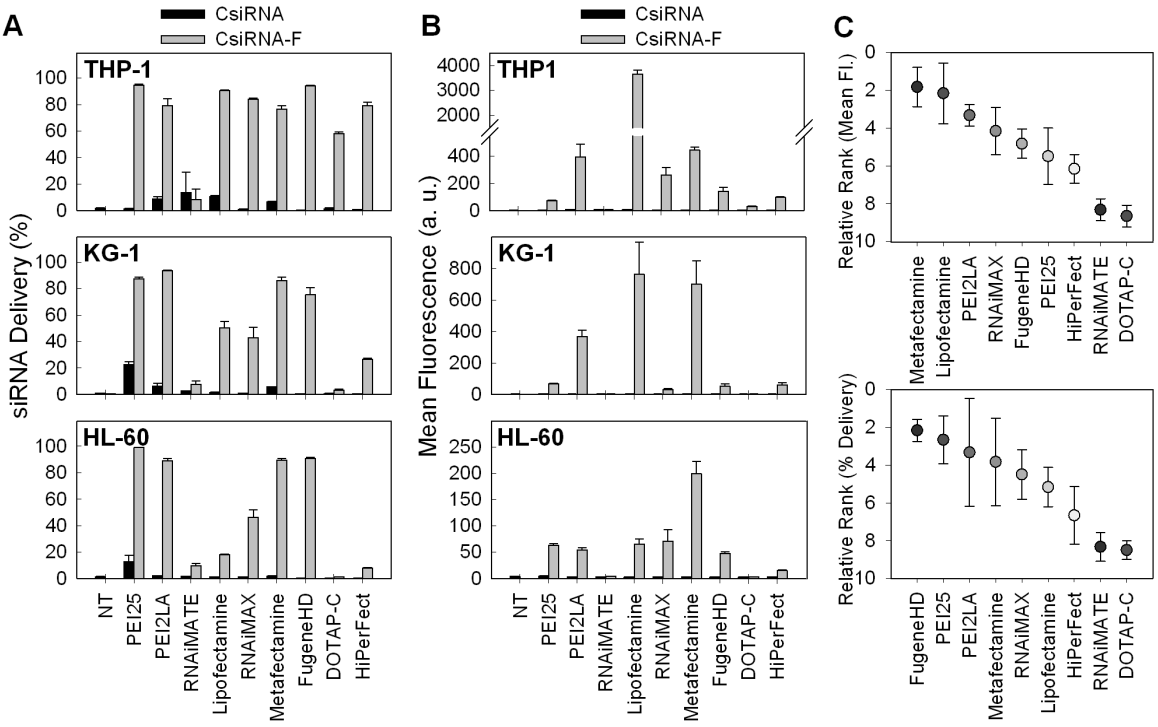


Figure 3.11 siRNA Delivery to THP-1, KG-1 and HL-60 Cells by Commercial Reagents
siRNA delivery after 24 hours was expressed as the siRNA-positive cell population (**A**) or mean fluorescence of cell population (**B**). (**C**) Relative ranking of various reagents based on mean fluorescence (top panel) or percentage of siRNA-positive cell population (bottom panel). Results from all three cell types were pooled for the ranking.

3.3.7 Silencing of Reporter (GFP) Gene Expression

To explore the silencing efficiency of the developed polymers, GFP-expressing THP-1 cells were used as a model system. The reduction in GFP expression was expressed as either a percent decrease in mean GFP fluorescence or percent decrease in

GFP-positive cells (**Figure 3.12A**). Note that the extent of GFP expression in the modified THP-1 cells was typically 3 logs higher than the background in unmodified cells (unmodified cells appeared in first quadrant of the histograms; not shown). The GFP silencing was evident by a leftward shift in the histograms (see **Figure 3.12A**), but at no point complete GFP silencing was obtained in this study. The control siRNA employed led to minor (insignificant) changes in GFP fluorescence of the cells at times, so that the changes in GFP fluorescence was normalized against CsiRNA treated cells (as described in Methods). The initial study focussed on comparing the PEI25 to two of the lipid substituted polymers (PEI2-CA and PEI2-LA) with higher siRNA delivery efficiency to THP-1 cells (from **Figure 3.9**). At the three polymer:siRNA ratios tested (2:1, 4:1 and 8:1), both lipid substituted polymers gave a higher silencing activity than PEI25, whose silencing efficacy was not significant. Unlike the delivery results, where LA substitution gave the most delivery, CA substitution was generally more effective in GFP silencing, with 20-26% GFP silencing achieved after 3 days of siRNA delivery. A dose-response curve was then explored with the lipid-substituted polymers at 4:1 and 8:1 polymer:siRNA ratios (**Figure 3.12C**). When silencing was based on mean GFP fluorescence, a gradual increase in GFP silencing was evident for some groups between 25 and 100 nM siRNA concentration (e.g., for CA and LA substituted PEI2 at 8:1 ratio), but not beyond the 100 nM siRNA concentration. Such clear dose-response curves were not evident for silencing based on percentage of GFP-positive cells (**Figure 3.12C**).

The silencing efficiency of the polymeric carriers (PEI2-LA and PEI2-CA) and three effective commercial reagents (Lipofectamine^{TM} 2000, Metafectamine and Fugene HD) were compared next (**Figure 3.12D**). GFP silencing was assessed over a period of 9

days after a single siRNA delivery. Although PEI25 was also included in this experiment, only a small fraction of cells (<5%) survived on the long run so that it was omitted from the analysis. The siRNA delivery resulted in most significant GFP silencing on day 3 for all delivery systems, and silencing was typically lost by 9 days (**Figure 3.12D**). Lipofectamine^{TM} 2000 gave the highest silencing (~62% on day 3 based on mean GFP fluorescence), followed by the two lipid-substituted PEIs and Metafectamine (~40% on day 3). Similar conclusions were reached when GFP silencing was analysed based on the decrease in GFP-positive cells.

However, a major difference was seen in cell numbers analysed by the flow cytometry; (i) while LA substituted PEI2 did not give any long term adverse effects on cells (i.e., cell numbers were equivalent to no-treatment controls), CA substituted PEI2 gave lower cell numbers especially after day 3; (ii) Lipofectamine^{TM} 2000 in particular resulted in gradual loss of cell survival to levels <5% of the un-treated cells, indicating long terms adverse effects on the cells, and (iii) Metafectamine and Fugene HD gave intermediate effects on the cells, where the cell numbers typically remained at the ~50% level to that of no-treatment controls. The long-term adverse effects on cells are obviously not desirable for systemic administration of delivery systems due to undesirable effects on healthy cells. The difference in the toxicity of CA and LA substituted PEI2 was noteworthy in the silencing studies and was not apparent in the initial studies (see **Figure 3.4**). The silencing studies employed cell concentrations from flow cytometry as a measure of toxicity, whereas the initial toxicity studies investigated cell viability by the MTT. While the results from CA substitutions agreed with both methods, results with LA substitution did not agree between the two methods. This issue

requires further investigation but it appears that LA substitution seems to be more desirable for longer exposure to the cells. Despite effective silencing, the toxicity of Lipofectamine^{TM} 2000 was considered prohibitive for *in vitro* use (since aberrant cellular physiology could complicate the investigated silencing phenomena) as well as *in vivo* use (too toxic for non-target cells and tissues). Such high toxicities were not evident in previous studies employing this reagent [11-16], since these studies were more concerned with elucidating the biological roles of specific targets, rather than safety and efficacy of the delivery system.

To confirm whether the silencing observed with GFP-positive cells also reflected silencing at the mRNA level, GFP mRNA levels in treated THP-1 cells was quantitated by PCR. A significant decrease in mRNA levels was observed with PEI2-CA delivered siRNA on day 1 and 2, after which insignificant change was seen on day 3 (**Figure 3.13A**). The cells exposed to PEI25 and PEI2-LA delivered siRNA did not yield as significant silencing at the mRNA level (**Figure 3.13A**). As before, silencing was additionally confirmed based on changes in GFP-positive cells and mean GFP levels, especially with PEI2-CA (**Figure 3.13B** and **3.13C**, respectively). It appeared that flow-cytometric assessment of GFP silencing was more readily detectable as compared to PCR-based assessment, given large variations observed with the latter assay. However, both PCR and flow cytometric based evaluation of silencing suggested the CA-substituted polymers to be more effective in functional siRNA delivery.

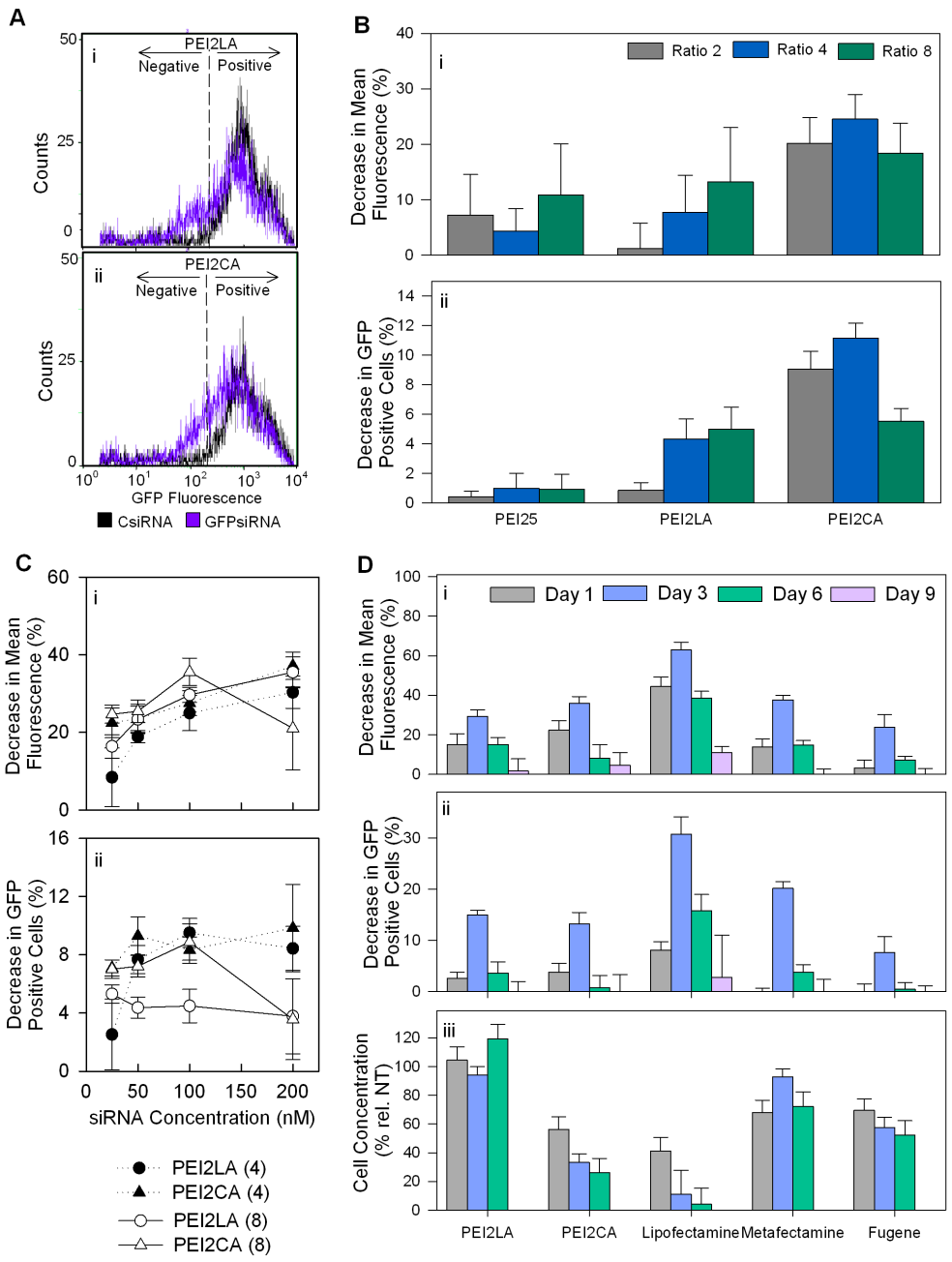


Figure 3.12 Silencing in GFP-Expressing THP-1 Cells.

(**A**) The cell counts as measured by flow cytometry (expressed as a percentage of non-treated cells). (**B**) Silencing was assessed after 3 days of siRNA treatment (50 nM) and expressed as decrease in mean GFP fluorescence or decrease in GFP-positive cells. The polymer:siRNA ratios were 2:1, 4:1 and 8:1. (**C**) Dose-response curves for GFP silencing between 25 and 200 nM siRNA treatment. The CA and LA substituted polymers were used at the polymer:siRNA ratios of 4:1 and 8:1, and silencing was assessed after 3 days of treatment. (**D**) Silencing by CA- and LA-substituted polymers and three commercial reagents (LipofectamineTM 2000, Metafectamine and Fugene HD). The extent of silencing was summarized over a course of 9 days and expressed as decrease in mean GFP fluorescence (top panel) or decrease in GFP-positive cells (middle panel). The lipid substitutions of the polymers used were 2.1 LA/PEI (PEI2-LA) and 6.9 CA/PEI (PEI2-CA).

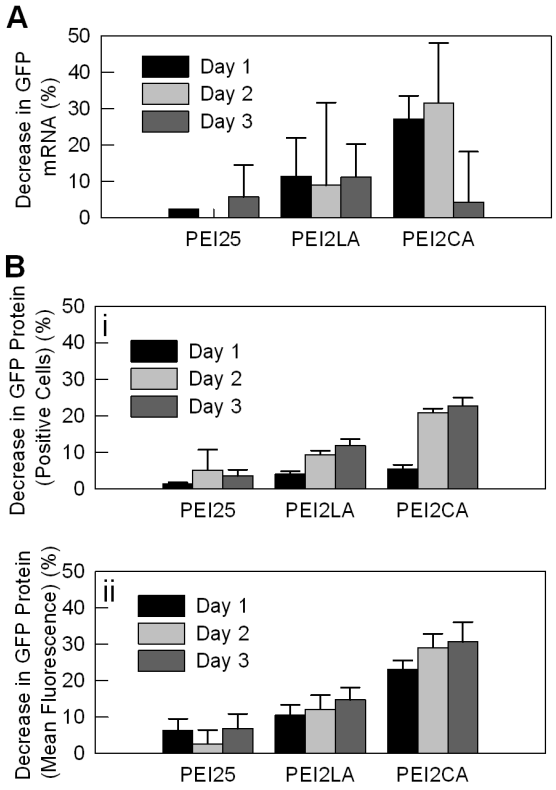


Figure 3.13 GFP mRNA and Protein Suppression in THP-1 Cells.
(**A**) Decrease in GFP mRNA levels, and (**B**) decrease in GFP protein levels (i: based on mean GFP fluorescence and ii: based on GFP-positive cell population). The GFP-positive THP-1 cells were treated with 50 nM GFP siRNA (or control siRNA) delivered with PEI25, PEI2-LA (2.1 LA/PEI) and PEI2-CA (6.9 CA/PEI; 4:1 = polymer:siRNA ratio) for 1 to 3 days, after which the cells were harvested for PCR (A) and flow cytometry (B).

3.3.8 Down-regulation of Endogenous CXCR4 Levels

The G protein-coupled chemokine receptor CXCR4 is an endogenous protein that has implications in abnormal proliferation of leukemic cells, migration and anchorage to the bone marrow [56] and with differential expression as a response to drug treatments including valproic acid (VPA) depending on maturation level of the cells [57]. Since THP-1 cells display high level of CXCR4 expression (>80% positive), we explored the feasibility of down-regulating the level of this endogenous protein since silencing

CXCR4 may prove beneficial in leukemia treatments. After treatment with 50 nM CXCR4-specific siRNA in THP-1 cells, a significant decrease in the mean CXCR4 level was achieved at day 2 with PEI2-CA and by day 3 with PEI2-LA (**Figure 3.14A**). Similar to the GFP silencing results, the most effective polymer was PEI2-CA, however, PEI2-LA was only slightly less efficient and PEI25 was not effective in this case. The extent of maximal decrease in the CXCR4 levels was 20-30%, a value similar to the extent of silencing observed with the GFP. A decrease in CXCR4-positive cell population occurred with a decrease of 8.9% for PEI2-LA20 and 6.8% for PEI2-CA20 (**Figure 3.14B**). Unlike the GFP, CXCR4 is highly dynamically regulated [57, 58] and it is possible that rapid regulation of CXCR4 levels could effect silencing in the cell population.

Considering all silencing results, it appeared that the designed polymers gave effective silencing (up to ~35% based on GFP silencing and ~30% based on CXCR4 levels) between 25 and 50 nM siRNA concentration and the benefit of employing higher siRNA concentrations was not immediately evident. Non-specific effects of siRNA treatment were investigated previously [59, 60]. Persengiev et al. reported an increase as well as a decrease in the expression of various mammalian genes in response to a luciferase siRNA treatment (where no natural target is expected to exist). They observed a concentration-dependent effect of siRNA in various genes with siRNA concentrations at >25 nM [59]. Semizarov et al. also observed off-target effects of siRNA at 100 nM, but not at 20 nM [60]. Therefore, the delivery formulations developed in this study appear to function favourably considering this constraint. Being a reporter protein, GFP silencing was not expected to lead to any functional changes and silencing specific targets for desirable functional changes are under study at the present time. CXCR4-silencing, on

the other hand, are expected to yield several functional outcomes, such as reduced migration, decreased proliferation and reduction in cellular anchorage to bone marrow [57], and thereby reduced cell survival. These effects will be the focus of further studies. We noted that previous silencing studies with leukemic cells rarely reported quantitative results due to the mechanistic nature of the studies. Some studies reported quantitative silencing outcomes; (i) >80% and >95% FADD protein silencing with Lipofectamine^{TM} 2000 (100 nM siRNA) in U937 [14] and K562 [15] cells, (ii) ~90% E2F1 protein silencing in Jurkat T-cells with HiPerFect (siRNA concentration not specified) [19], ~70% BCR-ABL protein silencing with Lipofectamine^{TM} 2000 (60-180 nM siRNA) and a Tat–LK15 peptide in K562 cells [16], and (iv) ~60% BCR-ABL mRNA with DOTAP (54 pM siRNA – an exceptionally low dose) in CML cells [20]. Our silencing results were not as high as these values, but it must be pointed out that our cells had exceptionally high GFP levels and complete silencing was not considered a realistic goal with this protein. The CXCR4 down-regulation, however, was not also as high as the values reported by other groups. The results from Lipofectamine^{TM} 2000 mediated silencing in this study as compared to other studies might serve as a good indicator of the differences in cell models used for silencing. The silencing efficiency in this study should be considered for comparison purposes for efficacy and toxicity among the carriers, and not in the context of therapeutic studies. Such studies with relevant molecular targets (including CXCR4) are currently underway in the authors' labs.

Finally, we explored the utility of our polymers for silencing in two additional cell types, the Hut78 cells (a T-cell lymphoma cell line) and the K562 cells (a chronic myeloid leukemia cell line) using the reporter GFP as the target. These cells display

constitutive GFP expression similar to the THP-1 cells extensively used in this study. Preliminary results indicated the feasibility of GFP silencing in the Hut78 cells, most effectively with PEI2-LA (**Figure 3.3SA**). The silencing in K562 cells was to a lesser extent with PEI2-LA, suggesting that the performance of the developed polymeric systems could depend on the specific cell type. Such a differential performance was also evident with the PEI25 mediated GFP-specific siRNA (**Figure 3.3SB**), where performance of PEI25 in K562 cells was superior to the Hut78 cells.

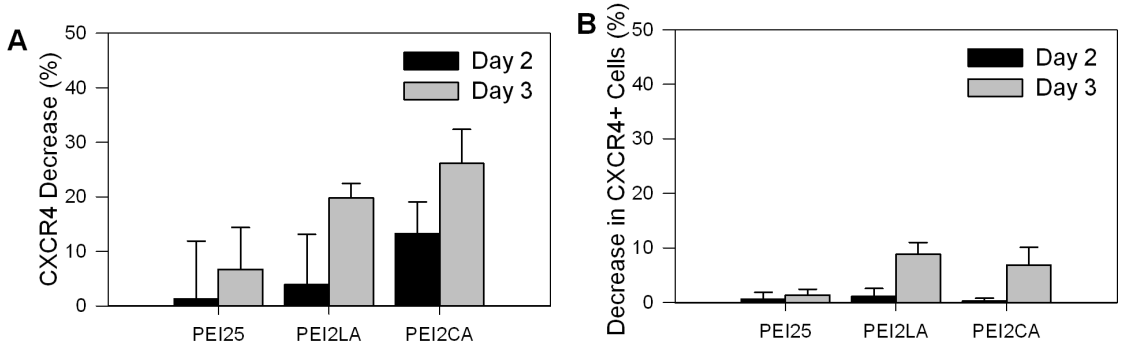


Figure 3.14 CXCR4 Silencing in THP-1 Cells.
Changes in CXCR4 levels based on (**A**) mean CXCR4 fluorescence intensity and (**B**) CXCR4-positive cell population. Silencing was assessed after 2 and 3 days of CXCR4-specific siRNA or control siRNA treatment (50 nM with polymer:siRNA ratio of 4:1). The polymers used were PEI25, PEI2-LA (2.1 LA/PEI) and PEI2-CA (6.9 CA/PEI).

3.4 CONCLUSIONS

Lipid modification of the ineffective polymer PEI2 clearly improved its ability to deliver siRNA to leukemic cells. Enhanced siRNA delivery was obtained with the appropriate choice of lipid for polymer substitution, whose delivery ability was dependent on (i) lipid substitution levels and (ii) polymer:siRNA ratio used for complex formation. Compared to the commonly used polymeric carrier PEI25, the leading lipid-

substituted polymer PEI2-LA gave siRNA delivery that was less dependent on serum concentration in medium. Among the three types of AML cells explored, cell-to-cell differences in siRNA delivery was evident, suggesting that optimization of siRNA complex formulations might be needed to maximize the delivery for individual cell types. The PEI2-LA compared favourably to several commercial siRNA delivery systems, and provided an effective silencing activity without significantly affecting the subsequent cell growth. Silencing was demonstrated by using a reporter (GFP) gene as well as the endogenous protein CXCR4 in THP-1 cells. Given the fully disclosed nature of the PEI2-LA (unlike most commercial reagents) and the versatility of polymeric delivery systems in general, the proposed polymers provide excellent possibilities for therapeutic delivery of siRNA in leukemia. The polymers could also serve as a platform to further improve siRNA delivery by incorporating functional groups such as cell targeting ligands and moieties facilitating endosomal release. The in vivo efficacy of the polymeric siRNA delivery systems was not explored in this study and it will be the next stage in evaluation of the proposed delivery systems.

3.5 ACKNOWLEDGEMENTS

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4. Targeting CXCR4/SDF-1 Axis by Lipopolymer Complexes of siRNA in Acute Myeloid Leukemia[x]

[X]A version of this chapter is to be published as:
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4.1 INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of disorders characterized by abnormal proliferation of myeloid blasts with reduced capacity to differentiate into mature cells. Little has changed with the AML treatment methods in the past decade and the standard treatment remains as chemotherapy, often in the form of cytarabine in combination with an anthracycline [1, 2]. Although conventional treatment yields high rates of complete remission, the majority of patients eventually relapse (more than 85%) due to the proliferation of drug-resistant leukemic blasts in the bone marrow [3, 4]. Besides high relapse rates, current therapies display immediate toxic side-effect, patient incompatibility with high-dose treatments, and undesirable effects on the long term [1, 2, 5, 6]. In addition, the five-year survival rates are only 4% in patients 65 years of age or older, and 31% in patients younger than 65 years of age [7]. The development of alternative, novel therapies for AML is therefore needed.

