Safe Polymeric Nucleic Acid Delivery Systems for Cancer Therapy:

Focus on Breast Cancer and Chronic Myeloid Leukemia

by

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ABSTRACT

RNA interference (RNAi) is a promising technology to specifically target the upregulated genes that provide uncontrolled proliferation and survival of cancers. The target specific silencing is accomplished by the delivery of small interfering RNA (siRNA), which require safe delivery systems to protect against serum nuclease degradation and cellular uptake. Lipid-substituted low molecular weight (LMW) polyethylenimine (PEI) polymers are excellent non-viral gene delivery systems which have the potential to establish safe delivery of the therapeutic gene in any cell type. This thesis investigates the potential of lipid-modified PEI delivery systems in the treatment of two different cancer types: breast cancer and chronic myeloid leukemia (CML) representing adherent and non-adherent cancers, respectively. The current challenge in the treatment of breast cancer is their metastatic ability to establish tumors in distant organs which necessitates the need to develop alternate therapeutic options. In this process of metastasis, cell adhesion molecules such as integrins are known to play a major role, especially the upregulation of integrin-β1 has strong correlation with breast cancer progression and metastasis. Hence, we hypothesized that the silencing of such overexpressed molecule would help to decrease breast cancer metastasis. Among the lipid substituted PEIs explored for breast cancer treatment, the linoleic acid (1.2PEI-LA6) substituent provided strong silencing of the overexpressed cell adhesion molecule integrin-β1 at the cell surface and mRNA transcript level. Silencing of integrin-β1 significantly decreased the attachment of breast cancer cells to fibronectin and human bone marrow stromal cells. High cytoplasmic localization of the siRNA achieved by the 1.2PEI-LA6 polymer helped to accomplish strong migration inhibition, reflecting the ability to decrease breast cancer metastasis. Using the same

delivery system, we screened a siRNA library of 496 genes targeting various cell adhesion and cytoskeleton molecules, to identify therapeutic targets for breast cancer treatment. We identified the endoplasmic reticulum heat shock protein 90B1 (HSP90B1) as a potential therapeutic target to decrease the growth and migration of breast cancer cells. Furthermore, the inclusion of hyaluronic acid as additives and the combinational silencing of integrin-β1 and HSP90B1 provided significant decrease in the migration of breast cancer cells. Whereas in CML, the treatment against drug resistance developed by the attachment of leukemic cells through integrin-β1 to the bone marrow stromal environment, activates signaling pathways independent of BCR-ABL whose treatment is a challenging task. Silencing of integrin-β1 in CML cells was achieved by thioester linked α-linoleic acid (tα-LA) substituted PEI polymers. Integrin-β1 silencing decreased its binding and increased its detachment from bone marrow stromal cells. A combinational silencing of integrin-β1 and BCR-ABL helped to overcome resistance mediated by fibronectin binding. Exploring the safety of these lipid-modified PEI delivery system revealed the wide dependence on the ratio of PEI and nucleic acid, through toxicity studies in T-lymphocyte and red blood cells. However, differences in the PEI and nucleic acid ratio and the concentration of nucleic acid does not alter the secretion of TNF-α, IL-6 and IFN-γ cytokines from pulmonary blood mononuclear cells. In addition, variations in the lipid type and the number of lipid substitution does not alter the cytokine secretion in comparison to the positive controls. Overall, we successfully established the therapeutic potential of PEI delivery systems among two different cancer types, in addressing breast cancer metastasis and CML drug resistance by siRNA delivery. In addition, we provided the safety information of these

PEI delivery systems which are vital during their clinical exploration and for further application in other cancer types.

PREFACE

Versions of the literature review and the research work presented in this thesis have been previously published as described below. All the chapters presented here were conceptualized, researched, and written with the supervision of Dr. Hasan Uludağ. Individual contributions and acknowledgments for each chapter are outlined below. Studies with human cells was performed under the approval of the University of Alberta Research Ethics Board, Project Name "Modification of Human Cell Lines and Primary Cells", No. Pro00081596. The ethics approval for animal research was obtained from the University of Alberta Research Ethics Board, Project Name "Gene Medicines for Cancer Treatment, No. AUP00000423 and the biosafety approval was obtained under UA file # RES0012356.

Chapter 1 provides a literature review on the various RNAi technology and highlights the success of siRNA therapy, the potential of low molecular PEI as non-viral gene delivery systems and the current knowledge on its safety. The role of cell adhesion molecules and specific intracellular molecules in breast cancer metastasis and the importance of BCR-ABL independent resistance observed in CML by its binding to bone marrow stromal environment are also reviewed in this chapter. Portions of this chapter are from a previously published review article Meenakshi Sundaram DN, Jiang X, Brandwein JM, Valencia-Serna J, Remant KC, Uludağ H, "Current outlook on drug resistance in chronic myeloid leukemia (CML) and potential therapeutic options" in *Drug Discovery Today*, vol. 24, pp. 1355-1369, July 2019. As the primary author, I was responsible for conducting literature review, analysis, and composition of the manuscript. Dr. Xiaoyan Jiang and Dr. Joseph M. Brandwein through their expertise in leukemia helped in

manuscript revision and ensured the accuracy of the paper. Dr. Juliana Valencia-Serna contributed by preparation of tables in the manuscript. Dr. Remant KC provided the information for BCR-ABL siRNA silencing as a figure. Dr. Hasan Uludağ was the supervisory author who was involved in revision of the manuscript.

Chapter 2 is a version of previously published research paper Meenakshi Sundaram DN, Kucharski C, Parmar MB, Remant Bahadur KC, Uludağ H, "Polymeric Delivery of siRNA against Integrin-β1 (CD29) to Reduce Attachment and Migration of Breast Cancer Cells" in *Macromolecular Biosciences,* vol. 17, 1600430, June 2017. This chapter focuses on the successful silencing of integrin-β1 by lipid-substituted polyethylenimine polymers as siRNA delivery systems and the therapeutic benefit of integrin-β1 silencing for breast cancer migration and attachment. As the primary author, I was responsible for concept formation, execution of experiments and writing of manuscript. Dr. Manoj Parmar helped in standardization of migration assays and Mr. Cezary Kucharski helped with RT-qPCR experiments, while Dr. Remant Bahadur KC synthesized the polymers for siRNA delivery and Dr. Hasan Uludağ assisted in interpreting the results and manuscript revisions.

Chapter 3 is a research article that will be submitted for publication as Meenakshi Sundaram DN, Remant Bahadur KC, Uludağ H, "Lipid Modified Polyethyleneimine is an Effective System for HSP90B1 Silencing to Treat Breast Cancer". This paper focuses on the identification of HSP90B1 as a potential target for breast cancer treatment. As the first author, I was responsible for concept formation, execution of experiments and manuscript writing. Dr. Bindu Thapa, Cezary Kucharski and Dr. Joaquin Lopez-Orozco assisted during

siRNA library screening. As the supervisory author, Dr. Hasan Uludağ helped in data interpretation and revision of manuscript.

Chapter 4 is a research paper that explored the therapeutic benefit of silencing integrin-β1 by lipid-substituted polyethylenimine polymers as siRNA delivery systems in CML. This research work will be submitted for publication as Meenakshi Sundaram DN, Kucharski C, Remant Bahadur KC, Ibrahim Oğuzhan T, Uludağ H, "Therapeutic Potential of Integrin-β1 Silencing to Address Drug Resistance in Chronic Myeloid Leukemia Implemented by Polymeric siRNA Delivery". As the first author I was responsible in conducting experiments, data collection and analysis and writing of manuscript. Cezary Kucharski assisted in the culturing and maintenance of human bone marrow stromal cells. Dr. Remant Bahadur KC synthesized the polymers used for siRNA delivery while Ibrahim Oğuzhan Tarman was responsible in conducting colony formation assay. Dr. Hasan Uludağ was the supervisory author who contributed by checking the accuracy of results and manuscript revision.

Chapter 5 contains the safety evaluation of the PEI delivery systems which will be submitted for publication as Meenakshi Sundaram DN, Plianwong S, Remant Bahadur KC, Uludağ H, "*In vitro* Safety Studies of Lipid-Substituted Low Molecular Weight PEIs Used in Gene Delivery Systems". As the lead author, I designed, performed, and analyzed the results, and wrote the manuscript. Dr. Samarwadee Plianwong conducted the isolation and maintenance of red blood cells and pulmonary blood mononuclear cells from whole blood samples. Dr. Remant Bahadur KC synthesized the polymers used in this study while Dr. Hasan Uludağ helped in data interpretation, checked the accuracy of results and manuscript revision.

Chapter 6 consists of the overall conclusion, discussion, and future directions. This chapter was primarily derived from the discussion and conclusions of previous chapters and provides the knowledge gained through the work presented in this PhD thesis.

DEDICATION

To my mom (Kalanithi), dad (Meenakshi Sundaram) and sister (Arul Jothi)

for their unconditional love, prayer, and support.

"Now faith is being sure of what we hope for
and certain of what we do not see"
(Hebrews 11:1)

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The following people have been a great inspiration and played key roles in helping me to pursue research and set an example with their dedicated work: former President of India Dr. A.P.J. Abdul Kalam, Dr. Luke Elizabeth Hanna, Dr. Devapragasam, Jacob Sir, Jayashree Miss, Mr. & Mrs. Xavier Poonolly and Bupesh Anna.

A special thanks to Thangaraj-Kalai family for their constant support, motivation, and love. Thanks to all my uncles, aunts, cousins and grandma for their love, support, and prayers. Thanks to my spiritual mentor and friend James Siebert and to all my IFG friends at the University of Alberta, who provided the much-needed break with lots of fun during the tough times. Thanks to my friends, Cyril, Abu, Chitra, and Praveen for their motivational and emotional support. Special thanks to Camila for her love, support, and constant encouragement.

Finally, this would not have been possible without the love, prayers and blessings of my mom, dad and sister who believed in me.

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17-AAG	17-allylamino-17-demethoxygeldanamycin
ABC	ATP-binding cassette
ADR	Adriamycin resistant
ADM	Adriamycin
AGO2	Argonaute 2
AGP	α1-acid glycoprotein
AHI-1	Abelson helper integration site-1
AML	Acute myeloid leukemia
AP	Accelerated phase
APC	Advanced pancreatic cancer
ApoB	Apolipoprotein B
b-PEI	Branched Polyethylenimine
BCL	B-cell lymphoma
BCL-xL	B-cell lymphoma-extra large
BCL2L12	Bcl2-like 12
BCR-ABL	Breakpoint cluster region-abelson murine leukemia virus oncogene homolog 1
BMM	Bone marrow microenvironment
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cells
BM-MSC	Bone marrow mesenchymal stem cells
BO	Bosutinib
BSA	Bovine serum albumin
B4GALT1	Beta-1;4-Galactosyltransferase 1
CA	Caprylic acid
CAA	Caproic acid
CAM	Cell adhesion molecules
CBA	Cystamine bisacrylamide
Cbl-b	Casitas-B-lineage lymphoma protein-b
CDC20	Cell division cycle protein 20
CDH	Cadherins
CFU	Colony-formation unit
Chol	Cholesterol
CLL	Chronic lymphocytic leukemia
Col	Collagen
CP	Chronic phase
CRS	Cytokine release syndrome
CSF	Colony-stimulating factors
CsiRNA	Control scrambled siRNA

DA	Dasatinib
DGCR8	DiGeorge syndrome critical region gene 8
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNMT3A	DNA methyltransferases 3 alpha
DOPC	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DS	Dextran sulfate
DsiRNA	Dicer-substrate siRNA
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EphA2	Eph receptor A2
ERK	Extracellular signal-regulated kinase
ERα36	Estrogen receptor alpha 36
FAK	Focal adhesion kinase
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
GAL-3	Galectin-3
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
Grp94	Glucose-regulated protein 94
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3β
HA	Hyaluronic acid
HAT	Histone acetyl transferases
hATTR	Hereditary transthyretin-mediated amyloidosis
hBMSC	Human bone marrow stromal cells
HBSS	Hank's Balanced Salt Solution
HDAC	Histone deacetylase
HeFH	Heterozygous familial hypercholesterolemia
HIF-1α	Hypoxia-inducible factor-1α
HIV	Human immunodeficiency virus
HMW	High molecular weight

HO-1	Heme oxygenase-1
hOCT1	Human organic cation transporter 1
HSC	Hepatic stellate cells
HSP	Heat shock proteins
IAP	Inhibitors of Apoptosis Proteins
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
ILK	Integrin-linked kinase
IM	Imatinib mesylate
IO	Ionomycin
ITGA	Integrin-α
ITGB	Integrin-β
JNK	c-JUN N-terminal kinase
LA	Linoleic acid
Lau	Lauric acid
LDL-C	Low-density lipoprotein cholesterol
LMW	Low molecular weight
LN	Laminin
L-PEI	Linear Polyethylenimine
LSC	Leukemic Stem Cells
MA	Myristic acid
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia 1
miRNAs	microRNAs
MMP	Matrix metalloproteinases
MPA	Mercaptopropionic acid
MRD	Minimal residual disease
MSC	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NI	Nilotinib
NK	Natural killer
nt	Nucleotide
OA	Oleic acid
OXPHOS	Oxidative phosphorylation
PA	Palmitic acid
PAA	Polyacrylic acid
PBMC	Peripheral blood mononuclear cells
PCDH	Protocadherin
PCSK9	Proprotein convertase subtilisin-kexin type 9

PDK-1	Phosphoinositide-dependent kinase-1
pDNA	Plasmid DNA
PE	Phycoerythrin
PEG	Polyethyleneglycol
PEI	Polyethylenimine
PGF	Placental growth factor
P-gp	P-glycoprotein
PH1	Primary hyperoxaluria type 1
PI3K	Phosphatidylinositol 3-kinase
PKN3	Protein kinase N3
PLGA	Polylactic-co-glycolic acid
PLK1	Polo-like kinase 1
PMA	Phorbol 12-myristate 13-acetate
PO	Ponatinib
PrA	Propionic acid
Pre-miRNA	Precursor-miRNA
Pri-miRNA	Primary miRNAs
PTEN	Phosphatase and tensin homolog
P8Am	Poly(N-(8-aminooctyl)-acrylamide)
RBC	Red blood cell
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RTK	Receptor tyrosine kinase
RT-PCR	Real time-polymerase chain reaction
SATB1	Special AT-rich binding protein
SDF-1	Stromal derived factor-1
shRNA	Short-hairpin RNAs
siRNAs	Small interfering RNAs
SOCS3	Suppressor of cytokine signaling
SOD1	Superoxide dismutase 1
St	Stearic acid
STAT	Signal transducer and activator of transcription
ST8SIA4	Sialyltransferase 8 (alpha-2; 8-polysialytransferase)
TGF-β	Transforming growth factor-beta
TKI	Tyrosine kinase inhibitor
TLR	Toll-Like Receptor
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

TRAP-1	Tumor necrosis factor receptor-associated protein
TRPV1	Transient receptor potential cation channel subfamily V member 1
UCB-MSC	Human umbilical cord blood mesenchymal stem cells
VCAM	Vascular cell adhesion molecule
VN	Vitronectin
XIAP	X-linked inhibitor of apoptosis protein
αLA	α-Linoleic acid

SCOPE

The key objectives of my thesis were to investigate the safe use of PEI-based gene delivery systems for the identification and therapeutic targeting for two different types of cancers, breast cancer and chronic myeloid leukemia (CML) representing adherent and non-adherent forms, respectively. The target selections were based on the specific need of the cancer type, as the major hurdle in breast cancer is their metastatic ability whereas in CML it is their drug resistance. Here, we examined the use of RNAi technology to silence the upregulated target genes by siRNA delivery. However, siRNA is highly susceptible to serum nuclease, hence lipid-modified low molecular weight polyethylenimine (PEI) non-viral systems were employed for safe intracellular siRNA delivery. The other main objective was to evaluate the safety of these PEI delivery system with respect to their toxicity to blood components and the ability to induce cytokine stimulation. In **Chapter 1**, a literature review on the practice of RNAi technology was provided, followed by the potential of PEI non-viral delivery systems, the success of lipid substitutions and the benefits of additives in complex preparation. Furthermore, the current knowledge on the safety of PEI delivery systems to the blood components and their ability to cause immune response were summarized. We then focused on key cell adhesion and intracellular molecules overexpressed in breast cancer and their role in its metastasis. Additionally, we explained the various resistance mechanisms observed in CML, particularly the BCR-ABL independent resistance which rely on cell adhesion to bone marrow niche.

In **Chapter 2**, we explored the therapeutic potential of integrin-β1 silencing in breast cancer metastasis by siRNA-based PEI delivery systems. The 1.2PEI-LA6 polymer emerged as the most successful delivery agent in decreasing integrin-β1 expression, and

the functional outcome was reflected with decreased binding to ECM protein and bone marrow stromal cells which is one of the common metastatic sites in breast cancer. Furthermore, strong inhibition in migration was observed following integrin-β1 silencing iterating the therapeutic benefit achieved by linoleic acid substituted PEI siRNA delivery (1.2PEI-LA6).

To further control breast cancer metastasis, **Chapter 3** aimed at identifying other potential targets using a siRNA library against various cell adhesion and cytoskeleton genes with 1.2PEI-LA6 as delivery agent. Following the siRNA library screening, and further validation of the identified targets, HSP90B1 emerged as a potential intracellular protein involved in breast cancer metastasis and survival. To further enhance the silencing outcomes, the inclusion of hyaluronic acid as additives improved the siRNA delivery efficiency. Finally, we optimized the combinational silencing of HSP90B1 and integrin-β1 to better address breast cancer metastasis.

The objective of **Chapter 4** was to address CML resistance, more specifically the BCR-ABL independent mechanism which rely on the binding of leukemic cells to the bone marrow niche through cell adhesion molecules. We demonstrated the high expression of integrin-β1 in CML cells and their binding to the ECM protein, fibronectin (FN). Successful reduction of integrin-β1 was achieved helping to decrease the binding and increase the detachment of leukemic cells from human bone marrow stromal cells. The detached leukemic cells can be easily targeted by tyrosine kinase inhibitors which are the first line of treatment for CML. Moreover, the resistance to BCR-ABL siRNA treatment provided by FN binding was overcome with a combination of integrin-β1 and BCR-ABL

silencing reflected by the significant reduction in leukemic cell proliferation. This study provides an alternative treatment option to address CML drug resistance.

With the successful establishment of PEI delivery agents for the treatment of two different cancer types, we evaluated the safety of these systems in **Chapter 5.** For a better understanding on the impact of lipid type and amount of lipid substitution, various other promising PEI polymers were also included in this study. This study revealed that the ratio of polymer and the nucleic acid is a critical driver for the toxicity experienced with T-lymphocytes and red blood cell (RBC). The additive, polyacrylic acid could be a promising compound to reduce any RBC lysis observed. Minimal differences were observed with variation in PEI to nucleic acid ratio and the concentration of nucleic acid in cytokine secretion with pulmonary blood mononuclear cells. No major cytokine upregulation was observed except for 0.6PEI with lauric acid substitution, however the levels were much lower in comparison to the positive controls. Furthermore, variations in the type and number of lipid substitution did not alter the cytokine production in any major way.

Finally, in **Chapter 6**, we render the overall conclusion of this thesis work and provide a promising and an alternate treatment option for cancer. To address breast cancer metastasis by targeting a cell adhesion molecule, integrin-β1 and an intracellular molecule, HSP90B1 and in CML drug resistance by the targeting of integrin-β1. We established the therapeutic efficiency of lipid modified PEI delivery systems which are safe at physiologically relevant formulations without eliciting any major toxicity and immune response. We also discuss and provide the future directions that would help to improve the cancer treatment as well as for the development of much safer PEI gene delivery systems.

1. MODIFIED POLYETHYLENIMINES AS NON-VIRAL DELIVERY SYSTEMS FOR CANCER THERAPY[1]

[1]A version of this chapter was published in: Meenakshi Sundaram DN, Jiang X, Brandwein JM, Valencia-Serna J, Remant KC, Uludağ H. Current outlook on drug resistance in chronic myeloid leukemia (CML) and potential therapeutic options. Drug Discov Today. 2019 Jul;24(7):1355-1369. doi: 10.1016/j.drudis.2019.05.007. Epub 2019 May 15. PMID: 31102734.

1.1 INTRODUCTION

Cancer is the uncontrolled proliferation of abnormal cells which can originate from any part of the body or tissue and has the property of invading the neighbouring organs making its treatment more challenging. At the cellular level, cancer is a multistep process which starts with mutations in the cell leading to enhanced proliferation, survival, invasion, and metastasis [1]. In Canada, it is the number one cause of death and according to the *Canadian Cancer Statistics 2019* one in two Canadian will develop cancer in their lifetime with an estimated 1 in 4 cancer related deaths [2]. Globally, cancer is the second leading cause of death with 1 in 6 deaths due to cancer as reported by World Health Organization (Geneva, Switzerland) along with an estimated 9.9 million deaths worldwide in 2020. Though there are a wide range of options available for cancer treatment based on their origin, consequently improving the overall survival of cancer patients, the mortality rate remains high which necessitates the identification and development of new therapies. In addition, due to the high cost and evidence of several drawbacks attached with the common types of cancer treatment such as chemotherapy, radiotherapy and surgery, alternate therapies are always being explored for cancer treatment. Moreover, the two major hurdles in cancer treatment are the emergence of drug resistance which develops following repeated exposure to conventional therapy and the targeting of metastatic sites which requires treatment methods with high specificity [3]. This highlights the need to develop new therapies with high feasibility in improving the lives of cancer patients

In this chapter, I will discuss on one such alternate therapy, RNA interference (RNAi), its discovery and their successful use in targeting the cancer-causing proteins while improving the overall survival of cancer patients. The mechanism of RNAi and

various categories of silencing the protein such as microRNAs (miRNAs), and short-hairpin RNAs (shRNA), with a special focus on the success of small interfering RNAs (siRNAs) for cancer treatment will be discussed. Since, the success of siRNA therapy is also dependent on the efficacy of the delivery system, the potential of polyethylenimine (PEI) derived non-viral systems for siRNA delivery will be discussed. The different PEI modifications, the employment of small anionic polymers as additives and their overall safety will be reviewed in detail with regards to its toxicity and immune response.

Finally, the current status and the various cell adhesion targets explored for arresting migration, initiating apoptosis and cell death as a measure to address metastasis of breast cancer as a model for solid cancer will be summarized. The present position of drug resistance in chronic myeloid leukemia (CML), as a model for liquid cancer and the various targets explored for the resistance, in particular the resistance mediated by bone marrow niche, for their successful eradication will also be reviewed.

1.2 POTENTIAL OF RNA INTERFERENCE (RNAI) FOR CANCER THERAPY

RNAi is a natural process that occurs in numerous organisms where silencing of a particular gene can be observed, however this mechanism was first identified in the nematode *Caenorhabditis elegans* when a double-stranded RNA (dsRNA) was injected by Andrew Fire and Craig Mello in 1998, leading to the mRNA degradation of the same sequences due to the dsRNA acting as triggers [4]. Later, this was also identified in protozoa along with higher eukaryotic organisms where the introduction of a small dsRNA of ~21 nucleotide (nt) in mammalian cells was able to trigger the RNAi response resulting in gene silencing [5] (**Fig 1.1**). Since its inception, RNAi has been rapidly explored to

harness this silencing machinery for the therapeutic intervention of a wide range of human diseases which requires the silencing of a specific protein to initiate a variety of responses depending on the target function [6].

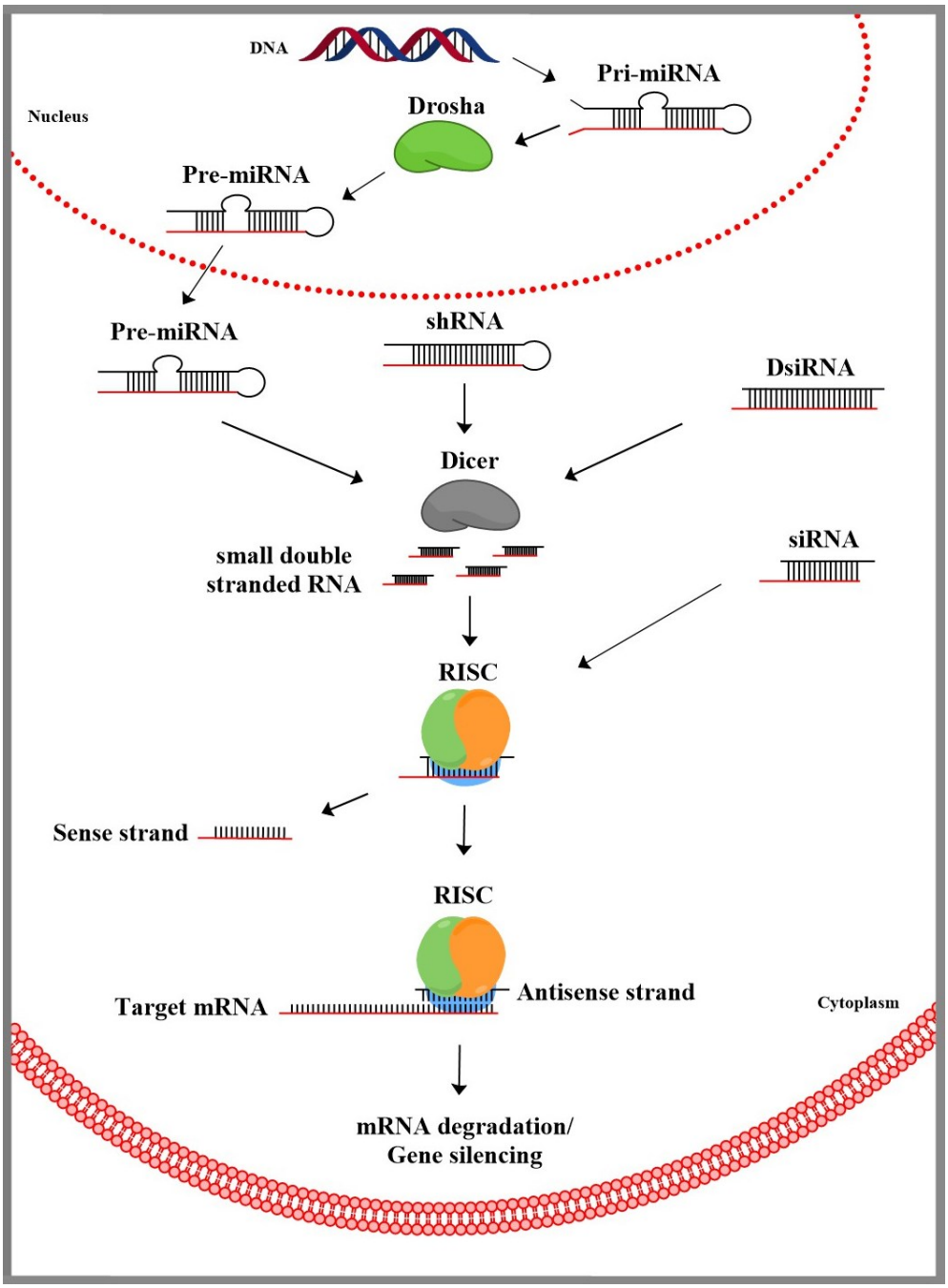


Figure 1.1: The RNAi silencing mechanism by the endogenous miRNA, and the artificially delivered shRNA, dicer substrate siRNA (DsiRNA) and siRNA with the common RNA-induced silencing complex (RISC) mediated degradation of target mRNA.