As an alternative therapeutic modality for AML, siRNA therapy provides the flexibility of choosing different targets and/or combining multiple targets under the same therapeutic approach. The requirements for siRNA therapy include a therapeutically responding (effective) target protein and a carrier to effectively deliver the siRNA as, without the protection of a carrier, the siRNA is readily degraded in the physiological milieu and is unable to enter the cell due to its relatively large size and negative charge. siRNA silencing in suspension cells, specifically leukemic cells, remains challenging [8-10]. Much of siRNA related work with leukemia has been performed with commercial carriers or by electroporation, which are both not clinically applicable [8-10]. The difficulties of siRNA delivery to leukemic cells are not well understood but recent

findings suggested that low expression of Caveolin 1 and 2 (with an important role in caveolae-mediated endocytosis) [9] or limited presence of extracellular matrix attachment proteins [11] maybe partly responsible. We have been developing polymeric carriers, namely lipid substituted low molecular weight polyethylenimines (PEIs), to be used in cancer therapy. Utilizing low molecular weight PEI (2 kDa) as the backbone of the siRNA carrier takes advantage of the positive aspects of the well known features of PEIs, which include effective siRNA binding due to its high charge density, electrostatic interaction with membranes needed for internalization, and endosomal escape mechanisms through a combination of buffering capacity and membrane interactions. Utilizing the lower molecular weight PEI in the backbone meanwhile minimizes the well known toxicity and limited biodegradability of the high molecular PEIs [12-14]. Without further modification, however, low molecular PEIs are not effective for siRNA delivery into cells, likely due to minimal charge of assembled complexes [15]. We have utilized lipid substitution via caprylic acid (as well as other lipids) of 2 kDa PEI to enhance the interactions with cellular membranes. A library of lipopolymers was shown to efficiently bind to siRNA to form distinct complexes, provide efficient siRNA delivery (comparable to commercial carriers) as well as to effectively silence a model protein (Green Fluorescence Protein, GFP) in leukemic cells [8]. In order to validate the utility of the proposed lipopolymer/siRNA delivery system for clinical use, further analysis was required on its ability to target a therapeutically useful protein in AML disease.

Targeting the CXC chemokine receptor 4 (CXCR4)/stromal-cell derived factor-1 (SDF-1) axis is a promising treatment for AML. The CXCR4-expressing leukemic cells have been found to migrate to bone marrow microenvironment as a result of bone

marrow stromal cells (BMSC) and endothelial cells releasing the chemo-attractant SDF-1. SDF-1 binds to cell surface located CXCR4, resulting in its activation through phosphorylation and endocytosis of surface-located CXCR4, followed either by ubiquitination and then degradation or surface re-location [16]. CXCR4 activation causes signaling through numerous pathways, including Scr family of tyrosine kinases, phospholipase C-β, PI3K/Akt, JAK/STAT, MAPK and NF-κβ, resulting in enhanced survival, increased proliferation, drug resistance, degradation of extracellular matrix and angiogenesis [16]. High level of CXCR4 expression has been demonstrated in many leukemia's including AML and its expression was increased as a response to cancer drugs [16, 17]. Current strategies targeting CXCR4 include small molecular antagonists and blocking antibodies [16], several of which are progressing through clinical trials [18]. Promising effects of the CXCR4 targeting antagonists in AML cell lines as well as primary AML cells have been reported and include decreased adhesion to BMSC/SDF-1, decreased proliferation, increased apoptosis, decreased survival support and decreased resistance to chemotherapy drugs [18, 19]. More importantly, in a phase I/II trial, CXCR4 antagonist AMD3100 (Plerixafor) was found to mobilize leukemia cells into the peripheral blood by 2-folds and provide chemosensitization with mitoxantrone, etoposide, and cytarabine treatment [18]. In addition, AMD3100 and TN140, used without chemotherapy drugs, caused regression in high CXCR4 expressing leukemic patient cells in a mouse model as well as increased apoptosis and increased mobilization in these cells [20]. The effects of CXCR4 antagonist on AML cells have been attributed to two separate mechanisms; (i) physical disruption of cell adhesion to drug resistance supporting bone

marrow microenvironment and (ii) prevention of signaling through the CXCR4 pathway that includes the pro-survival pathways PI3K/AKT and MAPK [18].

Downregulating CXCR4 expression with siRNA may provide a more beneficial therapeutic modality as compared to CXCR4 antibodies and small molecular inhibitors. siRNA is a highly targeted technology, specific for the mRNA of interest that results in decreased protein formation. The drawbacks of antibody therapies include complex and costly development, unpredictable toxicity, low efficacy/safety ratio and risk of immunogenicity [21, 22]. The challenges of inhibitors include lower specificity, short-half life, toxicity issues and varied treatment response due to target mutations, and complicated mechanism(s) of action [19, 21, 23]. Resistance to AMD3100 can also occur simply due to a specific single amino acid substitutions in a certain region of CXCR4 [24]. Through the CXCR4 antagonist binding mechanism, a signaling response through CXCR/SDF-1 pathways can be activated [19, 23]. Antagonists, such as AMD3100 and ALX40-4C, have been found to induce G protein signaling activation, as a result of being weak partial agonists, resulting in phosphorylation of some SDF-1/CXCR4 signaling molecules (MAPK p44/p42) [19, 23].

In this study, we investigated the impact of silencing CXCR4 and SDF-1 expression in AML cells with the lipopolymer-mediated siRNA delivery. We probed the effect of silencing with clinically relevant variables including the presence of human bone marrow stromal cells (hBMSC) and the chemotherapy drug, cytarabine. We show that silencing both CXCR4 and SDF-1 provide decreased leukemic cell survival and that CXCR4 silencing remains effective when the leukemic cells were co-incubated with hBMSC. Additionally, CXCR4 siRNA co-treatment with cytarabine provided an

enhanced anti-survival effect on AML cells, which is especially evident in the presence of hBMSC.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Two kDa polyethylenimine (PEI2; M_n: 1.8 kDa, M_w: 2 kDa), anhydrous dimethyl sulfoxide (DMSO), caprylic chloride (C8), 3-(4,5-demethyl-2-thiazoylyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue solution (0.4%), and Cytarabine (Cytosine β-D-arabinofuranoside; C1768-100MG) were purchased from Sigma-Aldrich (St. Louis, MO). Negative control siRNA (AM4635), DiI (Molecular Probes), and Hoechst (33258; Molecular Probes) was purchased from Life Technologies (Carlsbad, CA). CXCR4 siRNA (CAT: HSC.RNAI. N001008540.12.1) and SDF-1 siRNA (CAT:HSC.RNAI.N000609.12.1) were purchased from IDT Inc. (Coralville, IA). Hanks Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM; low glucose with L-glutamine; 11885), and RPMI Medium 1640 with L-glutamine (11835), penicillin/streptomycin solution (10000 U/mL/10 mg/mL), Minimum Essential Media (MEM) α medium, MEM non-essential amino acids (100x) and Trypsin EDTA Solution, 1x Liquid 0.25% Trypsin/1mM EDTA (25200-056) were from Invitrogen (Grand Island, NY). Accutase (SCR005) was from Millipore (Billerica, MA). Fetal bovine serum (FBS; A15-751) was purchased from PAA Laboratories Inc. (Etobicoke, ON). The PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody was from BD Pharmingen (Mississauga, ON).

4.2.2 Cell Models and Cell Culture

THP-1 cells (AML-M5; French-American-British (FAB) classification) were obtained from the American Type Culture Collection (Manassas, VA). THP-1 cells were maintained in RPMI medium containing 10% FBS (heat inactivated at 56°C for 30 min) and 1% penicillin/streptomycin under normal conditions (37°C, 5% CO_2 under humidified atmosphere) in suspension flasks at concentrations between 1-10x10^5 cells/mL (monitored by hemocytometer cell counts) and passaged by dilution after reaching 10x10^5 cells/mL. GFP-expressing THP-1 cells (THP-GFP) were obtained through retroviral transfection of enhanced GFP cloned into pMSCVpuro (Invitrogen), as described previously [8], and were cultured as above. Human BMSC (hBMSC) (35 years, male; isolation described in [25]; with informed consent and approval from the institutional health research ethics board) were maintained in αMEM with 1X non-essential amino acids, 10% FBS (heat inactivated at 56°C for 30 min) and 1% penicillin/streptomycin under normal conditions (37°C, 5% CO_2 under humidified atmosphere). The hBMSC were sub-cultured weekly at confluency (after trypsinization) by one-quarter dilution and used in the described experiments between the passages 3 and 7.

4.2.3 Preparation of Lipopolymer Carriers

Preparation of caprylic acid (CA) substituted PEI2 was completed for a range of substitution levels, the details of the synthesis and characterization have been described elsewhere [8, 26, 27]. In summary, caprylic acid was substituted onto the previously lyophilized PEI2 polymer by N-acylation of the amines. Caprylic chloride (varying amounts) was slowly added drop-wise to 100/400 mg of PEI in DMS0 for 24 h at

ambient temperature under N_2 producing a range of CA substitutions, which were dependent on the feed ratio of lipid:polymer. PEI2-CA polymers were then precipitated and washed with excess ethyl ether and dried under vacuum at ambient temperature overnight. The actual substitution ratios were determined by ^{1}H-NMR in D_2O (Bruker 300 MHz; Billerica, MA). Here the characteristic proton shifts of lipids (0.8 ppm; -C$\underline{H}_3$) and PEI (2.5–2.8 ppm; NH-$\underline{C}H_2$-$\underline{C}H_2$-NH-) were integrated and normalized to the number of protons in each peak (summarized in Table S1). The numbers of lipid methylenes substituted in each polymer (lipids/PEI2) were calculated by multiplying the level of lipid substitution (from ^{1}H- NMR) with the number of methylenes in each lipid. Percent lipid substitution of amines (% amine substitution) was calculated by dividing the number of lipid substituted with the number of amines (44 amines/PEI2). Lipopolymer concentrations used in the experiments were determined by dissolving the freeze-dried polymers with RNASE free/DNASE free water and performing a copper (II)/PEI assay on the solutions [28, 29].

4.2.4 Lipopolymer/siRNA Complex and Cytarabine Treatments

Lipopolymer complexes of siRNA were formed immediately prior to addition to the cells. First, the required amount of siRNA (e.g., 0.35 μg to give 50 nM final siRNA concentration in wells) was added to 150 mM NaCl solution in a 1.5 mL microcentrifuge tube. The polymers (dissolved in ddH_2O) were then added to the siRNA solutions at a 4:1 polymer:siRNA ratio (which corresponds to 31.6:1 N/P), and incubated, for 30 min (at room temperature) before addition, in triplicate, to the cells (15 μL/well containing 0.5 mL medium). In all cases, cells were seeded in the wells the day before the siRNA treatment. The concentration of siRNA in the wells was 50 nM, unless otherwise noted.

Lipopolymer complexes containing control siRNA were used in all incidences in order to rule out contributions due to any autofluorescence or physical effects caused by complex exposure to the cells. For the cytarabine treatment studies, cytarabine was prepared in HBSS at a stock concentration of 1 mg/mL before each experiment and stored at 4° C for a maximum of 2 days.

4.2.5 Detection of CXCR4 Silencing

THP-1 cells were seeded in 24-well plates (0.50 mL fresh medium/well) and allowed to acclimatize for 24 h under normal maintenance conditions prior to addition of lipopolymer/siRNA complex solutions as described above. At indicated time points, after complex addition, (see figures), the cells were transferred to microcentrifuge tubes and centrifuged at 1600 rpm (240 g). The supernatant was removed and re-suspended cells were stained with 4 μL of PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody in 90 μL of medium for 45 min at 4°C. When silencing was performed in the presence of hBMSC, unattached GFP+ THP-1 cells were first removed to the microcentrifuge tubes. The hBMSC and attached GFP+ THP-1 cells were then washed with HBSS, the supernatant was added to tubes and the attached cells were then removed with Accutase (100 μL/well) and added to the same tubes. Wells were rinsed with HBSS and the cells were centrifuged, stained with the labeled-antibodies at 4°C, as described above.

After antibody staining, cells were re-suspended in HBSS and fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by flow cytometry (FL2 channel) with Cell Lab Quanta^{TM} SC (Beckman Couter). When GFP positive THP-1 cells were used in the experiments, LSR-Fortessa SORP (BD Biosciences) was used for

simultaneous detection of PE antibodies (l_{EX} at 561 nm and l_{EM} at 586 nm) and GFP (l_{EX} at 488 nm and l_{EM} at 530 nm). GFP positive cells were used to clearly select for THP-1 population when grown in contact with hBMSC. Changes in mean CXCR4 levels (based on specific Ab fluorescence levels) and the CXCR4-positive cell population were calculated as a result of siRNA treatments. The cell population stained with non-specific antibody was used for flow cytometry calibration (i.e., designated as 1% CXCR4-positive population).

4.2.6 Cell Counts and Viabilities after CXCR4 Silencing

Relative cell concentrations were determined by counting in a flow cytometer (Cell Lab Quanta™ SC; Beckman Couter). Samples were prepared by a single centrifugation (unless further processing was necessary as in antibody staining) at 1600 rpm (240 g). The cells were suspended in clear HBSS and fixed by adding formalin for a final concentration of 1% formalin. Cells were added to 96-well plate (200 µL) for automated processing by the flow cytometer. When GFP-positive THP-1 cells were used in contact with hBMSC, cell concentration reported was from the GFP positive cells within the cell population region.

To visualize and detect nucleus fragmentation, Hoechst staining (250 ng/mL) was performed after the cells were fixed with 1% formalin (25 min). Images were taken with a FSX100 Olympus Fluorescent Microscope using both the FITC filter for GFP and the DAPI filter for Hoechst. Composite images were created with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.) where GFP positive cells (changed to black on

white background) and Hoechst stained nucleuses were combined. GFP-positive THP-1 nucleuses were then visually compared between the study groups.

4.2.7 BMSC Adhesion Assay

Cell adhesion to hBMSC was measured with both GFP-positive and DiI-stained THP-1 cells. The adherence assay was modified from Ref. [30]. For studies with GFP-positive cells, cells were treated with lipopolymer complexes containing CXCR4 siRNA or control siRNA (50 nM) for 48 h in 24-well plates as described above. Cells were then plated (330 μL of medium/well) on a hBMSC monolayer (which were seeded in 96-well plates the day before at 15000 cells/well). Non-treated cells were also added to wells without hBMSC, for control purposes. Cells were then incubated under normal conditions for 2 h to permit adherence to hBMSC. The 96-well plate was subsequently turned over and incubated for 2 h to allow for non-adhered cells to gravitate away from hBMSC. Supernatants were then collected with a pipette, while the plate remained inverted, and placed in a separate 96-well plate and then processed for flow cytometry (fixed in 300 μL of 1% formalin). The adhered THP-1 cells and hBMSC were also trypsinized and processed for flow cytometry (fixed in 300 μL of 1% formalin). Cell concentrations of the GFP-positive cells were then determined by flow cytometry, as described above, for THP-1 cells from supernatant and the portion adhered to the hBMSC. The percent adhered cells (%) were calculated as = 100-([cell conc. measured from supernatant]/[combination of conc. measured from supernatant and adhered cells] x100%.) No treatment adhered cells from hBMSC monolayer wells was 64.4 ± 4.2% where as adhered cells from wells without hBMSC was 1.5 ± 1.9% (not shown).

When DiI-stained parental cells were used, DiI staining was performed after CXCR4 silencing prior to incubating the cells with the hBMSC. For DiI staining, wells from the same group were combined and cells were resuspended in medium without serum with 1.25 μg/mL in normal growth conditions followed by 2X washing with HBSS and resuspension in normal medium. The DiI-stained THP-1 cells were then seeded in triplicate onto the hBMSC (330 μL medium), allowed to adhere to hBMSC, followed by the plate inversion, as described above. Non-attached cells were collected from the supernatant, when the plate was inverted. Fresh medium (330 μL) was added to the adhered THP-1 cells and the hBMSC. Medium only wells were added for base-line fluorescence measurements. Relative cells numbers were then determined by DiI fluorescence by a fluorescence plate reader (536EX/607EM). After subtracting the base-line fluorescence (medium-only wells) from the readings, the percentage of adhered cells was calculated as described above.

4.2.8 Cell Division Assay

To determine changes in cell proliferation, THP-1 cells were stained with 0.45 μM Cell Tracker™ Green CMFDA (Life Technologies) according to manufacturer's directions. Briefly, cells (12 x 10^5 cells/mL) were resuspended in FBS medium without serum and Cell Tracker™ Green CMFDA (10 mM in DMSO) was added for a final concentration of 0.45 μM. Cells were then incubated under growth conditions (37°C, 5% CO_2 under humidified atmosphere) in suspension flask for 30 minutes. Then, cells were centrifuged, medium was removed and fresh regular growth medium was added. Cells were then seeded in plates (with the pre-seeded hBMSC) at 1x10^5 cells/ml and allowed to acclimatize for 24h. Lipopolymer complexes containing CXCR4 or SDF-1 siRNA were

added in the presence of hBMSC as described above (designated as Day 0). Cells were then fixed (1% formalin) and processed for flow cytometry (as described above) for each subsequent time-point. The mean CMFDA fluorescence of the CMFDA positive population was detected via FL1 channel, as described above. The CMFDA concentration was chosen after testing a range of staining concentrations (0.50-20 μM). We determined that cell numbers over the time period of 0-4 days was negatively affected at dye concentrations of 10 and 20 μM and proliferation (as seen by change in CMFDA fluorescence of Cell Tracker^{TM} Green) was affected at the 20 μM dye concentration (**Figure 4.S1**). We also ensured that detectability of fluorescence was achievable for 4 days (not shown).

4.2.9 Statistics

All experiments were performed in triplicate with mean result displayed and error bars indicating the standard deviations. Statistical analysis was performed with GraphPad InStat v3.06 (GraphPad Software, San Diego, CA USA). One-way ANOVA with Bonferroni post-test was used to compare groups. Statistical significant difference when comparing to NT is indicated by +/ ++/ +++ and to lipopolymer complexes containing control siRNA is indicated by */ **/ *** where +/* indicates $p<0.05$, ++/** $p<0.01$ and +++/*** $p<0.001$. Other details, as required, are described in further detail in relevant figures.

4.3 RESULTS

4.3.1 CXCR4 Silencing in THP-1 Cells

We performed siRNA-mediated CXCR4 silencing utilizing the lipopolymers in the AML cell line, THP-1 cells, since they display high level of CXCR4 expression (>80%). We first accessed the ability of a PEI2 library, ranging in CA substitutions from 2.5 to 6.9 per PEI2, for down-regulating CXCR4. We investigated a range of CA modification levels to determine if a specific substitution provided an obvious improvement and if there was a correlation between the CA substitution and the silencing ability. With the prepared CA library, the maximal CXCR4 silencing achieved was up to 34% on day 2 and 32% on day 3 with the siRNA concentration of 50 nM (**Figure 4.1A**). However, there was very little decrease in CXCR4 expressing population (**Figure 4.1B**), suggesting that silencing was uniform among the cell population. The PEI2-CA5.4 (i.e., 5.4 CA substitution per PEI2) was chosen for the further studies as it demonstrated the most significant and consistent silencing on both day 2 and day 3. The extent of CXCR4 silencing did not correlate with the level of CA substitution (**Figure 4.S2**). Perhaps a trend would have been evident if lower siRNA concentration was used, or higher CA substitutions were obtained from the polymer library.

We next investigated the duration of CXCR4 silencing after a single treatment with lipopolymer/CXCR4 siRNA complexes of PEI2-CA5.4 over 5 days (**Figure 4.2**). Based on the mean CXCR4 levels, CXCR4 silencing was achieved from day 1 to day 3 but the silencing was lost by day 5 (**Figure 4.2A-B**). A small decrease in the percentage of CXCR4-positive cells was observed on day 1, but not afterwards (**Figure 4.2B**).

Serendipitously, we noted a decrease in the concentration of THP-1 cells (~30%) from day 1 to day 5 as a result of CXCR4 siRNA treatment (**Figure 4.2C**).

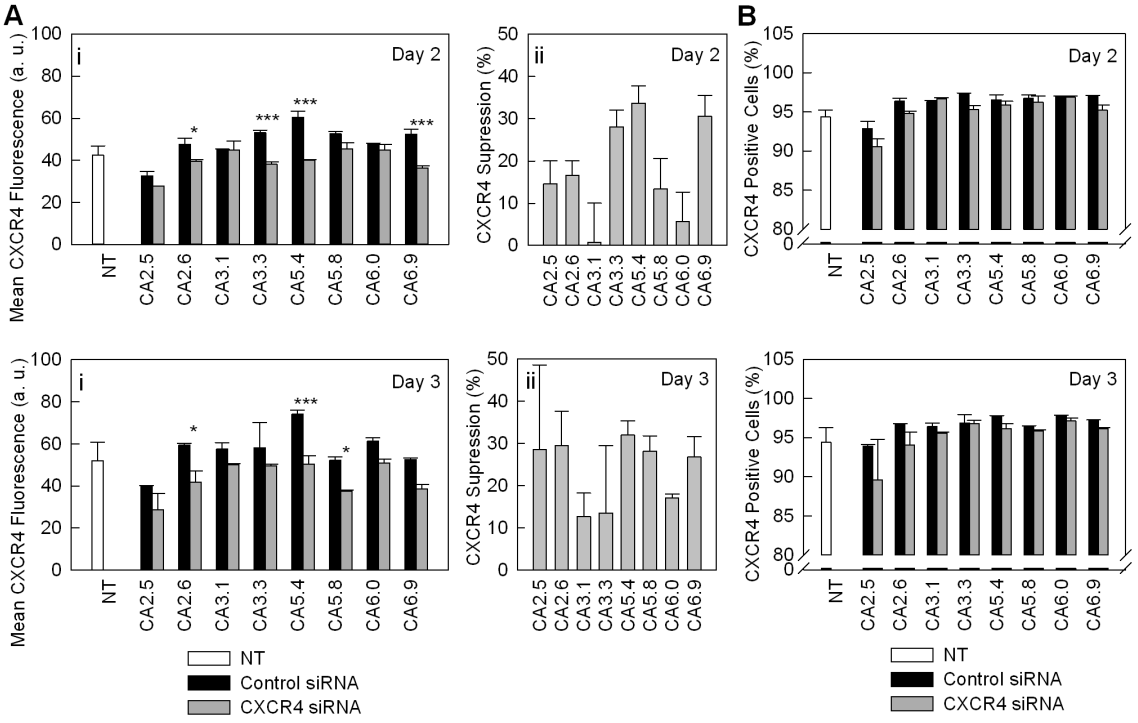

Figure 4.1 Effect of CA Substitution Level on PEI2 on CXCR4 siRNA Silencing Ability of Lipopolymer/siRNA Complexes.

(**A**) i. Mean CXCR4 levels based on antibody fluorescence (arbitrary units, a.u.) on day 2 and day 3. ii. Relative CXCR4 suppression levels with respect to control siRNA treated cells on day 2 and day 3 after lipopolymer/siRNA complex treatment. (**B**) CXCR4 positive cell population on Day 2 and Day 3 after lipopolymer/siRNA siRNA treatment. It was possible to obtain up to ~33% CXCR4 silencing (based on mean CXCR4 levels), without significant changes in the percentage of CXCR4-expressing cell population.

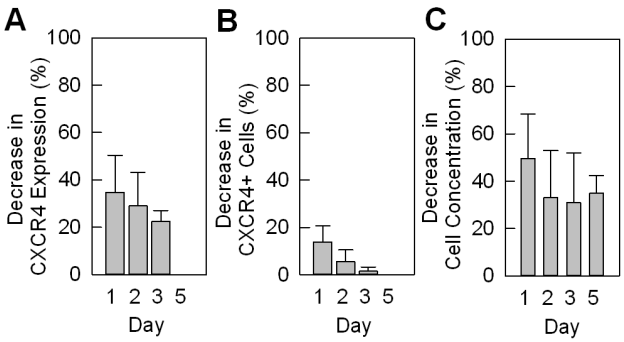

Figure 4.2 Time Course of CXCR4 Silencing with Lipopolymer/siRNA Complex Treatment.
(**A**) Percent change in mean levels of CXCR4 over 5 days. (**B**) Percent change in CXCR4-positive cell population over 5 days. (**C**) Change in cell concentration over 5 days. All values were expressed with respect to cells treated with PEI2-CA/control siRNA complexes. One time treatment of the cells resulted in CXCR4 silencing for 5 days and ~30% reduction in cell numbers.

4.3.2 Effect of CXCR4 Silencing on Cell Numbers

In our co-culture experiments with hBMSC, we utilized GFP-positive THP-1 cells in order to identify the THP-1 population from that of the hBMSC population. We therefore verified the ability of our polymers to silence CXCR4 in these cells as well (**Figure 4.3**). As before, maximal silencing from the lipopolymer/siRNA complex treatment was 29% on day 1 based on mean CXCR4 levels (**Figure 4.3Ai**), but the duration of silencing was shorter since no silencing was observed by day 3. This was less effective than what we have previously seen in the native THP-1 cells. A difference between the two cell types was also evident in the cell growth rates in regular culture passage, where the GFP-positive THP-1 cells appeared to have a faster proliferation rate than the native THP-1 cells (visual observation; not shown). It was possible, therefore, that the silencing was shorter duration as a result of faster proliferation of the cells. We then assessed the CXCR4 silencing in GFP-positive THP-1 cells in the presence of hBMSC. The extent of silencing was similar to the cells treated with the CXCR4 siRNA but in the absence of hBMSC (compare **Figure 4.3Ai** and **4.3Aii**). However, when the GFP-positive THP-1 cells were silenced in the presence of hBMSC, the silencing duration was longer, since we were able to detect silencing up to day 3 with the 50 nM

siRNA concentration. Although we normalized each silencing group with non-treated cells, we noted a significant increase in CXCR4 antibody staining when THP-1 cells were grown with hBMSC as opposed to without (1.7-fold higher on day 1 and 2.0-fold higher on day 2 and 3), suggesting an increase in CXCR4 levels when in contact with hBMSC.

The changes in cell number as a result of CXCR4 silencing with the lipopolymer/CXCR4siRNA complexes are summarized in **Figure 4.3B** for cells grown in the absence and presence of hBMSC. The toxicity of the control siRNA was evident on THP-1 cells at 50 nM where CXCR4 silencing did not lead to a specific reduction in cell numbers (i.e., that of beyond control siRNA treatment). A significant effect of CXCR4 silencing however was evident at the 25 nM siRNA concentration given the minimal toxicity of control siRNA observed at this concentration. The lipopolymer complexes with control siRNA did not appear to be toxic on the cells when they are treated in the presence of the hBMSC, and a more pronounced reduction in cell numbers were observed after CXCR4 siRNA treatment. The GFP-positive THP-1 cells grown with hBMSC were stained with Hoechst for visualization of nuclear fragmentation as a sign of cellular apoptosis. There was no visual indication of increased apoptosis based on nuclear fragmentation after CXCR4 silencing (**Figure 4.S3**).

We next compared the two effects of CXCR4 siRNA treatment, namely decrease in CXCR4 surface protein levels and cell concentration (**Figure 4.3C**). At 50 nM treatment of THP-1 cells without hBMSC (**Figure 4.3Ci**), a similar response was seen for the decrease in CXCR4 protein levels or cell concentration. At the lower concentration of 25 nM, a significant decrease in cell concentration was still seen despite minimal decrease in CXCR4 levels. In THP-1 cells co-incubated with hBMSC (**Figure 4.3Cii**),

the decrease in CXCR4 protein levels remained fairly constant between days 1 and 3, but the decrease in cell concentration was increased over this time to a maximum value of ~54% for 50 nM CXCR4 siRNA and ~35% for 25 nM CXCR4 siRNA.

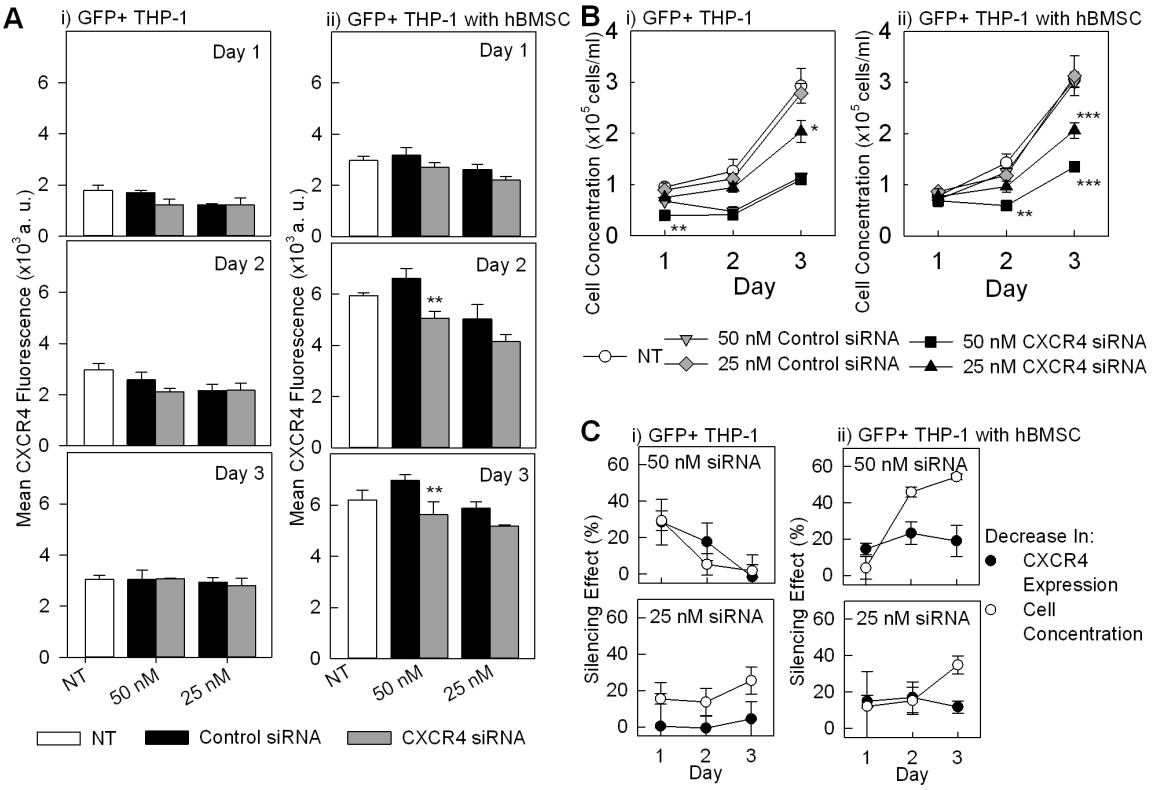

Figure 4.3 Lipopolymer/siRNA Complex Mediated CXCR4 Silencing in GFP-Positive THP-1 Cells Without and With Co-Incubation with hBMSC.
The cells were either untreated or treated with lipopolymer complexes containing control or CXCR4 specific siRNA (25 and 50 nM). (**A**) Mean CXCR4 levels without hBMSC (i) and with hBMSC (ii) co-incubation from day 1 to day 3. (**B**) Changes in cell concentration from day 1 to day 3 without hBMSC (i) and with hBMSC co-incubation (ii). (C) Comparison of silencing effect in terms of CXCR4 protein levels and decrease in cell concentration without hBMSC (i) and with hBMSC co-incubation (ii).

4.3.3 CXCR4 Silencing and Cytarabine Effect

We then investigated the effect of CXCR4 silencing with the lipopolymer/CXCR4siRNA complexes for 2, 3 and 4 days (50 nM) on subsequent

cytarabine treatment. The effect of cytarabine on THP-1 cells (in the absence of siRNA addition) was the same whether the cells were cultured with or without hBMSC (**Figure 4.4A**). When silencing CXCR4 in the absence of hBMSC (**Figure 4.4B**), a significant decrease in cell concentration (toxicity) was evident with control siRNA treatment on days 2, 3 and 4 in the absence of cytarabine. Increasing concentrations of cytarabine further reduced the cell concentration as expected. With CXCR4 siRNA treatment, further decrease in cell concentrations was evident on day 2, but not on day 3 and day 4. In the presence of hBMSC, CXCR4 silencing again demonstrated a more robust reduction in cell numbers (**Figure 4.4C**), partly due to reduced toxicity of the lipopolymer complexes containing control siRNA, which better revealed the specific effect of CXCR4 siRNA. When cytarabine was added after CXCR4 silencing, we observed a further decrease in cell concentration for all days and all cytarabine concentrations (except 5 μg/mL on day 2). The CXCR4 silencing sensitized the cells regardless of cytarabine concentrations (0.5-5 μg/mL) with a further 30-70% decrease in cell numbers compared to control siRNA treatment.

4.3.4 CXCR4 Silencing and Adhesive Properties of Cells

As CXCR4 binding to hBMSC via SDF-1 secretion is one mechanism of AML cell adhesion to hBMSC, we investigated the effect of CXCR4 silencing with the lipopolymer/siRNA complexes on the adhesion ability of THP-1 cells to hBMSC monolayers (**Figure 4.5**). We found a slight but significant decrease in cell adhesion after CXCR4 silencing with both GFP-positive THP-1 cells (10.6% vs. control siRNA) (**Figure 4.5A**) as well as DiI-stained THP-1 cells (13.7% vs. control siRNA) (**Figure 4.5B**), as compared to control siRNA treated and non-treated cells.

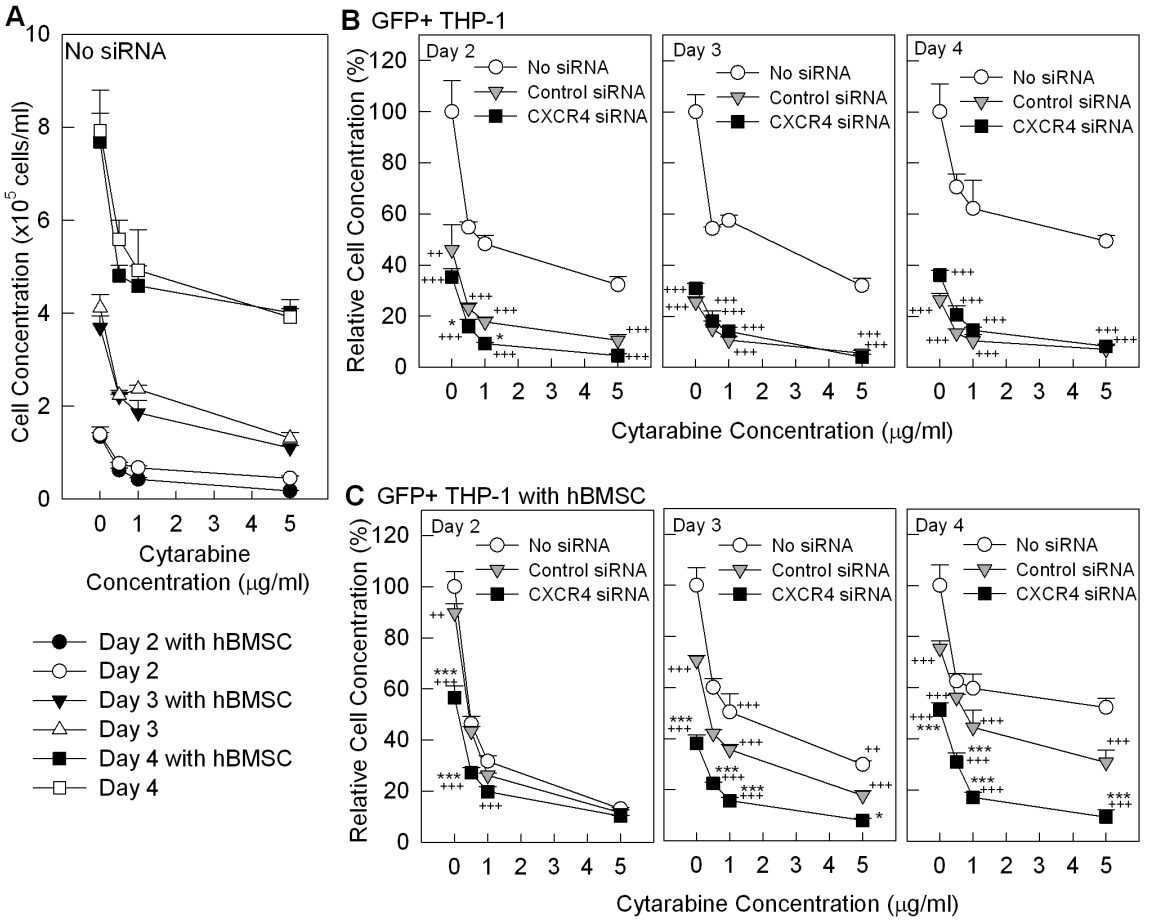

Figure 4.4 Effect of Cytarabine Treatment on GFP-Positive THP-1 Cell Concentration after Lipopolymer/siRNA Complex Mediated CXCR4 Silencing.

(**A**) Effect of cytarabine treatment on GFP-positive THP-1 cells concentration with and without hBMSC incubation. (**B**) Effect of cytarabine treatment after CXCR4 silencing without hBMSC. (**C**) Effect of cytarabine treatment after CXCR4 silencing with hBMSC co-incubation. Concentrations include both attached and unattached GFP-positive THP-1 cells. The lipopolymer/siRNA complex mediated treatment was performed at 50 nM for 48, 72 and 96 h with cytarabine treatment (at different concentrations) for the last 24 h of siRNA treatment. The resultant cell concentrations were expressed with respect to untreated cells (i.e., cells that received no siRNA or cytarabine).

4.3.5 Effect of SDF-1 Silencing

We next determined the effect of silencing SDF-1 (CXCL12), the ligand to CXCR4, in conjunction with CXCR4 silencing utilizing our lipopolymer/siRNA complexes with siRNA sequences targeting SDF-1 or CXCR4, (**Figure 4.6**). Although SDF-1 is secreted by hBMSCs, other cells including leukemia (THP-1) cells were also

shown to produce it [31, 32]. If CXCR4 requires interaction with SDF-1 for increased proliferation, then this interaction could still occur without hBMSC. In the absence of hBMSC (**Figure 4.6A**), silencing SDF-1 by itself appeared to give a similar decrease in cell concentration to that of silencing CXCR4 alone. There was also no enhanced effect when both SDF-1 and CXCR4 were silenced at the same time, which suggested that the silencing effect observed on cell numbers was the result of inhibiting the same pathway. The results were similar when CXCR4 siRNA treatment was performed in the presence of hBMSC (**Figure 4.6B**). It was possible that hBMSC produced SDF-1 was also decreased when silencing was performed in the presence of hBMSC but this was not verified in this experiment. Again, as previously seen, the toxicity of the non-targeting control siRNA containing complexes was lower when they were grown with hBMSCs, so that the effect of CXCR4 and SDF1 silencing was more clearly revealed in the co-culture experiment.

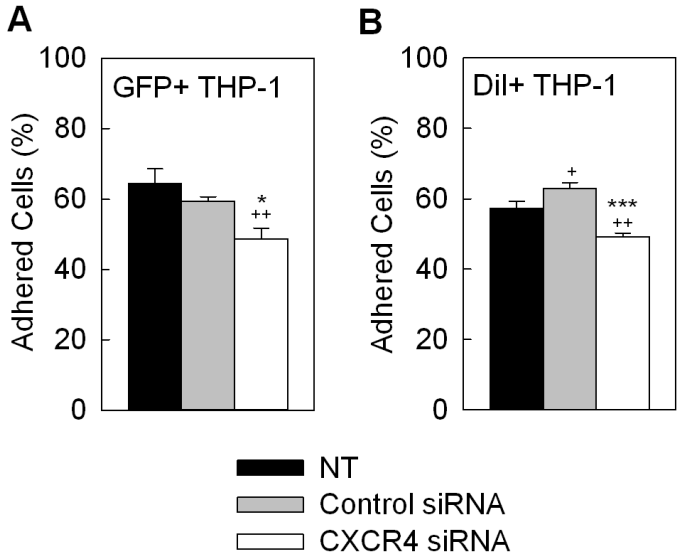

Figure 4.5 Effect of Lipopolymer/siRNA Complex Mediated CXCR4 Silencing on BMSC Attachment.
Cell attachment was assessed by (**A**) GFP-positive THP-1 cells and (**B**) DiI-stained THP-1 cells. The CXCR4 silencing causes a decreased in THP-1 hBMSC attachment in both methods.

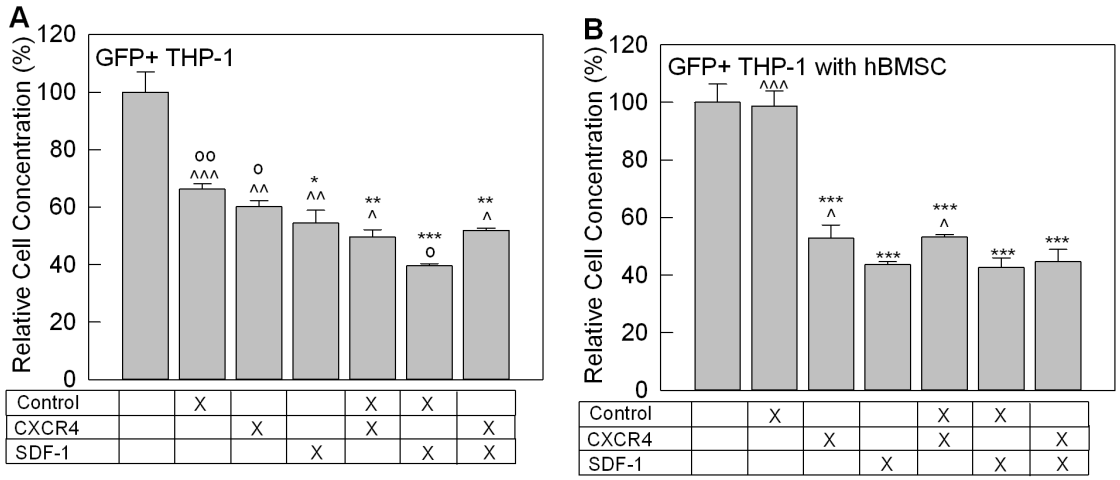

Figure 4.6 Effect of SDF-1 Silencing in Conjunction with CXCR4 Silencing with Lipopolymer/siRNA Complexes.

Silencing performed with (**A**) GFP-positive THP-1 cells without hBMSC, and (**B**) GFP-positive THP-1 cells with hBMSC co-incubation. In all treatments, the total siRNA concentration was 50 nM siRNA. '*' compares against control siRNA, '^' compares against control+SDF-1 siRNA, 'o' compares against control+CXCR4 siRNA. While CXCR4 and SDF-1 silencing was separately effective in reducing cell numbers, silencing both CXCR4 and SDF-1 simultaneously did not enhance the observed decrease in cell concentration.

4.3.6 Effect of Silencing CXCR4 and SDF-1 on Cell Division

In order to investigate the mechanism behind the effect of CXCR4 silencing on decreased cell numbers, we assessed cell proliferation by using an established dye-dilution assay with Cell Tracker™ Green CMFDA [33, 34]. After Cell Tracker™ Green CMFDA diffuses into the cell, it is converted into a cell impermeable form intracellularly, which is then diluted through cell division during passage onto daughter cells [34]. The THP-1 cells were initially labeled with an optimal concentration of Cell Tracker™ Green CMFDA prior to lipopolymer/siRNA complex treatments (**Figure 4.S1**). After silencing cells with the CXCR4 or SDF-1 siRNA with co-incubation with hBMSC, we observed a decline in fluorescence, providing a measure of cell division and subsequent CMFDA dilution (**Figure 4.7A**). The CMFDA dilution was less with THP-1

cells treated with 25 nM and 50 nM SDF-1 siRNA and 25 nM CXCR4 siRNA, indicating a decrease in proliferation after silencing these targets (**Figure 4.7A**). The decreased proliferation rate of CXCR4 and SDF-1 silenced cells compared to the control siRNA treated cells corresponded to the slower cell growth rates from direct cell counts (**Figure 4.7B**). The SDF-1 siRNA provided more significant effects in this experiment with a marked decrease in proliferation rates measured by CMFDA and cell concentrations.

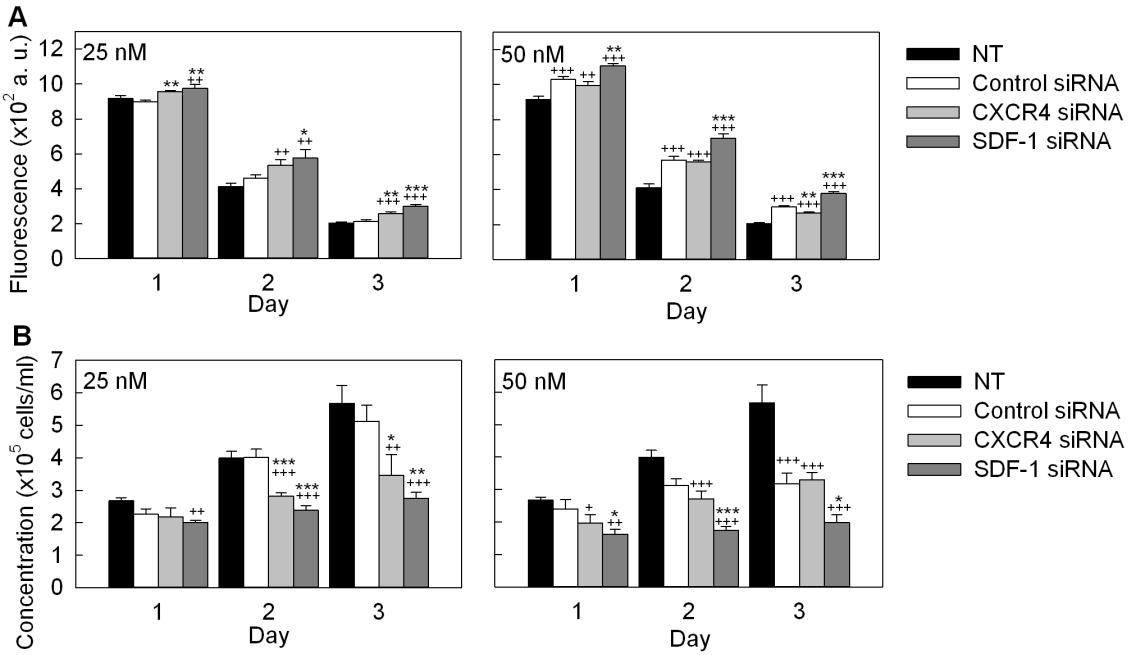

Figure 4.7 Effect of Lipopolymer/siRNA Complex Mediated CXCR4 and SDF-1 Silencing on Cell Proliferation with Co-Incubation of hBMSC.

The cells were either untreated (NT) or treated with control, SDF-1 and CXCR4 specific siRNAs at 25 and 50 nM concentration. (**A**) Cell proliferation as measured by the loss of CMFDA dye through cell division. The results are summarized as the mean (+SD) cell-associated fluorescence for 3 days following siRNA treatment. (**B**) Changes in cell concentrations as a result of siRNA treatment. A reduced proliferation was evident by increased intracellular fluorescence levels in A and decreased cell numbers in B.