RNAi is a multistep process and in mammals it involves the cytoplasmic ribonuclease III (RNase III)-like enzyme also called as Dicer that cleaves the dsRNA when introduced artificially or by any infectious pathogen into short dsRNA of ~21 nt, which in turn interacts with RNA-induced silencing complex (RISC), a multi-subunit ribonucleoprotein. The endonuclease argonaute 2 (AGO2) cleaves one of the strands, the passenger strand also known as sense strand and this AGO2 is a key component of the RISC. Whereas the other strand, the antisense strand remains attached to RISC guiding it to the target mRNA consequently resulting in its cleavage by the AGO2, causing posttranscriptional target gene silencing [7]. There are different types of small RNAs that perform gene silencing by RNAi machinery such as miRNA, shRNA, and siRNA which have been identified as natural and artificially induced mechanisms (**Fig 1.1**).

1.2.1 miRNAs

The microRNAs or miRNA are endogenous RNAs where the RNA polymerase II enzyme transcribes the miRNA gene to long primary miRNAs (pri-miRNA) of 60-70 base pairs containing hairpin loop structures which gets recognized by RNase III enzyme Drosha along with dsRNA-binding protein of DiGeorge syndrome critical region gene 8 (DGCR8) in the nucleus. The precursor-miRNA (pre-miRNA) formed from pri-miRNA is transported to the cytoplasm which gets cleaved by Dicer into small RNA duplexes of 19-25 base pairs [7]. This double stranded miRNA gets incorporated into the RISC initiating the RNAi silencing machinery and has the property to bind to hundreds of mRNA sequence leading to the degradation of different mRNA sequences (**Fig 1.1**). Imperfect matching and binding of miRNA can also result in the inhibition of mRNA expression in addition to

mRNA degradation by AGO2 splicing protein [8]. Recent developments on miRNA research has revealed the wide spectrum of its roles in cancer, for example, miR-200 family can act as an inhibitor for epithelial to mesenchymal transition (EMT), proliferation, cell division, differentiation of cancer stem cells, apoptosis induction and are also involved in prognosis of different cancer types [9]. In general, miRNAs as cancer therapeutics can be variable due to their dual role of being an oncogene or a tumor suppressor depending on varying situations, in addition to being diagnostic and prognostic biomarkers. Hence, in depth studies on miRNA expression is necessary for different cancer types combined with target validation as they also carry the disadvantage of eliciting off-target effects [10].

1.2.2 shRNAs

The shRNAs are artificial insertion of a RNA molecule targeting a particular gene sequence into a plasmid DNA (pDNA) vector, which are then delivered into the cells using viral or non-viral delivery systems and transcribed into a hairpin-like stem-loop structure in the nucleus. This structure containing the pre-shRNA is processed by the RNase III enzyme Drosha and DGCR8 like miRNA processing and gets transported to the cytoplasm which further undergoes cleavage by Dicer into double stranded siRNA. Following this, they are loaded into the RISC to initiate the degradation of target sequence [11] (**Fig 1.1**). shRNA has been successfully used for the silencing of various overexpressed genes such as special AT-rich binding protein (SATB1) in inhibiting the tumor growth for gastric cancer, silencing of survivin for ovarian cancer treatment, targeted reduction of B-cell lymphoma-extra large (Bcl-xL) for prostate cancer and even for effective gene silencing in brain [12–15]. Some of the challenges associated with pDNA delivery for shRNA

transcription would be their localization in the nucleus rather than in the cytoplasm [16]. Moreover, unregulated expression of shRNA could compete with the endogenous miRNAs and result in fatalities *in vivo* and on the other hand, due to cell division the shRNA expression can also be lost in the daughter cells [17]. Since the expressed shRNA carry similarities to miRNA, they also posses the potential to cause numerous off-target effects [18].

1.2.3 siRNAs

1.2.3.1 Clinical success of siRNA therapy

In the above-mentioned RNAi mechanisms mediated by miRNA and shRNA, the final product that performs silencing is a short, ~20nt double stranded siRNA which is inserted into the RISC and targets the specific mRNA based on the siRNA sequence for degradation (**Fig 1.1**). The direct use of these double stranded siRNAs as a therapeutic has been well explored since its discovery and its success is of broad spectrum. ONPATTRO (patisiran) is the first siRNA-based therapy approved by the Food and Drug Administration (FDA) for the treatment of peripheral nerve disease (polyneuropathy) caused by hereditary transthyretin-mediated amyloidosis (hATTR) where the abnormal amyloid proteins accumulate in peripheral nerves, heart and other organs causing death in most cases. 80% reduction in the serum levels of transthyretin protein was achieved, which was also evident at the gene and protein levels in the organs with an overall decrease in the disease progression, consequently improving the quality of life, daily living, and ambulation of the patients [19]. SYL1001 is another siRNA-based therapy for the treatment of Dry Eye Disease in Phase 3 clinical trials which has been successful in the targeted reduction of

transient receptor potential cation channel subfamily V member 1 (TRPV1) levels. This ophthalmic solution was shown to reduce ocular pain as well as conjunctival hyperemia associated with patients having dry eye [20]. The application of siRNA-based therapy can also be found in decreasing the levels of low-density lipoprotein cholesterol (LDL-C) using Inclisiran in patients with heterozygous familial hypercholesterolemia (HeFH) and since elevated LDL-C is also a major risk factor for cardiovascular failure, Inclisiran is also being tested for this purpose. Inclisiran has been successful in lowering the levels of proprotein convertase subtilisin-kexin type 9 (PCSK9) along with LDL-C in patients at high cardiovascular risk and is currently in Phase 3 clinical trails [21]. Another drug, Lumasiran (ALN-GO1) in Phase 3 clinical trials successfully depleted glycolate oxidase levels for the treatment of primary hyperoxaluria type 1 (PH1), an inherited rare disease of glyoxylate metabolism which leads to calcium oxalate crystal formation in the kidney and end-stage renal disease (kidney failure) being the outcome [22].

1.2.3.2 Current status of siRNA cancer therapy

For the treatment of advanced pancreatic cancer (APC), Atu027 was shown to inhibit the cancer progression and metastasis regression by silencing protein kinase N3 (PKN3) in the vascular endothelium, which is in a Phase 2 clinical trails [23,24]. siG12D-LODER is another siRNA-based system which targets KRAS gene (G12D) expression in pancreatic cancer as mutated KRAS gene is being linked with pancreatic cancer progression and advancement. Following its success in Phase 1 clinical trails, it is currently in their Phase 2 studies to evaluate their efficacy, safety, tolerability and pharmacokinetics [25]. There are various siRNA-based systems in Phase 1 clinical trials like EPHARNA for

targeting Eph receptor A2 (EphA2) which are overexpressed in various cancers like breast, prostate, lung, ovarian, endometrial and pancreatic are responsible for various functions such as proliferation, survival, invasion, migration and angiogenesis of the cancer cells [26]. APN401 is being tested for the treatment of recurrent or metastatic pancreatic cancer, colorectal cancer, or other solid tumors in which peripheral blood mononuclear cells (PBMCs) transfected with casitas-B-lineage lymphoma protein-b (Cbl-b) silencing by siRNA are infused into the cancer patients [27]. TKM 080301 is another siRNA-based therapy developed for the treatment of adrenocortical cancer by targeting Polo-like kinase 1 (PLK1) which has crucial role in tumor progression with its high expression associated to poor patient survival [28]. Such promising outcomes with RNAi therapy have shown the success of these systems and their ability to address wide range of disease which can be modified according to the requirement.

1.2.3.3 Combinatorial therapeutic approaches with siRNA delivery

In addition, siRNA-based systems have also been explored as combinational therapies with other drugs for cancer treatment. For example, Atu027 administered in combination with gemcitabine for the treatment of APC was more efficient with promising results and is being investigated to use Atu026 as a standard care for APC [24]. Similarly, siG12D-LODER in combination with gemcitabine and nab-paclitaxel in patients with APC was able to achieve disease control and improved survival in Phase 2 clinical studies [29]. siRNA and pDNA combinational strategies have also produced significant outcome for various cancer treatments. The co-delivery of a plasmid DNA encoding tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein with the silencing of *Bcl2-like*

12 (BCL2L12) or *superoxide dismutase 1* (SOD1) in breast cancer ameliorated the effects of TRAIL which was evident through *in vitro* and *in vivo* preclinical studies [30]. This approach was furthered with the inclusion of a drug cisplatin, a siRNA targeting P62/SQSTM1, an autophagy regulatory protein and a β5 plasmid DNA for the treatment of ovarian cancer drug resistance [31].

1.2.3.4 Challenges associated with siRNA-based therapy

For the successful delivery of siRNA, numerous challenges have to be overcome such as the anionic nature and size of siRNA makes it is impossible to cross the hydrophobic cell membrane by themselves [32,33]. Once taken up into the cells by endocytosis they have to exit the endosomes before degradation [34], along with proper maintenance and protection of intact siRNA against nuclease activity in the extracellular milieu which is warranted to perform its function [35]. It should also be able to avoid off-targets effects, immune system activation, and expelling of endogenous miRNA mediated silencing machinery thereby hindering the regular RNAi pathway in the cell [36,37]. Achieve high silencing efficacy and avoid rapid renal clearance which results in reduced half-life of the circulating siRNA, ultimately decreasing their silencing efficacy [38].

Some ways to overcome these challenges are by using low siRNA concentration which can mitigate off-target effects without saturating the RNAi machinery and one means to achieve this is by the delivery of pooled siRNAs that target the same gene thereby helping to achieve low siRNA concentration [39]. Additionally, the use of dicer substrate siRNA (DsiRNA) which are 25-30 nt can attain 100-fold higher silencing than the conventional 21 nt siRNA [40]. The additional nucleotides enable them to be processed by

the dicer enzyme which is part of the RNAi machinery generating 21-21 nt product and results in a more effective incorporation into the RISC due to its association with dicer improving target silencing [40,41]. They also possess the advantage of not eliciting any immune stimulation in comparison with its 21-mer counterpart since the dicer-substrate siRNAs undergo additional processing which is part of the endogenous mechanism and one step before the conventional siRNA [42].

Most of the other challenges associated with siRNA delivery can be overcome with the use of an appropriate carrier system that can bind to siRNA with high stability, protect against nuclease activity, traverse across the cell membrane, escape endosomolytic activity, accomplish cytoplasmic release of the functional siRNA, leading to target silencing without eliciting any immune stimulation and toxicity along with enhanced circulation time. In general, these carrier systems can be divided into viral and non-viral delivery methods, although viral systems have shown to exhibit high efficiency, their safety concerns for long-term use due to the implementation of viral integration, their immunogenic nature and to cause undesirable insertional mutagenesis warrants careful selection and simultaneous use of immunosuppressive therapy [43]. On the other hand, non-viral systems are relatively safer, cost effective and easy to manufacture, hence the next section of this review will focus on the use of PEI-polymeric delivery systems, especially the low molecular weight carriers for siRNA delivery.

1.3 POLYETHYLENIMINE NON-VIRAL DELIVERY SYSTEMS

Polyethylenimine is a cationic polymer which was first reported as an effective gene delivery system in 1995 by Boussif *et al*., due to its high cationic charge density aiding

in effective binding through electrostatic interaction, buffering capacity by the presence of amines and protection of the nucleic acid from nucleases [44]. PEI exist in linear (L-PEI) and branched (b-PEI) forms both of which have high as well as low molecular weight constituents. L-PEI carries terminal primary and secondary amines whereas b-PEI have primary, secondary and tertiary amines at a ratio of 1:1:1. These amines are key for its protonation at a wide range of pH values helping in its buffering capacity [45]. In general, PEI forms stable nano-sized polyplexes with the nucleic acid (pDNA/siRNA) having a positive surface charge that helps in its interaction with the anionic cell membrane and enters the cells through various endocytosis pathways while protecting the cargo from nuclease activity. Once inside the endosomes having an acidic pH, the amines undergo protonation leading to the influx of protons, chloride ions and water which eventually leads to the endosomal rupture because of increased osmotic pressure and swelling of the endosome, a process called "proton-sponge" effect [46]. This causes the release of the contents inside the cell, either in the cytoplasmic compartment for siRNA or in the nucleus for pDNA which are largely depended on the type of PEI used and the target cell [47].

Although PEIs share the above-mentioned common advantages, in addition to their structural difference of being linear or branched, the molecular weight significantly alters the degree to which they can perform its function. High molecular weight (HMW) PEI like 22 kDa (L-PEI) and 25 kDa (b-PEI) have been widely studied and shown to be successful for various gene therapies. For example, *jet*PEI™ which is a 22 kDa L-PEI has been effectively used for siRNA/pDNA delivery *in vivo* as well as in Phase I clinical trials for the treatment of solid tumors (NCT01274455, NCT03739138) [48]. On the other hand, low molecular weight (LMW) PEIs like 0.6, 1.2 and 2 kDa exhibits poor delivery efficacy but

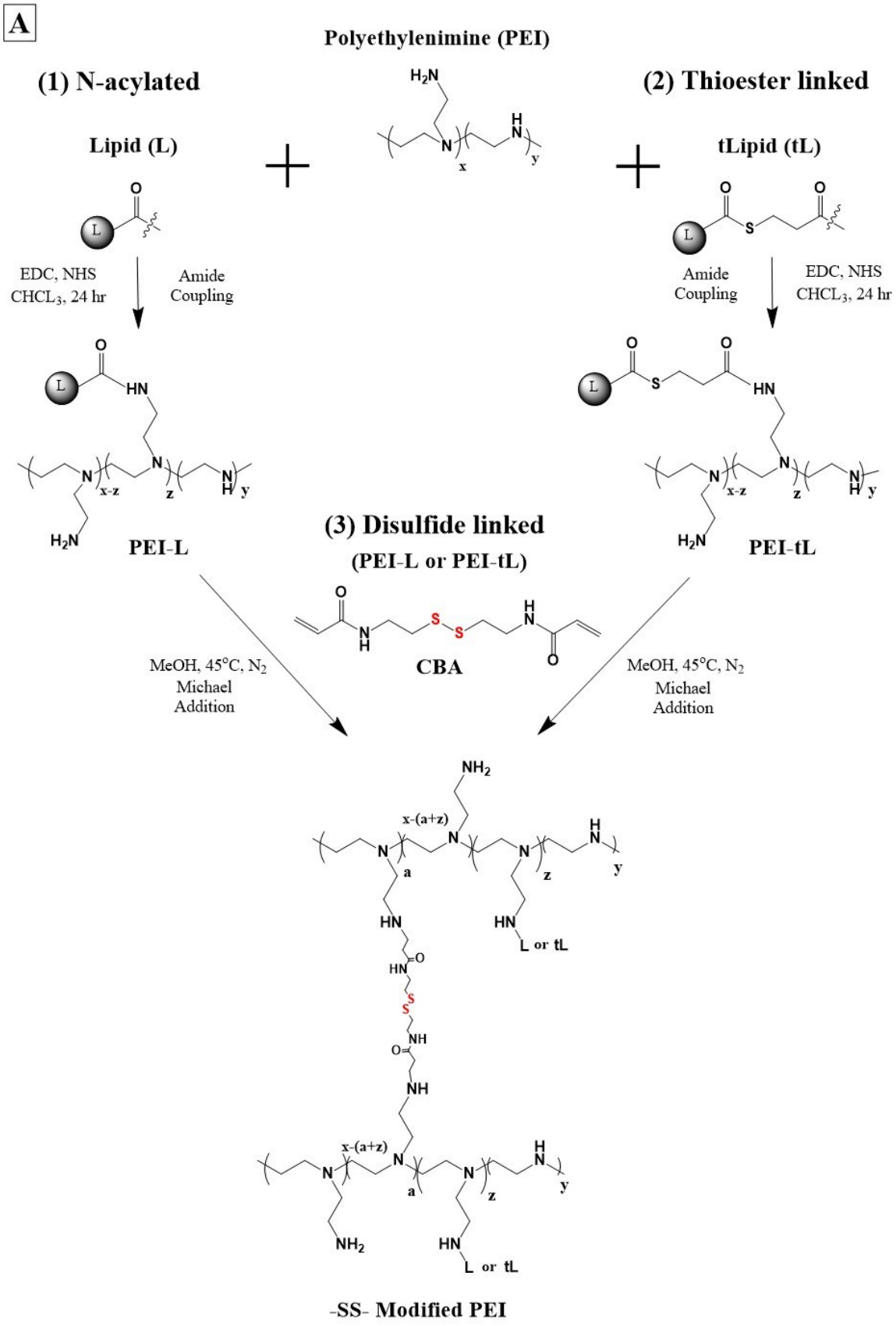
exert low toxicity when compared to HMW-PEIs which can lead to high damage to the cell membrane and has been documented by numerous studies [49]. Hence an inverse relation can be observed in the transfection efficiency and toxicity with respect to its molecular weight which is a major factor that governs the overall performs of PEI [50,51]. However, modification of LMW-PEIs has been investigated to improve their transfection ability, in particular the use of hydrophobic lipid substitutions has achieved tremendous success by Uludag group and others which will be discussed in the following section.

1.3.1 Lipid substitution in PEI

Hydrophobic modification of LMW-PEIs has been extensively studied in the past decade as it can improve its interaction with cell membrane, provide optimal binding with the nucleic acid, and increase the cellular uptake in addition to altering the size as well as surface charge of the polyplexes. The main rationale of using hydrophobic substitution was to improve the overall efficacy of LMW-PEIs and this has been successfully established for various disease conditions through both *in vitro* and *in vivo* studies (**Fig 1.2**).

Grafting of PEI with even a small hydrophobe propionic acid (PrA; C3) at low substitution amounts provided substantial increase in siRNA delivery and silencing compared to native PEI, which was attributed to their optimal surface charge, however with further increase in PrA a significant reduction in its performance was noted accompanied with much lower zeta potential, poor polyplex stability and decreased interaction with the cell membrane [52]. Such studies were carried out in attachment-independent K562 leukemic cell line as well as in attachment-dependent MCF-7 and MDA-MB-231 breast cancer cell lines for both siRNA and pDNA delivery [52,53]. Other hydrophobic lipids like

caprylic acid (CA; C8) and oleic acid (OA; C18) grafted PEI showed high siRNA uptake and silencing at higher substitution ratios in contrast to PrA findings [54,55]. Furthermore, lipids like lauric acid (Lau; C12), palmitic acid (PA; C16), linoleic acid (LA; C18), α-linoleic acid (αLA; C18), stearic acid (St; C18) and cholesterol (Chol) have also been successfully optimized and grafted onto LMW-PEI at varying amounts of substitutions. These modification have drastically improved their nucleic acid interaction, complex formation, polyplex stability, cellular internalization, intracellular release and achieved excellent silencing or expression of the therapeutic gene in various cell types and cancers *in vitro* and *in vivo* [54–60]. Due to the differences in the behaviour of different hydrophobic lipids and the number of substituents, in depth studies have been carried out exploring its role in gene delivery. Among the different lipids explored, LA and αLA substituted PEI have been widely successful for the silencing of numerous breast cancer genes like BCL2L12, SOD1, Myeloid cell leukemia-1 (Mcl-1), survivin, Signal transducer and activator of transcription-5 (STAT5), cell division cycle protein 20 (CDC20), P-glycoprotein, and TTK protein kinase [30,61–64]. Its success could also be noted in the silencing of CD44 in acute myeloid leukemia (AML) and BCR-ABL in CML [65,66].


A
Polyethylenimine (PEI)
(1) N-acylated
Lipid (L)
(2) Thioester linked
tLipid (tL)
EDC, NHS
CHCL3, 24 hr
Amide
Coupling
Amide
Coupling
EDC, NHS
CHCL3, 24 hr
PEI-L
PEI-tL
(3) Disulfide linked
(PEI-L or PEI-tL)
CBA
MeOH, 45°C, N2
Michael
Addition
MeOH, 45°C, N2
Michael
Addition
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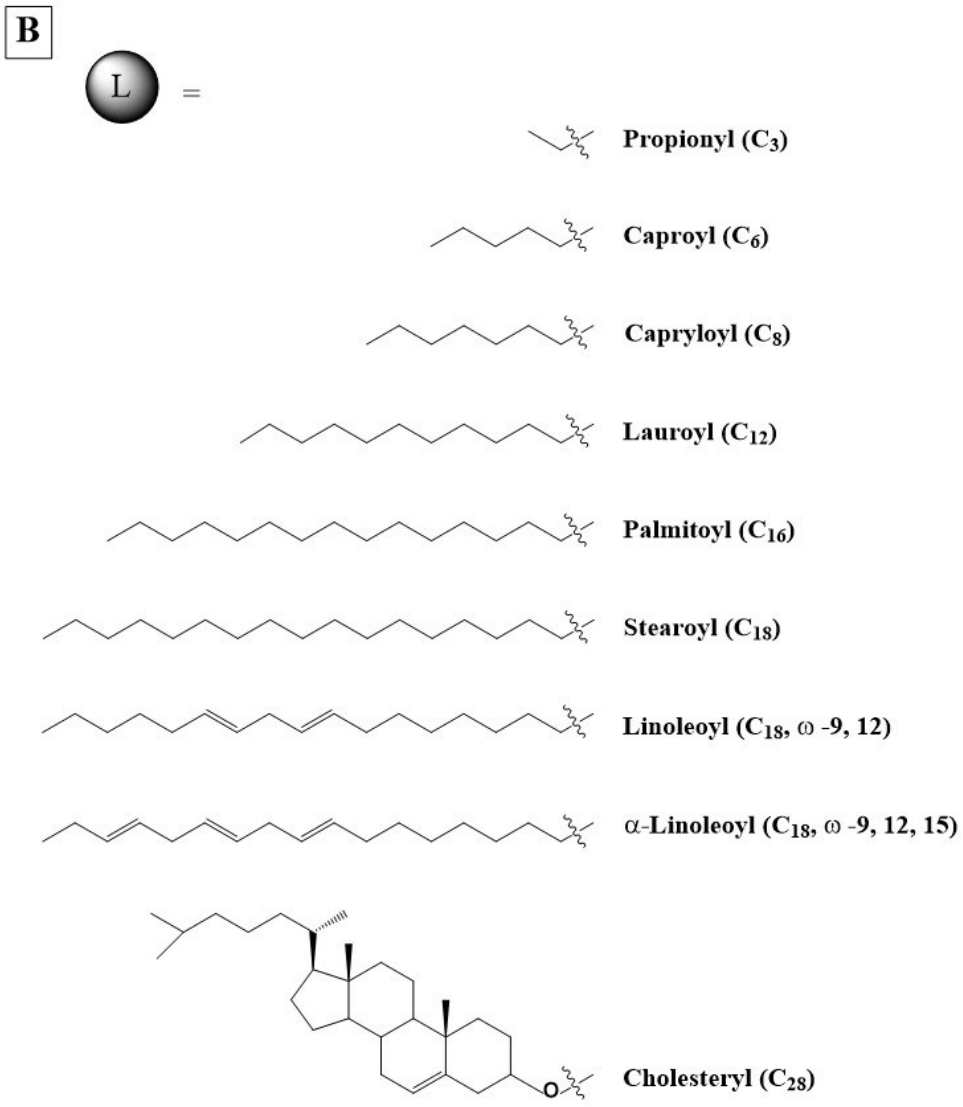

Figure 1.2: (**A**) Schematic representation of PEI lipid modification; (1) the regular method of N-acylation and (2) thioester linked lipid substitution (amide coupling). (3) disulfide linked PEIs following lipid substitution with either amide or thioester link (Michael addition). L-represents the type of lipid, t-represents thioester linkage and -SS- represents disulfide linkage. (**B**) Structure of the lipids with varying chain lengths used in polymer synthesis, with the number of carbon atoms (lower case number) and ω- representing the position of unsaturation.

1.3.1.1 Thioester linked lipid substitutions in PEI

The common method of grafting lipids to the PEI is by using *N*-acylation, creating an amide linkage between them, however, to further enhance the delivery efficiency of

these lipid grafted PEI, thio-ester linkage was introduced before the lipid (**Fig 1.2**). Hence a carboxyl end-capped LA and αLA on PEI through a thio-ester (-S-CO-) linkage forming tLA and tαLA have been explored recently. The thio-ester counterparts displayed better solubility, decreased particle aggregation, enhanced dissociation of nucleic acid with transfection efficiencies achieved similar or higher than commercial delivery agents in human umbilical cord blood mesenchymal stem cells (UCB-MSC) and bone marrow mesenchymal stem cells (BM-MSC) which are some of the hard to transfect cells [67]. Similar observation was seen in MDA-MB-231 breast cancer cells, where tLA and tαLA showed higher transgene expression along with better silencing owing to their enhanced dissociation in a co-delivery approach of BCL2L12 siRNA or SOD1 siRNA with pTRAIL study to induce apoptosis [30]. The success of thio-ester linked polymer have also been established in the silencing of oncogenes like Mcl-1, survivin and STAT5 in MCF-7 cell line, however the degree of lipid substitution was a key factor governing the performance of the delivery systems [61].

1.3.1.2 Disulfide linked PEI

One of the extensively studied methods to further improve the intracellular release of the nucleic acid is the inclusion of reducible disulfide (-SS-) linkage between the polymers (**Fig 1.2**). The principle behind this strategy is depended on the difference between the concentration of glutathione (GSH) between extracellular and intracellular milieu which range between 2-10 μM and 2-10 mM respectively. Such high concentration of GSH would help in the reduction of the disulfide bond leading to a better release and improve the overall performance of the delivery agent [68]. Success of -SS- linked LMW-

PEI can be found in the significant decrease of apolipoprotein B (ApoB) mRNA levels in B16F1 melanoma cells and hepatic stellate cells (HSC) for the treatment of liver cirrhosis using a PEI grafted-hyaluronic acid with -SS- linkage. The complexes formed by this system showed better stability even at high serum concentrations accompanied by improved silencing efficiency, effective cellular uptake and emerged as an effective therapeutic system *in vivo* with siRNA against transforming growth factor - beta (TGF-β) [69]. Similar findings can be observed with pluronic-diacrylate-PEI-SS polymer for pDNA delivery in the embryonic kidney cell line 293T, where the complexes showed low toxicity, high uptake and transfection efficiency, better stability against DNase I degradation due to the presence of disulfide linkage [70].

1.3.2 Inclusion of additives with PEI

The surface of cationic PEI complexes can be coated with specific polymers to ameliorate its efficacy and improve their biocompatibility. Polyethyleneglycol (PEG) is one such polymer which is known to decrease immune response, toxicity, improve stability, and retention time in circulation [71]. Recent developments have revealed many such compounds that carry low amount of negative charge which can be used as a coating or an additive with PEI complexes aiming to further the delivery and performance of gene delivery.

1.3.2.1 Hyaluronic acid

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan, an extracellular matrix molecule which are slightly anionic in nature. At optimal low HA additive

concentrations, the transfection efficiency was increased, cellular uptake was elevated with more sustained presence of intracellular siRNA, and better target silencing. Inclusion of HA as an additive can decreased the size of complexes at low amounts and further increase of HA result in a gradual increase in size, however with HA as coating the size was unchanged. Furthermore, the zeta potential was also reduced with low amounts of HA compared to complexes without HA, and further increase in HA turned the zeta potential to negative. Additional studies revealed the important role of CD44 receptor on the positive benefit of HA additives in MDA-MB-231 cells which express high CD44 on their surface compared to MCF-7 cells [72,73]. HA additives were also beneficial for pDNA delivery in UCB-MSC and BM-MSC cells with similar observations on zeta potential decrease and transfection improvement [67].