4.4 DISCUSSION AND CONCLUSIONS

Initial studies for the lipopolymers utilized in this paper focused on the physicochemical features of the polymer and properties of lipopolymer/siRNA complexes critical for intracellular delivery and silencing a model target (GFP) in AML cells [8, 15]. In this paper, we focus on demonstrating the therapeutic potential of the most promising lipopolymer carrier, caprylic acid modified low molecular weight PEI (PEI2-CA), in siRNA-mediated silencing of the CXCR4 receptor and its ligand SDF-1 (CXCL12) in AML. We demonstrated successful reduction of CXCR4 protein levels (by immunostaining) when siRNA delivery was undertaken with the PEI2-CA. The suppression of CXCR4 resulted in a decrease in overall cell survival, evident by a decrease in cell numbers during the experimental study period. The decrease in cell numbers was shown to be, at least in part, due to a decrease in proliferation by using a well-accepted dye dilution method. The SDF-1 is predominantly expressed by BMSC and binds to AML cells through the CXCR4 receptor, however it is also expressed and released by the AML cells and has been implicated in many roles besides chemotaxis [31, 32]. We also demonstrated a decrease in cell number as result of SDF-1 siRNA mediated silencing with the lipopolymer/siRNA complexes. Kim *et al.* have recently investigated the effect of silencing SDF-1 by siRNA with the commercial HiPerFect reagent in AML cells where suppressing SDF-1 resulted in decreased proliferation and decreased SDF-1 related signaling. All together their findings indicated a stimulatory (autocrine) role of SDF-1 in AML cells to enhance cellular proliferation [32]. Additionally, we observed no enhanced effect when simultaneously silencing CXCR4 and SDF-1, suggesting that the proliferative effect is a result of the same pathway. Kim *et al.* similarly suggested that

proliferative effects of CXCR4 and SDF-1 are a result of the same pathway with their observation that upregulation of cytoplasmic CXCR4 was observed as a result of SDF-1 silencing [32].

Besides CXCR4 and SDF-1, other adhesion proteins have also been found to result in decreased proliferation when suppressed with RNAi. In a shRNA screen *in vivo*, integrin-b3, (ITGB3) was found to decrease homing and BMSC adhesion of leukemic cells, as well cause decrease proliferation and differentiation of MLL-AF9 oncogene transduced granulocyte-monocyte progenitor cells (transplantable MLL-AF9 AML model) [35]. Furthermore, suppression of integrin-av (ITGAV), which forms a dimer with ITGB3, and suppression ITGAV pathway members Syk, Vav1, Rac2, Rhoa and CD47 showed similar results [35]. Similarly, shRNA suppression or antibody treatment for integrin-a6 (ITGA6), as well as ecotropic viral integration site-1 (EVI1) and integrin-b4 (ITGB4), have also been found to not only decrease leukemic cell adhesion to BMSC environment but also to decrease survival ability as well as to increase chemosensitivity in EVI1-high expressed AML [36]. siRNA suppression of insulin-like growth factor–binding protein 7 (IGFBP7), known as a tumor suppressor in solid tumors, was similarly found to decrease endothelial cell adhesion, migration, invasion as well as proliferation in U937 AML cells [37]. The involvement of proteins in both adhesion to bone marrow microenvironment as well as leukemia cell survival/proliferation was also observed in leukemic stem cell population (CD34+/CD38-), where suppression of CD82 (by siRNA and shRNA) was found to decrease adhesion as well as cell survival [38].

Determining potential targets to suppress in order to re-sensitize AML cells to cancer drugs is an important strategy for improved drug therapy. Previous siRNA screens

designed to determine potential siRNA targets that sensitize the cells to cytarabine included cell cycle check-point and DNA-damage and repair proteins [39, 40]. Other effective RNAi targets for re-sensitization to various AML treatments include anti-apoptosis proteins (Bcl-210 [41], Bcl-2 [42, 43], Mcl-1 [44-47], C-FLIP$_L$ [48]), epigenetic modifiers (LSD1 [49], HDACs 1 and 6 [50]), a protein involved in autophagy (S100A8 [51]), a molecular chaperone protein (NPM1 [52]), MEK/ERK signaling pathway proteins (MEK-1 [53], 4E-BP1 [47]), the oncogene Cot1 [54] and the kinases (Mnk1 and 2 [55]). We observed an enhanced anti-survival effect in THP-1 cells, when CXCR4 expression was suppressed by siRNA and then treated with cytarabine in the presence of hBMSC. The observed effect could be due to different mechanisms of cytarabine toxicity and anti-survival effect of CXCR4 suppression. Alternatively, CXCR4 pathways could mediate partial chemo-resistance to cytarabine exposure, and its silencing reduces cellular resistance to the drug [56-58]. CXCR4 activation by SDF-1 has been found to contribute to resistance to cytarabine through suppression of the microRNA let-7a, which activates Myc and Bcl-XL [56]. Secretion of unspecified soluble factor(s) from BMSCs, which could possibly include SDF-1, may additionally provide chemo-resistance to cytarabine (observed through decreased apoptosis) by causing decreased activity of drug transporter such as equilibrative nucleoside transporter 1 (ENT1) [57]. We did not however observe an increased resistance to cytarabine when AML cells were grown with the hBMSC, suggesting that other BMSC secreted factor(s) might not be significant in our culture system.

The decrease in cell numbers due to CXCR4/SDF-1 silencing with lipopolymer/siRNA complexes was observed both in the presence and absence of

hBMSC. However, co-incubation of cells with the hBMSC revealed more dramatic results of CXCR4 silencing. This was partly due to a decreased toxicity of the polymeric carrier system (i.e., control siRNA complexed with PEI2-CA) when THP-1 cells were treated in the presence of hBMSCs, suggesting a protective role of the hBMSC on the THP-1 cells. The decrease in toxicity did not however negatively affect silencing of the CXCR4 or its anti-survival response. It was conceivable that some of the siRNA complexes could be consumed by the hBMSC and THP-1 cells could be exposed to lower dose of siRNA in this way. Although this was not directly determined, no impediment was seen in the functional response to CXCR4 siRNA treatment. As noted above, BMSC environment was reported to provide protection against the drugs' toxic effects. This seems to be true for the cytotoxic effects of our PEI2-CA carrier system as well, but not for the specific effects of siRNA-mediated silencing.

The CXCR4 silencing demonstrated a significant although nondramatic decrease in cell attachment to hBMSC. We also did not achieve full silencing with CXCR4 (only ~30% decrease, based on cell surface immunolabeling) so that the remaining cell surface CXCR4 could mediate the observed binding to hBMSC. Additionally, CXCR4 expression levels ranges among the AML cells and THP-1 cells are among the high expressing cell types having >80% CXCR4-positive cell population. Also, THP-1 cells do have other adhesion molecules mediating their adhesion to BMSC besides CXCR4, such as the CXCR7 and CD44 [31, 59, 60], which were not targeted in this study. Therefore in order to completely prevent adhesion and dislodge leukemic cells from the protective bone marrow, multiple adhesion proteins may need to be targeted. Although displacing leukemic cell from the bone marrow environment is one of the main purposes

of targeting CXCR4, inhibition of adhesion may not be required for disruption of activating signaling through the CXCR4 pathway and resulting survival pathways [18].

It is foreseeable that other cell types will experience CXCR4 silencing with systemic siRNA delivery and that they will respond to CXCR4 silencing differently. This might be reminiscent of the potential side-effects of classical CXCR4 antagonists, including possible effects on normal hematopoiesis [61, 62]. If CXCR4 silencing did mobilize normal hematopoietic cells, they would be more susceptible to toxic effects of any co-treatments with chemotherapy drugs [61]. Disruption of CXCR4/SDF-1 mediated homing and trafficking of non-leukemic cells could negatively affect the immune system and hematopoietic functions [62], especially in case of long-term or repeat siRNA therapy. Future work should further explore the effects of CXCR4/SDF-1 silencing in other hematopoietic cells as well as bone marrow cells. In some cases, such as the leukemic stem cell population, silencing CXCR4 would likely remain beneficial. Further work could also employ use of targeting ligands to enhance specificity to leukemic cell populations.

Development of siRNA carriers and siRNA-mediated silencing as a therapy in leukemia, and specifically in AML, has not been explored in detail at the present time. In contrast to the multitude of CXCR4/SDF-1 antagonist studies on leukemic cells ranging from *in vitro* to clinical trials, very few studies utilized the siRNA technology as a therapeutic option for leukemia. Despite a lesser degree of focus in leukemia, siRNA therapy has been progressing into clinical trials as a cancer therapy [63, 64]. Additionally, the benefit of the CXCR4 target for siRNA therapies has been realized with solid tumors [65-67]. Here, we demonstrated a significant decrease in AML proliferation as a result of

silencing CXCR4 expression utilizing lipopolymer/siRNA complexes in THP-1 cells. This study represents the first polymeric system used specifically for CXCR4 and SDF-1 silencing in an AML model. We also show similar results following suppression of its main binding protein, SDF-1 (CXCL12). Decreasing CXCR4 and SDF-1 expression via siRNA could be a promising therapy and provides an additional option from the antagonists and blocking antibodies already in pursuit.

4.5 ACKNOWLEDGMENTS

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5. *Ex Vivo* Silencing in Primary Acute Myeloid Leukemia Cells and *In Vivo* Silencing in Acute Myeloid Leukemia Cell Line THP-1

5.1 INTRODUCTION

The CXC chemokine receptor 4, CXCR4, protein has garnered much attention in recent years as a potential target in treatment of AML. CXCR4 expression in leukemic cells leads to their migration to the bone marrow microenvironment as a result of the chemo-attractant stromal-cell derived factor-1, SDF-1, being released from the bone marrow stromal cells (BMSCs) and endothelial cells. Treatment of leukemic cells in the bone marrow microenvironment is difficult within this hard-to-reach tissue and chemoprotective environment. In addition, CXCR4 activation results in signalling through survival pathways resulting in enhanced leukemic cell survival and has been specifically implicated in causing increased proliferation, additional drug resistant, degradation of extracellular matrix and angiogenesis [1]. Development of small molecular antagonists and blocking antibodies against CXCR4 have shown potential for haematological malignancies including AML [2-4] as well as other cancers [5-7]. However the technologies continue to have their own challenges including specificity, toxicity, varied treatment responses, complicated mechanisms of action and possible resistance development [8-11]. siRNA mediated silencing of CXCR4 resulting in inhibition of the CXCR4 protein expression is a potential alternative to current approaches which block the already present CXCR4 protein.

However, progress in RNAi mediated therapies for AML has lagged behind other cancer types [12]. siRNA, once within the leukemic cell cytoplasm, can suppress the target protein production, however the requirement of a carrier to effectively deliver the siRNA intracellularly has been the principal hindering factor. As leukemic cells are within the ‘hard-to-transfect’ category of cells intended for gene therapy, effective

carriers for delivery to AML cells lag behind those developed for other types of cancers [12-14]. Despite some progress in novel siRNA carriers designed and tested in AML cell types, most studies are still performed with cell lines in *in vitro* cell culture [12]. Our recent work has shown successful silencing of CXCR4 [15] in the AML cell line THP-1 with our in-house lipid-PEI siRNA carrier and resulted in a decrease in cell proliferation and a significant decrease in adherence to BMSCs. We demonstrated clinically related outcomes, such as effective CXCR4 silencing in the presence of bone marrow stromal cells and improved efficacy of a leukemic chemotherapy drug (cytarabine) when co-treated with CXCR4 siRNA. With the same carrier we have also demonstrated silencing of CD44, another potential AML target, in AML leukemia stem/progenitor cell (LSPC) lines (KG-1 (CD34+/CD38+) and KG-1a (CD34+/CD38-)) as well a primary AML cells (WHO classification being 'AML without maturation'; CD34+ population (60-86%)) [16]. Although we have made attempts to include more clinically relevant *in vitro* studies and have demonstrated silencing in an CD34+ AML patient cells, there is a clear need to progress siRNA carrier development for AML to more clinically relevant studies, specifically to demonstrate: i) siRNA delivery in a larger AML patient cohort, ii) CXCR4 silencing and resulting therapeutic effect in patient cells, and iii) effective silencing in leukemic animal models.

siRNA silencing in *in vitro* experiments with novel carriers have been performed in AML cell lines but not in AML patient primary cells (except for our own work on CD44 silencing). In a SDF-1 siRNA target study (with a commercial reagent; HiPerFect) AML patient cells were tested for SDF-1 expression, but no attempts in silencing SDF-1 in the patient cells were made and instead cell lines were utilized for silencing

experiments [17]. A modified siRNA, for TLR9 targeted delivery, was delivered in AML and multiple myeloma (MM) patient cells where most TLR9+ positive cells displayed uptake of the FITC labeled siRNA, however again the silencing studies were limited to cell lines both in *in vitro* and in a xenograft model with these cell lines [18]. The fact that the AML patient cells were used in some studies related to siRNA delivery, but not for silencing or for measuring siRNA-mediated therapeutic effects, suggests limitations of siRNA mediated silencing with AML patient cells *in vitro* (i.e., it is likely that the outcomes were not successful and were not reported). AML patient cells are well known for difficulty for cultivating *in vitro*, requiring careful thawing process (if cryopreserved), use of deoxyribonuclease (DNase) and/or filtering to prevent clumping (as result of DNA released from dying cells), resulting in lower cell viabilities and usually restricting the studies to short-term culture (commonly less than 1 week). Whether RNAi machinery is still fully functional in these cells remains to be characterized.

We have previously demonstrated CD44 silencing in CD34+ AML patient samples (n=3) [16] and to our knowledge this was the first time siRNA mediated silencing was demonstrated in AML primary cells *in vitro* with a non-viral polymer carrier. However, the population tested is a specific AML type (CD34+ cells), a small sample size and therefore does not depict the variation of leukemic population in AML patients. Additionally, as CD44 is a different target with its own attachment properties, resulting silencing and effects on silencing will likely differ from the attachment receptor CXCR4. Determining the efficiency of the lipid-polymers in siRNA silencing of CXCR4 in AML patient cells should provide another step towards siRNA therapy reaching clinical application for AML. Patient-to-patient variation of the AML cell population

could result in varied siRNA uptake, siRNA silencing and siRNA mediated anti-leukemic effects and thus it is essential to determine effectiveness of the siRNA carriers in cells derived from numerous AML patients. This approach also provides insight into the frequency of CXCR4 over-expression in patient cells (i.e., how viable a target it is) and how often a therapeutic effect results from silencing this protein. Patient samples also can include LSC populations, the ideal siRNA target to prevent relapse in patients [19], thereby providing the opportunity in determining effects of silencing on sub-populations of the patients' leukemic cell population.

As *in vitro* testing does not always mirror *in vivo* results, experiments in animal models are another necessary step. However, few siRNA studies have been fully explored in leukemic *in vivo* models. Some of the current *in vivo* data available are derived from pharmokinetics and related studies in healthy mice. Other studies target only reporter genes such as luciferase. Some lack control (non-specific siRNA) treatments and definite description of carriers and scrambled siRNA, and often utilize high siRNA doses and numerous injections. As examples, a lipid nanoparticle designed for siRNA delivery to AML cells (as well as other hard to transfect cells) was tested in healthy mice (non-leukemic model) with demonstrated silencing of model targets (KIF11 and AHSA1) in spleen, bone and liver cells [13]. No leukemic cells were used in that study. A polyplex carrier (*in vivo*-JetPEI^{TM}) demonstrated silencing of luciferase positive leukemic cells in circulation, however siRNA concentrations and dosing scheme were relatively high (2.5 mg/kg every 48 h for 5 weeks) and no functional (therapeutic) outcome was explored [20]. A subcutaneous leukemia tumor silencing study was reported, however the use of the carrier and scrambled siRNA was ambiguous [21, 22]. Another subcutaneous tumor

study with the AML MV4-11 cell line demonstrated delivery and silencing (RRM2), but not therapeutic effects [23]. However, currently there does not appear to be any ideal demonstration of effective *in vivo* non-viral siRNA treatment for leukemia.

In the following experiments, we explore CXCR4 siRNA mediated silencing in AML patient cells and include a discussion on the challenges and potential techniques that can enhance leukemia patient studies *in vitro*. We then explore silencing and potential therapeutic effects when delivering CXCR4 siRNA with lipid-modified polymer carriers to a subcutaneous AML tumor model.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Two kDa polyethylenimine (PEI2; M_n: 1.8 kDa, M_w: 2 kDa), anhydrous dimethyl sulfoxide (DMSO), caprylic chloride (C8), linoleoyl acid (C18:2), trypan blue solution (0.4%) were purchased from Sigma-Aldrich (St. Louis, MO). Negative control siRNA (AM4635) and FAM labeled negative control siRNA (AM4620) was purchased from Life Technologies (Carlsbad, CA). AllStars Neg. siRNA labeled with Alexa Fluor 488 (SF488) (1027284) was purchased from Qiagen (Toronto, ON). CXCR4 siRNA (CAT: HSC.RNAI. N001008540.12.1) was purchased from IDT Inc. (Coralville, IA). Hanks Balanced Salt Solution (HBSS) and IMDM (12440) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS; 35-010-CV) was purchased from CORNING Cellgro (Manassas, VA). The PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody was from BD Pharmingen (Mississauga, ON). Ficoll-Paque™

PREMIUM a trademark of GE Healthcare Life Sciences was purchased from Fisher Scientific.

5.2.2 Cell Models and Cell Culture AML Patient Cells Harvest and Cell Culture

Peripheral blood (PB) or bone marrow (BM) samples were obtained from AML patients with active disease at diagnosis in University of Alberta Hospital. Patient characteristics are shown in **Table 5.1**. Written informed consent was obtained from patients according to the declaration of Helsinki. The project outlines and consent procedures were submitted and approved by the Ethic Committee of the University Of Alberta Hospital (#Pro00043783). All specimens were collected prior to treatment. PB was collected in heparinized vacutainer tubes and BM aspirates were collected in heparinized syringes. Mononuclear cell (MNC) fractions were obtained by density gradient centrifugation using Ficoll-Paque^{TM} PREMIUM (GE Healthcare).

Briefly, PB or BM samples were diluted 2 to 4 times with PBS/2%FBS and gently layered onto the Ficoll-Paque^{TM} PREMIUM solution and then centrifuged for 40 min at 400g with no brake at room temperature. Carefully, MNCs were aspirated from the Ficoll-plasma interface and washed with PBS/2%FBS at 200g for 10 min, twice and resuspended in PBS/2%FBS. For frozen samples, the cell suspensions were delivered to the Canadian Biosample Repository (University of Alberta) for freezing and storage.

MNCs obtained from frozen samples were thawed quickly at 37°C water bath (until almost completely thawed), wiped with 70% alcohol and 125 µl of filtered DNase (1 mg/ml) was added directly to cells. Cells were then immediately transferred slowly to 10 ml of IMDM medium (20% FBS) and centrifuged at 300 x g for 5 min. Supernatant was aspirated and 10 ml of IMDM medium (20% FBS) was added and then transferred to

cell culture flask and incubated under normal conditions (37°C, 5% CO_2 under humidified atmosphere). Trypan blue staining was used determine cell viability. When fresh MNCs were utilised, the MNCs were obtained on the day of harvest, ficoll-paque separated and then directly transferred to culture flask with IMDM with 20% FBS and incubated under normal conditions (37°C, 5% CO_2 under humidified atmosphere).

5.2.3 siRNA/Polymer Complex Transfection

AML mononuclear cells were counted and diluted to seeding concentrations (0.5-2x10^6 cells/mL) and incubated (37 °C, 5% CO_2). Polymer-siRNA complexes were prepared for reverse siRNA transfection (control siRNA (CsiRNA), FAM labeled control siRNA (FAM-siRNA), AlexaFluor 488 labeled siRNA (AF488-siRNA) and CXCR4 targeting siRNA (CXCR4siRNA)). Briefly, siRNA was added to IMDM medium followed by the polymer (final volume of 150 µL or 300 µL), briefly vortexed and incubated for 30 min at room temperature. After complex incubation, 100 µL of complex solution was added to 48-well plate and complete coverage of the surface was ensured. The pre-prepared AML MNCs were then added to each well (300 µL). CsiRNA final concentrations (75 nM) and polymer:siRNA weight ratios (4:1, 8:1 and 12:1) are as reported in figure captions.

5.2.4 Detection of siRNA Delivery

siRNA delivery experiments were performed with unlabeled control (scrambled) siRNA, FAM-labeled control siRNA and AlexaFluor 488-labeled control siRNA. One-day after siRNA transfection, cell suspensions were transferred to microcentrifuge tubes and centrifuged at 300g. Supernatant was removed and cells were resuspended in HBSS and then fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by

flow cytometry (FL1 channel) with Cell Lab Quanta^{TM} SC (Beckman Couter). Flow cytometry laser settings were kept constant for all samples. The gating for FL1+ region was however, adjusted to 1% of NT, on a per patient sample basis. To account for the change in cell surface area between patient samples, the mean fluorescence are also shown as normalized to cell surface area. Surface areas were calculated from the average diameter reported from the flow cytometer and assuming a spherical geometry (Cell Surface Area = $4\pi r^2$).

5.2.5 CXCR4 Silencing Experiments

siRNA silencing experiments were performed with siRNA targeting CXCR4 as well as negative control siRNA. At indicated time points after siRNA transfection, cell suspensions were transferred to microcentrifuge tubes and centrifuged at 300g. The supernatant was aspirated, cells were resuspended and stained with antibodies (4 μL of PE-labeled mouse anti-human CXCR4 (CD184) or mouse IgG isotype control antibody) in 90 μL of medium for 45 min at 4°C and then washed twice with HBSS. Finally, cells were resuspended in HBSS and fixed with 2.0% formalin (final concentration of 1% formalin) and analyzed by flow cytometry (FL2 channel) with Cell Lab Quanta^{TM} SC (Beckman Couter). To determine internal CXCR4 levels, cells were permeabilized prior to antibody staining, fixed with formaldehyde (1% final concentration) by incubation for 20 min at room temperature, after which they were permeabilized with 0.3% Triton X100 (in HBSS) for 20 min at room temperature in the dark and then centrifuged at 500g. Cells were stained with antibodies as described above, except in HBSS with 0.3% Triton X100 and 10% FBS instead of 10% FBS medium. Following staining, cells were washed twice

with 0.3% Triton X100 and then resuspended in HBSS. Flow cytometry was performed as described above.

5.2.6 CXCR4 Silencing in AML THP-1 Animal Model

GFP expressing AML (THP-1) tumors were established in 6 week old, male NCR nu/nu (Taconic Farms Inc.; average weight of mice 23.6 g on day of cell injection) following subcutaneous injection of THP-1 cells ($7x10^6$ cells/mouse) in the right flank. These studies were performed in accordance with the University of Alberta guidelines for the care and use of laboratory animals. During tumor establishment over the next 5 weeks, tumor volumes (by calliper) and mouse weights were measured twice a week. Ten μg of siRNA-polymer complexes (~0.33 mg/kg siRNA) at 1:4 siRNA:polymer PEI2-CA2.6 ratio (prepared as described above) were intratumorally injected every 3 days, 4 times after tumor establishment. Tumor volumes were measured by caliper every 3 days where the longest diameter (L) was measured as well as the diameter of the perpendicular direction (W) (prior to siRNA injections). Tumor volumes were estimated from measured diameters utilizing an ellipsoid volume formula (tumor volume = $L \times W^2 \times 0.4$) as previously described [24-26]. Three days after the last injection, mice were sacrificed (by CO_2 asphyxiation) and tumors were harvested. THP-1 cells from extracted tumors were obtained by gently using a pellet pistol motor on the tumor in HBSS solutions. The cell solution was passed through a sterile cell strainer (40 μm nylon mesh; Fisher Scientific). Cell concentrations were adjusted to be uniform between samples (~$5x10^6$ cells/mL). Antibody staining (PE-labeled CXCR4 Ab and Igg control Ab) to determine CXCR4 silencing was performed, as described above. As many replicates (1-4) per tumor sample as possible were used depending on total cell numbers recovered from the tumors. After

Ab staining, cells were analysed with the LSR-Fortessa SORP (BD Biosciences) for simultaneous detection of PE antibodies (λ_{EX} at 561 nm and λ_{EM} at 586 nm) and GFP (λ_{EX} at 488 nm and λ_{EM} at 530 nm). GFP positive cells were used to clearly select for GFP+ THP-1 population from the host murine cell population. The CXCR4 expression (PE antibody label) was based on the mean CXCR4 levels and the CXCR4-positive cell population determined within the GFP positive population. The cell population stained with non-specific antibody was used for flow cytometry calibration (i.e., designated as 1-2% CXCR4-positive population).

5.2.7 Statistics

All graphs display mean results and error bars indicate the standard deviations. Statistical analysis was performed with GraphPad InStat v3.06 (GraphPad Software, San Diego, CA USA). A two-tailed unpaired t-test was used unless otherwise stated (in figure captions) where '*' compares between control siRNA and CXCR4 siRNA groups with a given polymer, 'x' compares between FAM-siRNA and AF488-siRNA, '#' compares between PEI2-LA and PEI2-CA polymers used for siRNA/polymer complexes, and '+' compares between the relative tumor volume to the hypothetical mean of 1.0. Statistical significance is indicated utilizing symbols #/*/x/+ where #/*/x/+ indicates $p<0.05$, ##/**/xx/++ $p<0.01$ and ###/***/xx/++ $p<0.001$.

5.3 RESULTS

5.3.1 siRNA Delivery to Human Patient AML Mononuclear Cells (MNCs)

Two kDa polyethylenimine (PEI), modified with either caprylic acid (PEI2-CA5.4) or linoleic acid (PEI2-LA2.1) were previously demonstrated to be effective siRNA carriers for GFP (model target), CXCR4 and CD44 silencing in AML cell lines [15, 16, 27]. We first attempted siRNA delivery with the lipid-modified polymers, utilizing a FAM-labeled control siRNA to human patient AML mononuclear cells (MNCs), as shown in **Table 5.1**. Untreated AML patient samples (n=5) were selected based on high blast percentage, where the reported blast percentages were from 75% to >90%, ensuring that the majority of cells in the analysed cell population were in fact leukemic cells. The patient samples vary by age (45-77 years), cytogenetics, mutations, white blood cell (WBC) counts and location of harvest (peripheral blood or bone marrow). All of the AML patients tested were determined to have a poor prognosis based on their cytogenetics and mutations except Patient #1 (prognosis was determined as described in **Table 5.1**). It is important to also note the viability (measured after 24 hours) and recovery (calculated from the percentage of measured viable cells at 24 hours over the listed cell numbers per vial) ranged from 48-75% viability and 15-64% recovery, respectively, which are unfavourable numbers to begin experimental studies with.

Table 5.1 Mononuclear Cells Isolated From Untreated AML Patients.

Patient	Age (yr.)	Sex	BM / PB	Prognosis Category	Cytogenetics	Mutations		WBC	Blast %	Other Markers		State of Cells		Cell Diameter* ** (a.u)
						NPM1	FLT3-ITD			CD34	CD38	Viability (%)	Recovery (%)*	
#1	59	M	BM	Better	-Y	+	-	30	>80	-	+	75	48	9.63
#2	77	M	PB	Poor	Normal	+	+	268	>90	-	dim	56	28	7.87
#3	45	F	BM	Poor	Normal	+	+	10	75	-	dim	48	15**	8.42
#4	67	M	BM	Poor	+13,+19,+21	-	-	18	>80	+	dim	72	60**	7.50
#5	49	F	PB	Poor	del(3q),-7	-	-	107	78	NA	NA	54	64	11.88

Abbreviations: PB=peripheral blood; BM=bone marrow; NA=not available.

Prognosis category: *Better prognosis*: inv(16), t(16;16), t(8;21), t(15;17); Normal cytogenetics with NPM1 mutation or isolated CEBPA mutation, in the absence of FLT3-ITD. *Intermediate prognosis*: Normal cytogenetics, +8, t(9;11); other chromosomal abnormalities. *Poor prognosis:* -5, 5q-, -7, 7q-, 11q23 other than t(9;11), inv(3), t(3;3), t(6;9), t(9;22), complex findings (≥3 clonal chromosomal abnormalities), FLT3-ITD mutated (FLT3/ITD or FLT3/TKD). FAB and WHO classifications were not available. Viability was measured by trypan blue, 24h after thawing cells. *Recovery % = viable cell numbers (at 24hr)/listed cell numbers per vial. **Cells required filtration during thawing procedure due to cell clumping. *** Cell diameters are the averaged values reported by flow cytometry where standard deviations were ±0.14 (a.u) or less. Diameter readings corresponded with visual size observations.

Fluorescent labeled siRNA delivery to AML patients MNCs (n=5) demonstrated significant delivery. In **Figure 5.1,** siRNA delivery is shown for individual patients (i-v) and as an average for all AML patients (vi). The mean fluorescence, an indicator of the amount of siRNA per cell, is shown in **Figure 5.1A**. Here the amount of delivery was higher with increased polymer:siRNA ratio, as expected. Generally, PEI2-LA2.1 provided higher delivery amount compared to PEI2-CA5.4, except with one patient AML MNC sample (Patient #2) where the reverse occurred. Much higher mean fluorescence levels with Patient #5 and slightly higher mean fluorescence with Patient #1 was evident with the LA-substituted polymer (PEI2-LA2.1). Increased delivery with PEI2-CA5.4 with Patient #2 and Patient #5 in comparison to delivery with the other patient MNCs with the same polymer was also noted.

In **Figure 5.1B**, we display the mean fluorescence normalised by the average cell surface area, calculated from the mean diameter (shown in **Table 5.1**) as measured by the flow cytometer for each patient sample. Normalization was performed due to the large range in MNC cell sizes among patients, the largest cell size being ~1.6X larger (Patient #5) in diameter than the smallest patient sample (Patient #4), which amounts to ~2.5X or ~4.0X larger cell surface area or cell volume, respectively. Normalization with cell surface areas has been utilized in literature calculations for antibody staining for cell populations that differ in size (e.g., B cells, granulocytes and megakaryocytes), as the contact frequency of the antibody is dependent on total surface area of the cell [28]. In the case of polymer mediated siRNA delivery, size can effect the fluorescence levels due to increasing polymer-siRNA complex contact probability with increasing surface area. After normalizing with cell surface area, the mean fluorescence became more comparable

among patients, especially evident when considering Patient #5. Normalization with cell volume instead of cell surface area provided similar results (not shown)

In **Figure 5.1C**, we summarized the percentage of siRNA delivery to the AML patient cells. The percent delivery remained more consistent regardless of patient cell size. LA-substituted polymer gave a delivery percentage that ranged from 37.6% for the polymer:siRNA ratio of 4, to 55.5% for the ratio of 9 and to 64.2% for the ratio of 12. PEI2-CA5.4 delivery percentage was slightly (and statistically) lower with 20.6% for ratio of 4, 34.7% for ratio of 8 and 44.2% for ratio of 12. In Patient #2, where CA-substituted polymer (PEI2-CA5.4) had demonstrated higher mean fluorescence than LA-substituted polymer (PEI2-LA2.1), the percent delivery between the two polymers was much more comparable. Interestingly, the patient sample (Patient #5) with significantly larger cell size (as measured from flow cytometry diameters, **Table 5.1)** displayed the most significant siRNA delivery (regardless of surface area/volume normalization).

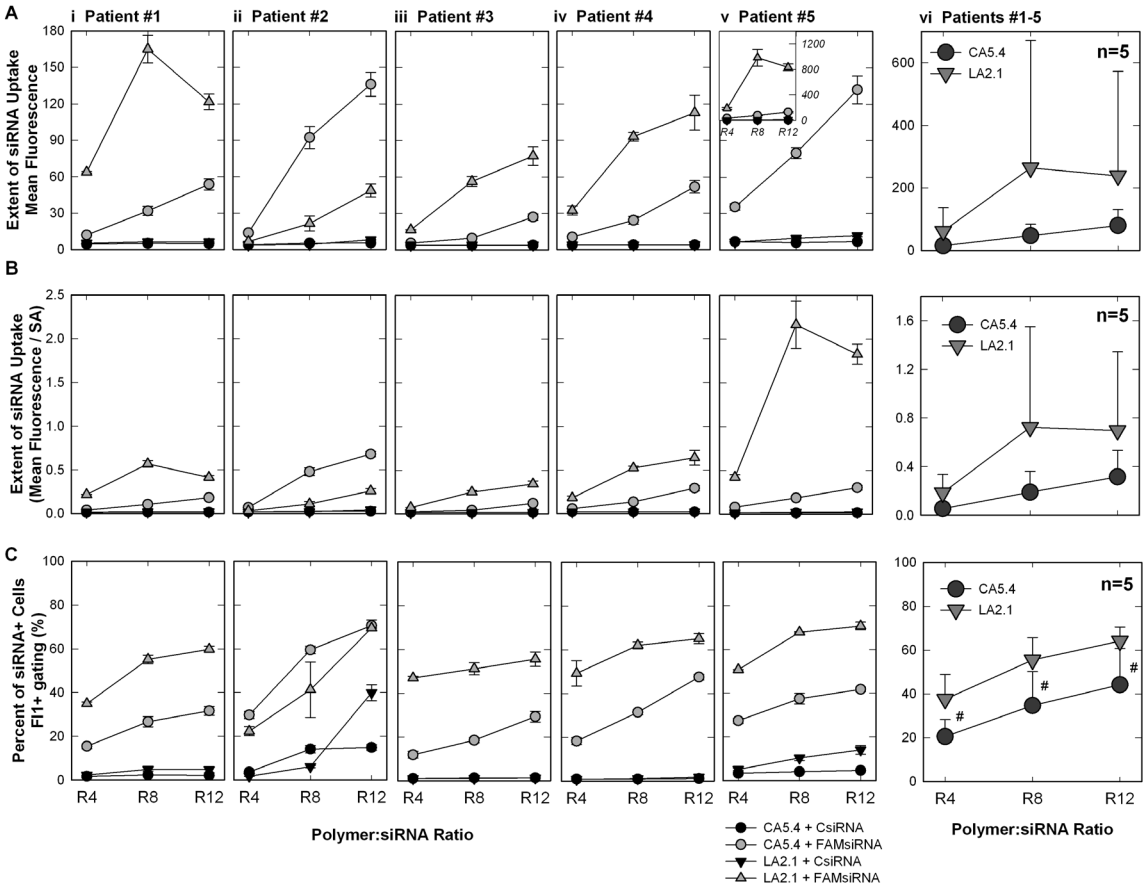


Figure 5.1 SiRNA Delivery to Human AML Patient Mononuclear Cells.
siRNA delivery in AML patient mononuclear cells (Patients 1-5) on day 1. siRNA delivery is presented as (**A**) the mean FAM siRNA levels, (**B**) the mean FAM levels normalised to cell surface area (SA) (to provide better comparison between patient samples), and the (**C**) percentage of siRNA positive cells. Delivery results are shown as either per patient (Patients 1-5) or as an average siRNA uptake in the AML patient samples (n=5). R8 (ratio 8) and R12 (ratio 12) are the polymer:siRNA weight ratios used in complex formulations. siRNA concentration was 75 nM. Both CA and LA provided significant siRNA delivery in all AML cells. LA was able to deliver to a higher percentage of the AML cells than CA. Significant difference in siRNA delivery between CA and LA polymer is indicated by '#' ($p<0.05$). CA5.4 refers to PEI2-CA5.4 and LA2.1 refers to polymer PEI2-LA2.1.

The fluorescent label used to detect siRNA delivery is expected to influence flow cytometric assessment; i.e., the minimum number of fluorescent siRNA molecules needed to detect intracellular uptake will depend on the label used. Thus, we investigated if by changing the fluorescent dye attached to the siRNA, the measured siRNA uptake would be affected. siRNA delivery was performed in Patient #5, with the FAM-siRNA as well as an AF488-siRNA (**Figure 5.2**). AF488 was utilised as it has similar

excitation/emission properties to FAM (AF488 Ex/Em 495/519 versus FAM Ex/Em 394/520 nm) but it is considered to have superior emissivity and improved photo-stability. In Patient #5, with PEI2-CA5.4, AF488-siRNA provided higher mean fluorescence levels suggesting better detection of siRNA uptake, when considering mean fluorescence of the two labels (**Figure 5.2A**). The mean fluorescence of the positive cells remained comparable between AF488- and FAM-siRNA (**Figure 5.2B**). The percentage of siRNA-positive cells increased by ~19.5% when AF488-siRNA was used instead of FAM-siRNA, producing percent uptake ranges of 44.2-62.3% instead of 27.4-41.7% depending on the ratio of the formulation used (**Figure 5.2C**). This increase in siRNA-positive cells is likely due to lower siRNA levels detected with the AF488 label. If AF488 provides better detection, it would make sense to observe a higher overall mean fluorescence value when considering the entire cell population and a higher percentage of cells positive for siRNA with AF488-siRNA but not necessarily an increase in the mean fluorescence of the siRNA positive population. With PEI2-CA5.4, we did make such observations, however this was not the case with PEI2-LA2.1, which may have reached the saturation limit for uptake already with the FAM-siRNA.

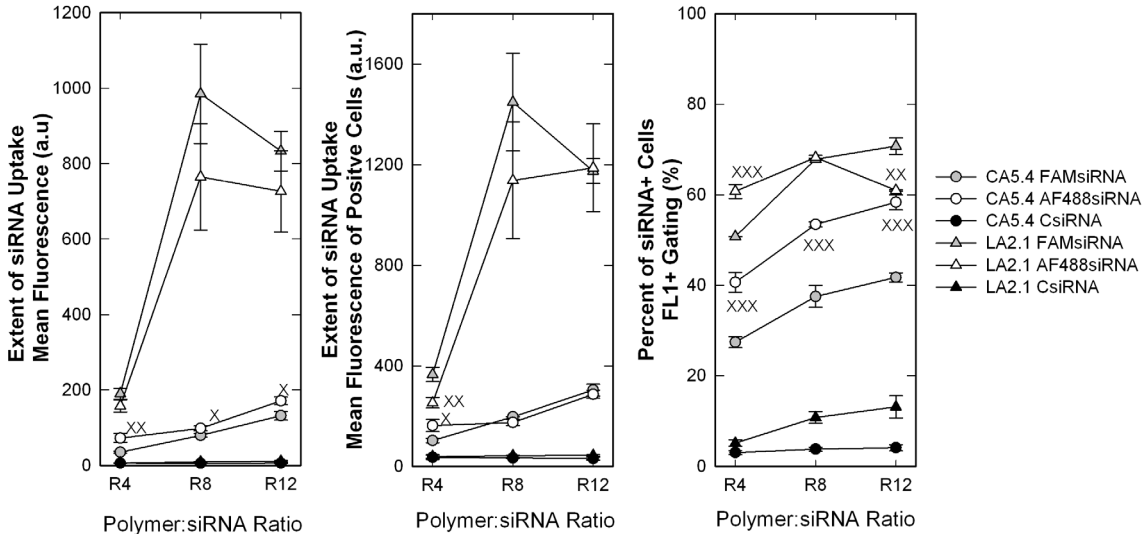


Figure 5.2 Comparing Detection of siRNA Delivery to AML MNCs with Fluorescence siRNA Labels.

siRNA delivery in AML Patient #5 mononuclear cells on day 1 with 5'-carboxyfluorescein (FAM) or Alexa Fluor 488 (AF488) fluorescent labels on control siRNA. siRNA delivery is presented as (**A**) the mean FAM siRNA levels, (**B**) the mean FAM siRNA levels of the siRNA positive cell population (FL1+) and (**C**) percentage of siRNA positive cells. Significant difference in siRNA delivery between AF488 and FAM siRNA with the same polymer is indicated with 'x' ($p<0.05$), 'xx' ($p<0.01$), 'xxx' ($p<0.001$). AF488 siRNA provided better detection than FAM siRNA when considering delivery with CA lipid-polymer. Similar delivery detection was apparent with LA lipid-polymer likely due to its already high delivery in this particular patient. CA5.4 refers to PEI2-CA5.4 and LA2.1 refers to polymer PEI2-LA2.1.

5.3.2 CXCR4 siRNA Silencing in Human Patient AML Mononuclear Cells (MNCs)

CXCR4 silencing was attempted in two patient cells utilized in the siRNA delivery studies, Patient #4 and #5. The samples were chosen for CXCR4 silencing solely based on available cells, as silencing experiments were performed in conjunction with siRNA delivery experiments. A single polymer:siRNA formulation was utilized, again due to limited cell numbers available. No significant CXCR4 silencing nor effect on cell numbers was observed in Patient #4 (**Figure 5.3A**). In Patient #5, clear CXCR4 silencing was apparent with the use of the PEI2-CA5.4 (**Figure 5.3B**). Here the overall mean fluorescence decreased by 19.4%, the mean fluorescence of the CXCR4 positive cells decreased by 13.8% and the over-all percentage of CXCR4-positve cells decreased from

29.6% to 25.0% resulting in 4.6% less CXCR4+ positive cells and percent decrease of 15.5%. A decrease of 30.7% in cell numbers was also observed as compared to control siRNA treated group (which was equivalent to no treatment group). Silencing with PEI2-LA2.1 substituted polymer was less apparent, with only a slight indication of silencing when considering the mean fluorescence of the CXCR4 antibody in the CXCR4-positive cells (8.2%) (**Figure 5.3Bii**) as well as an effect on cell concentrations (**Figure 5.3Biv**). Silencing with PEI2-CA5.4 was clearly stronger than LA-substituted polymer in Patient #5, despite the lower siRNA delivery ability in the same sample (**Figure 5.1v**).

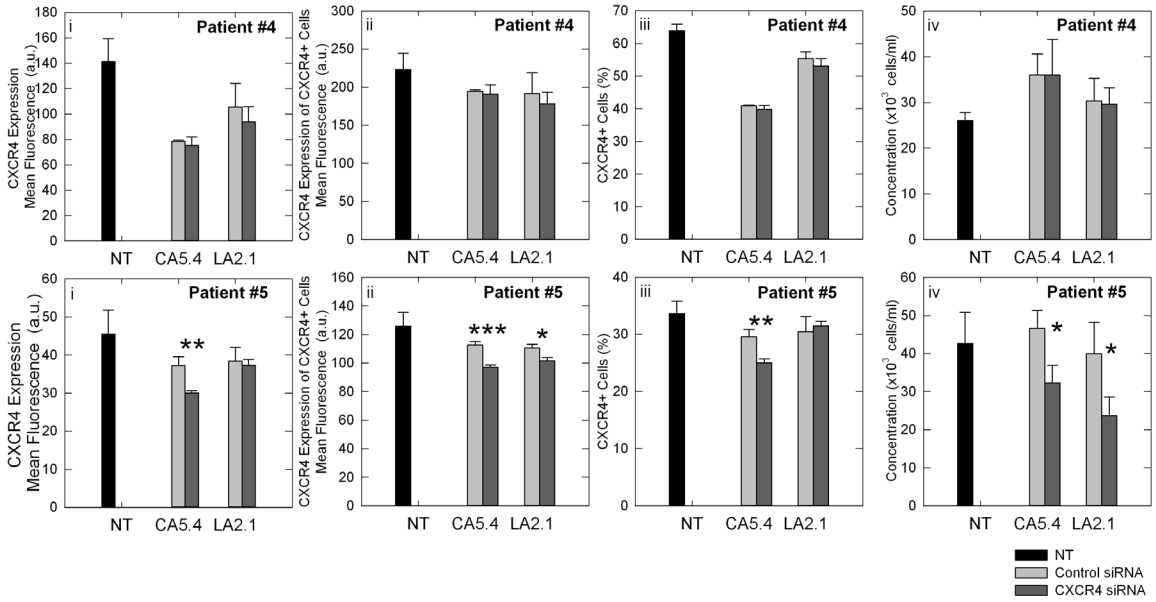


Figure 5.3 siRNA Mediated CXCR4 Silencing in Human AML Patient Mononuclear Cells Ex Vivo.

CXCR4 silencing in selected AML patient samples on day 2. Effect of CXCR4 silencing is indicated based on (i) mean CXCR4 antibody fluorescence levels, (ii) mean CXCR4 antibody fluorescence levels in CXCR4 positive cells, (iii) the CXCR4 positive cell population and (iv) the cell concentrations. Significant differences between control siRNA and CXCR4 siRNA for both CA and LA polymers is indicated by '*' ($p<0.05$), '**' ($P<0.01$) and '***' ($P<0.001$). R8 (ratio 8) with 75 nM siRNA was utilized for silencing experiments. CA demonstrated significant CXCR4 silencing in Patient #5 but not Patient #4. Despite higher siRNA delivery in both Patient #4 and Patient #5 than CA, LA did not demonstrate very strong silencing. CA5.4 refers to PEI2-CA5.4 and LA2.1 refers to polymer PEI2-LA2.1.

5.3.3 Human Patient AML Mononuclear Cells (MNCs) Viability Issues

The viability of the human AML patient cells after thawing from storage, in the above studies, was relatively low with a range of 48-75% 24 hours after cell thawing, **Table 5.1**. In addition, CXCR4 expression can be affected by cold temperature as well as ficoll-paque separation technique [29]. In an especially low viable sample (Patient #6; stored for 14 years), we obtained low surface CXCR4 expression with much higher internal CXCR4 levels on day 3, **Figure 5.4A**. Here, the poor cell viabilities may have contributed to the lack of external CXCR4 expression recovery. CXCR4 silencing with this sample was evident in the CXCR4+ population using CA-substituted polymer at ratio 8 ($p<0.05$) when internal CXCR4 staining was utilised (data not shown). CXCR4 expression, when affected by cell processing (ficoll separation) can be recovered, as shown in Patient #7, which was tested for CXCR4 expression from day 0 to day 3 immediately following patient harvest, **Figure 5.4B**.

Utilizing fresh (unfrozen) AML leukemic samples would clearly allow for higher cell viability. Initial CXCR4 silencing studies (not shown) were performed on fresh (unfrozen) untreated leukemic patient cells simultaneously with diagnosis of leukemic patients, such that leukemic patient status was unconfirmed at the start of the experiment. In these studies, 2 out of 4 patients were determined to have approximately 0% blasts, 1 had 30% blasts and diagnosed with erythroleukemia (AML-M6) and 1 had 88% blasts but belatedly diagnosed with B-ALL. In addition, in all fresh patient samples, contamination with RBCs (to varying degrees) was an issue as a result of the ficoll separation technique. It is unclear what the impact RBCs would have on the silencing process. Ideally, silencing would be done regardless of the presence of RBCs, however the over-all

negative charge of RBCs could result in binding to the positive charged siRNA complexes. Collectively, these studies provided less than ideal patient cells for our studies so that they were not considered beneficial.