1.3.2.2 Polyacrylic acid

Polyacrylic acid (PAA) is another anionic polymer widely utilized in the cosmetic and pharmaceutical industry which can be used as additives with PEI complexes [74]. PAA as an additive can increase the size and decrease zeta potential of the complexes at low amounts which in turn is reflected with an increase in siRNA uptake in MDA-MB-231 cells. Complexes with PAA additive at low amounts were also able to silence genes like CDC20, survivin more effectively which was evident by the decrease in cell growth and increase in caspase-3 activity among various breast cancer cell lines. Ideal release of siRNA due to the presence of PAA additive at optimal amount is a key reason for achieving such positive outcome with such systems [72]. A similar study employing PAA in a PEI/pDNA complex coated with PAA, followed by an additional poly (N-(8-aminooctyl)-

acrylamide) (P8Am) forming a multilayer polyplex delivery system showed high cellular uptake and endosomolytic activity in human cervix carcinoma cell line (HeLa) and human hepatoma cell line (HepG2) [75].

1.3.2.3 Dextran sulfate

Dextran sulfate (DS) is a polysaccharide widely found in different organs like liver, spleen, and kidney. DS has been mostly explored by studies as conjugates for PEI mediated gene delivery and at low amounts it has shown to decrease toxicity, improve stability, increase buffering capacity, with lower surface charge and particle size [76–78]. In addition, DS as additives at low amounts helped in better siRNA release from the PEI complexes with a moderate success in improving the siRNA uptake compared to complexes without DS in MDA-MB-231 cells [72].

1.4 SAFETY OF PEI DELIVERY SYSTEMS

The next important aspect of gene therapy is the overall safety, although non-viral systems are considered safe in comparison to viral vector, due to the inherent nature of the human body, any artificially injected drug or therapeutic molecule will be treated as ‘foreign’. Hence, it is of utmost importance that PEI delivery systems remain ‘stealth’ in biological system and evade the immune system in addition to being non-toxic.

1.4.1 Toxicity

One of the principal reasons to employ LMW-PEI against HMW-PEI is the difference in toxicities [79]. However, due to modifications of LMW-PEI, while improving

the overall efficiency, the toxicity of these systems can also be altered [51,52]. Once inside the physiological environment, the PEI complexes having positive surface charge can interact with the anionic serum proteins and the red blood cells which then undergo aggregation leading to cell surface binding and destabilization, causing toxicity [80]. In general, the observed toxicity can be of immediate effect mostly associated with free PEI and delayed effect caused by the processing of the PEI complexes inside the cell [81]. However, LMW-PEI display no or negligible toxicity in addition to being poor gene delivery agents [79]. On the other hand, lipid modification of LMW-PEI which helps to achieve transfection efficiencies similar or higher than the commercial delivery agents, can also affect the toxicity exerted on the cells [53,67,82]. Specific factors contributing towards the toxicity for the lipid modified LMW-PEI are the type of lipid being grafted on the PEI [53,67,82], the degree of lipid substitutions on PEI [53], the cell type in which the studies are carried out [52,53], the ratio of PEI to pDNA or siRNA [53,56], and the size [82,83] as well as surface charge of the complexes [72,84].

1.4.2 Hemolytic ability

Interaction of PEI complexes having cationic surface property with red blood cells (RBC) having negatively charged surface in circulation is part of the natural proceedings which could lead to its lysis, releasing hemoglobin into the circulation [85]. In general, a delicate balance exists between the intact RBCs and the amount of free hemoglobin present extracellularly, however during pathological conditions, and interactions with therapeutic molecules, hemolysis might occur introducing high quantities of hemoglobin into the circulation causing vascular dysfunction, renal, hepatic, and splenic failure. The circulating

hemoglobin also possess the tendency to become a redox active protein further contributing towards organ failure [86]. Furthermore, the therapeutic complexes can also bind to the released hemoglobin resulting in their rapid elimination [87]. Strong interaction between the PEI complexes and the RBCs can lead to its low availability resulting in poor therapeutic performance as observed in clinical drugs [85]. In addition, due to the similarities between the RBC membranes and endosomal environment, the hemolytic ability also provides information on the endosomolytic behaviour of the therapeutic compound [88]. Although, the key reason for the hemolytic behavior is mainly associated to the positive surface charge of the complexes [89] in contrast to anionic particles that are nontoxic [90], other factors like the number of free amine groups [91], and the dose of compound used, also contribute towards RBC toxicity or hemolysis [89,92].

1.4.3 Immune stimulation

The next vital step when the therapeutic compound is injected into the system is the possibility of being recognized by the immune cells which is an inherent response of the human body to any foreign agent or pathogen. The key indicator for immune stimulation is the secretion of cytokines which are proteins of small molecular weight, from the immune cells and these cytokines are the molecules that govern the duration and intensity of the immune response to the external stimuli. Also, the type of cytokine produced is greatly dependent on the treatment duration and the type of therapeutic molecule used. Apart from playing key roles in protecting the human body from pathogenic invasion, they can also hinder the effect of the therapeutic compound injected due to immune recognition

and elimination. Additionally, either inadequate or exorbitant cytokine production can aid in the progression of various disease conditions [93].

In general, cytokines are important in various signaling activities depending on the target cell and the type of cytokine secreted, which are (i) interferons (IFN) like the type II IFN-γ mediates adaptive and innate immunity, (ii) interleukins (IL) that govern leukocyte growth and differentiation can either be anti- or pro-inflammatory in nature, (iii) tumor necrosis factor (TNF) that are pro-inflammatory, (iv) chemokines that control chemotaxis and, (v) colony-stimulating factors (CSF), as the name suggest they are mostly involved in hematopoietic progenitor cell stimulation, proliferation and differentiation [94].

Some of the key cytokines that are released following the administration of any therapeutic compound are TNF-α, IL-6 and, IFN-γ although numerous other types of cytokines can be released under the above mentioned three major types (TNF, IL and IFN). The detection of these 3 cytokines from the blood, following treatment are considered as primary steps to understand the proinflammatory response concerning cytokine release syndrome (CRS), which is the sudden and excessive accumulation of a variety of cytokines in the circulation causing rapid organ failure and could lead to the eventual fatality of the patient [95]. In addition, under the guidelines and recommendations provided by the FDA for a new therapeutic compound, detection of these 3 cytokines have been presented as key CRS predictors *in vitro* and *in vivo* [96]. Much of the studies on the immune response by PEI have been focused on HMW and very few knowledges exist for the LMW-PEI, hence a general cytokine response with PEI will be discussed.

The delivery of negative control siRNA as well as the functional siRNA targeting human epidermal growth factor receptor (EGFR) in SPCA-A1 (human lung

adenocarcinoma cell line) xenograft mice with a linear HMW-PEI did not induce TNF-α secretion as tested in the serum [48]. Similar observations were reported with other research groups using HMW-PEI following intravenous (i.v) or intraperitoneal (i.p) injection of PEI-complexes *in vivo* [97,98]. Even with branched PEI of 10, 25 and 70 kDa, no upregulation of TNF-α could be detected through *in vivo* studies [99]. However, contrasting observation can also be found were TNF-α was detected in mice serum following i.v administration of PEI complexes [100]. The secretion of the pro-inflammatory cytokine, TNF-α from T-cells, activated macrophages and natural killer cells (NK) is strongly associated with serious liver toxicity, drastic changes in the coagulation of blood and, stimulation of oxidative stress [101–103]. Moreover, its role in cardiovascular diseases and in the progression of various cancers like leukemia, prostrate cancer and ovarian cancer has been well demonstrated [104–107].

Similar to TNF-α, the IL-6 induction by HMW-PEI had contrasting findings, where some reports of no upregulation could be seen *in vitro*. However, intravenous delivery of DNA as well as siRNA using HMW-PEI was able to induce IL-6 production in mice serum [97]. Similarly, IL-6 secretion could be detected in the supernatant of mouse bone marrow cells even with low concentration of HMW-PEI showing significant difference compared to the control treatment [108]. Other *in vitro* studies also reported the induction of IL-6 with HMW-PEI which was concentration dependant [109]. In addition to its contribution towards liver toxicity and in the progression of various cancers like prostate cancer, breast cancer, ovarian cancer and leukemia, high stimulation of IL-6 can also have detrimental effects in neuronal and cognitive functioning [104–106,110,111]. Furthermore, increase in IL-6 can cause high accumulation of reactive oxygen species resulting in muscle atrophy

with major changes in the proper functioning of various types of muscles and tissue degeneration [112].

The upregulation of IFN-γ was not detected while delivering pDNA with HMW-PEI *in vivo* and even repeated delivery of the complexes did not elicit any immune response [113,114]. Similarly, no IFN-γ production was observed with HMW-PEI/siRNA complexes during i.v administration [97]. Contrary to these findings, IFN-γ secretion was noted in mice serum with pDNA complexes, which were drastically upregulated [99] and high amounts of IFN-γ can exhibit multiple negative effects on erythropoiesis causing anemia and disrupt differentiation of hematopoietic cells [115]. Due to the direct impact of IFN-γ on the production of TNF-α, the observed deleterious effects of cytokine upregulation can be further enhanced resulting much higher toxicity [116]. In addition, high IFN-γ can influence cancer progression and aid in autoimmunity *in vivo* [117–119].

Blocking of these cytokines using antibodies has been explored for cancer therapy, while shielding of complexes with additional polymers like PEG have also shown beneficial effects in reducing the stimulation of cytokines and improve transgene expression [120–123]. Treatment with optimal concentration of the complexes is another vital component in reducing unwanted immune stimulation [109]. These findings highlight the importance to study and understand the overall safety of non-viral delivery systems and the essential components which requires close attention while employing them for therapeutic purpose.

1.5 BREAST CANCER METASTASIS AND TREATMENT OPTIONS

A critical step in cancer development is the induction of metastasis, which significantly reduces the chances of survival for patients. As in other cancers, breast cancer is a complex, molecular disease that originates due to gene mutations causing uncontrolled proliferation and metastasis of the cancer cells. According to Canadian breast cancer statistics 2020, breast cancer is the most common type of cancer among Canadian women and is the second most common cause of death with an estimated 1 in 8 women developing breast cancer in their lifetime. Approximately 27,000 women would be diagnosed for breast cancer which accounts for 25% of all new cancer cases in women for the year 2020. Improvements in breast cancer diagnosis has helped in early detection of the cancer which are followed by various treatment options like chemotherapy, radiation therapy and surgical resection, all of which have improved the overall survival of the patients although suffer with various drawbacks of their own [124]. However, the major challenge associated with breast cancer treatment is their metastatic ability and to stay dormant and later result in cancer relapse [125]. More specifically, the highly metastatic triple-negative breast cancer which are devoid of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2, display high heterogeneity representing 10-15% of all breast cancers are more challenging to treat. The process of metastasis is a multistep process which reflects the ability of the cancer cells to leave its primary site, travel through the circulation evading immune recognition, reach a distant organ (e.g., bone, brain), and stay dormant only to re-emerge later [126]. As a result, the treatment for metastatic cancer proves to be challenging with a poor 5-year survival rate of 22%. The multistep process of metastasis starts with (i) the epithelial to mesenchymal transition (EMT) of the tumor at

the primary site, (ii) degradation of extracellular matrix (ECM), (iii) intravasation, (iv) escape from the host immune response, (v) migration and (vi) extravasation followed by attachment and survival at the distant metastatic site [127] (**Fig 1.3**). In this complex process of metastasis, numerous molecules are activated as well as upregulated, contributing to each of the above-mentioned steps. In particular the cell adhesion molecules present on the membrane of the cancer cells play significant roles ultimately aiding in its attachment and metastasis and in the overall breast cancer progression [128].

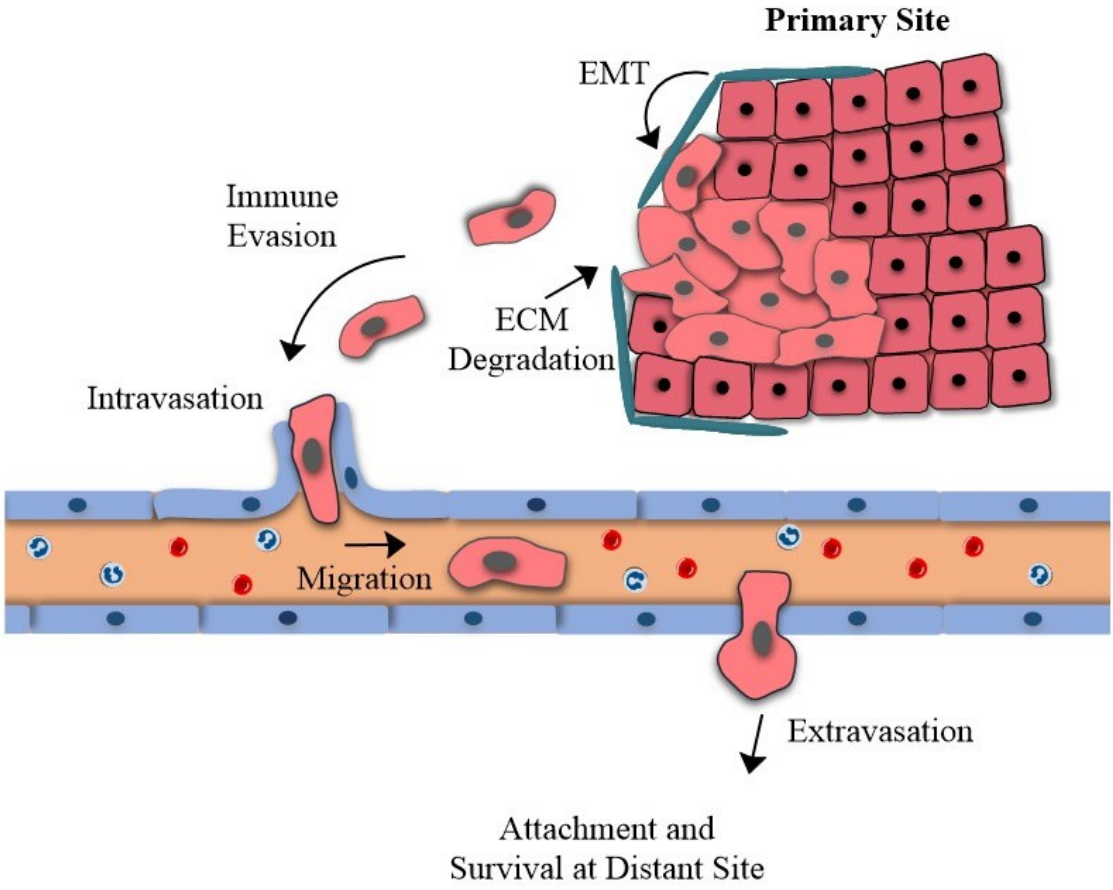

Figure 1.3: Schematic of the process of breast cancer metastasis which involves epithelial to mesenchymal transition at the primary site, ECM degradation, immune system evasion, intravasation, migration, extravasation, and attachment at the distant site for survival.

1.5.1 Cell adhesion molecules

Cell adhesion molecules (CAMs) are mostly transmembrane receptors on the cell surface and structurally consist of an extracellular domain that interacts with external stimuli including various extra cellular matrix components, a transmembrane domain across the membrane and an intracellular domain that mediates various signaling pathways inside the cells. Some of the CAMs reported for their involvement in breast cancer metastasis include cadherins, integrins and selectins.

1.5.1.1 Cadherins

Cadherins are calcium dependent cell adhesion proteins that are instrumental in maintaining the tissue structure by attaching cells together and are base molecules that are required for tissue remodeling, morphogenesis, and proper preservation of the tissue integrity. The presence of Ca^{2+} aids in the formation of stable cell-cell adhesion termed as homotypic adhesion which when disrupted can cause the metastasis [129]. Till date more than 20 members of cadherin molecules have been reported which are expressed on specific cell types like E-cadherin (epithelial), N-cadherin (neural), VE-cadherin (vascular endothelial), P-cadherin (placental) and R-cadherins (retinal tissues) [130].

The expression of E-cadherin is vital in the proper maintenance of the cancer at its primary site as its loss can be observed in 50% of the metastasis cases of breast cancer along with poor prognosis and exhibit strong contribution in overall cancer progression [131]. The observed detrimental outcome of E-cadherin downregulation is not only associated with its loss of adhesion at the extracellular space but also due to the intracellular signaling linked with β-catenin in the cytoplasmic domain of the cadherin. Specifically,

any disruption of the E-cadherin-β-catenin complex can result in multiple outcomes all of which aid in the metastasis. In addition to the weakened cell-cell interaction that leads to cellular motility, the free cytoplasmic β-catenin can be phosphorylated by the glycogen synthase kinase-3β (GSK-3β) resulting in its degradation. Furthermore, the activation of the Wnt signaling pathway due to GSK-3β downregulation helps in the nuclear translocation of β-catenin which can further activate various target genes linked to breast cancer advancement [132]. The loss of E-cadherin expression has shown to be accompanied by upregulation of N-cadherin in breast cancer and is known to aid in its attachment to the stromal cells fulfilling the distant metastasis. In addition N-cadherin can also independently assist in metastasis, irrespective of E-cadherin expression [133].

Other than the classical cadherins which have five domains, protocadherins are the largest subgroup of cadherins which are structurally different having seven cadherin domains. In breast cancer, numerous protocadherins have been identified with an array of functions, all of which impacts cancer progression in different ways. The expression of protocadherin-7 (PCDH7) was shown to influence the brain and bone metastasis of breast cancer through *in vitro* and *in vivo* studies [134]. In contrast the expression of protocadherin PCDH8 was found to be downregulated in breast cancer and its restoration was able to reduce the migration and proliferation of the cancer cells. Similarly, the suppression of PCDH17 was detected in breast cancer cells and in tumors whereas its overexpression through plasmid DNA helped to decrease the proliferation, migration and the epithelial to mesenchymal transition which was dependent on the Wnt/β-catenin signaling pathway [135]. Other protocadherins like PCDH10 and PCDH20 have also been found to be tumor suppressors in various other cancer types [136].

1.5.1.2 Integrins

Integrins constitute the largest group of cell adhesion molecule that have been studied extensively in breast cancer and proven to exhibit broad range of functions in its progression. Integrins are heterodimeric transmembrane cell surface molecules consisting of one α- and one β- subunit. Unlike cadherins that are mostly involved in intracellular signaling through the binding of β-catenin in the cytoplasmic domain, integrins can carry out both "outside-in" and "inside-out" signaling making it a more complex and crucial molecule for proliferation, survival and migration of cancer cells [137]. Till now 18 α- and 8 β- subunits have been identified in humans which heterodimerizes to form 24 different integrin combinations which carryout various functions including its binding to ECM components like fibronectin (FN), laminin (LN), collagen (col), fibrinogen and vitronectin (VN) as well as activation of complex intracellular signaling pathways. Several integrin subunits like α3, α5, α6, αv, β1, β3 and heterodimers like α4β1, α5β1, αvβ3, α6β4, αvβ5 and αvβ6 have shown to be strongly upregulated helping in the process of metastasis to distant organs [138–140].

Integrin-β1. Integrin-β1 is a highly expressed molecule in breast cancer and has strong correlation with poor disease-free survival rate in patients [141]. They also play key roles in the dormancy to metastasis switch and in the invasiveness of breast cancer [142]. The significance of integrin-β1 in cancer progression was highlighted by its *in vivo* deletion which resulted in significant decrease in breast cancer growth [143]. Integrin-β1 is also a critical molecule which is required during the process of tumor formation, EMT, metastasis initiation, proliferation and re-attachment to distant organs as evident through various

studies [144,145]. Among patients experiencing breast cancer recurrence, high expression of integrin-β1 was observed [146]. *In vivo* integrin-β1 deletion in ErbB2 tumors (receptor tyrosine-protein kinase erbB-2) was able to significantly decrease the tumor volume, improve tumor cell death and decrease its lung metastasis [147]. The binding of integrin-β1 ligand, FN to its extracellular domain activates the intracellular signaling involving FAK and AKT impacting the proliferation, migration, and survival of cancer cells with a negative effect on the expression of epidermal growth factor (EGFR) [148–150]. Furthermore, other signaling pathways like phosphatidylinositol 3-kinase (PI3K) and serine/threonine kinase AKT activated by integrin-β1 was shown to aid in drug resistance [151]. Other major signaling pathway components like the focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and c-JUN N-terminal kinase (JNK) can be activated through integrin-β1 which helps to overcome drug induced apoptosis, support tumor growth, induce the expression of various matrix metalloproteinases (MMP) like MMP-2, MMP-9 and MMP-13 which are prominent molecules largely identified as drivers of cancer progression [152,153]. Hence, cross-talk between integrins and receptor tyrosine kinase (RTK) signaling is considered as a key phenomenon in the progression of breast cancer [154]. Studies on the heterodimerized form of integrin-β1 subunit with other α- subunits has provided invaluable information on their significant role in breast cancer advancement. The expression of α2β1 was closely related with bone metastatic breast cancer cells [155]. Similarly, α3β1 was found to be essential in the attachment of the cancer cells to cortical bone matrix through collagen [156]. Other integrin-β1 heterodimers like α4β1 and α5β1 that bind to fibronectin were critical in

mediating Adriamycin resistance helping in their uncontrolled proliferation and survival emphasizing the significance of integrin-β1 expression in breast cancer metastasis [157].

Integrin-β3. Similar to integrin-β1, the expression of integrin-β3 is upregulated among breast cancer cells in comparison with the non-malignant cells. They are involved in the adhesion of cancer cells to the distant site, most notably to bone metastasis. Particularly, αvβ3 integrin expression was strongly upregulated in breast cancer cells that displayed high bone metastasis and their elevated expression was also detected in different patient tumors experiencing bone metastasis [158]. The role of the αvβ3 in breast cancer bone metastasis has been reported by various studies both *in vitro* and *in vivo* [159]. Moreover, the binding of its ligand VN can initiate various signaling pathways which can initiate EMT, increase MMP expression affecting cell migration and invasion [160].

Integrin-β4. Integrin-β4 overexpression can be observed in patients experiencing more aggressive breast cancers and can act as a marker to identify cancer stem cells [161]. Having a long cytoplasmic tail helps in the activation of various signaling pathways and upregulates ErbB2/3 and EGFR which provides resistance towards tamoxifen and other anti-RTK therapy. Furthermore, binding to LN in the ECM enhances PI3K, AKT and MAPK signaling molecules showing the RTK cross-talk with integrin-β4 [162,163]. Till date α6β4 is the only integrin-β4 molecule that has been identified and their cooperation with P-cadherin can induce stem cells and influence the invasive potential of breast cancer cells [164,165]. Finally, high expression of α6β4 was also shown to be critical in the lung metastasis of breast cancer cells [166].

Integrin-β5. Integrin-β5 expression was closely related to the tumor forming ability, migration, adhesion to VN ECM protein, as well as in their proliferation and survival through the activation of FAK and ERK signaling pathways in breast cancer *in vitro* and *in vivo* studies [167]. αvβ5 is shown to interact with EGFR promoting the metastasis and influencing the invasiveness of other cancers like lung cancer, pancreatic cancer and melanoma, but less information is available in breast cancer [168,169].

1.5.2 Essential intracellular molecules

Overexpression of numerous intrinsic intracellular molecular targets have also been identified contributing towards the proliferation, metastasis, drug resistance and invasiveness of breast cancer. Especially the importance of upregulated heat shock proteins (HSP), apoptosis and cell division regulatory proteins like BCL2L12 and cell-division cycle protein 20 (CDC20) in the overall progression of breast cancer have been highlighted by various studies.

1.5.2.1 Heat shock proteins

Heat shock proteins (HSP) are molecular chaperones that governs the proper folding of the proteins in the cell which is required in the functioning of normal cells. HSPs continue to perform their main function in protein folding even in cancerous cells by chaperoning the mutated as well as upregulated proteins which further worsens the disease outcome [170]. There are six main HSP families, named based on their molecular weight: HSP27, HSP60, HSP70, HSP90 and HSP100. One of the main characteristic differences

between the high and small MW HSPs is the dependance on ATP in the former (high MW) whereas small HSPs functions in an ATP-independent manner [171]. The upregulation of HSPs like HSP90 and HSP70 has been recorded in various cancers aiding in the proliferation, apoptosis, differentiation and even in poor prognosis like breast cancer [172].

HSP90. HSP90 is one of the most abundant HSPs and is also called as glucose-regulated protein 94 (Grp94), or gp96, or endoplasmin or ERp99. They are highly involved in apoptosis regulation and possess various client proteins like TLRs, integrins and insulin-like growth factors (IGFs) [173]. Currently five isoforms of HSP90 have been identified which are HSP90α (HSP90AB1) and HSP90β (HSP90B1) in the cytoplasm, HSP90B1 in the endoplasmic reticulum, tumor necrosis factor receptor-associated protein 1 (TRAP-1) in the mitochondria and HSP90N in the plasma membrane [174,175].

The overexpression of HSP90B1 can be seen in a variety of cancers governing a wide range of functions such as proliferation, metastasis and apoptosis regulation in the cells. More specifically, its upregulation was linked with poor overall survival in lung cancer, in the tumor growth of osteosarcoma through the activation of PI3K/ AKT/ mTOR signaling, in the proliferation of hepatocellular carcinoma, B-cell lymphoma, myeloma and in chronic lymphocytic leukemia (CLL) drug resistance [176–181]. Analysis of breast cancer patient samples for HSP90B1 expression displayed strong correlation with their overall survival and distant metastasis. Patients with low expression had higher survival compared to those having high expression who experienced poor survival and metastasis [182]. These findings were also confirmed at the protein and mRNA levels of HSP90B1 and their correlation with poor patient survival by other research groups [183]. One of the

key roles of HSP90B1 is their overexpression in breast cancer metastasis, specially to the brain compared to bone and lung. HSP90B1 helps in the survival of the brain metastasized cancer cells in hypoglycemic environment. Moreover, HSP90B1 overexpression was also confirmed in brain metastatic breast cancer cell lines with the upregulation of apoptosis related proteins like BCL-2, cellular inhibitor of apoptosis protein (HIAP1) and X-linked inhibitor of apoptosis protein (XIAP) [184]. Additionally, the upregulation of the autophagy-mitophagy genes like BNIP3 and BNIP3L were also observed as they are survival mechanisms in cancer growth, particularly in brain metastasis. HSP90B1 upregulation can also be manifested in breast cancer stem cells especially in the CD44hi/CD24lo phenotype and in cells that are oxidative stress-resistant helping in their survival, migration and recurrence resulting in aggressive breast cancer progression [185,186]. More evidence of HSP90B1 overexpression in patients who underwent chemotherapy compared to their initial stage and those experiencing cancer recurrence provides strong evidence on their multifaced role in breast cancer advancement [186].

1.5.2.2 Apoptosis and cell division regulatory proteins

BCL2L12. BCL2L12 is a member of the BCL2 family of anti-apoptotic proteins which is overexpressed in breast cancer cells. In glioblastoma, BCL2L12 was shown to play anti-apoptotic role by inhibiting caspase-3 and -7 activity [187]. In breast cancer, knockdown of BCL2L12 led to improved activity of doxorubicin and cisplatin *in vitro* but contrasting finding were also observed in which cisplatin-resistance was acquired following its knockdown [188,189]. Other studies have shown the importance of BCL2L12 in breast

cancer, where its silencing improved TRAIL-induced apoptosis *in vitro* and decreased tumor growth *in vivo* [30,190].

CDC20. The cell division cycle protein 20 is a key regulatory protein which is overexpressed in many cancers including breast cancer. Its overexpression promoted the metastatic ability of breast cancer cells and was also shown to exhibit an important role in cellular proliferation [64,191]. Multiple studies have highlighted the therapeutic significance of CDC20 in tumorigenesis and progression of breast cancer [192].