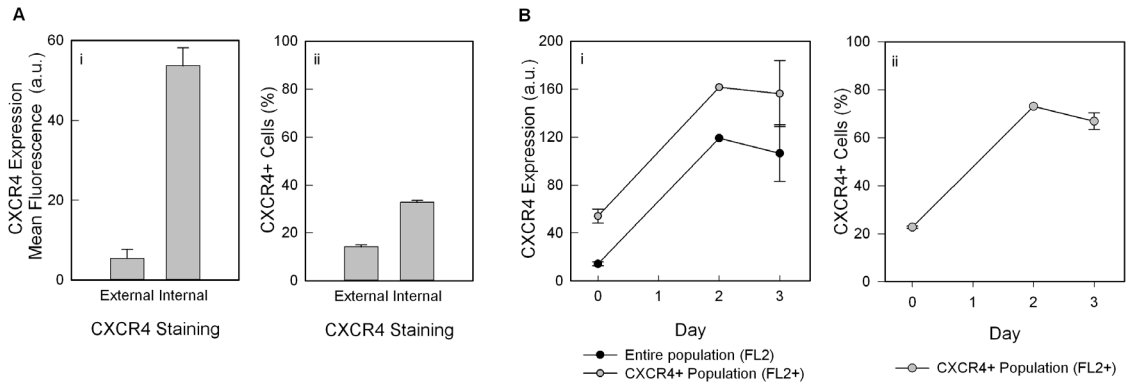


Figure 5.4 CXCR4 Expression Issues in AML Patient MNCs.
(**A**) CXCR4 external and internal expression on day 3 in AML MNCs of Patient #6 and (**B**) CXCR4 expression from day 0 (date of harvest) to day 3 of *in vitro* cell culture of AML MNCs of Patient #7. Presented as (i) mean fluorescence of the CXCR4 (**ii**) percentage of cells positive for CXCR4 expression.

5.3.4 CXCR4 siRNA Silencing *In Vivo*

GFP-expressing AML (THP-1) tumors were established in nude mice following subcutaneous injection of cells in the right flank. Tumor establishment was not ideal, with a slow establishment (five weeks until first injection in first treatment set), although the same mice established breast cancer tumors with ease [30]. Due to the slow tumor establishment, siRNA treatment experiments were performed over three different time periods, where each treatment group (control siRNA and CXCR4 siRNA) were tested in three mice for the first treatment and in a single mouse for the second and third treatments (**Figure 5.5** and **Figure 5.6D**). Ten out of thirty cell-injected mice established acceptable tumors by the end of the third treatment set, where the actual volume of the

tumors varied greatly at the start of treatment (**Figure 5.6D**); the average (±SD) size for all tumors was 80.2±66.2 mm^3, for size of the tumors selected for control siRNA treatment was 92.4±83.6 mm^3 and tumor size selected for CXCR4 siRNA treatment was 68.0±49.1 mm^3 on day 0. The main outliers were two large tumors (used in the control siRNA-1 and CXCR4 siRNA-1 treatments) with volumes on day 0 (first day of siRNA treatment) being, 233.0 mm^3 and 146.6 mm^3, respectively. All other tumor volumes were less than 100 mm^3 on day 0.

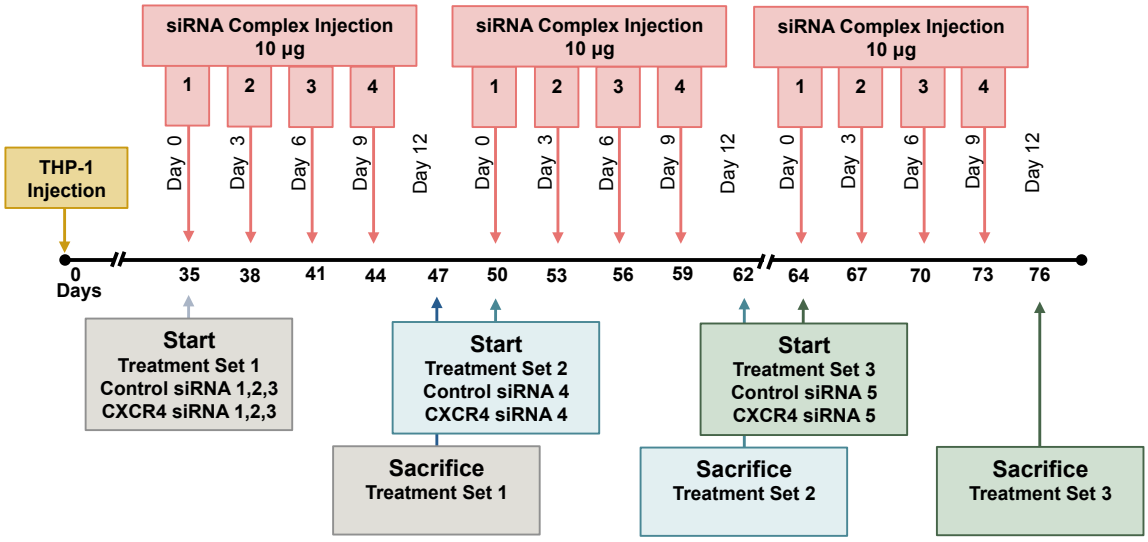


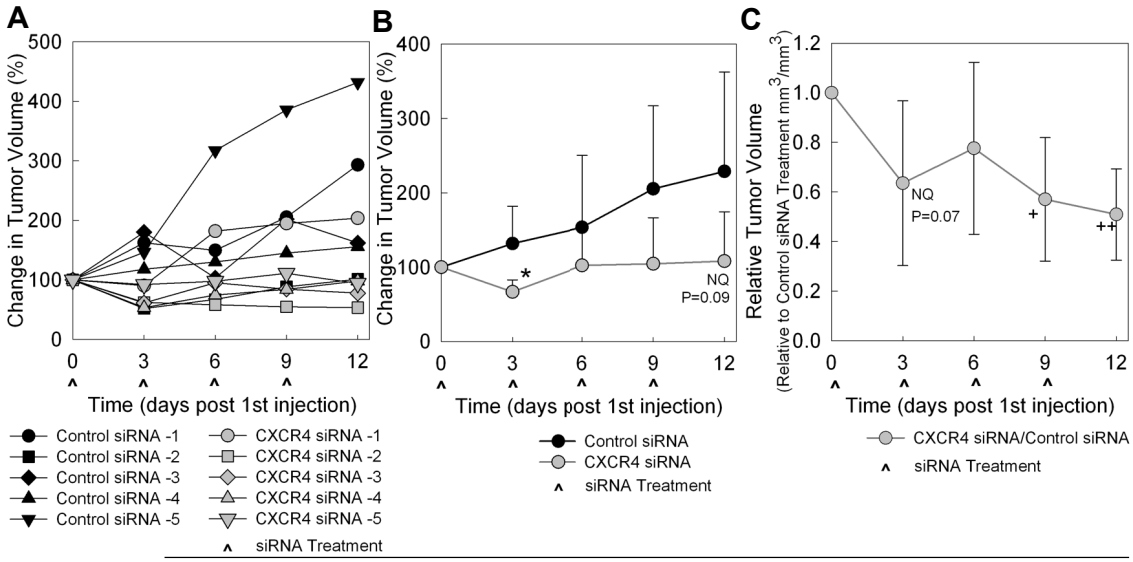
Figure 5.5 Schedule of siRNA Treatment of THP-1 Subcutaneous Tumors.
Due to the slow tumor establishment, siRNA treatment experiments were performed over three different time periods (Treatment Set 1, Treatment Set 2 and Treatment Set 3). Ten µg of siRNA-polymer complexes (1:4 siRNA:polymer PEI2-CA2.6) were intratumorally injected every 3 days, 4 times after tumor establishment, for each treatment set. Tumor volumes were measured every 3 days, and harvested on Day 12.

Ten µg of siRNA-polymer complexes (1:4 siRNA:polymer PEI2-CA2.6) were intratumorally injected every 3 days, 4 times after tumor establishment, **Figure 5.6**. Note that PEI2-CA2.6 was used as opposed to PEI2-CA5.4, due to the quantities required for the mouse xenograft model, but was also previously shown to be effective for CXCR4

silencing (**Chapter 4 - Figure 4.1**). The dosage of 10 μg siRNA (~ 0.33 mg/kg siRNA calculated based on average weight of mice (30.71 g) at start of treatment) was based on the successful range (1.5-6.5 μg) used for subcutaneous treatment of breast cancer xenografts utilizing similar lipopolymers carriers (PEI2-LA and stearic acid-substituted poly-L-lysine) [24, 30] and the ranges reported for in vivo leukemia-related studies in literature (2-100 μg or 0.1-30 mg/kg) [12]. We present the growth rates of individual tumors in **Figure 5.6A,** means of the treatment groups in **Figure 5.6B** and a relative tumor volume compared to the control siRNA groups (calculated per treatment set) in **Figure 5.6C.** Statistical analysis comparing CXCR4 siRNA treatment to the control siRNA treatment group indicated statistical significant difference when comparing the average tumor sizes in the two groups on day 3 but not at later time-points ($p>0.05$; **Figure 5.6B**). However, the different treatment periods and range in tumor volumes is thought to have impacted the results. The different treatment periods and tumor volumes may impact the tumor rate of growth and the tumor volumes also affect the siRNA dosage received by the grafted cells (total siRNA/tumor volume). The relative tumor volumes (CXCR4 siRNA treatment tumor volumes relative to control siRNA treatment tumor volumes) were calculated and then averaged to take into account variations in treatment periods, initial tumor size (CXCR4 siRNA-1 and control siRNA-1) and the non-GFP positive THP-1 cells used in some cases (CXCR4 siRNA-3 and control siRNA-3) (**Figure 5.6C**; formula shown in figure caption). Based on relative tumor volumes, compared to a hypothetical mean of 1 (expected if there was no difference between CXCR4 siRNA treatment and control siRNA treatment groups), statistical analysis

showed a significant decrease in volume with CXCR4 siRNA on day 9 ($p<0.01$) and day 12 ($p<0.05$).

The CXCR4 silencing was measured by analyzing surface CXCR4 levels of THP-1 cells from the tumors harvested on day 12. The GFP-positive population (ensuring selection of grafted THP-1 cells) was analyzed for change in CXCR4 expression (percentage of CXCR4 positive population, mean fluorescence (of CXCR4 antibody), and mean fluorescence of CXCR4 positive population (**Figure 5.7).** The leukemic cell population selection for a representative sample is shown in **Figure 5.7Ai**, selection of GFP positive subpopulation in **Figure 5.7Aii** and CXCR4 expression analysis, of the GFP positive population in **Figure 5.7Aiii**. A significant decrease ($p<0.05$) in CXCR4 positive population (~21% decrease in CXCR4 positive cells) was observed in GFP positive THP-1 cells harvested from the tumors (**Figure 5.7B**). However, there was no significant difference in the mean CXCR4 levels in the total and CXCR4-positive population. In later treatment periods (second and third treatment sets), GFP expressing cells were not detected by flow cytometry (due to loss of GFP expression in the cells or actual loss of GFP-expressing cells), so the CXCR4 suppression was only analyzed in the first treatment set. Loss of GFP expression was unexpected, however we also noted this occurrence in GFP-positive CML *in vivo* subcutaneous tumor studies (Valencia-Serna, personal communication). Multiple staining groups for each tumor sample for both PE-CXCR4 and PE Igg control were completed, as available cell numbers would allow, to ensure staining variations were not due experimental variations, **Table 5.1S**.



D

Absolute Tumor Volume (mm3)/Normalised Tumor Volume (%)###

Time (days post 1st injection)

	Day 0	Day 3	Day 6	Day 9	Day 12
Control-1#	233.0 / 100.0	378.5 / 162.4	349.0 / 149.8	478.6 / 205.4	683.3 / 293.3
Control-2	71.4 / 100.0	37.1 / 51.9	48.1 / 67.3	63.2 / 88.5	72.4 / 101.3
Control-3##	91.7 / 100.0	165.2 / 180.2	94.8 / 103.4	185.2 / 202.0	148.8 / 162.3
Control-4	50.9 / 100.0	59.9 / 117.8	66.2 / 130.2	73.8 / 145.1	79.3 / 156.0
Control-5	14.9 / 100.0	21.9 / 146.4	47.4 / 317.4	57.6 / 385.8	64.5 / 431.8
Control-Ave	**92.4 / 100.0**	**132.5 / 131.8**	**121.1 / 153.6**	**171.7 / 205.4**	**209.7 / 228.9**
Control-Stdev	**83.6 / 0.0**	**148.5 / 50.2**	**128.8 / 96.7**	**179.4 / 111.7**	**266.9 / 133.6**
CXCR4-1#	146.6 / 100.0	132.6 / 90.4	266.7 / 181.8	285.9 / 195.0	298.7 / 203.7
CXCR4-2	65.4 / 100.0	40.6 / 62.2	38.1 / 58.3	35.8 / 54.7	34.9 / 53.4
CXCR4-3##	23.3 / 100.0	14.2 / 61.2	22.2 / 95.3	19.6 / 84.4	18.1 / 77.7
CXCR4-4	74.4 / 100.0	39.8 / 53.5	55.6 / 74.6	62.6 / 84.1	73.1 / 98.2
CXCR4-5	30.2 / 100.0	27.9 / 92.2	29.5 / 97.7	33.6 / 111.1	28.5 / 94.2
Control-Ave	**68.0 / 100.0**	**51.0 / 71.9**	**68.0 / 68.0**	**68.0 / 68.0**	**90.7 / 90.7**
Control-Stdev	**49.1 / 0.0**	**46.8 / 18.1**	**103.7 / 47.7**	**112.0 / 53.7**	**118.1 / 57.7**

Figure 5.6 Effect of CXCR4 silencing in THP-1 Subcutaneous Tumor.

Tumor growth rates are shown as (n=5) (**A**) individual tumor growth (B) means of treatment groups and (**C**) CXCR4-siRNA tumor growth relative to control siRNA tumor growth within the same treatment set and tumor type (large or small) (i.e. Relative tumor volume=CXCR4 siRNA-n/control siRNA-n where n=1-5; see D). (**D**) Established tumor absolute volumes and calculated normalized tumor volume percentages. '^' Indicates siRNA injections. Statistical difference between CXCR4-siRNA treatment to control siRNA treatment is indicated by * for P<0.05. NQ refers to 'Not Quite" statistically significant used for 0.05>P<0.10. For (D) a two-tailed one-sample t-test comparing to a hypothetical mean of 1 was used to determine significant decrease in tumor volume by CXCR4 siRNA treatment indicated by + and ++, for p<0.05 and p<0.01. #Significantly Larger Tumor; ##Non-GFP+ THP-1 Cells, ###Ratio relative to control siRNA = (CXCR4 siRNA-n)/(Control siRNA-n) where n=1,2,3,4 or 5.

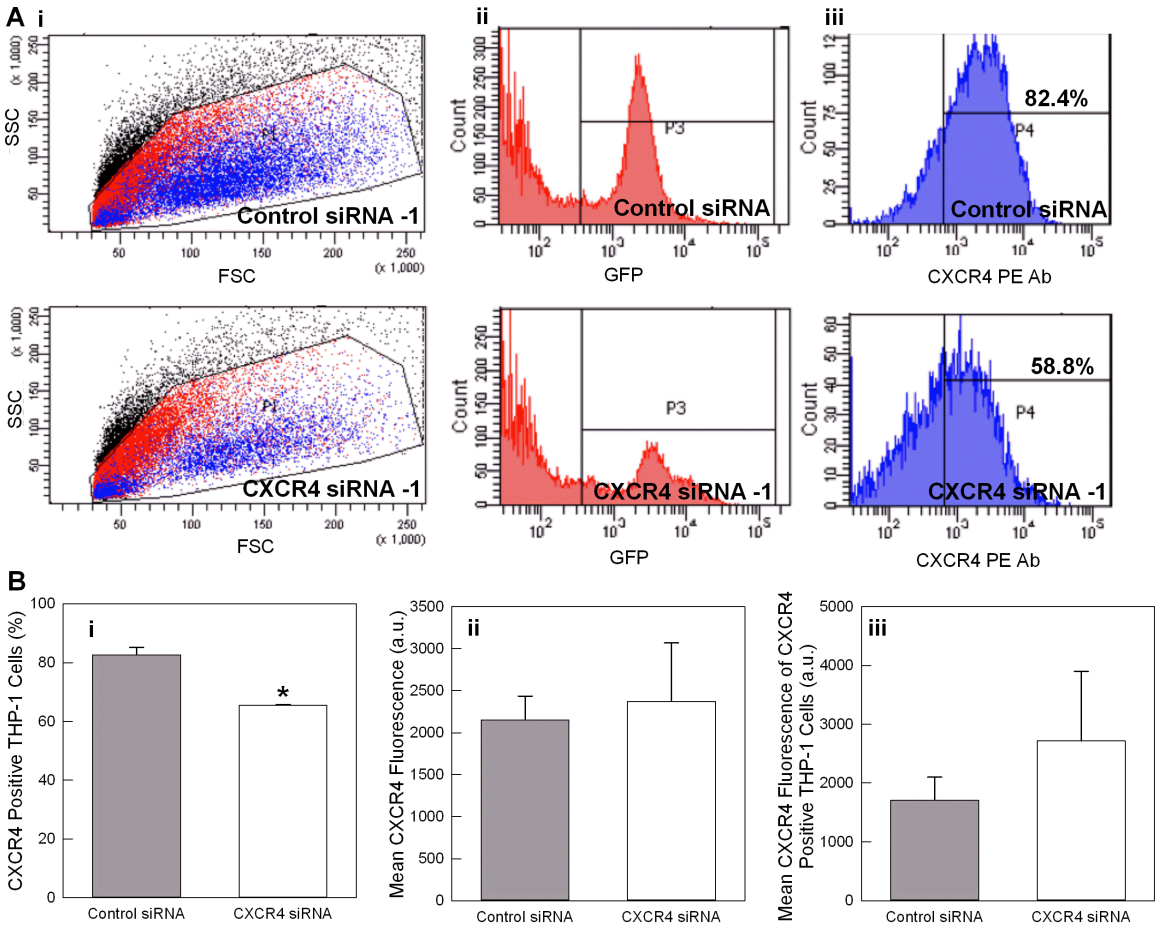


Figure 5.7 CXCR4 Silencing of GFP+ THP-1 Tumors.
CXCR4 silencing was verified after CXCR4 siRNA treatment on Day 12. (**A**) Displays examples of (**Ai**) selection of GFP+ population, (**Aii**) gating of GFP+ THP-1 cells (**Aiii**) CXCR4 expression within the GFP+ gate for Control siRNA and CXCR4 siRNA treatments. Flow cytometry silencing analysis is presented as (**Bi**) the percentage of CXCR4 positive cells in the GFP+ population (**Bii**) the mean fluorescence of the CXCR4 antibody in the GFP+ cell population and (**Biii**) the mean fluorescence of the CXCR4 antibody in the GFP+/CXCR4+ cell population. The percentage CXCR4 positive cells decreased by day 12, however there was no significant change in mean values of CXCR4 expression in THP-1 and CXCR4+ THP-1 cells. Statistical difference between CXCR4-siRNA treatment to control siRNA treatment is indicated by * for $P<0.05$

5.4 DISCUSSION AND CONCLUSIONS

In order to progress siRNA technology for leukemia therapy, there is a need for further in-depth evaluation of the technology in more clinically relevant scenarios. A thorough assessment in patient cells and employing *in vivo* models of leukemia are the

next steps currently needed. Towards this end we show that effective siRNA delivery was achieved across all five patients tested with both PEI2-CA5.4 and PEI2-LA2.1 polymers. PEI2-LA2.1 consistently demonstrated higher delivery in all but one of the five tested patient MNCs. Normalizing mean fluorescence values (from the fluorescence labeled siRNA) to the cell surface area was performed due to the large variation in cell size between the human AML MNC samples. As normalizing to surface area resulted in more comparable mean fluorescence between patient samples, we can surmise that the higher mean fluorescence (from the fluorescence labeled siRNA) seen in the larger samples may primarily be result of their larger size, which thereby provides a larger surface area for complex interaction and uptake into the cell. However, the absolute mean fluorescence is more appropriate when comparing the amount of siRNA delivered and thus the large sized AML patient MNCs (Patient #1 and Patient #5) had more total siRNA per cell.

CXCR4 silencing was achieved in some patient samples (1 out of 2 samples that were pre-diagnosed), where silencing corresponded with a strong decrease in cell concentration, similar to what we have seen in THP-1 cells in culture [15]. Interestingly, silencing was achieved in the largest cell-sized AML MNC sample (Patient #5) and not in the smaller sized AML MNC sample (Patient #4). Although, a greater number of patient samples would need to be tested to determine a correlation between cell size and resulting silencing, silencing in larger AML cells may be more effective simply due to increased siRNA delivery due to their larger surface area, enabling more interaction with the siRNA-polymer complexes. Other factors such as CXCR4 expression levels may have also contributed to the difference in CXCR4 silencing between to the two patients. CXCR4 expression was much higher in the non-responding AML sample (Patient #4)

than the sample that demonstrated CXCR4 silencing (Patient #5); Patient #4 had 64.0% CXCR4 positive cells while Patient #5 had 33.6% CXCR4 positive cells) and 1.8 times higher surface expression on a per CXCR4 positive cell basis. A larger sample set would be required in the next stage to fully determine the range of effectiveness of our polymeric siRNA carriers.

As CXCR4 surface expression can be altered by numerous factors, it is likely that CXCR4 proteins are internalized during freeze-thawing and/or ficoll separation, and then depending on the cell viability, varying degree of surface CXCR4 recovery can occur. The low cell viability might also affect cell processes including the RNAi mechanism. It is likely that challenges in utilizing cryopreserved AML cells, which include a delicate thawing procedure to optimize recovery, clumping of cells after thawing (a result of DNA release from damaged cells which can be prevented/minimised with DNase and filtering), resulted in low initial viability in *in vitro* culture. We noted that CXCR4 surface expression was low on Day 0, after patient harvest and after ficoll separation in our fresh cells that did not undergo cryopreservation. Cell processing such as the use of ficoll gradient process has been found to affect chemokine receptor expression (to varying degrees depending on the chemokine), causing them to internalize [29]. In the referenced study, it was found the ficoll decreased CXCR4 expression levels (mean fluorescence) but not significantly the percentage of cells expressing CXCR4 (94.5-95.2%) compared to non-ficoll treated monocytes in peripheral blood when gating for CD14+ cells in both cases. Other chemokines were more dramatically affected including CCR2, CCR6 and CXCR3 demonstrating drastic drops in the percent positive populations as well as their expression levels (mean fluorescence). Chemokine surface expression recovery,

monitored with CCR2 and CXCR3, was not found to be fully recoverable after 1 day nor under differentiation and activation conditions (1 day and 7 days) during *in vitro* cell culture and thus their internalization was considered irrecoverable, however some recovery (incomplete) still occurred. In the present study, fresh AML MNCs (Patient #7) more than doubled their surface CXCR4 levels and percentage of CXCR4-positive population from day 0 to day 3 (**Figure 5.3A**). If the surface expression of CXCR4 were being recovered (i.e., protein trafficking) at the same time CXCR4 silencing is attempted, some interference with silencing as well any resulting therapeutic effects would be expected. We did not have sufficient samples to undertake PCR analysis to assess silencing at the mRNA level but such an analysis would have revealed the extent of silencing better, even though surface CXCR4 levels could vary independent of silencing.

Future *in vitro* studies with AML MNCs could be improved in order to make silencing and resulting therapeutic outcomes more attainable and consistent. As mentioned the state of the cells at the onset of the experiment could effect silencing and resulting therapeutic effects due to viability effects on (1) RNAi mechanism, (2) CXCR4 surface levels and (3) CXCR4 surface recovery occurring during the experiment. One way to deal with initial low viability is removing the non-viable cells (propidium iodide (PI)+) by fluorescence-activated cell sorting (FACS) prior to beginning the experimental studies (utilizing the PI-viable population) as is done with extremely low viable CML patient cells [31, 32]. However, the cell sorting process could also contribute to additional toxicity as well as to deplete available cell numbers, due to loss of viable cells during the sorting process. Thus cell-sorting to increase cell viability is likely not the ideal choice. As mentioned previously, leukemic patient cells typically cannot be cultured *in vitro* for

long-term but progress on this front has recently been made. Using medium supplemented with recombinant growth factors, 7 of 31 AML patient cells with leukemic stem cells (LSCs) demonstrated long-term (35 days) culture *in vitro* [33]. Additionally, a further 3 patient samples could be grown for long-term with co-culture with endothelial cells. It may be beneficial to utilize such culture conditions for testing of siRNA silencing system as this would likely include viable and 'normal-functioning' leukemia cells as well as a functioning stem cell population. Longer *in vitro* culture would also allow time for the cells to recover after harvesting and sorting processes, and lead to a more stable cell population. As we have demonstrated effective silencing of CXCR4 in the THP-1 cells co-cultured with hBMSCs [15], it is likely that co-culture with endothelial cells (and perhaps hBMSC) should not interfere with siRNA silencing in the AML patient cells. It was also noted that long-term surviving cells may also reduce the possibility of contamination with normal hematopoietic cells and would allow for AML MNC patient samples with lower blast percentages to be more easily utilized [33]. As the LSC/progenitors population could propagate preferentially *in vitro*, it would also be interesting to determine the effect of siRNA silencing of selected targets (e.g., CXCR4) on long-term *in vitro* survival itself. Despite the strong recovery of CXCR4 (observed for patient #7, recovery during silencing will complicate analysis, and different separation methods (instead of ficoll) could be utilized (such as the MACs system, which separates the white blood cells from human whole blood or bone marrow (Miltenyi Biotec, Auburn, CA). Implementation of other separation process may be difficult, as the collaborative nature of the projects requires obtaining patient cells from other labs, where the source lab may not require non-ficoll separation methods for their own studies. Thus

implementing a longer term *in vitro* cell culture process is likely the preferred choice in future studies.

We end with a promising demonstration of CXCR4 silencing and therapeutic effect in subcutaneous THP-1 xenografts. These results are a significant improvement with the realisation that limited siRNA therapeutic studies have been performed *in vivo* [13, 18, 20-23, 34-40]. However, much progress still needs to be made. A repeat of the presented *in vivo* study with larger treatment groups would be beneficial to ensure (i) the desired therapeutic effect (i.e., slowing of tumor growth) and (ii) the CXCR4 silencing is reproducible and could include further dosage optimization. Future *in vivo* studies could be improved upon to achieve a more clinical relevant model. We could replace intratumoral injection in subcutaneous tumors with better routes of delivery. Other injection options include subcutaneous in the vicinity of tumors, intraperitoneal and intravenous injections. Establishment of orthotopic leukemia models will be preferred utilizing cell lines, patient cells and leukemic stem cell (such as MLL-AF9 oncogene expressing granulocyte-monocyte progenitor cells [41]) to fully demonstrate an effective system. Although non-intratumoral injections (such as intravenous injections) have proven effective in breast cancer tumors [30], success is largely dependent on the vascularization of the leukemic subcutaneous tumor, and thus these options may not be feasible for AML subcutaneous models. Additionally, the siRNA dosage of 10 μg (~0.33 mg/kg) for 4 injections (totalling 40 μg) is within the lower range reported for *in vivo* leukemic studies [13, 18, 20-23, 34-40], thus a higher dosage/number of injections might increase the therapeutic response in future studies.

Through siRNA delivery and CXCR4 silencing in human AML MNC samples *ex vivo* and AML subcutaneous tumor *in vivo,* we demonstrated progression into more clinical relevant scenarios for siRNA-based therapy of leukemias. With further enhancements as discussed above, we expect a greater silencing response to be achievable in the future.

5.5 ACKNOWLEDGEMENTS

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6. Overall Conclusions, Discussion and Future Directions[x]

[x]Versions of sections of this chapter was published in:
H.M. Aliabadi, B. Landry, B, C. Sun, T. Tang, and H. Uludağ, "Supramolecular assemblies in functional siRNA delivery: where do we stand?" *Biomaterials*, vol. 33, issue 8, 2546-69.

[x]Versions of sections of this chapter to be published as:
B. Landry, J. Valencia-Serna, H. Gül-Uludağ, X. Jiang, A. Janowska-Wieczorek, J. Brandwein, and H. Uludağ, "Progress in RNAi Mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia."

6.1 CONCLUSIONS

This thesis explored non-viral siRNA therapy approach for silencing key proteins implicated in resistance to current cancer drugs as well as the malignant cell's elevated survival ability. Currently employed non-viral carriers include liposomes, lipoplexes, cationic polymers and peptides. These carriers have been designed to deliver the siRNA to the cytoplasm to allow for siRNA to function through the RNAi mechanism by overcoming many barriers from siRNA encapsulation and protection, penetration of cellular and endosomal membranes and functional release of the siRNA. Through extensive review of these current obstacles, focusing on the intracellular barriers to delivery, and the mechanisms employed to overcome these obstacles in **Chapter 1**, it is understood that each individual siRNA carrier must be investigated and developed without assumptions based on other developed carriers. Further in **Chapter 1**, we identify leukemia, particularly acute myeloid leukemia (AML), as a cancer to be potentially treatable by siRNA therapy, which has lagged behind in conventional drug development as well as in development of siRNA therapies. We then introduced a carrier library, namely lipid-modified 2 kDa PEIs, first demonstrating their potential in adherent cell lines through silencing of numerous protein targets including the house-keeping gene GAPDH (for functional demonstration) as well as effective cancer targets P-gp and BCRP (involved in drug resistant) and survivin (cancer cell survival), as summarized in **Chapter 2**. We characterized the ability of the same lipid-modified polymers in AML cells, to determine the most effective lipid modification and formulations for effective silencing of the reporter protein, GFP (**Chapter 3**), which highlighted caprylic acid and linoleic acid modified 2 kDa PEIs, at formulations of 4:1 and 8:1 polymer:siRNA weight

ratios, to be the most promising carrier systems. We then chose a potential therapeutic target in leukemia, CXCR4, and report the effects of silencing this protein with the lipid-polymer carriers; decrease in cell proliferation and a small but significant decrease in adherence to bone marrow stromal cells (BMSCs). We furthermore showed clinically related outcomes, such as silencing in the presence of bone marrow stromal cells and co-treatment with a leukemia chemotherapy drug (cytarabine), which improved the efficacy of the drug, as summarized in **Chapter 4**. Further testing in more clinically relevant models was then performed, particularly in AML patient MNCs and in an *in vivo* subcutaneous tumor model in mouse using AML THP-1 cells (**Chapter 5**). siRNA delivery studies demonstrated significant delivery to all AML patient samples tested. Silencing of CXCR4 in AML patient cells *ex vivo* was demonstrated, albeit not in all AML patient samples. In an *in vivo* subcutaneous tumor model, effective decrease in THP-1 tumor growth as well as measured decrease in CXCR4 expression surface expression were achieved. Despite significant contribution to siRNA therapy, specifically for AML, our research has highlighted many barriers that need to be addressed both for siRNA carrier systems (Section **6.2**) and particularly for treatment of AML (Section **6.3**).

6.2 DISCUSSION AND FUTURE DIRECTIONS IN SIRNA CARRIER SYSTEMS

6.2.1 Better Evaluation of Developed Carrier Systems

Biomaterials and pharmaceutical scientists have taken enormous strides to create a diverse array of functional carriers that can assemble siRNA in supramolecular complexes. However, most practitioners in the field are in desperate need of developing good comparisons among the available carriers. One needs to understand their relative

performance in well-controlled experimental systems, with the purpose of identifying carriers with the highest potency. The latter could be defined based on the dose of siRNA needed for effective silencing or amount of carrier to be employed for therapeutic efficacy. Dose-response studies clearly revealing the IC_{50} of the developed systems will clarify some of the confusion in the literature on the relative efficacy of promising delivery systems. This is needed not only in *in vitro* studies but also in preclinical studies (similar to any pharmacological agent to be developed for clinical testing). With siRNA, however, non-specific effects of carriers and/or siRNA exposure need to be further assessed; dose-response studies ought to be carried out with non-functional (scrambled) siRNA sequences along with functional siRNAs to better reveal the magnitude of the observed side-effects.

6.2.2 siRNA Delivery Pharmacokinetics on Cellular and Intracellular Levels

Independent studies have overwhelmingly demonstrated the feasibility of siRNA-mediated down-regulation using both non-viral and viral vectors, but complete knockdown is rare. What happens to sub-populations of cells where the molecular target is not silenced is an open issue in the literature. Will those cells display selective resistance to therapy and take over the pathophysiology, ultimately creating a phenotype resistant to the therapy? Studies focusing on reason(s) for lack of complete down-regulation will be needed to better understand this issue. If inherent reasons prevent siRNA action (e.g., overwhelming the RISC pathway), other silencing agents, such as miRNA or anti-sense oligonucleotides that employ different mechanisms of actions, could complement the siRNA action. If delivery issues are limiting effective silencing, we need to urgently focus on mechanistic studies revealing critical impediments to the

delivery. Understudied areas on this front include: (**A**) role of extracellular matrix in supporting or impeding access of supramolecular complexes to cell surface (more applicable to adherent cell types), (**B**) intracellular dissociation of supramolecular complexes, and (**C**) long term fate of dissociated carriers. Quantitative studies on the fate of intracellularly delivered siRNA will better reveal how effective the silencing attempts are. If one can probe whether all delivered siRNA molecules are used up in silencing (highly unlikely) and what fraction remains 'unfunctional' or sequestered, one can then assess the need for improved carriers that can present siRNA to the biochemical machinery more effectively. Degradable and environmentally-sensitive carriers are likely to form the foundation of such carriers, but one has to assess the desired functional properties *in situ* and relying on *in vitro* characteristics (or functional responses) are likely to lead to misinterpretation of the perceived mechanisms of actions.

6.3 DISCUSSION AND FUTURE PROGRESS IN SIRNA CARRIER SYSTEMS FOR AML

6.3.1 Improvement of AML siRNA Therapy Efficacy

The siRNA therapies need to be effective in the 20-50 nM range in culture for a practical translation to preclinical animal models. It is typical for most reported leukemia related studies to employ siRNA concentrations beyond this range, including our own work [1]. Concerted efforts to lower efficacious doses will be needed as we move forward. It is interesting to note that effective dose of siRNA therapies (whether formulated with a carrier or delivered naked) did not significantly change over the years (**Figure 6.1**), despite increased diversity in the nature of carriers developed. An

improvement in effective doses should be expected with each newly developed carrier. Employing more effective siRNAs, such as multimeric, cell-penetrating or nuclease-resistant siRNAs could be one approach to improving efficacy. Even large scale screening of many siRNAs (with different sequences) should be performed as not all sequences have comparable efficacy and it is difficult to predict the efficacious sequence [2]. Targeting specific isoforms of the given protein target could also enhance efficacy and improve specificity. For example, CXCR4 has many isoforms [3] but we utilised a particular CXCR4 siRNA designed to target regions that are common to multiple splice forms. Even mixing siRNAs that target single isoforms may be more potent that a signal siRNA targeting all isoforms. Evidently much optimization can be done on the siRNA design alone. Employing microRNA instead of siRNA is appealing due to its promise to regulate gene networks (rather than single targets), however this may result in less over-all control of the therapy, similar to the less specific effects of small molecule drug inhibitors. In a limited set of studies, the effective doses of the microRNAs used in culture for leukemia treatment were also not superior to siRNAs: e.g., 100 nM with a lipoplex [4], 100 nM with Darmafect 2 [5], and 45 nM with electroporation [6]. Although discernible, efficacy may also be improved by simply finding the ideal protein target or protein target combinations as carrier efficiency (usually measured by silencing percent and therapeutic effect) can vary depending on the protein target as well as if multiple protein targets are utilized [7]. Although, the effect of carrier characteristics such as molecular size, degree of substitution (or modification) and optimal balance of lipophilicity-cationic charge is routinely elucidated on siRNA delivery/silencing

efficiency, this needs to be investigated also in the context of toxicity, intracellular trafficking and cell specificity.

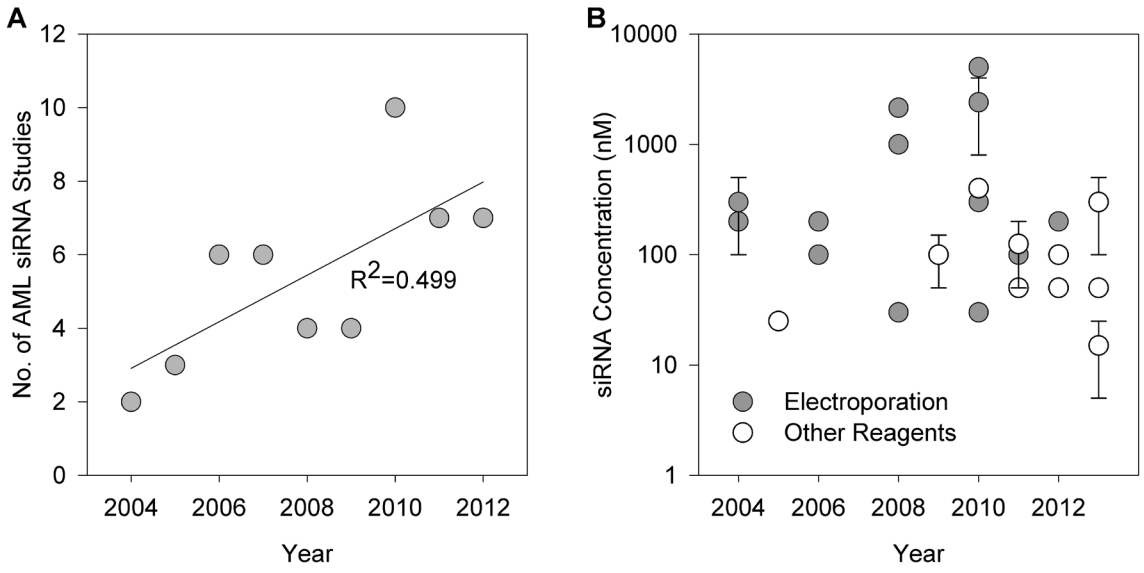

Figure 6.1 Graphical Representation of siRNA Studies Performed in AML Cells.
(**A**) Number of studies published between 2004-2012 involving siRNA delivery in AML (original data from Table 1.6 (published in [8])). (B) Effective in vitro siRNA concentrations utilized (if reported) in the studies outlined in Table 2. For clinical purposes, one would like to have an effective concentration less than 50 nM. Error bars are displayed to indicate the siRNA concentration range used in a given study.

6.3.2 What defines leukemic cells as 'difficult-to-transfect'?

It is clear that leukemic cells are within the 'difficult-to-transfect' category when it comes to delivering and utilizing nucleic acids intracellularly. Here silencing efficiency is often reported to be less than 50% with both commercial and non-commercial reagents [9-11] compared to adherent cells that can often achieve much higher levels (e.g., 90% silencing is typical). Reasons for decreased delivery and silencing have been elucidated [8, 12] such as deficiency in Ca^{2+}-dependent cell surface ligands and decreased endocytosis rates compared to adherent cells. Uptake of various carriers has been shown

to occur along numerous endocytosis pathways (clathrin-mediated and clathrin-independent pathways) where the RNAi activity efficiency can vary greatly depending on the route. As suspension cells are generally more difficult to transfect than adherent cells [1], one can even envision the physical shape of the cell to be an impeding factor. An adhered cell is flatter (with exposed surface area or higher surface area/volume ratio) where as a suspension cell is more spherical; the surface shape may impede attachment and penetration of the complex similar to how the curvature of nanoparticles have been shown to effect uptake and endosomal escape [13-15]. The volume and depth (distance from cell membrane to nucleus) of the cytoplasm (which would change depending on adherence and cell type) may also affect the efficiency of the siRNA activity due to trafficking and location in the cytoplasm. Various effective carriers (liposomes, peptides and dendrimers) were found to locate to the perinuclear region and locating to the perinuclear location seems to correlate with increased RNAi activity [16-18]. Intracellular trafficking itself can also vary on cell type, thus perhaps siRNA carriers are being trafficked non-optimally in leukemic cell and would benefit from intracellular targeting. RNAi activity itself can depend on the location of the delivered siRNA, the availability and location of RISC complex components, and the physiological stress on the cell due to the carrier [18-21]. A clear picture of which barriers prevent siRNA silencing in leukemia cells is needed. As this difficulty is observed in a wide range of carriers, it is reasonable to assume that there are biological features of leukemic cells that are creating the issue (rather than specific carrier-related issues). Thus determining these barrier(s) could provide an opportunity to help all types of carriers under development become more efficient in leukemic cells.

6.3.3 Targeting Leukemic Cells

Carriers designed for siRNA delivery specifically to leukemic cells, an under-studied area, could be a significant pursuit. Specificity is additionally important to prevent down-regulation of targets critical in normal physiology of hematopoietic cells, be it stem, progenitor or differentiated, as seen with AS-ODN in a clinical setting [22]. Given the cationic nature of 'typical' NPs, they could bind to a multitude of cells after administration in patients and, to overcome this, non-interacting NPs will be needed by tailoring neutral particles, and/or including sterically-protected surfaces (e.g., PEG). Regardless of carrier design, future work should assess effects of silencing in location related cells such as normal hematopoietic cells (including stem cells) as well as bone marrow cells. A modular design could be envisioned where a delivery system optimized for (**A**) minimal binding to non-target cells, (**B**) improved binding to leukemic cells (and/or specifically leukemic stem cells), and (**C**) rapid penetration and efficient trafficking intracellularly. One can envision the benefit of multiple-targeting strategies utilizing siRNA carriers that incorporate cellular targeting specifically to leukemic cells (i.e., progeny of LSC) and LSCs as well as locational targeting to bone marrow microenvironment.

6.3.4 Enhanced AML siRNA Treatment Strategies

Since leukemia cells are considered 'difficult-to-transfect' cells, it may be necessary to accept a lower efficacy in therapy (barring significant improvements in enhanced targeting and further determination of siRNA therapy barriers as discussed above). Instead, we might have to choose siRNA targets with complementary strategies in mind:

(**A**) siRNA therapy could act in conjunction with current clinically-employed drugs to improve their effectiveness or re-sensitize the cells to current drugs;

(**B**) A more specific co-treatment would be to use small molecular drug inhibitors or antibodies that target the same protein as the siRNA. The siRNA treatment would stop production of new target proteins, whereas the drugs can stop the activity of the existing protein target. By minimizing the protein activity in malignant cells by two different mechanisms, inhibition of the protein and suppression of protein synthesis, two different 'pools' of the target protein can be curbed at the same time; the active surface expressed protein as well as those that would be newly synthesized which should result in an intensified therapeutic response. Co-treatment would be beneficial for both inhibitor and siRNA strategies as they both could benefit from the enhanced (complementary) efficacy [11, 23, 24];

(**C**) Targeting nanoparticles to over-expressed surface proteins specific to leukemic cells, which are also therapeutic targets for siRNA, could provide an effective and highly specific siRNA therapy system. For example, a CXCR4 siRNA/carrier complex can be targeted to cells expressing surface CXCR4 with an anti-CXCR4 antibody. It is conceivable that this system could function as a super-efficacious inhibitor due to both physical targeting and provide protein silencing with high specificity to target-expressing cells;

(**D**) Targeting multiple therapeutic targets, an effective strategy demonstrated in adherent cells, such as for breast cancer [7], is another possible method to enhance efficacy. Novel siRNA targets could prove beneficial for use in combination with established targets, such as BCR-ABL in CML and FLT3 in AML or two similarly function targets such as

the adhesion receptors, CXCR4 and CD44, which both have a role in leukemic cell survival. However, choosing two targets that are directly active along the same pathway may not be beneficial, as we did not observe any enhanced effects when silencing the adhesion receptor protein CXCR4 and its correspond ligand simultaneously (**Chapter 4**). The combinational siRNA delivery addressing different signalling pathways will probably yield more efficacious therapy, and possibly more specific outcomes, and;

(**E**) Lastly, a treatment that is directed at leukemic cells as well as non-leukemic cells such as BMSCs within bone marrow niches (such as the endosteal and vascular hematopoietic stem cells (HSC) niche) may provide a more complete treatment strategy. The importance of the bone marrow niches in supporting leukemic cells (through leukemic-niche crosstalk), and leukemic disease associated changes that occur in non-leukemic cells within the bone marrow is a possible approach to therapy currently under investigation [25-27]. Mobilizing leukemic cells away from the niche and/or preventing their survival, through RNAi targeting of adhesion/chemotaxis proteins (i.e., CXCR4) followed by RNAi-mediated (or drug) therapy of leukemic-niche cell crosstalk may provide a strategy to more completely eliminate leukemia. The need to also focus on non-leukemic bone marrow stromal niche cells is highlighted by recent demonstrations of induction of myeloid malignancies, including AML-like disease, through mutations of cells (such as osteoblasts) contained in the niche [28, 29].

Recently developed siRNA screens are optimal for combinational system discovery (although challenging to set-up for suspension cells) as they can provide the opportunity to provide a less biased screen of potential siRNA targets. It can be used to determine single siRNA targets, combinational siRNA targets, and siRNA targets that can

provide synergistic effects with small molecular inhibitors, antibodies and standard chemotherapy treatments. Such screens have only been demonstrated in leukemic cells, to our knowledge, with siRNA/shRNA screens (kinase screen [30]) without combinational treatment, in conjunction with standard chemotherapy treatment using cytarabine [31, 32] or siRNA/shRNA screens with complementary screens (proteomic or small-molecule) [33, 34]. The Uludağ group also explored this with an Mcl-1 siRNA and a kinase library for breast cancer therapy [7], but work in the context of leukemia has not be reported. With further work with bone marrow stromal cell (BMSC) *in vitro* co-culture (as described further below), determining additional targets within a more bone marrow-like microenvironment may also be possible through siRNA screens (and may also be more easily set-up due to adhesion of the leukemic cells to the BMSCs).

6.3.5 Clinically Relevant Evaluation of siRNA Carriers in AML

6.3.5.1 Human AML Patient Samples Ex Vivo

While one wishes to identify universal carriers suitable for all types of leukemias, current evidence suggest that tailoring of carriers will probably be needed for specific types of leukemias and it might even be needed for individual patients. No information exists on patient-to-patient variations in siRNA delivery and evaluating off-target effects of delivered siRNAs and cytotoxic effect of the carriers will be warranted. While cell lines are preferred (due to practical reasons) to optimize cellular delivery initially, endocytosis rate and intracellular trafficking pathways in primary cells may be significantly different from cell lines. Misleading directions could be avoided by employing primary cells early on in the siRNA therapy development process. Although leukemic patient samples can be harvested and then immediately tested *ex vivo*, as they

do not normally survive long in *in vitro* culture, this model is far from ideal. Discernably, leukemic cells that are struggling to survive *in vitro* may not function normally as they are already pre-disposed to cell death pathways and RNAi mechanism can me inhibited in stress conditions and have delayed recovery following removal of stress conditions [19] (note that I am not aware of any studies on the changes in RNAi machinery as a result of *ex vivo* culture). However, long-term *in vitro* culture is possible utilizing conditions that provide long-term (~4-6 weeks) survival with use of recombinant growth factors and/or feeder layers such as endothelial or bone marrow stromal cells, albeit not every patient sample is successful with reported success rate of ~32 or 100 depending on the study [35, 36]. Utilizing a more viable *in vitro* system for AML cell culture also provides the opportunity for testing treatment of a functioning LSC population, which propagate the long-term survival.