1.5.3 Treatment options for breast cancer

The current treatment of breast cancer includes surgical resection of the cancer tissue from the breast (lumpectomy) or the removal of the entire breast (mastectomy) which aims to remove the maximum amount of cancer. Surgical removal can be followed with radiation therapy, chemotherapy, hormone therapy and targeted therapy. Some of the commonly used chemotherapeutic agents include anthracyclines such as doxorubicin and taxanes such as paclitaxel [193]. All these drugs exhibit various side effects which can be significant in the short-term or long-term. Some of the short-term side effects are nausea, vomiting, diarrhea, hair loss, changes in body weight, loss of appetite, decrease in blood cell count and fatigue [193]. The effects of these therapies can also be observed as changes in the menstrual period, infertility, birth defects, bone loss, osteoporosis, and increased risk of cardiomyopathy [193].

On the other hand, specific targeting of the overexpressed protein is a promising strategy that has the potential to exhibit low side effects and toxicity as their effects on normal cells would be minimal. The success of targeted therapy by RNAi has been

mentioned earlier and the aim of this thesis is to establish therapeutic outcome by the targeting of overexpressed integrin cell adhesion molecule as well as the intracellular heat shock protein to overcome metastasis and achieve proliferation inhibition in breast cancer as a model for solid tumor using the non-viral PEI gene delivery systems.

1.5.3.1 Targeting cell adhesion molecules

Function-blocking N-cadherin antibody was able to reduce the migration and invasion. Peptide based antagonist like ADH-1 has been evaluated in the treatment of prostate cancer but not in breast cancer [194]. Treatment with E-cadherin activating antibody was shown to inhibit the progression of metastasis and the formation of mouse tumor [195]. A siRNA-based approach was implemented by the commercial transfection reagent Lipofectamine 2000 to silence PCDH7 which resulted in reduced migration of breast cancer cells [134].

Targeting of integrin-β1 has been carried through a wide range of therapeutic strategies. The integrin-β1 inhibitory antibody AIIB2 was successful in decreasing the growth of cancer cells and inducing apoptosis without altering the viability of non-malignant cells. Moreover, the combination of AIIB2 with ionizing radiation helped to increase cancer apoptosis [196–198]. Clinical studies with PF-04605412 to block α5β1 suffered from acute-infusion related complications and eventually was ineffective in decreasing tumor growth [199]. Combinational targeting of integrin-β1 were also explored to overcome trastuzumab resistance, in which AIIB2 antibody along with siRNA treatment through electroporation provided beneficial outcome [200]. In addition, due to the cross-talk between integrin-β1 and EGFR, antibody mediated targeting of either of these targets

decreased the expression of both and reverted the breast tumors to non-malignant forms [201]. Use of integrin-β1 blocking peptides like ATN-161 helped to reduce the tumor growth as well as the metastasis *in vivo* and achieved prolonged stable disease in patients [202,203]. Peptide mediated targeting of α2β1was also able to reduce the tumor volume, bone metastasis and enhanced survival. Further combinational treatment of this cyclic chondroadherin (CHAD) peptide with doxorubicin showed beneficial effect *in vivo* [155].

The selective upregulation of αvβ3 in bone metastasis increased the interest in its targeting. Hence the use of a nonpeptide PSK1404 helped to reduce the bone tumor burden accompanied by a strong decline in bone destruction *in vivo* [204]. The monoclonal antibody etaracizumab targeting αvβ3 could inhibit proliferation, invasion and the migration of breast cancer cells [158,205]. Similarly, treatment with the peptide S247 decreased the lung metastasis of breast cancer cells *in vivo* in addition to inhibition of bone metastasis [159].

Targeting of α6β4 helped to hinder their cross-talk with P-cadherin thereby reducing the invasiveness of breast cancer cells achieved by siRNA delivery by HiPerFect transfection reagent [165]. Likewise targeting of α6β4 with inhibitory antibody drastically reduced the lung metastatic property of breast cancer cells [166].

Although antibodies targeting integrins have advanced to clinical trials, they have numerous limitations such as high production costs, possible adverse interaction(s) with immune system and other unwanted short-term or long-term side effects.

1.5.3.2 Targeting the essential intracellular molecules

Numerous HSP90 inhibitors have been developed and tested in various types of cancers. PU-H71 was able to successfully induce caspase-dependent apoptosis in drug sensitive as well as drug resistant myeloma cells [179]. The therapeutic potential of PU-H71 was also shown by their anti-tumor activity in B-cell lymphoma [180]. Furthermore, PU-H71 exhibited prolonged *in vivo* activity for the treatment of hepatocellular carcinoma accompanied by significant cell cycle arrest and apoptosis induction [181]. Its success in the treatment of breast cancer can be manifested by the prolonged downregulation of various oncoproteins that play key roles in proliferation, survival, and invasiveness. Decrease in various signaling molecules like AKT, ERK2 and PKC were also observed leading to enhanced apoptosis induction [206]. Treatment with PU-H71 was able to improve the sensitivity of breast cancer cells to radiation therapy, however an inflammatory response was also documented warranting the need for careful future investigation before proceeding further [207]. Numerous other HSP90 inhibitors like ganetespib, 17-allylamino-17-demethoxygeldanamycin (17-AAG), AT-533, BJ-B11, DCZ3112 and TAS-116 have been explored for breast cancer treatment, however reports of significant side effects and failures due to non-specific effects in clinical trials can be observed [208–211]. HSP90B1 knockdown using Lipofectamine 20000/siRNA in the brain metastatic breast cancer cells as well as in animal studies helped to decrease the viability of cancer cells and improved the survival of the animals [212]. Similar studies of RNAi based silencing of HSP90B1 was achieved, improving doxorubicin sensitivity of the cancer cells, but by using high siRNA concentrations [213].

1.6 CHRONIC MYELOID LEUKEMIA DRUG RESISTANCE AND TREATMENT OPTIONS

As an alternative cancer model, this thesis work also involved a leukemia model, namely Chronic myeloid leukemia (CML), which is distinct from the solid tumors characterized by breast cancer. CML is a multi-lineage myeloproliferative neoplasm that originates from hematopoietic stem cells. It affects 1 to 2 per 100,000 adults worldwide. CML is characterized by uncontrolled proliferation of hematopoietic cells, particularly an excessive number of granulocytes is produced in the bone marrow. The cytogenetic hallmark of CML is a reciprocal chromosomal translocation that occurs between the long arms of chromosome 9 and 22, t(9;22)(q34;q11) forming a shortened chromosome 22, known as the Philadelphia chromosome (Ph) found in 95% of CML patients. The resulting breakpoint cluster region-abelson (*BCR-ABL*) fusion gene, encodes an oncoprotein ($p210^{BCR-ABL}$) with increased tyrosine kinase activity, substantially contributing towards uncontrolled proliferation, growth factor independent survival, modified cell adhesion and apoptosis inhibition [214–216]. The CML progresses in 3 phases: a chronic phase (CP) representing 85% to 90% of patients at diagnosis, but it can progress to an accelerated phase (AP) and then either a myeloid or lymphoid blast crisis. With the introduction of BCR-ABL tyrosine kinase inhibitors (TKIs), the overall survival of CML patients in CP has been drastically improved with imatinib mesylate (IM) becoming the gold standard for first-line of treatment and has already became available as a generic drug in many countries [217,218]. However, the emerging IM resistance and therapeutic failure have led to development of second and third-generation TKIs with increased potency in treatment of CML, but they also elicit inadequate responses and fail to prevent disease progression in

some patients [219–222]. Further failure modes of TKI monotherapies have provoked comprehensive studies exploring various BCR-ABL-dependent and BCR-ABL-independent mechanisms of drug resistance. Here we will discuss the mechanisms of drug resistance in CML, the range of therapeutic solutions available to overcome resistance and ultimately help to improve the CML treatment with special focus to address the bone marrow mediated BCR-ABL independent resistance.

1.6.1 Drug resistance in CML

The growth dependence of CML on constitutively activated BCR-ABL tyrosine kinase allows its targeting by TKIs, of which the first-generation IM has become the frontline therapy. An 8-year follow up study on CP-CML indicated an estimated event-free survival rate of 81% and overall survival rate of 93% [223].

However, drug resistance in various forms was described through *in vitro* and *in vivo* studies (**Fig 1.4**), which was shortly followed by clinical reports of resistance to IM and development of second-generation TKIs dasatinib (DA), nilotinib (NI), bosutinib (BO) and third-generation ponatinib (PO). The drug resistance in patients can be classified into two major groups as (i) BCR-ABL dependent and (ii) BCR-ABL independent mechanisms.

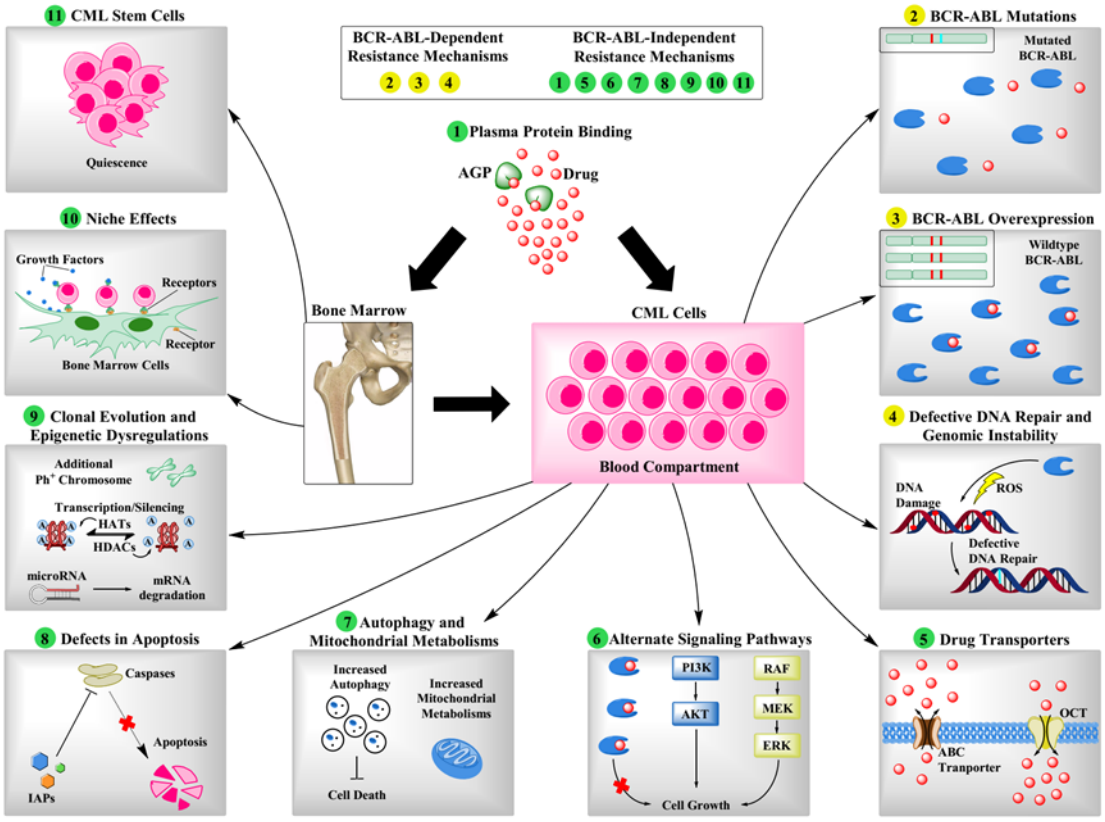

Figure 1.4: Resistance mechanisms in CML. BCR-ABL dependent mechanisms indicated by yellow circles that include (2) BCR-ABL mutations, (3) BCR-ABL overexpression, and (4) Defective DNA repair and genomic instability. BCR-ABL independent mechanisms indicated by green circles that include (1) Plasma protein binding (AGP, α1-acid glycoprotein), (5) Drug transporters, (6) Alternate signaling pathways, (7) Autophagy and mitochondrial metabolism, (8) Defects in apoptosis (Inhibitors of Apoptosis Proteins – IAPs), (9) Clonal evolution and epigenetic dysregulations, (10) Niche effects and (11) CML stem cells.

1.6.1.1 BCR-ABL dependent resistance

This mechanism of resistance could arise due to mutations at the BCR-ABL kinase domain, mutations outside BCR-ABL kinase domains, compound mutations, defective DNA repair mechanism and amplification/over-expression of BCR-ABL, which all ultimately impair the effectiveness of TKI treatment in patients.

Mutations in BCR-ABL Kinase Domain. Mutations in ABL kinase domain and other domains that control the conformation of the kinase domain hinder the binding of drug affinity by altering the BCR-ABL conformation or by hampering the binding all together leading to resistance. Mutations are the most common resistance mechanism with >100 different mutations identified to-date. They can be observed at various structural subunits of the kinase domain and classified into four categories affecting (i) the ATP-binding P-loop, between amino acids (aa) 244 to 255, (ii) the IM direct binding site between aa 315 to 317, (iii) C-loop (catalytic domain) between aa 350 to 363 and (iv) A-loop (activation loop) between aa 381 to 402 [224]. The widely observed and highly resistant 'Gatekeeper' T315I mutation (4-15%) in the IM binding site is a result of a single nucleotide-change from C to T at position 944 in ABL kinase, which leads to replacement of threonine at position 315 with isoleucine. T315, the gatekeeper residue for ABL that aids in the formation of H-bonding with the TKIs, is changed with a bulky isoleucine that hinders the interaction of all second generation TKIs [225–227].

Mutations outside BCR-ABL Kinase Domain. The SH2, SH3 and Cap domains are involved in the autoinhibition of ABL kinase [228]. The T212R mutation in the SH2 domain stabilizes the active conformation of BCR-ABL kinase, thereby inhibiting IM binding [229]. The *BCR-ABL* mutations can also arise prior to TKI treatment and that, not all mutations lead to drug resistance [230].

Defective DNA Repair Mechanism and Genomic Instability. DNA repair is an important component of DNA damage response by which cells can recover, although its efficacy and

accuracy vary, affecting cell cycle, induction of malignancy, cell death and transfer of genetic information. The production of reactive oxygen species (ROS), inhibition of DNA glycosylase (UNG2) activity by BCR-ABL protein can cause DNA damage and faulty DNA repair, leading to accumulation of mutations [231]. Finding from multiple studies suggest that BCR-ABL–induced genomic instability may originate in the most primitive TKI-refractory LSCs that contribute to drug resistance and disease progression in CML [232–234]

Over-expression of BCR-ABL. Genomic amplification of *BCR-ABL* gene as well as over-expression of BCR-ABL transcripts have been detected in patients with drug resistance [225,235]. The over-expression of *BCR-ABL* in 50% of TKI-resistant cell lines was the initial mechanism of resistance identified, as no mutations emerged in the absence of *BCR-ABL* over-expression. However other modes of resistance were also described [236]. The level of *BCR-ABL* expression correlated with mutation emergence in patients, but it may not translate to increased leukemic burden [237]. Although, contrasting evidence on *BCR-ABL* amplification has been reported earlier where *BCR-ABL* amplification was absent in IM resistance, a follow-up study in a 68-year-old patient who developed secondary resistance to IM, displayed not only genomic *BCR-ABL* amplification but also an increase in *BCR-ABL* transcripts, which were also demonstrated in other studies [238–240].

1.6.1.2 BCR-ABL independent resistance

Though BCR-ABL-dependent resistance mechanisms are most common, other mechanisms of resistance include activation of alternate pro-survival signaling pathways,

drug influx-efflux activity, clonal evolution, epigenetic modifications, inherently-resistant stem cells, bone marrow stromal microenvironment and elevated levels of inhibitors of apoptosis proteins.

Alternate Pro-survival Pathway. One of the earliest findings from studies of BCR-ABL-transduced hematopoietic cells was the activation of an autocrine mechanism which could confer partial or complete growth factor autonomy [215,241]. This finding was then demonstrated in most $CD34^+$ CML patient cells that display a constitutively activated production of IL-3 and granulocyte-colony stimulating factor (G-CSF), which accounts for increased STAT5 phosphorylation [242]. In primitive CML cells, this is silenced when they become quiescent and is then reversed when they begin to proliferate. Increased levels of GM-CSF protected the CML cells against IM and NI, through the activation of JAK2/STAT5 pathway, independent of *BCR-ABL.* The GM-CSF levels were elevated in CML patients exhibiting IM-resistance independent of any *BCR-ABL* mutation, suggesting a possible contribution towards IM and NI resistance [243,244]. IM treatment can induce PI3K/Akt/mTor signaling pathway, which is essential in mediating early IM-resistance [245]. The overexpression of (i) FOXO1, which is downstream of PI3K pathway, (ii) PRKCH a member of Protein Kinase C (PKC) and an activator of c-RAF (RAF/MEK/ERK signaling) and (iii) SRC family kinase (SFK)-LYN also contribute towards BCR-ABL independent resistance [246–248].

Over-expression of various proteins has been documented among the TKI resistant CML cells, including (i) PFKFB3 [249], (ii) nuclear β-catenin, NFκB-p65 and Akirin-2 protein [250], (iii) tumor progression locus Tpl2 (COT1 kinase, MAP3K8) along with

SFKs NFκB and MEK/ERK [251]. Other findings indicated the involvement of exosomes (30-120 nm vesicles) with the transfer of miR-365 from resistant to sensitive cells [252]. It is interesting to note that some of the signaling pathways involved in BCR-ABL dependent survival, overlaps with alternate signaling pathways independent of BCR-ABL, such as JAK/STAT and RAF/MEK/ERK, which could be 'overlapping' therapeutic targets.

Changes in Drug transporters and Plasma Protein Binding. The activity of transporters is associated with TKI resistance, as the availability of intracellular drug is crucial to achieve a clinical outcome. Membrane influx pumps such as human organic cation transporter 1 (hOCT1), a key transporter required for IM uptake and ATP-binding cassette (ABC) members that encode key efflux pumps play important roles [253]. However contrasting evidences can be seen with OCT1 content and IM response [254,255]. Similarly, the ABC efflux transporter P-glycoprotein (P-gp or MDR1 protein) can reduce intracellular IM levels when over-expressed, adversely affecting the therapeutic efficacy and is true with various other ABC transporters [256,257]. The importance of such transporters have also been shown in $CD34^+$ CML patient cells [258–264].

Clonal Evolution and Epigenetic Dysregulations. Additional chromosomal abnormalities in Ph^+ cells are often detected in 30% of patients in the AP and 80% of patients in the blast crisis. The frequently reported clonal abnormalities include an additional Ph+ chromosome, trisomy 8 and chromosome 17 abnormalities, all of which contribute to diminishing the effect of IM [265,266]. Detection of these in Ph- cells in CML patients during IM treatment has also been reported, although this is not limited to IM resistant

patients as it could be detected in patients undergoing interferon-α or DA treatments [267]. Interestingly, changes in gene expression regulated by epigenetic modifications such as DNA methylation, histone modification and non-coding RNAs can also contribute to drug resistance in CML. Hyper- and hypo-methylation of numerous genes including tumor suppressors affecting proliferation, differentiation, cell-cycle regulation, DNA-repair and apoptosis induction has been well documented [268,269]. In general, the number of methylated genes was higher for drug resistant and intolerant CML patients and CpG island in the promoter region was the most recognized mechanism of DNA methylation leading to gene suppression [270,271]. Histone modifications such as acetylation by histone acetyl transferases (HATs) and hypoacetylation by histone deacetylase (HDAC) leading to gene transcription and silencing respectively, can also contribute to drug resistance [272,273]. Non-coding RNAs were recently recognized as epigenetic regulators that can manage mRNA and protein levels via several mechanisms. The mRNA binding miRNAs can silence or activate gene expression and some of the dysregulated miRNAs identified in CML are summarized in **Table 1.1**.

Table 1.1: Recently identified miRNAs that are dysregulated (down- or upregulated) in CML leading to drug resistance.[a,b]

microRNA	Dysregulation	Cells	Outcome	Target	Refs
miR-101	Downregulation	K562 cells	Overexpression inhibited proliferation and induced apoptosis	Decreased antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, XIAP, survivin), proliferative genes (c-Myc, CCND1)	[274]
miR-124-3p	Downregulation	K562, KU812, patient BMNCs	Inhibition of SOCS3, cell proliferation and drug resistance	Decreased expression of B4GALT	[275]
miR-146a	Downregulation	K562/ADM	Overexpression re-sensitized cells to Adriamycin (ADM)	Increased expression of CXCR4	[276]
miR-181c	Downregulation	K562/ADR, CML/MDR	Overexpression re-sensitized cells to ADM	Decreased expression of ST8SIA4	[277]
miR-199b	Downregulation	CML patients	IM resistance	Caused by deletion in 9q34.1 region (ABL)	[278]
miR-212	Downregulation	K562 cells	Inhibition improved cell viability, reduce apoptosis, decrease cytotoxicity caused by IM treatment	Upregulation of ABCG2 efflux pump	[279]
miR-217	Downregulation	K562 cells	IM resistance	Increased expression of DNMT3A	[280]
miR-3142	Overexpression	K562/ADR, CML/MDR	Increased colony formation. Enhanced resistance to ADR	Decreased expression of PTEN and activation of PI3K/Akt pathway	[281]
miR-451	Downregulation	CML patients	Inhibition leads to IM resistance	Increased levels of MYC	[282]
miR-574-3p	Downregulation	K562 cells	Overexpression inhibited proliferation, induced apoptosis	Suppression of IL-6/JAK/ STAT3 pathway	[283]
miR-9	Downregulation	K562/ADR, CML/MDR	Overexpression re-sensitized cells to multiple drugs also in vivo.	Decreased expression of ABCB1 efflux pump & P-gp proteins	[284]

a Summary of the ability of miRNAs to alter other targets along with specific cell types used in the studies

b Abbreviations: ABC, ATP-binding cassette transporter; ADR/ADM, adriamycin resistant; B4GALT1, beta-1;4-Galactosyltransferase 1; DNMT3A, DNA methyltransferases 3 alpha; PTEN, phosphatase and tensin homolog; SOCS3, suppressor of cytokine signaling; ST8SIA4, sialyltransferase 8 (alpha-2; 8-polysialytransferase)

Leukemic Stem Cells (LSC). CML stem cells are a rare population that have been established as inherently-resistant to TKIs and a key population for driving relapse and disease progression. Strong evidence indicates that LSCs possess multiple features expected to promote TKI resistance, including deregulated expression of *BCR-ABL*, a high

degree of genetic instability and *BCR-ABL*-independent survival [233,260,285]. Independent groups have reported that *BCR-ABL* expression is highly elevated in the most primitive subset of $lin^{-}CD34^{+}CD38^{-}$ CML stem cells and is then rapidly and progressively reduced as these cells differentiate [260,285,286]. Interestingly, the levels of *BCR-ABL* transcripts present in CML LSCs are much higher than those in quiescent cells, which seems not dependent on BCR-ABL kinase activity for survival and explains why all FDA-approved TKIs are ineffective against primitive quiescent CML cells [287–289]. The resistance of LSCs could also arise due to the increased expression of PRKCH thereby activating RAF/MEK/ERK signaling pathways. High BCR-ABL activity by phosphorylated-CrkL could be another reason for the observed resistance in this population. [248,285,290]. The presence of external factors such as autocrine production of cytokines and stromal-support microenvironment indeed enhance survival of CML LSCs irrespective of BCR-ABL inhibition by IM. It has been reported that AHI-1 (Abelson helper integration site-1), a scaffold oncoprotein, is highly deregulated in LSCs and interacts with multiple kinases and other proteins (BCR-ABL, JAK2, β-catenin, DNM2 and PP2A) to enhance leukemia-initiating activity and resistance to TKIs [291–293]. Combinations of TKIs with a JAK2 inhibitor or a PP2A inhibitor to disrupt the AHI-1-mediated protein complex sensitize drug-insensitive LSCs to TKIs both *in vitro* and in preclinical xenotransplant models [293,294]. In addition, the high expression of human estrogen receptor alpha 36 (ERα36), an alternative splicing variant of ERα66, has been demonstrated in $CD34^{+}$ CML cells and T315I mutant cells and is abnormally localized in plasma membrane and cytoplasm, which can be another factor in the observed TKI resistance [295].

Activation of various pathways like Wnt/β-catenin, Hedgehog signaling, JAK/STAT, and expression of BCL-6 can support cell survival independent of BCR-ABL in CML stem cells [296–298] The innate as well as acquired mechanisms that make CML stem cells resistant to TKIs has thus prompted considerable interest in developing strategies to target these cells more effectively.

Bone Marrow Microenvironment. Cell-cell contact mediated by various receptors in bone marrow stroma along with cytokines, chemokines and growth factors secreted by stromal cells can contribute towards drug resistance [299]. Stromal Derived Factor-1 (SDF-1, or CXCL12) which acts through the CXCR4 receptor can modulate CML cell survival; the CXCR4 expression is altered with BCR-ABL activity, leading to defective adhesion of CML cells to bone marrow stroma. The IM treatment can induce CXCR4 and BCL-XL expression leading to migration/homing to bone marrow and protection from drug-induced cell death. Bone marrow samples collected from IM resistant patients displayed an increase in the protective FGF2 levels without any BCR-ABL mutation [300]. High levels of IL-7 in the bone marrow microenvironment, secreted by mesenchymal stem cells (MSC), was shown to provide protection against IM-induced apoptosis [301]. IL-1β in high levels can help migration of cells towards the stroma [302]. The production of placental growth factor (PGF) by the stromal cells stimulates proliferation of *BCR-ABL* cells via Flt1 (VEGFR1) and help to overcome the effects of IM [303]. Studies with stromal conditioned media were shown to cause and/or enhance resistance towards IM *in vitro,* associated with an increase in the levels of STAT3 target genes *BCL-xL*, *MCL-1* and *Survivin* [304]. Co-culture of CML cells with bone marrow stromal cells (BMSCs) can induce the expression of heme

oxygenase-1 (HO-1, heat shock protein 32) in the later and galectin-3 (GAL-3) in the former, providing anti-apoptotic protection, multidrug resistance, proliferation and bone marrow homing [305,306].

Integrins play a major role in cell adhesion mediated drug resistance. Various intracellular signaling pathways can be regulated via 24 different receptors that are formed by dimerization of 18α and 8β integrin subunits whose expression vary significantly from cell to cell [307]. The well-studied β_1 integrins VLA4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) expressed on CML cells can bind to VCAM-1 and fibronectin expressed on BMSC and extracellular matrix and could activate or downregulate multiple genes to confer drug resistance and act as a sanctuary for minimal residual disease (MRD) [299,304,308,309]. Activation of integrin-linked kinase (ILK) can lead to direct interaction with β integrins promoting survival via a myriad of signaling pathways such as AKT/PI3K, ERK1/2, STAT3 and Notch1/HES [310]. A subpopulation of CML cells exhibiting IM resistance with high adhesion ability and invasiveness were observed following continuous IM treatment; this was attributed to enhanced expression of $\alpha_V\beta_3$ integrin along with the activation of focal adhesion kinase (FAK)/AKT and ERK1/2 pathways [311] (**Fig 1.5**).

The cytoplasmic domain of Ca^{++}-dependent adhesion molecule Cadherin can bind to intracellular β-catenin, which is linked to actin cytoskeleton, stabilizing cell-cell adhesion. Co-culture of $CD34^+$ CML cells with MSC can activate WNT-β-catenin signaling and due to its association with N-cadherin, protects CML cells from TKIs [312]. The direct interaction between MSC and CML cells by CXCR4 receptor effectively protected the leukemic cells from IM-induced cell death and home CML cells bone marrow [313]. The overexpression of hyaluronan-receptor CD44, selectins and osteopontin also

promotes homing and engraftment of CML cells in bone marrow which bear N-selectins on the endothelium [314].

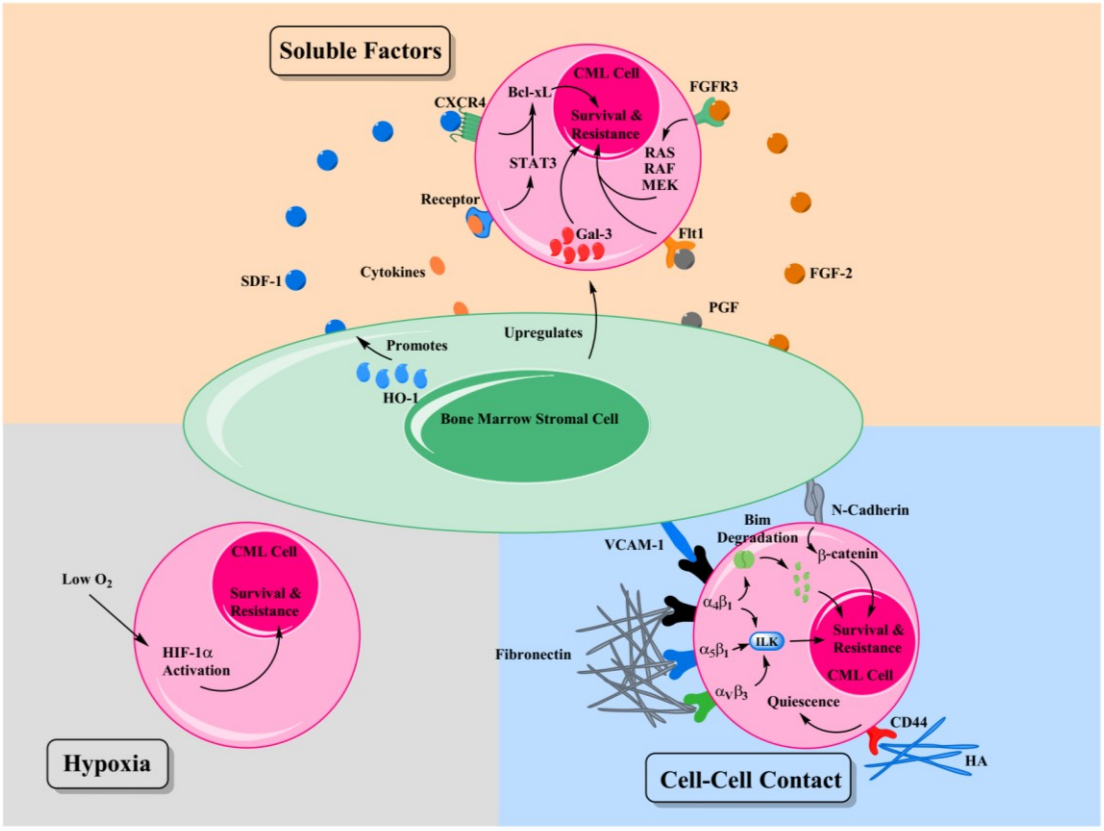

Figure 1.5: Resistance mechanisms in the Bone marrow microenvironment. Overview of select soluble factors, cellular receptors and their mediators altering different pathways along with hypoxia in bone marrow microenvironment (BMM) that act as a protective niche and contribute to drug resistance. Abbreviations: stromal derived factor-1 (SDF-1), heme oxygenase-1 (HO-1), signal transducer and activator of transcription-3 (STAT3), galectin-3 (GAL-3), placental growth factor (PGF), fibroblast growth factor-2 (FGF-2), fibroblast growth factor receptor-3 (FGFR3), vascular cell adhesion molecule-1 (VCAM-1), integrin-linked kinase (ILK), hyaluronan (HA), integrins $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ and hypoxia-inducible factor-1α (HIF-1α).

The inherently hypoxic bone marrow environment can prolong survival of CML cells during TKI treatment. The hypoxic environment leads to activation of hypoxia-inducible factor-1α (HIF-1α, a transcriptional factor), aiding in survival of CML cells

despite a BCR-ABL reduction by IM treatment and evading IM-induced apoptosis. Gene profiling studies indicated up-regulation of various pro-survival genes, which were partially attenuated by IM treatment, however several genes still promoted survival CML cells in stromal environment [315]. These observations strongly support the role of bone marrow niche as a sanctuary for cells responsible for MRD as well as drug resistance (**Fig 1.5**).

Defects in Apoptosis (Inhibitors of Apoptosis Proteins -IAPs). IAPs proteins such as XIAP, survivin, MCL-1 can inhibit drug-induced apoptosis and its up-regulation in TKI resistant CML cell lines as well as primitive patient cells has been documented which can also be attributed to their ability to inhibit caspase activity [316,317].

Autophagy and Mitochondrial Metabolisms. Recent evidence on enhanced autophagy as a survival mechanism in IM treated CML cell lines and patient cells has identified key autophagy proteins such as autophagy related 5 (ATG5), ATG7 and ATG4B and their role in CML drug resistance [318,319]. Reports of increased dependence on mitochondrial oxidative phosphorylation (OXPHOS), as evident by high ROS levels and DNA damage in CML LSCs when compared to normal hematopoietic stem cells (HSC), could provide an additional specific treatment strategy to treat CML drug resistance [320,321].

1.6.2 Therapeutic approaches against resistance

CML patients who do not respond to IM treatment with 400 mg/day dose in CP and 600 mg/day for AP are considered as treatment failures. Increasing IM dose from 400

mg/day to 800 mg/day could be tolerated and therapeutically beneficial. Multiple studies have reported the therapeutic outcome of dose escalation among CML-CP patients experiencing suboptimal response or cytogenetic relapse (appearance of Ph^+ metaphases), patients with mutations that exhibit low-level of resistance, and patience who experience resistance due to inadequate levels of IM in the plasma and BCR-ABL gene amplification. However, this approach appears to be ineffective for all other cases of IM resistance [265,322]. Inhibition of the drug efflux transporter P-gp helped to increase the intracellular concentration of IM which in turn addressed IM resistance. Reversin 205, a P-gp inhibitor, was able to decrease the IC_{50} of IM significantly in IM resistant cell lines, thus re-sensitizing the cells to IM and this approach can also be implemented for treating LSC [323]. However, this approach might fail in T315I mutant cells where IM cannot bind to BCR-ABL irrespective of its intracellular concentration. Here, we summarize the treatment options that have been explored in clinical trials, *in vivo* and *in vitro,* which have the potential to address different BCR-ABL independent resistance mechanisms.

1.6.2.1 Targeting BCR-ABL independent resistance

Interferon-α (IFNα). IFNα is a cytokine that was the gold standard for treatment of CML before the TKIs and was able to improve the median survival time to five years. Several groups have reported the benefit of using IFNα combined with TKIs in drug resistant T315I patients. IFNα exhibits antiviral, anti-proliferative, and immunomodulatory activity along with its ability to induce cells differentiation. The PEGylated IFNα when administered with IM/DA was effective in T315I mutants and was also able to successfully eradicate this mutation in CML patients [324,325].

Targeting Signaling Pathways. Targeting the JAK pathway by ruxolitinib effectively inactivated STAT5 and, with NI, provided beneficial outcome in growth inhibition of CML stem and progenitor cells, which were resistant to TKIs previously [326]. Blocking JAK-2 pathway led to decreased levels of GM-CSF-induced STAT5 activation, thereby inhibiting proliferation of drug resistant cells [243]. A combinational treatment targeting STAT3, STAT5 and HO-1 was shown to overcome a range of resistances including CML stem cells, highly resistant *BCR-ABL* sub-clones and T315I-inclusive mutations. In this study, the STAT3 inhibitor CDDO-Me (bardoxolone methyl) in combination with TKIs showed synergistic effect on growth inhibition and apoptosis induction in drug resistant CML cells. The outcome was more effective when combined with ZnPP (zinc protoporphyrin IX) that inhibits HO-1, leading to increased apoptosis [327]. Another study targeting STAT3 with BP-5-087, a salicylic acid-based inhibitor, in combination with IM reduced the survival of BCR-ABL independent resistant CML cells [328].

Inhibition of mTOR (the mechanistic target of rapamycin) with RAD001 (everolimus) was shown to prevent IM-induced AKT activation and resistance development [245]. More recently, mTOR inhibition with NVP-BEZ235 in PO-resistant cell line, which exhibit BCR-ABL independent activation of mTOR, was shown to induce cell death. Moreover, inhibition of autophagy by hydroxychloroquine helped to sensitize these cells to mTOR inhibitor treatment *in vitro* and *in vivo* resulting in higher cell death [329].

A class-I PI3K inhibitor, GDC-0941 (pictilisib) was able to inhibit the growth and induce apoptosis in a dual TKI (IM and DA) resistant cell line by downregulating the transcription factor FOXO1. The BCR-ABL independent resistant cells were also sensitive

to a combinational treatment of DA and GDC-0941 along with a significant inhibition of AKT substrates [246]. Another inhibitor, LY294002 in combination with IM or alone, was able to induce apoptosis and autophagy in *BCR-ABL* positive cells by causing endoplasmic reticulum stress, which could be further explored for IM-resistant cells [330]. Inhibition of phosphoinositide-dependent kinase-1 (PDK-1), which is key in AKT signaling, using OSU-03012 was able to re-sensitize resistant cells to IM due to lower AKT phosphorylation [331].

The simultaneous inhibition of RAF/MEK/ERK pathway by the MEK inhibitor trametinib (GSK1120212) and IM exhibited synergistic outcome in BCR-ABL independent, IM resistant CML patient cells [248]. Another study using the MEK inhibitor U0126 in combination with DA (SFK and BCR-ABL inhibition) significantly decreased the survival of IM resistant cells, whereas U0126/IM combination did not have any beneficial effect, highlighting the importance of targeting both MEK-ERK and SFK [251].

Targeting bone marrow microenvironment

The rise of drug resistance has made HSC transplantation (HSCT) an important option for CP patients with T315I mutation who fail to respond to two TKIs and for AP patients [332,333]. The growth of CML stem cells independent of BCR-ABL and their inherent resistance to TKIs has led to targeting numerous intracellular pathways critical in bone marrow milieu. The MEK inhibitor, trametinib targeting the RAF/MEK/ERK pathway was successful in inducting apoptosis in both murine and human stem cells with minimal effect on normal HSCs [248]. Targeting the up-regulated ERα36 in TKI-resistant $CD34^+$ CML stem cells and T315I mutant cell line by the flavonoid Icaritin (SNG162)

impeded cellular growth and induced apoptosis. The outcomes were synergistically improved with TKIs, and a detailed mechanistic study revealed the disruption of BCR-ABL/Tyr177/GRB2 complex interactions along with RAS/MAPK pathway inhibition as this is activated by the binding of GRB2 at the SH2 domain, initiated by the phosphorylation of Tyr177 by ABL [295]. The β-catenin signaling in CML stem cells can be counteracted by (i) antibodies blocking the interaction of CD70 and CD27, (ii) PADs (Protein Phosphatase 2A-Activating Drugs) such as FTY720 leading to activation of PP2A and degradation of β-catenin, and (iii) 15-lipoxygenase inhibitors (15-LOi) that can down-regulate β-catenin. In addition, a combinational treatment of NI and 15-LOi was synergistic in TKI resistant CML stem cells. The smoothened (SMOs) inhibitors LDE225 (Sonidegib) and GDC-0449 were able to inhibit the Hedgehog signaling in $CD34^+$ CML-CP and T315I mutant BCR-ABL cells respectively in combination with TKIs, without affecting the normal HSCs [296]. The STAT3 SH2 domain binding inhibitor BP-5-087 was also able to restore the TKI sensitivity without any toxicity on normal HSCs [328]. The BCL6 inactivator peptide RI-BPI effectively targeted the primary $CD34^+$ as well as the more primitive $CD34^+CD38^-$ CML population. It was unable to cause any significant change in the viability of cells on its own, but its combination with IM improved the activity [297].

The CXCR4 antagonist plerixafor (AMD3100) in combination with NI provided substantial improvement in the survival of mice. More importantly, plerixafor was effective in reducing the NI resistance in CML cells induced by bone marrow stromal cells which could be a promising to eliminate MRD [334]. The interruption of CXCR4/SDF-1 interaction by plerixafor can also downregulate the expression of the anti-apoptotic protein BCL-XL in resistant cells [313]. The protection exerted by FGF-2 from bone marrow

microenvironment was successfully neutralized by PO, which was also able to decrease the FGF-2 concentration high in resistant patients [300]. Increased expression and signaling mediated by IL-1R in CML stem cells was successfully treated using IL-1R antagonist in combination with NI, resulting in decreased cell growth, cell division and colony formation. The same approach can be extended to other 'protective' interleukins (e.g., IL-1β and IL-7) at bone microenvironment [302,335].

The anti-PIGF monoclonal antibody 5D11D4 helped to extend the survival of CML mice that were resistant to IM treatment [303]. A monoflavonoid oroxylin A (isolated from the root of *Scutellaria baicalensis Georgi*) effectively re-sensitized the IM-resistance exerted by the presence of stromal derived factors. Oroxylin A was able to inhibit the STAT3 pathway, thereby improving the IM sensitivity *in vitro* and *in vivo* [304]. The anti-apoptotic protection against IM treatment by HO-1 expression can be counteracted by HO-1 inhibitors metalloporphyrins like zinc (ZnPPIX), tin (SnPPIX) and chromium protoporphyrin (CrPPIX) [336]. Similarly, the expression of GAL-3 can be encountered by GCS-100, a GAL-3 antagonist and can be used in combination with other TKIs [306].

The binding of CML cells to bone marrow niche via specific surface receptors can be abrogated using blocking antibodies such as Ha2/5 targeting integrin-β1, RG7356 targeting CD44, various antibodies targeting $\alpha_V\beta_3$, GC-4 targeting N-cadherin, bicyclams, such as plerixafor targeting CXCR4, RGD (Arg-Gly-Asp) peptides blocking integrins and RNAi targeting specific receptors that has been successfully used in acute myeloid leukemia, example: siRNA/polymer targeting CXCR4 [299,308,337]. As the binding of leukemic cells to the BM niche through integrin-β1 can cause IM resistance, activating various survival proteins like MCL-1 BCL-xL and survivin preventing apoptosis, integrin-

β1 is explored as a potential therapeutic target to address BCR-ABL independent resistance in CML particularly with the BM mediated mechanism.

1.7 RATIONALE AND HYPOTHESIS

The potential of siRNA therapy for treatment of various disease conditions including solid tumors and leukemias by PEI-based delivery system is encouraging due to the possibility of target specific downregulation of the cancerous gene. Breast cancer and CML have been chosen as model systems representing adherent and non-adherent cancers to explore the therapeutic potential of PEI delivery systems.

1.7.1 Rationale

The major hurdle in the treatment of breast cancer is their metastatic ability, after which the survival of the cancer patients is very minimal warranting the urgent need to limit the spread. Cell adhesion molecules have found to be critical regulators aiding in the migration and attachment at distant organs resulting in secondary tumor formation. Especially, the overexpression of integrin-β1 in various cancer including breast cancer has found to be crucial in the process of metastasis making it a valuable therapeutic target. Therefore, targeting these overexpressed integrin-β1 cell adhesion molecule in the highly metastatic TNBC will help to reduce their migration and attachment, thereby decreasing the metastatic ability. In addition to molecules at the cell surface, various intracellular proteins are also upregulated which not only helps in the survival and drug-resistance, but also aid in the metastatic process. In that regard, heat shock proteins are known to chaperone various essential proteins for cancer progression. Particularly, the

overexpression of HSP90B1 in the more aggressive and invasive breast cancer, in patients who underwent chemotherapy and experiencing cancer recurrence has been documented making HSP90B1 a valuable target. Hence, targeting these highly expressed HSP90B1 which also play a major role in brain metastasis will help to decrease the viability of cancer cells as well as result in migration inhibition.

Like breast cancer, the expression of integrin-β1 in CML has been found to be critical helping in the BCR-ABL independent resistance to TKI therapy as the attachment of leukemic cells to bone marrow environment activates various signaling pathways that operate independent of BCR-ABL. Though TKI inhibitors are the first-line of treatment for CML targeting the BCR-ABL whose expression is a hallmark trait of CML, some percentage of CML patients experience resistance and requires specialized treatment options. Hence integrin-β1 targeting using siRNA-based PEI delivery system can provide the needed benefit to overcome resistance.

Finally, the modification of PEI, especially the LMW polymers which has shown to improve its delivery efficacy, can alter their various physicochemical properties like the surface charge, size, intracellular localization, the binding, and dissociation ability of the of the complexes. These changes are dependent on the type of lipid modification, degree of lipid substitution, concentration of PEI, amount of therapeutic nucleic acid and the ratio of PEI to nucleic acid used for treatment. Moreover, the inclusion of negatively charged polymers like HA and PAA as additives have been shown to further improve the delivery efficiency. In this process of nanoparticle engineering, there is a lack of information on the overall safety of these lipid-modified LMW PEI used for gene delivery. Especially the need

to remain less toxic during its interaction with the blood components and to not exhibit any immune response is of paramount importance.

1.7.2 Hypothesis

1) Targeting of the highly expressed integrin-β1 cell adhesion molecule in the TNBC model cell line MDA-MB-231 using target specific siRNA delivered by lipid modified PEI systems will contribute towards a decrease in migration and binding ability.
2) Reducing the levels of the overexpressed endoplasmic reticulum HSP90B1 in MDA-MB-231 breast cancer cell line by PEI/siRNA delivery system will result in decreased cell viability and migration inhibition.
3) Decreasing the integrin-β1 levels in the CML model cell line K562 will allow the detachment of the cancer cells from human bone marrow stroma, decrease the binding ability, and inhibit the proliferation of the cells.
4) Employing LMW-PEI with different lipid substituents and varying levels of substitutions in evaluating their hemolytic ability, toxicity and cytokine stimulating ability in T-lymphocytes will help to understand the *in vitro* safety of these non-viral delivery systems.

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2. POLYMERIC DELIVERY OF siRNA AGAINST INTEGRIN-β1 (CD29) TO REDUCE ATTACHMENT AND MIGRATION OF BREAST CANCER CELLS[2]

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2.1 INTRODUCTION

Metastasis of breast cancer to various organs such as brain, liver, lung, and bone makes the disease almost incurable. Metastatic breast cancer is the second leading cause of cancer deaths following lung cancer [1]. The process of metastasis is initiated with (i) EMT of the tumor at the primary site, (ii) extracellular matrix degradation, (iii) intravasation, (iv) migration, (v) evasion from host cell defense mechanism and (vi) extravasation followed by attachment to the distant metastatic site [2]. The cell surface integrins expressed on tumor cells are vital for attachment of metastasizing cells to other organs. The integrins are cell surface receptors which are essential for cell-cell, cell-extracellular matrix interactions and play a pivotal role in migration, invasion, proliferation, survival of tumor cells and also in growth factor receptor signaling [3]. In mammals, there are 18 α-subunits and 8 β-subunits in the integrin family which can form 24 different integrin receptors and bind to various extracellular matrix components such as fibronectin, collagen, laminin and vitronectin [4, 5]. Over expression of integrins and their involvement in cancer progression has been demonstrated in different cancer types.

In breast cancer, integrin-β1 (CD29) activation contributes to dormancy-to-metastatic shift in *in vitro* and *in vivo* models [6, 7] and its over-expression is shown to be associated with invasive potential of the cancer cells and a significant decrease in the overall disease-free survival [8-11]. Antibodies targeting integrin-β1 were shown to result in induction of apoptosis as well as decrease in tumor growth. One such promising antibody, PF-04605412 targeting α5β1, displayed acute-infusion related reaction and it failed to inhibit the tumor growth in clinical trials [12]. The use of CRISPR/Cas9 to study the importance of integrin-β1 on EGFR (epidermal growth factor receptor) tyrosine kinase

inhibitor (gefitinib) resistance revealed reduced migration of cells and negative regulation of EGFR activation upon integrin-β1 knockout [13]. The use of CRISPR/Cas9 for therapeutic intervention, however, may not be desirable, given the permanent deletion of integrin-β1. Resistance to trastuzumab, a monoclonal antibody targeting HER-2 (human EGF receptor 2) was addressed by targeting integrin-β1 using a combination of a blocking antibody (AIIB2) and specific siRNAs (delivered by electroporation) [14]. In addition to antibodies, ATN-161, an integrin-β1 binding peptide, reduced the metastasis and tumor growth in animal models and its clinical trials resulted in prolonged stable disease in patients [15, 16]. The importance of integrin-β1 during tumor progression was further emphasized by ablating integrin-β1 in a mouse model, which interfered with the proliferation of cancer cells and mice deficient in integrin-β1 exhibited a drastic reduction in mammary lesion [17]. Evidence of integrin-β1 as the major factor for the attachment of breast cancer cells has been shown by binding studies to fibronectin. Attachment to fibronectin activates various intracellular signaling pathways which enhances cancer cell proliferation, migration, survival and helps to confer increased drug resistance [18-20].

These studies supported the importance of integrin-β1 as a potential therapeutic target to reduce breast cancer metastasis. Though several integrins have been identified and their inhibitors interrupted breast cancer metastasis, the pre-clinical and clinical outcomes of such drugs had little impact to improve the survival rate of the patients [21]. There is a strong need to develop more efficient approaches to overcome integrin-mediated metastasis of breast cancer. An alternative approach to target integrin-β1 is to employ RNA interference (RNAi) mechanism where a target protein can be endogenously silenced post-transcriptionally. It seems possible to silence any protein target at will using RNAi. A

recent study focused on integrin-β1 by using lentiviral short hairpin RNA (shRNA); integrin-β1 knockdown resulted in reduced attachment of MDA-MB-231 and MDA-MB-231 BO (bone metastatic) cells to human osteoblastic cell-derived matrices and led to reduced cellular migration without affecting the proliferation of cells [22]. Employing viral mechanisms to implement RNAi, however, is not desirable in a clinical setting. A more acceptable approach is to employ a pharmacological mediator of RNAi, namely short interfering RNA (siRNA), with non-viral carriers to achieve silencing.

In this study, we further explored the therapeutic prospect of integrin-β1 using a simpler and efficient intervention based on the polymeric delivery of a dicer-substrate siRNA targeting integrin-β1. As the successful entry of intact siRNA into the cells is plagued by barriers such as the anionic cell membrane and degradation by RNase A, a safe non-viral delivery system was employed to protect and deliver it into the cells. For this purpose, we utilized polyethylenimine (PEI) polymers of low molecular weight (1.2 kilo Dalton PEI – 1.2PEI) which has been substituted with lipids to improve the interaction of the cationic PEI/siRNA complexes with the anionic cell membrane. The use of dicer substrate siRNA (rather than conventional 21 nt double-stranded RNA) previously showed improved silencing by being incorporated into dicer enzyme in RISC complex [23] and was additionally demonstrated in a previous study from our group [24]. We successfully silenced integrin-β1 with minimal siRNA concentration which in turn reduced the migration of breast cancer cells as well as its binding ability to human bone marrow stromal cells and fibronectin coated surface. This treatment had very minimal effect on the proliferation of the breast cancer cells, thus providing a promising as well as a specific approach for breast cancer metastasis.

2.2 MATERIALS AND METHODS

2.2.1 Materials

The 1.2PEI, fetal bovine serum (FBS), anhydrous dimethyl sulfoxide (DMSO), fibronectin, bovine serum albumin (BSA), formaldehyde, chloroform and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Hank's Balanced Salt Solution (HBSS), trypsin/EDTA, Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin and UltraPure DNase/RNase-free dH_2O were obtained from Fisher Scientific (Ottawa, Canada). Phycoerythrin-labeled mouse anti-human CD29 was from BD Biosciences (Oakville, Canada). Dicer substrate siRNA (CD29_1 sense: 5'-AGUUAACAGUGAAGACAUGGAUGCT-3', antisense: 5'-AGCAUCCAUGUCUUCACUGUUAACUUC-3'; CD29_2 sense: 5'-GCAAAUUCUAGCAAUGUAAUUCAGT-3', antisense: 5'-ACUGAAUUACAUUGCUAGAAUUUGCAG-3' and primers for PCR analysis from IDT (Coralville, USA). Trizol used for total RNA extraction was from Invitrogen (Carlsbad, CA). Corning Costar Transwell plates (6.5 mm inserts with 8.0 μm pore size PET membrane) was from Fisher Scientific. Cell lysis buffer (BML-KI117-0030) was purchased from Enzo Life Sciences (Farmingdale, NY).

2.2.2 Cell models

The metastatic breast cancer cell line, MDA-MB-231 were kindly provided by Dr. Judith Hugh (Faculty of Medicine and Dentistry, University of Alberta, Edmonton) and confirmed to be mycoplasma-free. The cells were maintained in DMEM supplemented

with 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2. Cells were passaged after reaching 80% confluency, using 0.25% Trypsin/EDTA for 2 min at room temperature. Cells were collected by adding complete DMEM and centrifuged at 600 rpm for 5 min. They were allowed to grow for 24 hours prior to treatment. Human bone marrow stromal cells (hBMSCs) were isolated from patients (between 25-50 years of age) based on a procedure [25] approved by the Research Ethics Board of University of Alberta. These hBMSC cells were maintained in DMEM with 10% FBS, 100 U/ml penicillin, 100 µg/mL streptomycin and 5 ng/mL basic fibroblast growth factor (bFGF) at 37°C and 5% CO_2.

2.2.3 Polymer synthesis and siRNA-polymer complex preparation

The 1.2PEI modified with thiol-ester containing α-linoleic acid (tαLA, 2.73 αLA substitutions/PEI; 1.2PEI-tαLA), amide-linked lauric acid (Lau, 4.6 Lau substitutions/PEI; 1.2PEI-Lau) and amide-linked linoleic acid (LA, 6 LA substitutions/PEI; 1.2PEI-LA), were synthesized based on a previously published protocol [26, 27, 41] and the degree of substitution was determined through ^{1}H-NMR. The polymer-siRNA complexes were prepared in serum-free DMEM and incubated for 30 min at room temperature before adding to the cells (in 10% FBS). Complexes were prepared at 4:1 and 8:1 polymer to siRNA weight/weight ratio (corresponding N:P ratios were 15:1 and 30:1). 40 nM and 80 nM of siRNA concentrations were used in culture treatments for dose optimization experiments. All other experiments were carried out at 4:1 polymer to siRNA (weight/weight) with 40 nM siRNA concentration. The Lipofectamine™ 2000-siRNA complexes were prepared at 2:1 lipid to siRNA (weight/weight) ratio (as suggested by the

manufacturer) with similar siRNA concentrations and incubated for 30 min at room temperature.

2.2.4 Flow cytometry for integrin-β1 analysis

The MDA-MB-231 cells were seeded 24 hours prior to the experiments. The cells were treated with the polymer/siRNA complexes for 72 hours (day 3) following which they were collected using Accutase (1:1 diluted with HBSS), washed using HBSS and stained with PE-anti-CD29 for 1 hour at room temperature. The cells were washed twice with HBSS to remove excess antibody. For study groups involving day 6 and day 9 assessment, the media was replenished with fresh complete DMEM medium on day 3 and were incubated further, which was followed by staining as mentioned above. Cells were fixed with 2% formaldehyde prior to analysis with BD LSRFortessa (Becton-Dickinson, San Jose, USA). The extent of integrin-β1 expression was expressed as either (i) mean FI (fluorescence intensity) levels per cell (in arbitrary fluorescent units) or (ii) as percentage of cell population positive for integrin-β1. Cells with no treatment (unstained) were designated as 1% positive population.