6.3.5.2 Enhanced In Vitro Bone Marrow Microenvironment Models

Enhanced *in vitro* bone marrow microenvironment models are not only beneficial for improved survival of AML patient cells, but can be also used to match more closely to actual bone marrow microenvironment, now known to be major participant in leukemic disease. Co-incubation of leukemic cells with BMSCs or endothelial cells is the fundamental step towards modelling a bone marrow microenvironment *in vitro*, and is beginning to be commonly used. Additionally, the use of BMSCs from AML patients may provide an even better model for assessment of AML therapies, as BMSCs from AML patients have genetic abnormalities which results in altered signalling and cross-talk within the bone marrow microenvironment [37-39]. Commonly used for establishment of subcutaneous tumors (including with AML cells), Matrigel^{TM} (contains

basement membrane proteins and growth factors) gels at 37°C and allows for three-dimensional cell culture *in vitro*, which has been demonstrated to support the growth of many cell types including stem cells [40-43]. *In vitro* cell culture studies have been performed with Matrigel to study morphological response, invasion through the matrix, adherence to the matrix and cell differentiation [40, 44] as well as co-culture with BMSCs and leukemic cells forming spheroid structure that can better model the bone marrow multi-dimensional niches [43]. Other three-dimensional bone marrow models have been also developed such as co-culture of leukemic cells with BMSCs in a synthetic polyglycolic acid/poly-L-lactic acid scaffold developed for *in vitro* chemotherapy drug testing [45]. It is anticipated that AML patient cells would respond well to culture in a bone-marrow model *in vitro*, similar to their better viability when grown with BMSCs and specific growth factors. As human AML cell numbers per patient are usually very limited, in addition to viability benefit in increasing *in vitro* cell culture duration, better expansion of leukemic cells will enhance the scope and the number of experiments that can be performed on these samples.

6.3.5.3 In Vivo Leukemic Models

Although subcutaneous tumor models are a reasonable first model to determine siRNA delivery, protein silencing and resulting effects *in vivo*, there is an obvious need for use of more clinically relevant models. The bone marrow niche(s) plays a complex role in leukemogenesis and should ideally be incorporated into the leukemic model (as similarly mentioned in the *in vitro* studies). Typical leukemic engraftment (primarily in the peripheral blood, bone marrow and the spleen) can be performed with human AML patient cells (10^5 to 10^7 cells/mouse) and in some AML cell lines (such as HL-60, KG-1,

Kasumi) in NOD/SCID (NS) and/or NOD/SCID/IL-2Rγnull (NSG) mice with and without pre-irradiation through intravenous (and sometime intraperitoneal) injection of the AML cells [37, 46, 47]. NOD/SCID mice have impaired T and B cells lymphocyte development and natural killer cell function whereas the addition of the IL-2Rγ deficiency further impairs development of the natural killer cells and has additional negative effect on innate immunity. Engraftment of human AML MNCs has reported ranges which can be over 70%. Better engraftment is usually observed with poor prognosis. But engraftment of AML cell lines varies greatly, dependent on mouse model utilized and the specific leukemic cell line [37, 46, 47]. *In vivo* engraftment models would allow for intraperitoneal and intravenous injection of our polymer-siRNA complexes and resulting examination of response of established leukemic populations within the mouse peripheral blood and bone marrow environments. Other potential models also include a leukemic stem cell mouse model, MLL-AF9 oncogene expressing granulocyte-monocyte progenitor cells [48], which would also provide further testing specifically in stem cell type cells *in vivo*. Another possible leukemic *in vivo* model is only a step away from subcutaneous AML model utilized in our studies (**Chapter 5**); establishment of a subcutaneous extramedullary bone marrow environment (trabecular structure similar to human long bone) with mesenchymal stromal cells and endothelial colony-forming cells in NSG mice utilizing Matrigel™ with subsequent engraftment of murine HSCs, human HSCs and/or human leukemic cells (MOLM13) [37]. It might be possible to use the extramedullary bone marrow environment to study (i) engraftment of pre-CXCR4 suppressed AML cells, (ii) CXCR4 suppression in leukemic cells, and (iii) changes in leukemic cell population/numbers within the extramedullary bone after subcutaneous

injection of CXCR4siRNA-polymer complexes. The extramedullary bone model utilizes human BMSCs, endothelial cells and leukemic cells instead of using murine bone marrow environment with human leukemic cells; a possible benefit as interactions between mouse and human cells may differ from interaction seen in solely between human cells [37]. Additionally, this model allows the use of bone marrow cells from AML patients, which have recently been found to contain genetic abnormalities (as mentioned above) and thus are a more clinically relevant choice [37].

Appendix

A. APPENDIX FOR CHAPTER 3

Table 3.S1 Lipid Substituted 2kDa PEI Library (PEI2-Lipids)

Polymer	Substitution	Feed Ratio	Lipid/PEI[a]	Methylene/ PEI[b]	% Substitution[c]
PEI2-CA0.1	Caprylic Acid (CA)	0.012	0.5	3.7	2.9
PEI2-CA1		0.066	1.1	8.8	6.9
PEI2-CA10	C8:0	0.100	2.4	19.0	14.8
PEI2-CA20		0.200	6.9	56.8	43.4
PEI2-PA0.1	Palmitic Acid (PA)	0.012	0.3	4.9	1.9
PEI2-PA1		0.066	0.6	9.5	3.7
PEI2-PA10	C16:0	0.100	0.8	12.6	4.9
PEI2-PA20		0.200	1.1	18.0	7.0
PEI2-OA0.1	Oleic Acid (OA)	0.012	0.3	4.6	1.6
PEI2-OA1		0.066	1.0	18.1	6.3
PEI2-OA10	C18:1	0.100	1.7	30.0	10.4
PEI2-OA20		0.200	2.5	44.1	15.3
PEI2-LA0.1	Linoleic Acid (LA)	0.012	0.2	4.3	1.5
PEI2-LA1		0.066	1.0	17.3	6.0
PEI2-LA10	C18:2	0.100	1.8	33.2	11.5
PEI2-LA20		0.200	3.2	57.7	20.0
PEI2-StA0.1	Stearic Acid (StA)	0.012	0.2	3.2	1.1
PEI2-StA1		0.066	0.5	8.4	2.9
PEI2-StA10	C18:0	0.100	3.6	66.6	22.8
PEI2-StA20		0.200	4.9	89.0	30.9
PEI2-MA0.1	Myristic Acid (MA)	0.012	0.4	5.2	2.3
PEI2-MA1		0.066	0.6	8.3	3.7
PEI2-MA10	C14:0	0.100	1.7	24.1	10.8
PEI2-MA20		0.200	1.5	20.8	9.3

a. Actual number of lipids substituted per PEI2 (calculated from ^{1}H-NMR analysis).

b. Lipid carbon (C) substitutions per PEI2 were calculated based on the number of Cs present in each lipid and the number of lipids substituted per PEI2.

c. Percent substitution refers to the percentage of primary amines modified with the corresponding lipids.

Table 3.S2 Linear Regression Analysis of Complex Cytotoxicity

Polymer	THP-1		KG-1		HL60	
	r^{2a}	P^b	r^{2a}	P^b	r^{2a}	P^b
PEI25	**0.9537**	**0.0234**	**0.9641**	**0.0181**	**0.9845**	**0.0078**
PEI2	0.1693	0.5885	**0.6821**	0.1737	0.8850	0.0593
CA1	**0.9987**	**0.0007**	0.0005	0.9778	0.5822	0.2370
CA10	**0.8166**	0.0964	0.2286	0.5219	**0.9866**	**0.0067**
CA20	**0.9930**	**0.0035**	**0.9200**	**0.0408**	**0.9701**	**0.0151**
PA1	**0.8948**	0.0541	0.07418	0.7276	**0.9550**	**0.0228**
PA10	0.7470	0.1357	0.04244	0.7940	0.1294	0.6403
PA20	**0.9571**	**0.0217**	0.1482	0.6151	**0.9898**	**0.0051**
OA1	0.3485	0.4096	0.4284	0.3455	**0.9924**	**0.0038**
OA10	0.2134	0.5380	0.03309	0.8181	**0.9252**	**0.0381**
OA20	0.4570	0.3240	0.2687	0.4816	0.6200	0.2125
LA1	**0.9133**	**0.0444**	**0.9964**	**0.0018**	0.8184	0.0954
LA10	**0.9193**	**0.0412**	**0.8908**	0.0562	**0.9327**	**0.0343**
LA20	0.8408	0.0831	**0.7874**	0.1126	**0.9951**	**0.0024**

a. Linear regression r^2 values b. Calculated to determine if slope was significantly different from zero
* Significant values are bolded.

Table 3.S3 Trends Between Complex Cytotoxicity and Lipid Substitutions

Concentration (µg/ml)	Lipid	THP-1		KG-1		HL60	
		r^{2a}	P^b	r^{2a}	P^b	r^{2a}	P^b
10	ALL	0.05520	0.4397	0.2776	0.0643	0.1185	0.2493
	CA	**0.9790**	**0.0106**	**0.9621**	**0.0191**	**0.5470**	0.2604
	PA	**0.9123**	**0.0449**	**0.7009**	0.1628	**0.8228**	0.0929
	OA	0.4665	0.3170	0.1190	0.6551	0.0056	0.9249
	LA	**0.6978**	0.1647	0.0448	0.7884	0.1150	0.6609
5	ALL	0.1119	0.7309	0.1837	0.1440	0.0006	0.9364
	CA	**0.8374**	0.0849	**0.9179**	**0.0419**	0.1876	0.5669
	PA	**0.6975**	0.1648	0.4946	0.2967	**0.5650**	0.2483
	OA	0.4842	0.3042	**0.9321**	**0.0346**	0.0454	0.7869
	LA	**0.6464**	0.1960	0.1360	0.6312	0.0575	0.7602

a. Linear regression r^2 values b. Calculated to determine if slope was significantly different from zero
* Significant values are bolded.

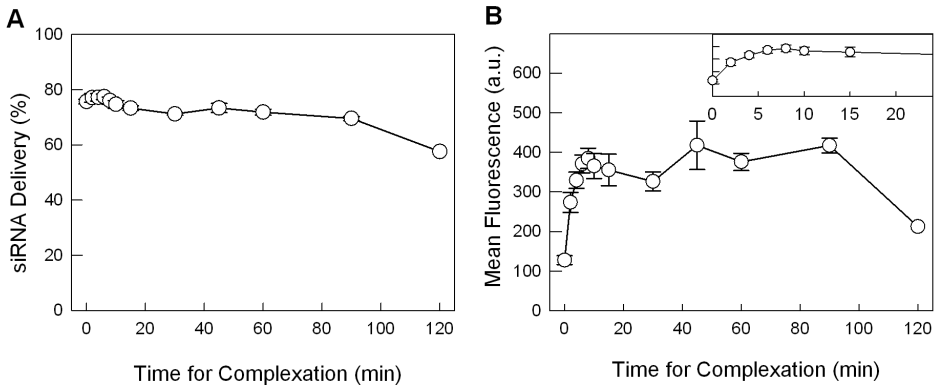

Figure 3.S1 Effect of Complexation Time on siRNA Delivery.
PEI2-LA20 complexes were delivered after incubated at room temperature from 0-120 minutes. (**A**) SiRNA delivery remained relatively constant regardless of incubation time, with a slight decrease in uptake starting at 90 min. (**B**) Mean fluorescence, providing an indication on amount of siRNA/cell plateaued by 10 minutes and a decrease (corresponding with a decrease in uptake) at ~90 minutes. This indicates that our 30 minute incubation time (described in the methods) is an appropriate incubation period and also provides leeway without significantly affecting results.

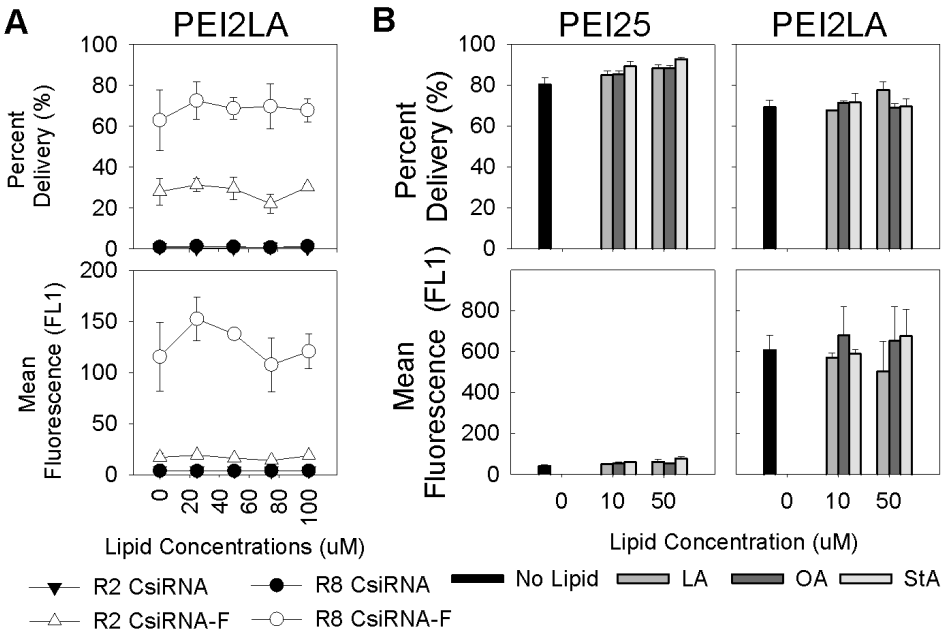

Figure 3.S2 Effect of Free Fatty Acids on siRNA-Polymer Delivery.
(**A**) Fatty acids were pre-treated for 24h with LA and then incubated with FAM siRNA/polymer complexes (2:1 and 8:1 polymer:siRNA ratios) for 24h. (**B**) Various Fatty acids were delivered with siRNA-polymer (25 nM at 8:1 polymer:siRNA ratio) treatments simultaneously to THP-1 cells for 24h.

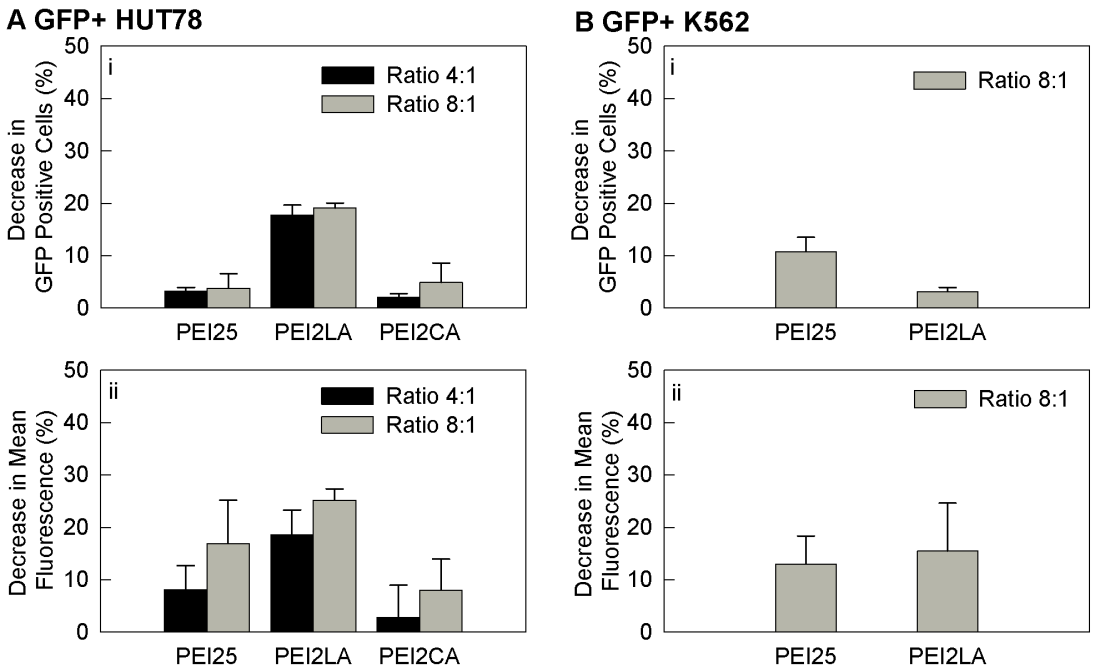

Figure 3.S3 GFP Silencing in GFP-Positive Hut78 and K562 Cells.
GFP silencing was measured 3 days after siRNA treatment with (**A**) 25 nM (Hut78 cells) and (**B**) 36 nM (K562) GFP siRNA (or control siRNA) at indicated polymer:siRNA ratios. Percent decrease in GFP-positive cells are indicated in the top graphs whereas percent decrease in the mean GFP levels are indicated in bottom graphs. *Thanks to J. Valencia-Serna and B. Sahin for the data to create this figure.*

B. APPENDIX FOR CHAPTER 4

Table 4.S1 Caprylic Acid (CA) Substituted 2 kDa PEI Carrier Library

Polymer	Feed Ratio ($M_{Lipid\text{-}Chloride}$: M_{PEI2})	Lipids/PEI	% Amine Substitution
CA2.5	2.995	2.46	15.86
CA2.6	3.057	2.60	16.77
CA3.1	4.730	3.05	19.70
CA3.3	4.766	3.34	21.55
CA5.4	6.094	5.36	34.64
CA5.8	4.92	5.79	37.38
CA6.0	6.333	5.99	38.68
CA6.9	8.18	6.9	56.8

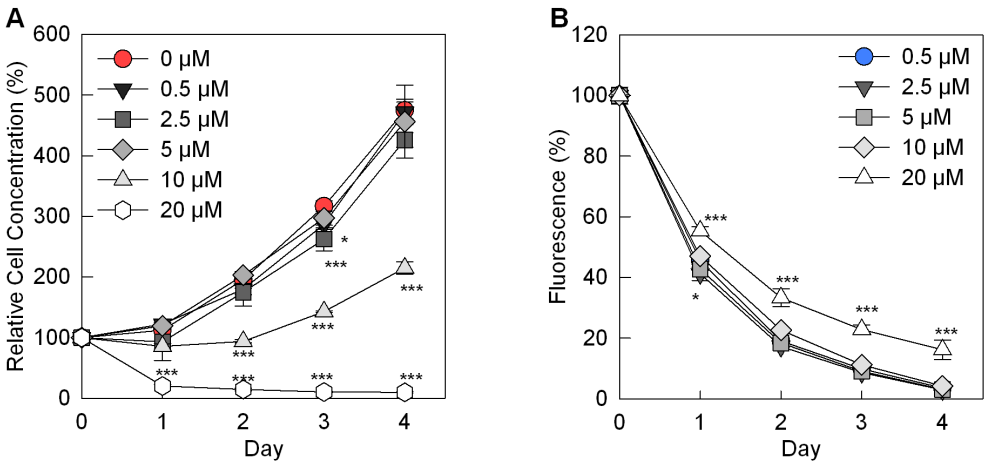

Figure 4.S1 Effect of Cell tracker™ CMFDA on THP-1 Cell Growth.
(**A**) Cell concentration by flow cytometry counts and (**B**) cell division as measured by the CMFDA fluorescence per cell. Statistical analysis (*) compares to lowest concentration of dye used, 0 and 0.5 µM in (A) and (B) respectively.

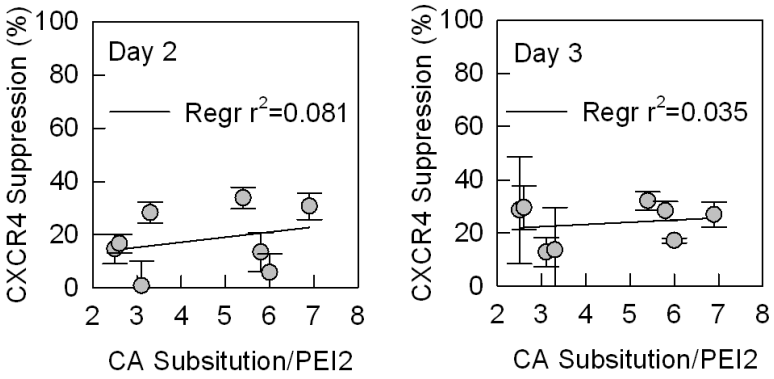

Figure 4.S2 Comparison between CA Substitution Level and Resulting CXCR4 Suppression Utilizing Lipopolymer/siRNA Complexes.
No clear correlation between the level of CA substitution and the extent of CXCR4 silencing was evident.

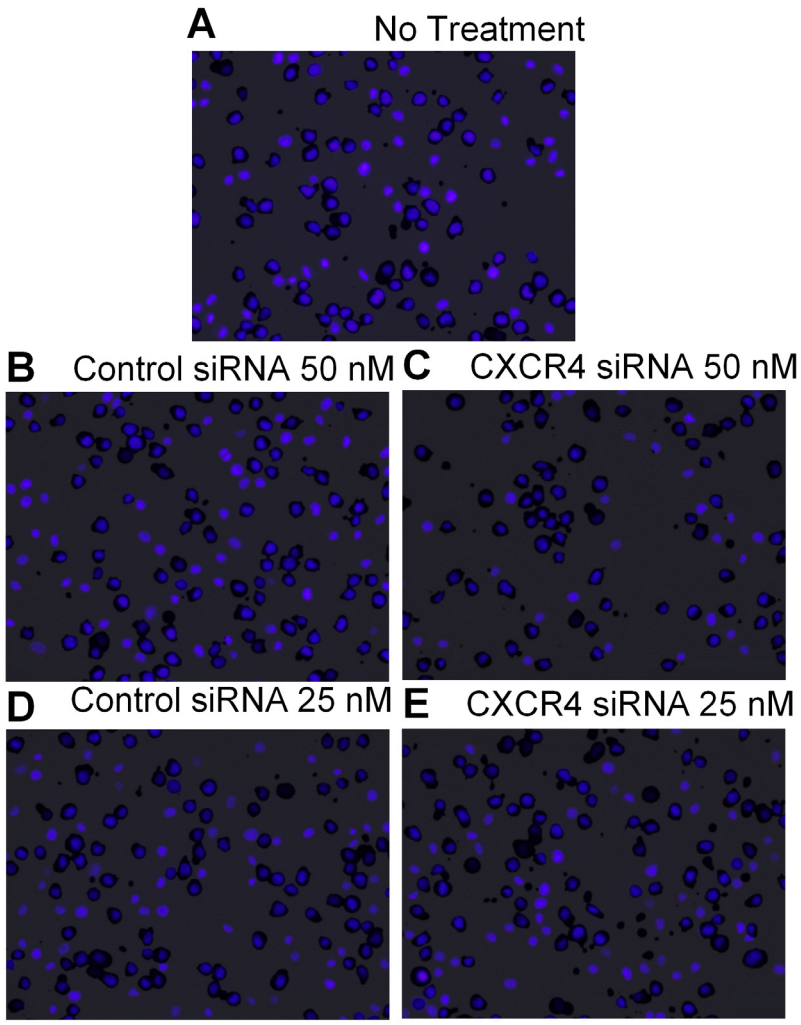

Figure 4.S3 Effect of Lipopolymer/siRNA Complex Mediated CXCR4 Silencing on Nucleus Morphology in Presence of hBMSC.

Cell nuclei were stained with Hoechst, 24 hours after CXCR4 silencing. GFP fluorescence (black) was over-laid with nucleus images to determine which nuclei were GFP-positive THP-1 cells as opposed to hBMSC nucleuses. No clear fragmentation of nuclei was evident from this analysis. Images for day 2 and day 3 were comparable to day 1 (not shown).

C. APPENDIX FOR CHAPTER 5

Table 5.S1 CXCR4 Expression in GFP+ THP-1 Cells Isolated from Tumors.

CXCR4 expression was determined by antibody (Ab) labeling of the surface expressed CXCR4 protein. Tumor samples were divided into 1-4 groups, depending on cell numbers in sample, for antibody staining. Resulting percentage of expression and mean fluorescence levels were then averaged for each tumor.

Sample	IGg Ab				CXCR4 Ab					
	% CXCR4+		Ave.	Std. Dev.	% CXCR4+				Ave.	Std. Dev.
Control siRNA -1	1.8	2.6	2.2	0.57	81.9	82.4	80.3	78.7	80.8	1.68
Control siRNA -2	X	X	X	X	84.4	X	X	X	84.4	X
CXCR4 siRNA -1	0.8	0.8	0.8	0.00	71.1	60.3	X	X	65.7	7.61
CXCR4 siRNA -2	X	X	X	X	65.3	X	X	X	65.3	X

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