2.2.5 Quantitative real time-polymerase chain reaction (qRT-PCR)

To quantify the integrin-β1 mRNA levels, qRT-PCR was performed for which cells were seeded 24 hours prior to treatment. Complexes prepared as mentioned earlier were added to the MDA-MB-231 cells. Later, cells were collected at three different time points (day 3, 6 & 9) as mentioned in the previous section and total RNA was extracted using Trizol reagent based on the manufacturer's instruction. 2 μg of total RNA was reverse

transcribed to synthesize cDNA by using 2 µl of mix-1 containing 0.5 µl random hexamer primer, 1 µl (10mM) dNTP's, 0.5 µl Oligo (dT) and heated at 65°C for 5min. Following which 8 µl of mix-2 containing 4 µl synthesis buffer (5x), 2µl DTT (0.1M), 1µl RNase out and 1µl M-MLV RT enzyme were added and incubated at 25°C for 10 min, 37°C for 50 min, 70°C for 15 min. Real time PCR was carried out on a StepOnePlus RT-PCR system with human β-actin (Forward: 5′-GCG AGA AGA TGA CCC AGA T-3′ and Reverse: 5′-CCA GTG GTA CGG CCA GA-3′) as the endogenous housekeeping gene and for Integrin-β1 (Forward: 5′-CCG CGC GGA AAA GAT GAA T-3′ and Reverse: 5′-TGA GCA AAC ACA CAG CAA ACT-3′). 10 µl of reaction mixture containing 5 µl master mix SYBR Green, 2 µl of 10 µM primers and 3 µl of 5 ng/µl cDNA template were added in triplicates to MicroAmp Fast Optical 96-well reaction plate. The reaction mixtures were heated at 95°C for 10 min before proceeding through 40 cycles of denaturation step 95°C for 15 s and annealing/elongation step 60°C for 1 min. $\Delta C_{T,}$ $\Delta\Delta C_T$ and Relative Quantity (RQ) of mRNA were calculated with endogenous gene and the NT-no treatment group as reference points.

2.2.6 Scratch assay

MDA-MB-231 cells were plated in 48-well plates with four replicates and cultured for 24 hours. The prepared 1.2PEI-LA polymer/siRNA complexes were added to the cells for 48 hours and scratches were made using a 200 µL pipette tip, following which cells were washed to remove the floating cells. Images were obtained before incubating (0 hour) the cultures at 37°C for 24 hours, after which images were obtained once again. The open wound area was measured using TScratch software (available from http://cse-

lab.ethz.ch/software/) and the percentage of migration was calculated by subtracting values of 0 hour from 24 hour and values were plotted relative to the no treatment group.

2.2.7 Transwell migration assay

The MDA-MB-231 cells were grown on 6-well plates, 24 hours prior to the treatment. 1.2PEI-LA polymer/siRNA complexes were added to the cells and incubated at 37 °C for 24 hours. The cells were then washed gently with HBSS to remove the serum content and fresh serum free media was added and further incubated for 24 hours. This was followed by collection of cells using trypsin and approximately 1 - $2x10^5$ cells were re-suspended in 100 µL of serum free medium and were added to transwell inserts. The lower bottom of the wells containing the inserts were filled with medium containing 20% serum as a chemoattractant. This setup was incubated for an additional 24 hours at 37°C. The cells present on the upper surface of the inserts (the cells that did not migrate) were removed gently using a cotton swab. The inserts were then fixed with 3.7% formaldehyde for 20 min and stained with 0.1% crystal violet for 1 hour. The inserts were provided with 3-4 washes with HBSS before imaging under a microscope. The dye was subsequently solubilized with 10% acetic acid and absorbance was measured at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments).

2.2.8 Fibronectin binding assay

The binding assay was carried out based on a published protocol [28] with minor modifications. Briefly, fibronectin was coated onto 96-well flat bottom plates at 4 °C overnight with 50 µL of 5 µg/mL of fibronectin. The plates were then blocked with 2%

BSA in PBS for 1 hour at room temperature. MDA-MB-231 cells treated with polymer/siRNA complex for 72 hours were collected by trypsinization and equal number of cells were added in triplicates to the fibronectin coated plates and incubated at 37°C for 1 hour with a total volume of 300 to 350 µl HBSS in each well. The plates were subsequently inverted and further incubated for 3 hours at 37°C. Cells were fixed with 3.7% formaldehyde followed by staining with 0.1% crystal violet for 1 hour and washed with HBSS. The dye was solubilized with 10% acetic acid and the absorbances were measured at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments).

2.2.9 hBMSC adhesion assay

Human bone marrow stromal cells were seeded in 96-well flat bottom plates and were maintained for 2-3 days to reach confluency [29]. MDA-MB-231 cells were treated with desired siRNA/polymer complexes, harvested after 72 hours of treatment (i.e., siRNA complexes incubated continuously with cells during this time) and stained with DiI (carbocyanine dye) for 20 min. These cells were added to the confluent hBMSC monolayers in 96-well plates and incubated for 1 hour at 37 °C followed by which the plates were placed upside down and further incubated for 3 hours at 37 °C. The non-adherent cells were removed by washing and the fluorescence was recorded at excitation: 549 nm and emission 565 nm using Fluoroskan Ascent plate reader (Thermolab systems).

2.2.10 siRNA uptake

FAM (6-carboxyfluorescein) labeled control siRNA was used to study the uptake of siRNA in MDA-MB-231 cells. The cells were allowed to grow for 24 hours, following

which FAM-siRNA/polymer complexes (polymer/siRNA ratio of 4:1 and 40 nM in solution; prepared as above by replacing unlabeled/specific siRNAs with FAM-labeled siRNA) were added to the cells and incubated for 24 hours. Cells were trypsinized, washed twice with HBSS and fixed with 3.7% formaldehyde. Cells treated with unlabeled control siRNA was used as negative population and the uptake was quantified using BD LSRFortessa (Becton-Dickinson, San Jose, USA). The extent of siRNA uptake was expressed as either (i) mean siRNA levels per cell (in arbitrary fluorescent units) or (ii) as percentage of cell population positive for FAM-labeled siRNA. Cells treated with unlabeled siRNA complexes were designated as 1% positive population.

The siRNA uptake was also investigated by confocal microscopy as a complementary tool to flow cytometry analysis. After treating the cells grown on coverslips with siRNA complexes for 24 hours, the cells were washed thoroughly with HBSS and were fixed with 4% paraformaldehyde (PFA) for 20 min at 37 °C. Cells were once again washed with ddH_2O and stained with 1 µg/ml wheat germ agglutinin (WGA)-Texas Red conjugate for 5 min at room temperature and were washed three times. 6 µl of mounting medium with DAPI (4,6-diamino-2-phenylindole) was used to mount the coverslip onto the slides. Samples were imaged under 40X 1.3 plan-apochromat lenses in Laser Scanning Confocal Microscopy (LSM710, Carl Zeiss AG, Oberkochen, Germany). The captured images were analyzed using the software ImarisCell v 8.3, BITPLANE.

2.2.11 Caspase activity

Following 3 days of treatment with polymer/siRNA complex, cells were collected, counted, and lysed using a cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 5 mM DTT,

0.1 mM EDTA; pH 7.4) with incubation on ice for 5 min. The supernatants were centrifuged and 50 µl of reaction buffer prepared by mixing 10 µl of Ac-DEVD-AFC substrate (20 mM) with 1 ml of HEPES buffer were added to the cell lysate at 0.2 mM.(final concentration). Fluorescence was recorded at Ex: 400 nm / Em: 505 nm using a Fluoroskan Ascent plate reader (Thermolab systems) for different time points (0, 30, 60, 90 and 120 min).

2.2.12 Statistical analysis

The results are summarized as mean ± standard deviation and unpaired Student's *t*-test was used to assess the statistical differences between the group means with p-value < 0.05 considered as statistically significant. Where specified, the number of independent experiments used to generate the data (n) are indicated.

2.3 RESULTS

2.3.1 Low concentration of siRNA is sufficient to reduce integrin-β1 levels

To undertake effective delivery of siRNA across the cellular membrane, we employed 3 in-house prepared PEI polymers that were modified with (i) thiol-ester containing α-linoleic acid (tαLA, 2.73 substitutions/PEI): 1.2PEI-tαLA, (ii) amide-linked lauric acid (Lau, 4.6 substitutions/PEI): 1.2PEI-Lau and (iii) amide-linked linoleic acid (LA, 6 substitutions/PEI): 1.2PEI-LA. These polymers where selected based on their silencing efficiency from an initial library screening where an anti-integrin-β1 siRNA was used (**Fig 2.S1**) to lower cell surface integrin-β1 levels. The size of the polymer:siRNA

complexes were in the range of 300 to 350 nm with positive zeta potentials and conferred complete siRNA protection which has been reported previously [41].

The siRNA uptake studies using flow cytometry with the polymers, displayed similar levels of internalization (~50% of cell population) with all 3 polymers, which was equivalent to siRNA delivery with Lipofectamine™ 2000 (**Fig 2.1A**). However, the mean fluorescence, corresponding to mean amount of siRNA associated per cell, was ~2-fold higher for 1.2PEI-LA and 1.2PEI-Lau polymers, followed by similar levels of fluorescence by the 1.2PEI-tαLA polymer and Lipofectamine™ 2000 (**Fig 2.1B**).

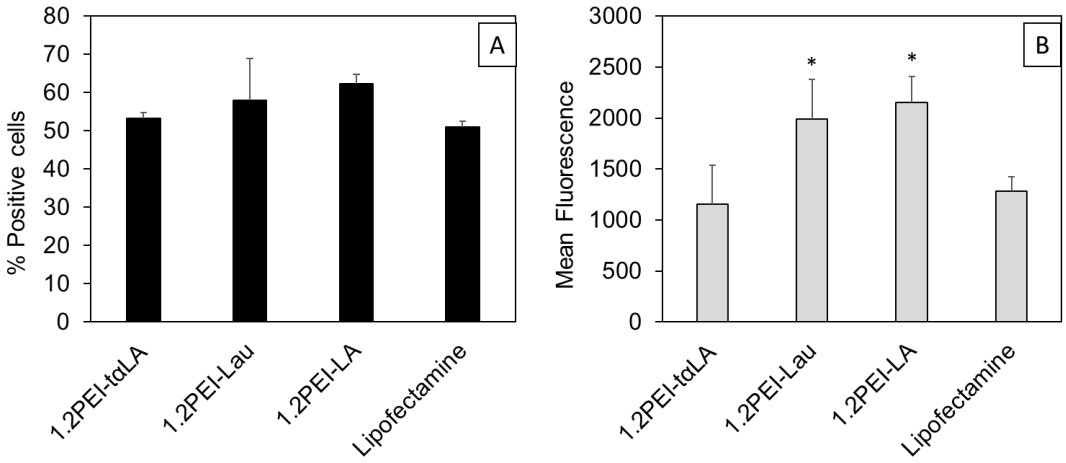
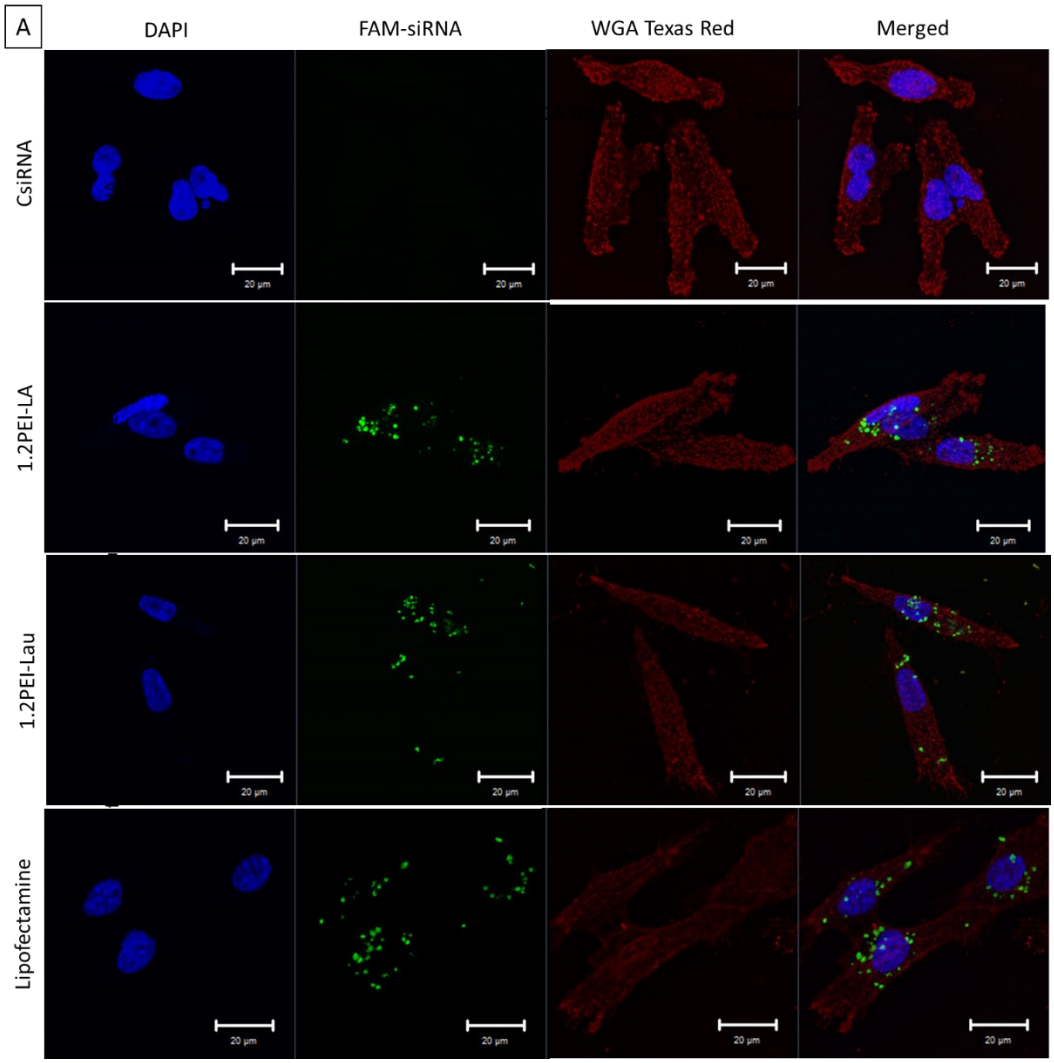


Figure 2.1: Uptake of FAM-labelled siRNA analyzed by flow cytometry. The uptake of FAM-labelled siRNA with 1.2PEI-tαLA, 1.2PEI-Lau, 1.2PEI-LA and Lipofectamine™ 2000 after 24 hours of treatment. (**A**) Displays the percentage of positive cells for FAM-siRNA/polymer complex and (**B**) Displays its corresponding mean fluorescence calculated relative to the no treatment cells (cells without treatment). Significant difference in mean fluorescence compared to 1.2PEI-tαLA, * $p \leq 0.03$ (n=2).

The localization of polymer/siRNA complex inside the cell was analyzed using confocal microscopy. The cytoplasmic localization was higher with 1.2PEI-LA polymer when compared to 1.2PEI-Lau and Lipofectamine™ 2000 ($p \leq 0.0001$) with relatively few complexes in the nucleus (**Fig. 2.2A, B**). The overall internalization was slightly lower with 1.2PEI-Lau but it still exhibited higher cytoplasmic localization than in nucleus unlike

Lipofectamine™ 2000, which exhibited equivalent localization between the cytoplasm and the nucleus. The overall internalization of siRNA delivered with 1.2PEI-Lau and Lipofectamine™ 2000 appeared to be similar. Thus, the higher cytoplasmic localization of 1.2PEI-LA/siRNA complexes was considered beneficial since the site of action of siRNA delivery is cytoplasmic.



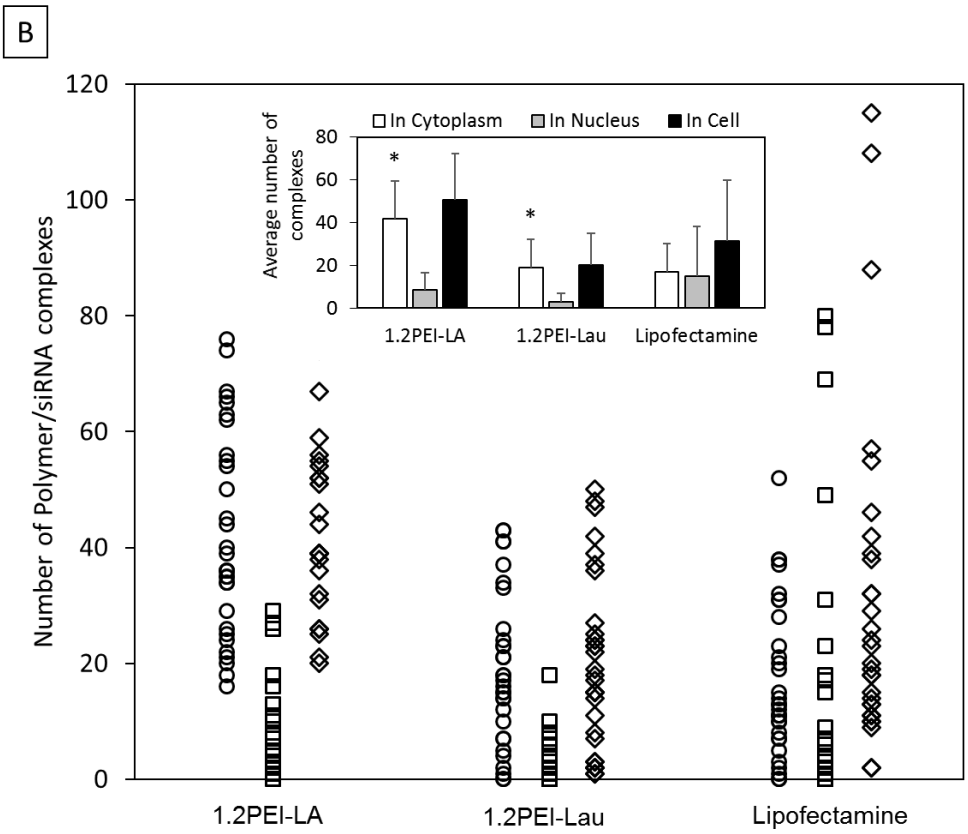


Figure 2.2: Cellular uptake of FAM-labelled siRNA in MDA-MB-231 cells. (**A**) The uptake of FAM-labelled siRNA with 1.2PEI-LA, 1.2PEI-Lau and Lipofectamine^{TM} 2000 after 24 hours of treatment. Representative pictures are shown where the siRNA particles were visualized as green, cell membrane as red and nuclei as blue. Unlabeled CsiRNA was used as a negative control. (**B**) The number of polymer/siRNA complexes in cytoplasm (circle), nucleus (square) and total cell (diamond) was analyzed using Imaris software. The insert represents the average number of polymer/siRNA complexes in each cell (+ SD) with significant difference in cytoplasm compared to the nucleus, * $p \leq 0.0001$ (n=31 for each group). Treatment with unlabeled siRNA (CsiRNA: Control scrambled siRNA) was used as control.

Since optimal silencing depends on siRNA concentration as well as polymer:siRNA ratio used in making the complex formulations, we performed a dose optimization study for reducing cell surface integrin-β1 levels with 2 Dicer-substrate siRNAs (CD29_1 and CD29_2) targeting 2 different regions in the integrin-β1 gene. The siRNA concentrations were 40 and 80 nM, while the complexed were formed at 4:1 and

8:1 weight/weight ratio (polymer to siRNA) (**Fig 2.3A, B, C [i]**). The CD29_2 siRNA was not effective in silencing integrin-β1 with any of the polymers as the integrin-β1 levels remained similar to the CsiRNA treatment. On the other hand, CD29_1 siRNA displayed significant silencing at the lowest ratio of 4:1 with 40 nM siRNA in complexation with all polymers. Further increasing the concentration of siRNA or the polymer:siRNA ratio did not influence the silencing effect. Lowering the siRNA concentration as low as 10 nM was also effective in silencing and with gradual increase in concentration, the effect was maximum between 20 and 40 nM (**Fig 2.3C [ii]**). The lower doses of siRNA are advantageous as it reduces the possibility of off-target effects [30]. Failing to observe any increase in silencing after adding higher doses of siRNA or polymer:siRNA ratio, further studies were conducted at 4:1 polymer:siRNA ratio and with 40 nM of CD29_1 siRNA.

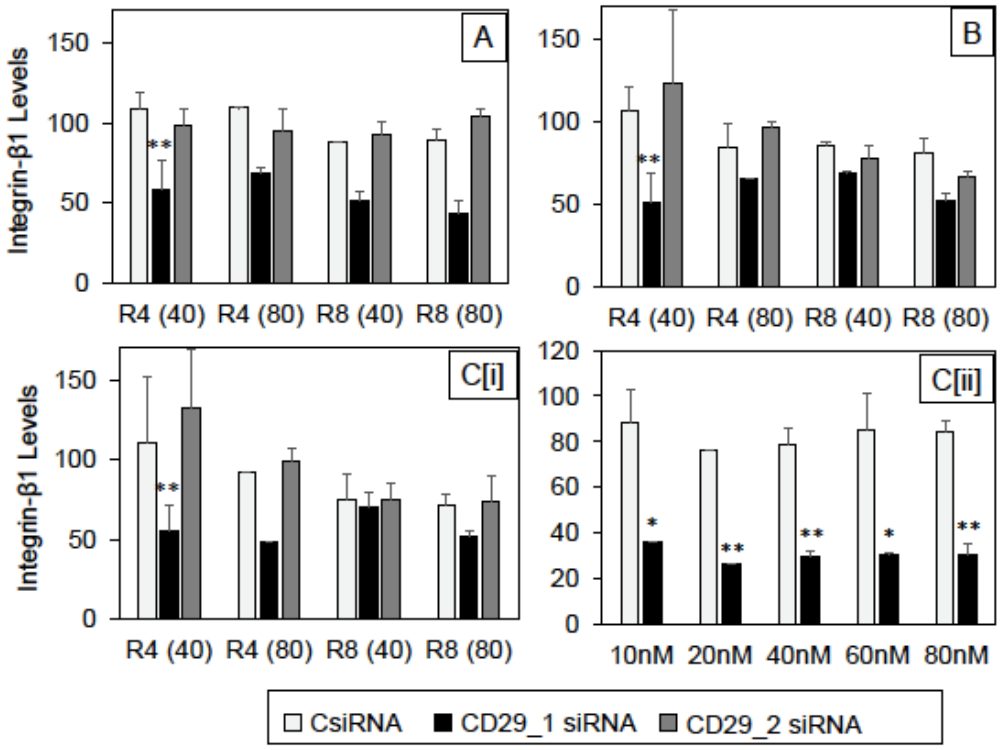


Figure 2.3: Integrin-β1 silencing with siRNA delivery. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment group - cells without any treatment) were measured after 3 days of siRNA treatment for polymers 1.2PEI-tαLA

(**A**), 1.2PEI-Lau (**B**), and 1.2PEI-LA (**C [i]**). The polymer: siRNA (weight/weight) ratio of 4 (R4), and 8 (R8) were used at 40 and 80nM of siRNA concentration with two different siRNAs (designated as CD29_1 and CD29_2 siRNAs). (**C** [**ii**]) Dose response curve for integrin-β1 levels with CD29_1 siRNA delivered with 1.2PEI-LA polymer with increasing siRNA concentrations. CsiRNA: Control scrambled siRNA. Significant difference compared to the respective CsiRNA treatment. * $p \leq 0.05$; ** $p \leq 0.01$ (n=2).

Using direct cell counts (**Fig 2.4**), integrin-β1 siRNA delivery with 1.2PEI-tαLA and 1.2PEI-LA polymers gave significant decrease in cell numbers after 3 days of siRNA treatment. The cell number were drastically reduced for both integrin-β1 siRNA and CsiRNA treated sample of 1.2PEI-Lau, which was indicative of non-specific toxicity of the complexes on the cells.

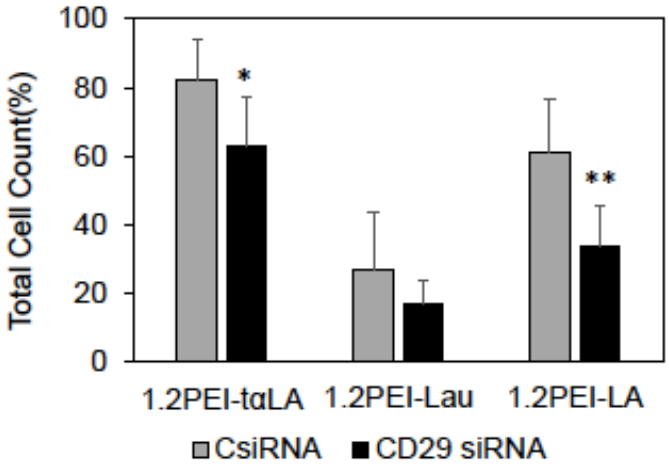


Figure 2.4: Effect of integrin-β1 silencing on cell number. Cell counts were obtained after 3 days of treatment with the 3 polymers at a ratio of 4 and at 40 nM CD29 siRNA concentration (given by percentage of cell count relative to no treatment group – cells without any treatment). CsiRNA: Control scrambled siRNA. * $p \leq 0.05$; ** $p \leq 0.007$ (n=3) compared to CsiRNA treatment for each polymer.

2.3.2 Polymer mediated siRNA delivery sustains the silencing effect.

We examined the duration of silencing over a period of 9 days after treating the MDA-MB-231 cells with polymer/siRNA complexes. All 3 polymers exhibited similar

extent of silencing (40-50% based on mean integrin-β1 levels) on day 3 when compared to the CsiRNA treated cells (**Fig 2.5**). With 1.2PEI-tαLA, the integrin-β1 levels relapsed back to the levels observed for CsiRNA treated samples at day 6 and 9. The 1.2PEI-Lau polymer was able to sustain the silencing until day 6, but the effect was not significant on day 9. The cells treated with 1.2PEI-LA polymer exhibited prolonged and significant silencing from day 3 to day 9. The reference reagent Lipofectamine™ 2000 was also capable of achieving significant reduction in integrin-β1 levels, but the silencing effect was not significant on day 9.

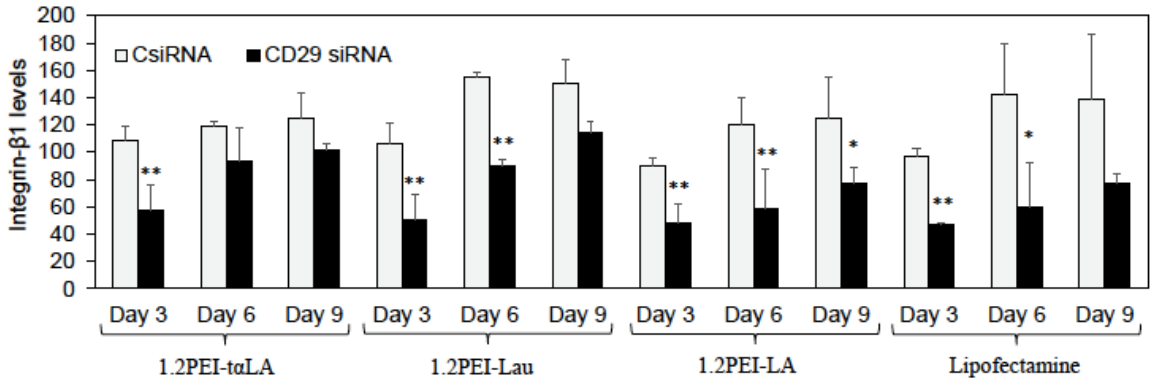


Figure 2.5: Integrin-β1 silencing with siRNA delivery at longer durations. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment group) were measured after 3, 6 and 9 days of siRNA treatment for polymers 1.2PEI-tαLA, 1.2PEI-Lau, 1.2PEI-LA and Lipofectamine™ 2000. A polymer:siRNA (weight/weight) ratio of 4 with 40 nM of siRNA concentration was used. * $p \leq 0.04$; ** $p \leq 0.01$ (n=2). Significant difference compared to the respective CsiRNA treatment.

2.3.3 Polymer mediated siRNA delivery provides strong knockdown of Integrin-β1 mRNA.

The reduction of integrin-β1 mRNA levels was assessed through qRT-PCR after treating the cells with siRNA/polymer complexes for 3 days. All 3 polymers displayed significant reduction in the mRNA levels after day 3, but the knockdown efficacy was

much higher with the 1.2PEI-LA delivered siRNA (~90%). Though all the polymers expressed similar levels of silencing in the flow cytometry analysis on day 3 (**Fig 2.5**), the integrin-β1 mRNA levels were reduced to a different extent (**Fig 2.6A**). The silencing was significantly higher with siRNA delivered with 1.2PEI-LA (90%) than 1.2PEI-tαLA (70%) and 1.2PEI-Lau (40%). It was interesting to note that treatment with 1.2PEI-Lau complexes resulted in elevated integrin-β1 levels with CsiRNA, and this increase could be due to the presence of lauric acid. Lauric acid (at very high concentrations) was shown to regulate the expression of NF-κB in macrophages [31] and in colon epithelial cells [32]. The transcription factor NF-κB binds to integrin-β1 promoter region and could account for our observation in this experiment. We selected 1.2PEI-LA polymer to study the silencing duration; siRNA delivery with this polymer gave strong integrin-β1 knockdown until day 9 (**Fig 2.6B**), which was consistent from day 3 to day 9.

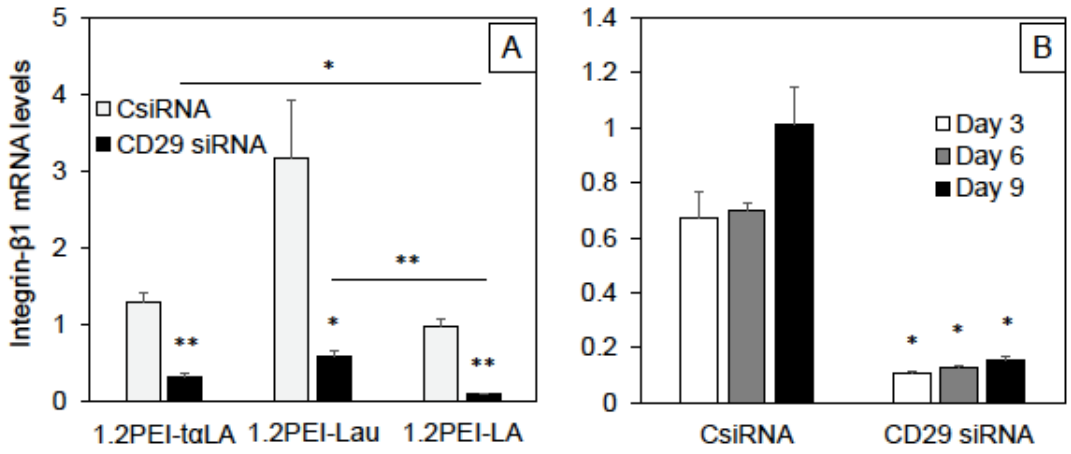


Figure 2.6: Integrin-β1 mRNA levels after siRNA treatment. (**A**) The mRNA levels of integrin-β1 after 3 days of siRNA treatment with the polymers 1.2PEI-tαLA, 1.2PEI-Lau, 1.2PEI-LA were quantified through qRT-PCR (values are plotted relative to no treatment group). (**B**) Integrin-β1 mRNA levels after treatment with 1.2PEI-LA polymer for 3, 6 and 9 days. Significant difference compared to the respective CsiRNA treatment, * $p \leq 0.005$; ** $p \leq 0.0002$. CsiRNA: Control scrambled siRNA.

2.3.4 Integrin-β1 silencing reduces binding of breast cancer cells to fibronectin and hBMSCs

Following successful knockdown of integrin-β1, we checked the functionality of treatment by assessing binding of siRNA treated cells to primary integrin-β1 receptor fibronectin. After treatment with the polymer/siRNA complexes for 3 days, all polymers showed significant reduction in cell binding, consistent with integrin-β1 silencing. The cells treated with 1.2PEI-LA/siRNA complexes, which presented the highest knockdown efficiency among the polymer complexes, showed higher decrease in binding to fibronectin (**Fig 2.7A**). Treatment with increasing siRNA concentrations showed significant and steady decrease in fibronectin binding starting from 10 nM to 40 nM, but additional increase in siRNA concentration (60 and 80 nM) had no further effect on binding (**Fig 2.7B**).

Binding to hBMSCs was additionally explored for siRNA treated MDA-MB-231 cells, where the treated cells were collected and allowed to adhere to a monolayer of hBMSC. Significant reduction in hBMSC binding was observed with 1.2PEI-LA/siRNA complex treatment (~20%) whereas other polymers did not inhibit the binding of treated cells to hBMSCs (**Fig 2.7C**).

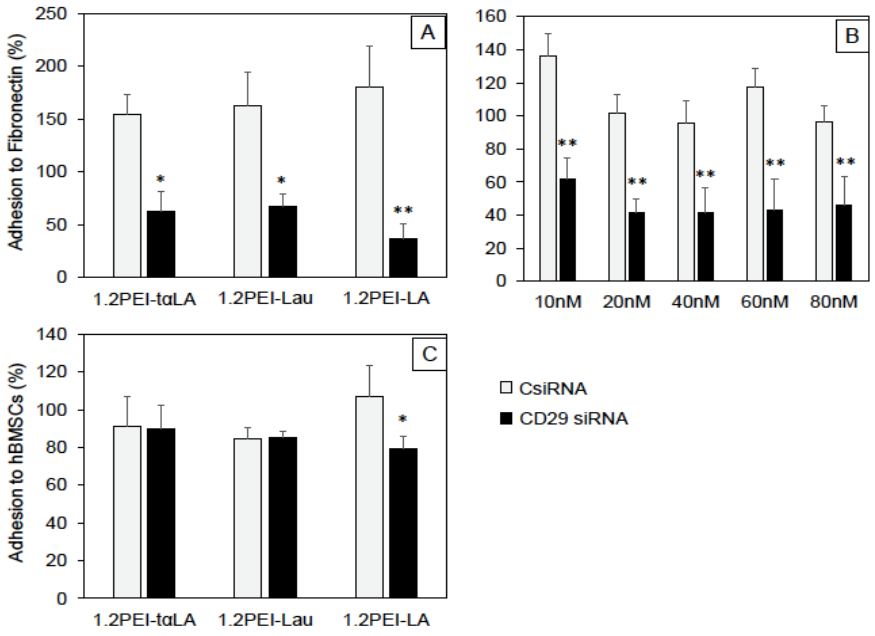


Figure 2.7: Effect of integrin-β1 silencing on cell adhesion. (**A**) Adhesion on fibronectin-coated surfaces: Percentage of MDA-MB-231 cells binding to fibronectin relative to non-treated cells calculated following 3 days of treatment with siRNA using the polymers 1.2PEI-tαLA, 1.2PEI-Lau and 1.2PEI-LA. (n=2). (**B**) Adhesion to fibronectin after treatment with 1.2PEI-LA at different siRNA concentrations. (n=2) and (**C**) Adhesion towards hBMSCs: Percentage of MDA-MB-231 cells binding to human bone marrow stromal cells (hBMSCs) relative to non-treated cells calculated after 3 days of siRNA treatment with all 3 polymers. * $p \leq 0.03$; ** $p \leq 0.001$ (n=5) compared to CsiRNA: Control scrambled siRNA for individual treatment group.

2.3.5 Integrin-β1 silencing inhibits migration of breast cancer cells

The migration of MDA-MB-231 was studied using the well-established 'scratch' assay following silencing of integrin-β1. The percentage of open wound area was calculated using image analysis after allowing the cells to recover for 24 hours. We employed only 1.2PEI-LA polymer to deliver the integrin-β1 siRNA as it showed strongest silencing at the protein and mRNA levels (data from **Figs 2.5, 2.6**). We observed ~60% migration inhibition with the integrin-β1 siRNA delivery as compared to the CsiRNA treated samples (**Fig 2.8A, B [i]**). A small but significant decrease in cell numbers (assessed

by the MTT assay) was noted for integrin-β1 siRNA treated cells (**Fig 2.8B [ii]**), similar to results in Fig 2.4. A dose effect study with different siRNA concentrations (low to high) showed a gradual and steady drop in the migration of cells with increase in the concentration of siRNA (**Fig 2.9 and Fig 2.S2**).

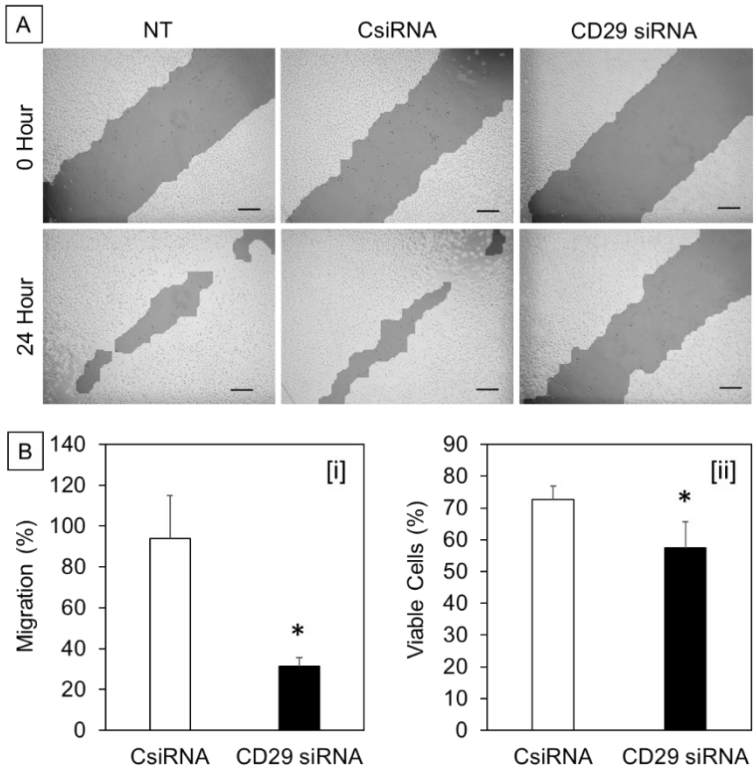


Figure 2.8: Effect of integrin-β1 silencing on cell migration by scratch assay. Scratch assay was performed after 2 days of siRNA treatment with 1.2PEI-LA polymer. (**A**) Shows the representative images of scratches captured at 0 and 24 hours and the inhibition in migration of cells. Scale bar = 200 µm. (**B)** (i) Percentage of migration relative to no treatment (NT) was calculated using TScratch software and (ii) its corresponding cell viability through MTT assay. * $p \leq 0.0003$ (n=3) compared to CsiRNA: Control scrambled siRNA.

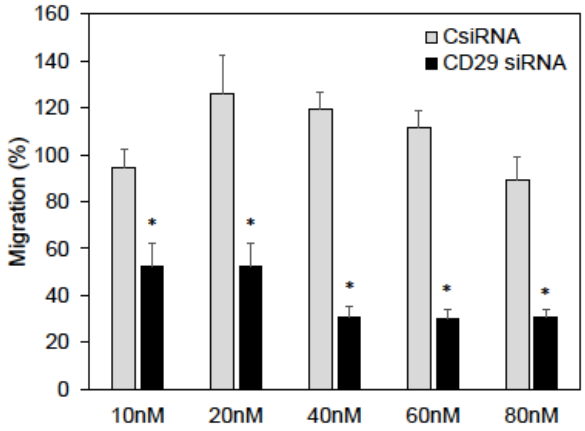


Figure 2.9: Percentage of migration at different siRNA concentrations. Percentage of migration after 3 days of treatment with 1.2PEI-LA polymer at different siRNA concentrations was calculated using TScratch software through 0 hour and 24 hours images from scratch assay relative to no treatment cells. * $p \leq 0.0005$ compared to CsiRNA.

To further validate the scratch assay results, we performed a Transwell migration assay with the same conditions as above. Using crystal violet staining, representative images showed a decline in cells that have migrated to the lower surface of the insert upon integrin-β1 siRNA treatment (**Fig 2.10A**). Upon quantitating the cell numbers, the CsiRNA treated cells in the absence of serum displayed ~26% migration, whereas the CsiRNA treated cells in complete medium (i.e., with 20% serum) exhibited ~85% migration relative to no-treatment (**Fig 2.10B**). The siRNA delivery against integrin-β1 resulted in ~50% reduction in migration, compared to CsiRNA treated cells.

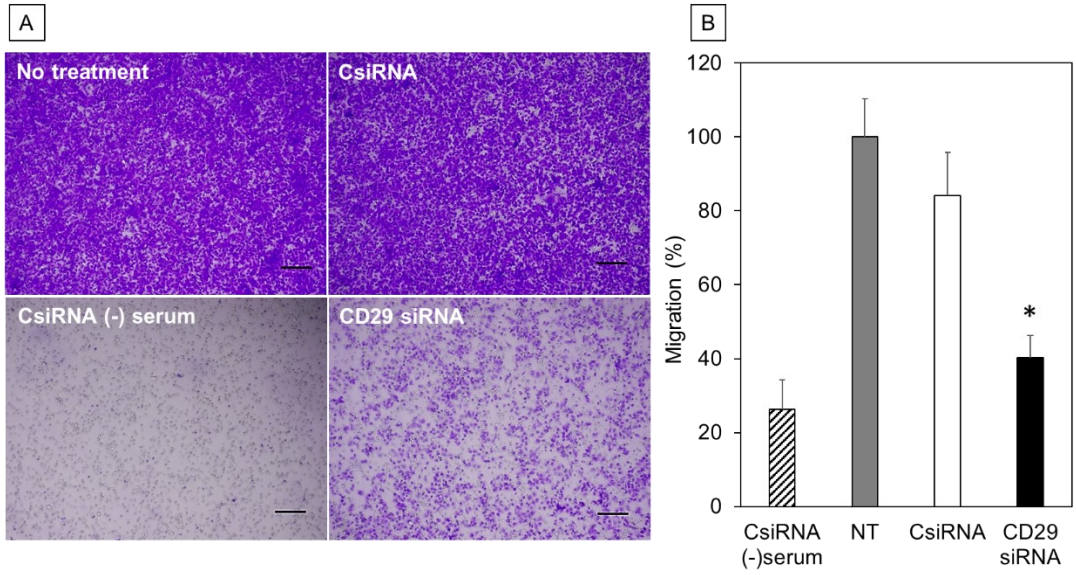


Figure 2.10: Effect of integrin-β1 silencing on cell migration by Transwell migration assay. Transwell migration assay was performed after 24 hours of siRNA treatment with 1.2PEI-LA polymer. (**A**) Representative images of the crystal violet stained cells that have migrated and (**B**) The corresponding absorbance of the migrated cells after solubilizing the crystal violet in 10% acetic acid (absorbance values are relative to no treatment (NT) group). Significance compared to CsiRNA treatment * $p \leq 0.012$. (n=2). Scale bar = 200 µm.

2.4 DISCUSSION

The primary interactions between the cancer cells and their microenvironment, hence their propensity to metastasize and establish distant colonies, are significantly influenced by cell surface integrins [2, 3]. Cellular interactions mediated by integrin-β1 were therefore explored as a potential therapeutic approach to prevent metastasis of breast cancers [33]. Peptides and antibodies were designed to block this receptor-mediated tumor growth and metastatic invasiveness in several xenograft studies [34]. The blocking antibodies was able to inhibit the adhesion of breast cells to human osteoblast cells and also to ECM proteins by targeting integrins β1, α1, α2, and α3 [35, 36]. Antibodies

targeting integrins have advanced to clinical trials but have numerous limitations such as high production costs and possible adverse interaction(s) with immune system, and their efficacy has not been proven. Hence, we envisioned to employ siRNA as a promising approach to target integrin-β1. We have shown for the first time that a simpler approach, based on delivery of integrin-β1 siRNA with lipid-modified PEI polymers, is effective in reducing the migration and adhesion of the breast cancer cells to extracellular proteins. The use of dicer substrate siRNA leads to the direct interaction of the siRNA with the dicer enzyme before incorporating into RISC assembly which helps to increase the silencing effect by employing the natural siRNA silencing pathway [37]. Three different 1.2PEI polymers, each modified with a unique lipid (i.e., 1.2PEI-tαLA, 1.2PEI-Lau and 1.2PEI-LA) were functional in undertaking siRNA delivery against the integrin-β1, although significant differences in their performance and non-specific effects were observed.

We successfully reduced cell surface integrin-β1 levels for relatively long durations (9 days) with a single treatment of siRNA on MDA-MB-231 cells. We could observe the silencing to be much more effective at the mRNA level, which displayed 75-90% silencing up to 9 days. This silencing of integrin-β1 was attained using a relatively low siRNA concentration of 40 nM (in fact as low as 10 nM siRNA was also effective, albeit to a lower extent) and at a polymer to siRNA ratio (4:1) that was lower than other targets utilized in our lab [38]. We have previously reported that hydrophobic lipids on low molecular weight PEI helped with the better interaction of polymers with siRNA as well as DNA in multiple studies, which in turn increased the transfection efficiency when compared to the unmodified PEI [38–40]. An initial library of PEI polymers having different lipid modifications and varying levels of substitution was screened by looking into the surface

integrin-β1; only 3 polymers (1.2PEI-tαLA, 1.2PEI-Lau and 1.2PEI-LA) out of 32 polymers showed promising effect, indicating that the details of polymer design (such as molecular weight of backbone, the nature of lipid substitution and extent of lipid substitution) was crucial in ultimate silencing efficiency. Minimal use of siRNA for cell treatment is crucial as lower siRNA concentration is known to display lower off-target or nonspecific effects [42, 43] and we consider the 40 nM of siRNA used here to be sufficiently low enough to perform significant silencing. The siRNA CD29_1 was effective whereas CD29_2 was not functional as these sequences were selected from a pre-existing library of commercial siRNAs targeting the same gene at different sites. Clearly, the silencing efficiency was dependent on the target site, and it might be possible to further improve the efficacy of integrin-β1 silencing by targeting different regions of the mRNA. The levels of integrin-β1 started to relapse with time, for 1.2PEI-tαLA and 1.2PEI-Lau polymers, but the silencing effect was significant and stable for the 1.2PEI-LA treatment. The presence of linoleic acid on the PEI is shown to improve the interaction of polymer with siRNA/DNA which in turn has helped in better interaction with the cell membrane resulting in higher cellular uptake [39, 41, 44–45]. In addition to this, better cytoplasmic localization as well as higher intracellular release of siRNA could have contributed for the effective silencing. As the aim of our study was to validate the therapeutic potential of integrin-β1, we did not explore the role of different substituents in detail in this work. We note that published work from our lab has previously explored mechanistic insight into the beneficial effect of LA substitution in the context of delivery of siRNAs against other oncotargets [29].

Migration ability of breast cancer cells after silencing integrin-β1 was investigated *in vitro* as a model of metastatic response. The well-established scratch assay was performed after treating the cells with 1.2PEI-LA/siRNA complex, which showed 60% inhibition in the migration of the MDA-MB-231 cells after 24 hours of scratch formation. It was earlier reported that integrins β1 and β4 were critical during the process of tumor formation and also during epithelial to mesenchymal transition in the initials steps of metastasis [47, 48]. The knockout of integrin-β1 using CRISPR/Cas9 reported 50% inhibition in migration through scratch assay in a previous study [13], so that siRNA-mediated approach in this study yields a similar level of efficiency. While CRISPR/Cas9 causes a permanent deletion of the target gene, its high potency is worrisome if the target gene is silenced in other tissues. A transwell migration assay was additionally employed to evaluate the impact of integrin-β1 silencing on migration, which supported the scratch assay results with 50% inhibition, and provided a stronger confirmation of the therapeutic potential of silencing integrin-β1 with specific siRNAs. A cyclic peptide targeting integrin-$\alpha_2\beta_1$ showed a relatively lower ~25% inhibition in wound healing (scratch assay) as well as in migration assays, while its inhibition in tumor growth and bone metastasis *in vivo* was significantly higher [49]. The benefit of siRNA therapy using lipid-modified polymers could be more significant *in vivo*, albeit this issue remains unexplored in the current study.

As the colonization of secondary tumor is initiated with the attachment of cancer to distant organs, we checked the ability of integrin-β1 silenced MDA-MB-231 cells to adhere to fibronectin for which integrin-β1 is a primary receptor. The knockdown of the cell surface integrin-β1 using 1.2PEI-LA led to 65% inhibition in the binding of MDA-MB-231 cells to fibronectin, while the other polymers displayed lower (~40%) inhibition of

fibronectin binding. As low as 10 nM siRNA treatment was sufficient to influence the fibronectin binding. It is likely that other receptors might have participated in fibronectin binding, so that complete inhibition could not be achieved solely by the integrin-β1 siRNA. We further examined the adhesion ability by allowing the treated breast cancer cells to interact directly with hBMSC, which express a wide range of adhesion protein on cell surface and aid in tumor cell interactions leading to the adhesion of cells and colonization [50]. Only the most effective polymer (1.2PEI-LA) treatment displayed significant inhibition of binding. However, this reduction in binding to BMSC was relatively low (~20%). Such a difference in the adhesion studies between fibronectin and hBMSCs could be attributed to the involvement of other adhesion molecules including other members of the integrin family of proteins. A recent study (retroviral transduction of targeting integrin-β1) with MDA-MB-231 cells also reported similar levels of binding inhibition towards human osteoblasts derived matrices. Our study indicates the possibility of achieving similar functional outcomes by targeting integrin-β1, but without the need for viral carriers [23]. Thus, silencing of integrin-β1 solely in the metastatic MDA-MB-231 cells could be more beneficial to reduce cellular migration rather than adhesion of cells to other bone-marrow resident cells.

Treating the cells with AIIB2 antibody led to a drastic decrease in the total cell number along with decreased proliferation and increased apoptosis without affecting non-malignant cells [50, 51]. A combinational treatment of AIIB2 along with ionizing radiation enhanced the apoptosis in cells [33]. Earlier reports on the inhibition of paclitaxel-induced apoptosis due to the presence of integrin-β1 explains its critical roles on cell viability [50]. In this study, we also evaluated the effect of integrin-β1 silencing on the cell numbers; we

observed a small but significant effect when compared to CsiRNA treated cells. The induction of apoptosis (by caspase assay) was also not evident in our hands (**Fig 2.S3**). Previous studies with antibodies and peptides intended to block integrin-β1 have reported apoptosis in breast cancer cells [19, 50], but one other approach using integrin-β1 lentiviral shRNA had no significant difference in apoptosis when compared to control treatment [22]. Similarly, the CRISPR/Cas9 approach to study the role of integrin-β1 did not report its involvement in apoptosis; rather, a strong dependency in the cell growth at lower cell density and suppression of cell growth at higher cell density was observed [13]. Such contrasting observations could be explained by the different approaches used for targeting integrin-β1 and different cell types used in the studies. Perhaps the model of inhibition, i.e., post-transcriptional vs. post-translational, rather than the extent of integrin-β1 levels on cell surfaces, might be a causative factor for apoptotic response. This issue needs to be clarified in future studies.

The downstream integrin-β1 signaling via phosphatidylinositol 3'-kinase (PI3K) and serine/threonine kinase AKT provides resistance towards drug-induced apoptosis and is a key survival pathway in drug resistant breast cancer [53]. Integrin-β1 adhesion to ECM proteins upregulated FAK, ERK, p38 MAPK and JNK expression in MDA-MB-231 cells, which also induced expression of matrix metalloproteinase-13 [36]. The knockout of integrin-β1 comprehensively decreased the expression levels of FAK and AKT in MDA-MB-231 cells, affected cell migration, and revealed a negative effect on the expression of epidermal growth factor receptor (EGFR), suggesting an inverse relationship between integrin-β1 and EGFR [13]. It is likely that siRNA-mediated integrin-β1 silencing in MDA-MB-231 cells also down-regulated FAK, PI3K or AKT in this study.

The importance of integrin-β1 during the EMT, angiogenesis, migration, proliferation, and re-attachment at distant sites has been explored in various studies. As the process of metastasis starts with EMT followed by migration and later re-attachment, our study that demonstrated reduced cell attachment, might curtail initial propensity to migrate along with reduced adhesion towards fibronectin and hBMSC could be a promising approach. During the execution of these studies, we also focused on the possibility of cell detachment from the tissue culture plates; we did not observe any significant difference in the number of floating cells in wells between the CsiRNA and integrin-β1 siRNA treated cells (**Fig 2.S4**), so that silencing integrin-β1 does not seem to alter the initial adhesiveness once the cells are attached to a substrate. The siRNA-treated cells in circulation can consequently fail to adhere to fibronectin/hBMSC and might pose less threat to form a secondary tumor, as they have lost the ability to bind to target sites as a result of integrin-β1 silencing. It appears that the possibility of increased metastasis from the primary site (due to inhibition of ECM binding after siRNA silencing) should not be a concern with our approach. This was also not the case in antibody and peptide inhibition studies involving integrin-β1, where no incidence of increased metastasis was observed as the *in vivo* studies with these systems showed reduced metastasis to various organs and a decrease in the tumor volume which was also reported by retroviral-shRNA and CRISPR/Cas9 knockout studies [13, 14, 15, 50].

Thus, for the first time, we have shown that therapeutic silencing of integrin-β1 was possible by using linoleic acid modified PEI polymers and using dicer-substrate siRNA delivery. Such an approach reduces the migration of metastatic breast cancer to a large extent, in addition to significant inhibition of cell adhesion to fibronectin and human bone

marrow stromal cells. Little effect on cell viability and cell numbers were observed after integrin-β1 silencing. Thus, silencing of a single integrin subunit can have multiple inhibitory effects on the breast cancer cells and careful selection of addition integrin subunits could be more beneficial to inhibit its attachment to different metastatic sites.

2.5 ACKNOWLEDGMENTS

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3. LIPID MODIFIED POLYETHYLENEIMINE IS AN EFFECTIVE SYSTEM FOR HSP90B1 SILENCING TO TREAT BREAST CANCER

3.1 INTRODUCTION

Breast cancer is one of the four commonly diagnosed cancer types along with lung, colorectal and prostate cancers, according to the most recent *Canadian Cancer Statistics 2020*. It accounts for 25% of new cancer cases and 13% of cancer deaths among Canadian women [1]. The metastatic ability of breast cancer to bone, lung, liver, brain, and lymph nodes makes its treatment challenging, even though the diagnosis and therapy has improved significantly in recent years, extending the survival of the patients [2]. Specifically, the treatment of the more aggressive and highly metastatic, triple-negative breast cancer (estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 negative) remains a formidable task and this necessitates the identification of novel targets for a better outcome. In addition, triple-negative breast cancer (TNBC) which is represented by 10-15% of all breast cancer cases, exhibits high heterogeneity which also acts as stumbling blocks for its successful treatment [3].

The process of breast metastasis is a complex, multistep cascade which depends on the ability of cancer cells to abandon the primary site, navigate through the circulation while evading the immune system, to reach a distant site. The cells navigate and survive through various distant organs and the bone environment and subsequent re-emergence after establishment [4]. The treatment of metastatic breast cancer is most challenging that is evident by the poor 5-year survival rate of 22% (according to Canadian Cancer Society). In this intricate process, numerous genes are activated, contributing to each step of metastasis, especially the cell adhesion molecules [5]. Some of these cell adhesion molecules include cadherins (CDH) which are vital in the interaction and signaling between the cells, especially in breast cancer the impaired expression of E-cadherins were

associated with enhanced invasiveness and decreased survival of breast cancer patients [6]. In addition, protocadherins (PCDH) which are the largest subfamily of cadherins are shown to play diverse role in breast cancer metastasis. For example, overexpression of PCDH-7 was observed in breast cancer bone metastasis, whereas PCDH-17 was identified as a tumor suppressor gene through the reduction of β-catenin levels, which also highlights the crucial role of catenins in breast cancer invasiveness through its association with cadherins [7–9]. Another major type of cell adhesion molecule involved in breast cancer metastasis are integrins. Numerous integrins have been identified to be upregulated during the metastatic process by the activation of various intracellular signaling pathways. For example, the expression of integrins α5β1, α6β4 and αvβ3 displayed strong correlation with the metastatic progression of breast cancer [10]. The expression of various cytoskeleton components like actin, dynein, tubulin and laminin have been additionally shown to contribute in the complex metastatic process and even provide resistance to chemotherapy [11]. Finally, other intrinsic intracellular targets like the heat shock proteins (HSP) that govern the proper protein folding, the apoptosis regulators like BCL2 family and survivin, and cell cycle regulatory protein like cell-division cycle protein 20 (CDC20) help in the survival, proliferation, and anti-apoptotic property of breast cancer cells. Hence the successful downregulation of such metastasis-associated genes, either alone or in combination, will have strong beneficial outcome in addressing breast cancer progression and metastasis.

One of the most promising approaches to interfere with the functioning of the oncogenes is the use of short interfering ribonucleic acids (siRNA) to silence there *in situ* expression. Since the discovery of siRNA 2 decades ago, it is now possible to safely silence

the gene of interest without affecting other targets, which is also dependent on its sequence specificity as well as the type of delivery system used [12,13]. For nucleic acid delivery, non-viral systems have been considered as a safe alternative to viral methods [14], however they suffer from poor delivery efficiency. Especially with the polyethylenimine (PEI) systems, low molecular weight PEIs (< 5 kDa) have shown to be less toxic but also exhibit low delivery efficiency of siRNA, hence lipid modifications have been employed to increase its capacity [15]. We have successfully designed such delivery systems in our research group and demonstrated its therapeutic usefulness in a wide range of cancer types [26–30].

Here, we used a linoleic acid substituted PEI polymer to deliver siRNA in the metastatic breast cancer cell line, MDA-MB-231. We screened a siRNA library targeting various cell surface and cytoskeleton genes to identify new targets that could inhibit the growth and migration of breast cancer cells. Out of the 496 genes silenced from the siRNA library using our delivery system, we observed that knockdown of HSP90B1 was able to inhibit cell growth effectively and decrease in migration was observed by the silencing of ITGA4, ITGA6 and ITGAX genes. We further investigated the promise of the identified targets for which dicer-substrate siRNAs were used with HA as additives in the complex preparation process. Different weight ratios (siRNA to PEI-LA to HA) were explored to obtain the optimal condition for effective silencing, and the weight ratio of 1:5:0.15 was the most successful in this study with HSP90B1 reduction. Given the critical role of HSP90B1 in metastasis to brain, which has also been reported by different research groups, this approach could be a valuable alternative treatment as we were able to show a significant reduction in the cell growth as well the migration of the breast cancer cells. We

achieved strong silencing of HSP90B1 with a low concentration and single dose of siRNA which was adequate to exhibit significant functional outcomes. Given the important role of integrin-β1 (ITGB1) in the migration of breast cancer cells from our previous studies, the combinational silencing of integrin-β1 (ITGB1) along with HSP90B1 could be more advantageous to effectively reduce the migration as well as cell growth [20]. Hence, we were able to show a promising alternative to address breast cancer metastasis with a more simple and feasible approach.

3.2 MATERIALS AND METHODS

3.2.1 Materials

1.2 kDa Polyethyleneimine (PEI) purchased from Polysciences (Warrington, Pennsylvania) was used for linoleic acid (LA) substitution through *N-acylation* exhibiting 6 LA per PEI, as assessed by ^{1}H-NMR spectroscopy. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anhydrous dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), chloroform and hyaluronic acid (HA) 300 kDa were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), trypsin/EDTA, penicillin, streptomycin, UltraPure DNase/RNase-free dH_2O, coomassie brilliant blue and TRIzol were procured from Fisher Scientific (Ottawa, Canada).

3.2.2 Cell models

Human metastatic breast cancer cell lines, MDA-MB-231-WT and MDA-MB-231-GFP cells were obtained from Dr. Judith Hugh (Faculty of Medicine and Dentistry, University

of Alberta, Edmonton). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin until they covered 80% of the culture flask. Subsequently, cells were washed with HBSS and incubated with 0.05% Trypsin/EDTA for 2 min at 37°C. Cells were collected by centrifugation at 600 rpm for 5 min and allowed to culture for 24 hours before its utilization for studies.

3.2.3 Polymer/siRNA complex preparation

Serum free DMEM was used to prepare complexes at siRNA to 1.2PEI-LA6 polymer weight/weight ratio of 1:5 for siRNA library studies and incubated for 30 min at room temperature. For the preparation of additive complexes using HA, siRNA was mixed with HA in serum free DMEM, which was followed by the addition of polymer and incubated as mentioned above. These complexes were prepared at various siRNA to polymer to HA weight ratios, using 30 nM and 60nM of siRNA concentrations. In combination studies, different siRNAs were mixed, prior to HA and polymer addition.

3.2.4 Human cell adhesion and cytoskeleton siRNA library screening

Screening of the siRNA library targeting 496 genes of cell adhesion and cytoskeleton (MISSION siRNA Human Cell Adhesion and Cytoskeleton Panel; SI05100, Sigma-Aldrich) was performed in MDA-MB-231-GFP cells. Briefly, the cells were dispensed into 96-well plates using a Perkin Elmer Automated Workstation and allowed to grow for 24 hours. 18 ul of the siRNA for a final concentration of 50 nM was added to a new 96-well plate followed by 18 ul of 1.2PEI-LA6 polymer solution at a weight ratio of 1:5. Each treatment group had three replicates, with each well containing 10ul of the prepared

complex solution. After 3 days of treatment at 37°C, the suspended/dead cells in the media were removed and to the attached cell, MTT solution was added, and cell viability was calculated relative to no-treatment group. Each plate contained a negative control siRNA from the library, a dicer substrate control siRNA (DsiRNA), and a positive control BCL2L12 siRNA. A similar experimental setup was carried out to study the migration of the cells following the siRNA library treatment. After 2 days of treatment, the monolayer of cells was scratched using a 200ul pipette tip and the cells were washed with complete DMEM to remove the detached cells in the media. Further, the cells were allowed to migrate for 24 hours after which they were stained with coomassie brilliant blue, fixed and scanned. The migration (wound closure percentage) of the cells were determined based on the open wound area and was compared to the no-treatment well. For this study, CDC20 siRNA and ITGB1 siRNA were included as additional positive controls and doxorubicin (5ug/ml) was included in both the siRNA library screening studies due to its ability to inhibit cell growth and migration.

3.2.5 Validation of targets

The identified targets, ITGA4, ITGA6, ITGAX and HSP90B1 from the siRNA library were further validated for cell growth and migration inhibition, for which dicer substrate siRNA sequences were procured from IDT (Coralville, USA). The following combination of siRNA: polymer: HA weight/weight ratios at 30 nM and 60 nM siRNA were used in this study along with the negative control siRNA (CsiRNA), CDC20 siRNA, BCL2L12 siRNA and ITGB1 siRNA as positive controls.

	Polymer			
siRNA	**5**	**7.5**	**10**	**HA**
1	1:5:0	1:7.5:0	1:10:0	**0**
	1:5:0.15	1:7.5:0.15	1:10:0.15	**0.15**
	1:5:0.3	1:7.5:0.3	1:10:0.3	**0.3**
	1:5:1	1:7.5:1	1:10:1	**1**
	1:5:3	1:7.5:3	1:10:3	**3**

Cell viability of MDAMB-231-WT was assessed after 3 days of complex treatment using MTT assay with a final concentration of 1 mg/ml and 2 hours incubation at 37°C. The inhibition in migration was also studied by calculating the wound closure percentage using scratch assay for which images (0 hour) were acquired after making scratches following 2 days of treatment. Images were again taken after 24 and 48 hours of scratch formation and the open wound area was calculated using T-scratch software. Migration was calculated using the following:

$$\text{Migration \%} = [(A_{t=0h} - A_{t=\Delta h})/A_{t=0h}] \times 100\%$$

Where, $A_{t=0h}$ is the measurement of wound at 0 hours (immediately after scratch formation) and $A_{t=\Delta h}$ is the measurement of wound at 24 or 48 hours after scratching. In both these studies, doxorubicin (0.5ug/ml) was used as an additional positive control.

3.2.6 Quantification of mRNA Levels

The silencing of the target genes at the mRNA level was evaluated by reverse transcription – quantitative real time polymerase chain reaction (RT-qPCR). Following 3 days of treatment with HA additive complexes at ratios 1:5:0, 1:5:0.15, 1:5:0.3 in 6-well plates,

MDA-MB-231-WT cells were collected, and the total RNA was extracted using TRIzol. SensiFast™ cDNA synthesis kit (Bioline Meridian Bioscience) was utilized to convert RNA to cDNA according to the manufacturer's protocol. Following which, PCR was performed on StepOnePlus™ Real-Time PCR system with the below mentioned genes and their respective primers:

Gene	Forward	Reverse
B-Actin	5'-GCGAGAAGATGACCCAGAT-3'	5'-CCAGTGGTACGGCCAGA-3'
HSP90B1	5'-CAGTTTGGTGTCGGTTTCTATTC-3'	5'-TGTGCTGGGTATCGTTGTT-3'
ITGB1	5′-CCGCGCGGAAAAGATGAAT-3′	5′-TGAGCAAACACACAGCAAACT-3′
CDC20	5'-CGCTATATCCCCCATCGCAG-3'	5'-GATGTTCCTTCTTGGTGGGC-3'
BCL2L12	5'-CCCGCCCCTATGCCCTTTTT-3'	5'-ATAACCGGCCCAGCGTAGAA-3'

Experiment was performed in triplicates with a total of 10 µl reaction mixture volume, of which 5 µl constituted the SYBR Green master mix, 2 µl of 10 µM primers with 3 µl of 5 ng/µl cDNA template. MicroAmp Fast Optical 96-well plates were used for this assay and the setup was heated to 95°C for 10 min before progressing into 40 cycles of denaturation at 95°C for 15s and an annealing/elongation at 65°C for 30s. Following the completion of the cycles, the ΔC_T, $\Delta\Delta C_T$ and Relative Quantity (RQ) of mRNA were calculated with B-actin as the reference gene and relative to NT-no treatment group. The expression of ITGB1, CDC20 and BCL2L12 was also calculated following HSP90B1 silencing.

3.2.7 Combination therapy

Combinational siRNA therapy using HSP90B1 siRNA and ITGB1 siRNA were performed in MDA-MB-231 cells at four siRNA concentrations (total concentration), (10+10) 20 nM, (20+20) 40 nM, (30+30) 60 nM and (40+40) 80 nM. Each of these targets were treated in

combination with CsiRNA as controls. The effect of silencing was evaluated at mRNA level, inhibition in wound closure and cell viability by RT-qPCR, scratch assay and MTT respectively, as described above.

3.2.8 Statistical analysis

All results were summarized as mean ± standard deviation and the unpaired Student's *t*-test was employed to show the statistical difference between the group mean. Significance (p-value < 0.05) was determined in comparison with the respective control groups. Where specified, the number of independent experiments used to generate the data (n) are indicated.

3.3 RESULTS

3.3.1 siRNA library screening to identify targets for breast cancer treatment

A siRNA library targeting various cell adhesion and cytoskeleton genes (496 siRNAs) was screened in the TNBC cell line MDA-MB-231 to identify targets that could inhibit cell growth. 1.2PEI-LA6 polymer was used to deliver the siRNAs at a concentration of 50 nM and cell growth was assessed by the MTT assay after 3 days. A siRNA against BCL2L12, which was previously reported to exhibit significant cell growth inhibition, was included as a positive control [21]. BCL2L12 in this screen showed 28% of growth inhibition and this was considered as cutoff to shortlist successful hits from the 496 targets. We could observe 2 targets that exhibited ≥28% cell death, cadherin-9 (CDH9) at 34% and heat shock protein 90B1 (HSP90B1) at 29% (**Fig 3.1A**).

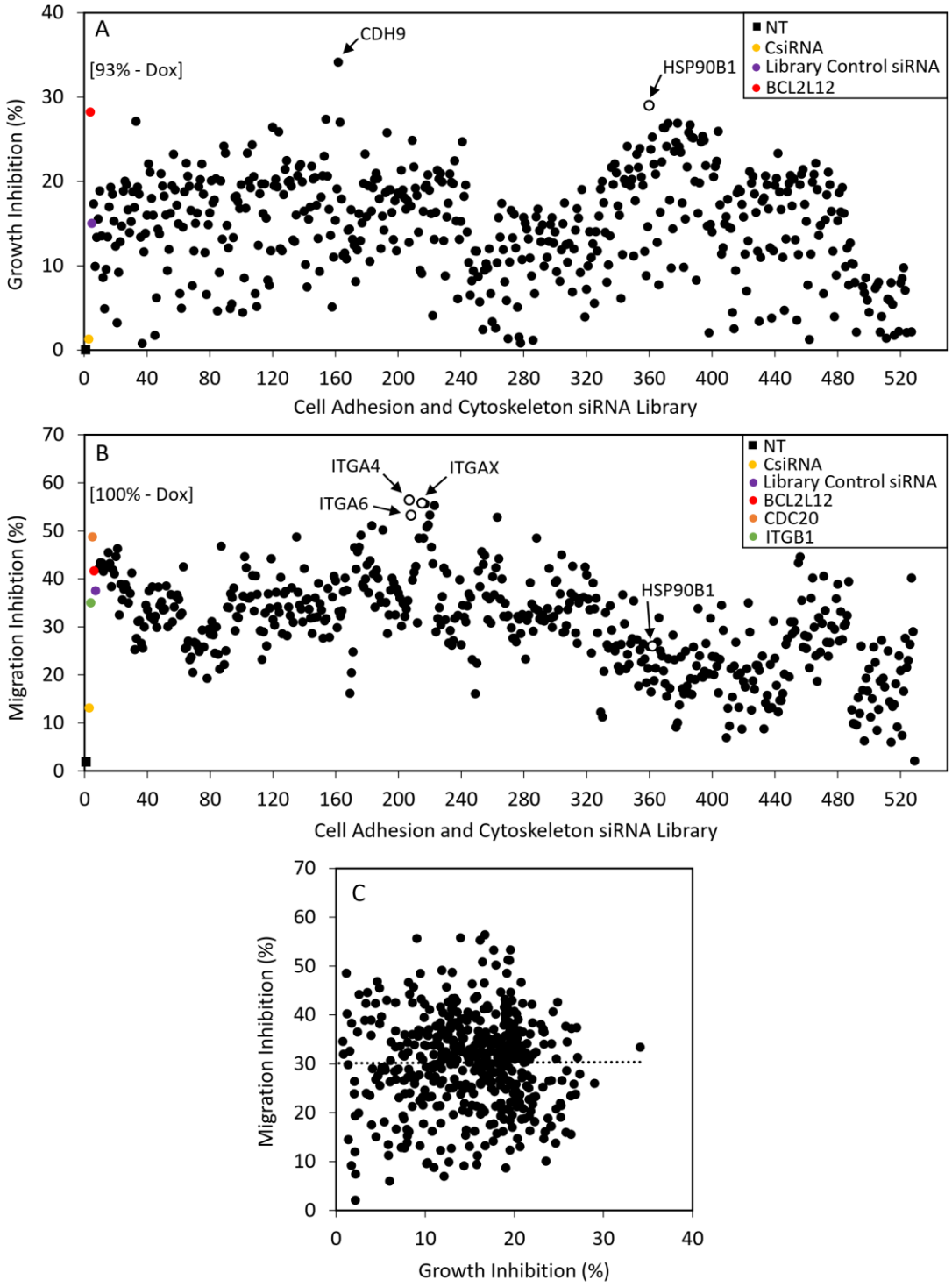

Figure 3.1: Cell adhesion and cytoskeleton siRNA library screening in MDA-MB-231 cells. (**A**) Growth inhibition (%) evaluated by MTT assay following 3 days of siRNA library treatment calculated relative to no-treatment cells. Solid black square represents no treatment (NT), yellow dot represents control scrambled siRNA (CsiRNA), purple

represents library control siRNA and red represents BCL2L12. Doxorubicin (Dox) treatment gave 93% cell growth inhibition. (**B**) Migration Inhibition (%) by siRNA library treatment for 24 hours; calculated relative to no-treatment cells. A CDC20 siRNA, represented by orange dot and an ITGB1 siRNA represented by green dot were also included. Dox treatment gave 100% inhibition of cell migration. (**C**) Correlation between growth inhibition (%) and migration inhibition (%) are shown.

A migration assay was also performed using the same library to identify targets that can inhibit cell migration into a 'wound' area. In this study, we included 2 positive control siRNAs, namely ITGB1 and CDC20 specific siRNAs, that were previously shown to decrease the migration in breast cancer cells [20,22]. Three integrin genes appeared to inhibit the migration effectively, integrin-a_4 (ITGA4), integrin-a_6 (ITGA6) and integrin- a_X (ITGAX), with migration inhibition percentages of ~56% (**Fig 3.1B**). HSP90B1 which showed 30% inhibition in cell growth also showed ~30% inhibition in migration studies (**Fig 3.S1**). We also explored any correlation between the cell growth inhibition and migration inhibition in this assay format, but we did not observe a positive correlation among the siRNAs in this library (**Fig 3.1C**).

3.3.2 Validation of targets for inhibition of cell growth

The identified targets ITGA4, ITGA6, ITGAX and HSP90B1 were further validated by the delivery of specific siRNAs at different ratios and siRNA concentrations of 30 nM and 60 nM. The previously used positive controls ITGB1, CDC20 and BCL2L12 siRNAs were also included and the inhibition in cell growth was assessed by the MTT assay after 3 days of treatment. Silencing of HSP90B1, significantly and consistently reduced the cell growth; formulations at siRNA:polymer ratio 1:5 with 3 additive ratios gave 55%, 67% and at 87% cell viability at 30 nM siRNA concentration. The other identified targets for migration

inhibition had lesser effect on cell growth as expected (ITGA4: 20% and ITGAX: 17% cell growth inhibition). At 60 nM, the outcome was more pronounced at similar ratios as observed with 30 nM, although the control scrambled siRNA (CsiRNA) also showed some non-specific activity (toxicity). Altogether, we recorded 40%, 45% and 56% cell growth with the 3 additive ratios gave (**Fig 3.2A**). With respect to nanoparticle formulations at 1:7.5 siRNA:polymer ratio, the overall effect was less in comparison to 1:5 ratio, however HSP90B1 silencing still resulted in significant drop in cell viability (**Fig 3.2B**), whereas with 1:10 no significant growth inhibition was documented (**Fig 3.2C**).

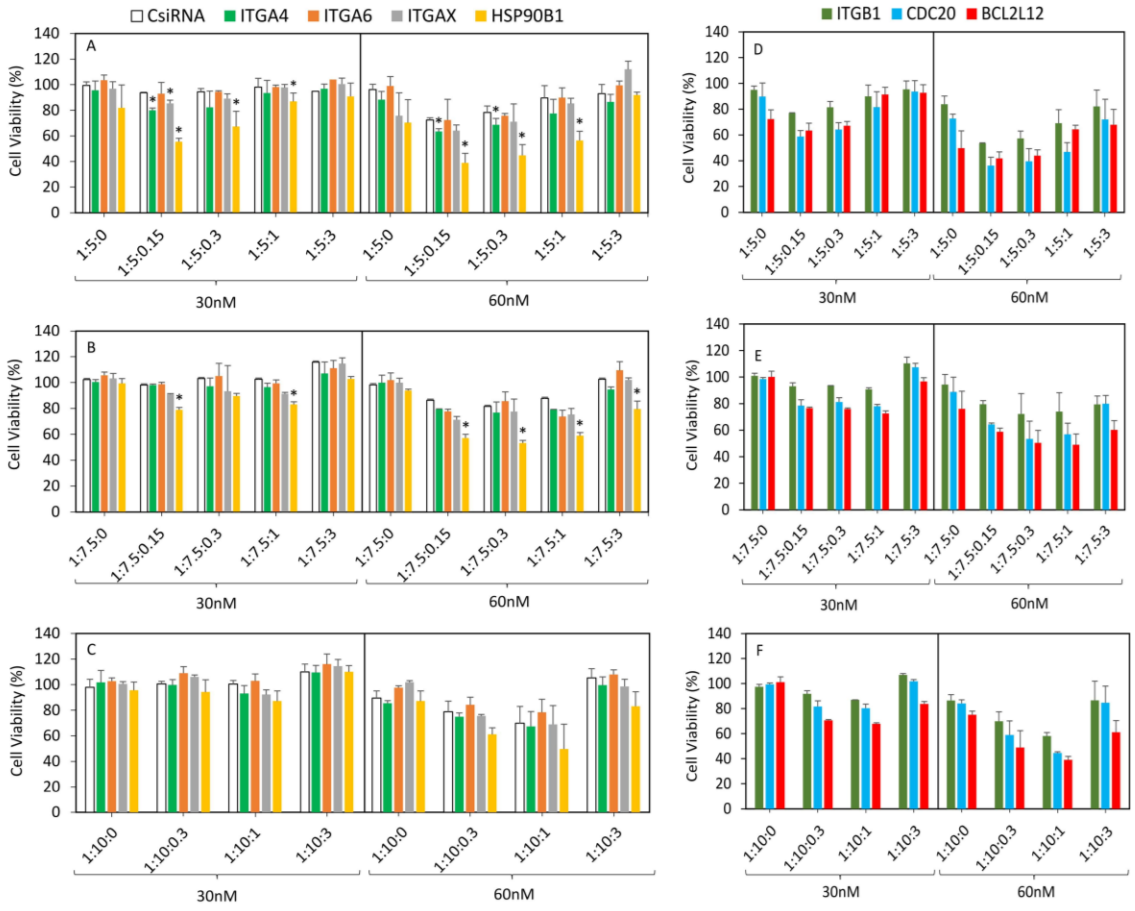

Figure 3.2: Validation of targets by the MTT assay after 3 days of treatment with (**A** & **D**) 1:5 siRNA:polymer complexes having 1:0.15, 1:0.3, 1:1 and 1:3 ratios of siRNA:HA w/w ratio. (**B** & **E**) 1:7.5 siRNA/polymer complexes having 1:0.15, 1:0.3, 1:1 and 1:3 ratios of

siRNA:HA w/w ratio. (**C** & **F**) 1:10 siRNA/polymer complexes having 1:0.3, 1:1 and 1:3 siRNA:HA w/w ratio. Complexes were prepared with 30 nM and 60 nM of siRNA concentration. Significance compared to CsiRNA treatment in each group * $p \leq 0.05$. The results are summarized from 2 independent experiments.

3.3.3 Validation of targets for inhibition of migration

Inhibition of migration was investigated using nanoparticle formulations with 1:5 siRNA:polymer ratio and 60 nM siRNA concentration, as this formulation showed a better effect in inhibition of growth. After 24 hours, the control siRNA treatment led to 45% of migration, while ITGA4 and HSP90B1 siRNAs showed significant inhibition with migration values of 28% and 20% respectively, at 1:5:0.15 formulation. Other targets, ITGA6 and ITGAX did not manifest any notable reduction irrespective of the ratios employed (**Fig 3.3A**). The previously observed reduction at 24 hours with HSP90B1 silencing at 1:5:0.15 was prolonged until the 48-hour time point (**Fig 3.S2**). Overall, HSP90B1 showed a significant reduction in migration (43%) when compared to CsiRNA (88%) after 48 hours (**Fig 3.3B**). Similar to the cell viability studies, low amount of HA (siRNA:HA ratio of 1:0.15) appeared to be the optimal formulation for reducing the migration (**Fig 3.S2**). Using higher amounts of HA abolished the inhibitory effects of the specific siRNA on migration. With regards to the positive controls, CDC20 siRNA was significantly effective with 1:5:0.15 weight ratio at 24 hours. At 48 hours, the 1:5:1 was the most effective for all the positive controls, ITGB1, CDC20 and BCL2L12 siRNAs.

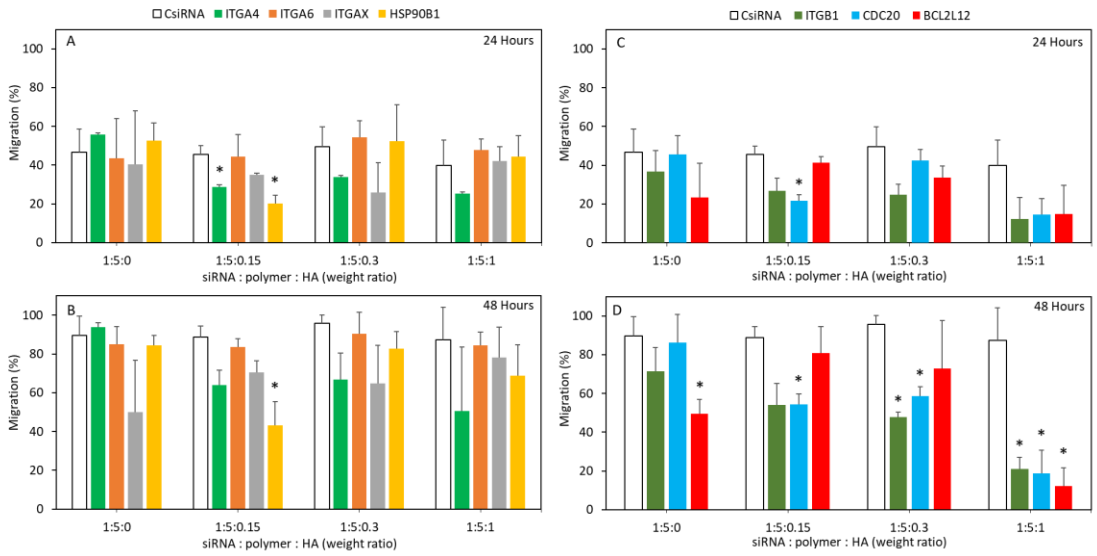

Figure 3.3: Validation of targets by scratch assay after 2 days of treatment followed by wound formation with images taken at 0, 24 and 48 hours. Cells were treated with 60 nM of siRNA at 1:5 siRNA/ polymer complexes having 1:0.15, 1:0.3 and 1:1 ratios of siRNA:HA w/w ratio. Migration (as of percentage of no treatment) was determined at 24 hours (**A** & **C**) and at 48 hours (**B** & **D**) after siRNA treatment. Significant difference compared to the CsiRNA in each treatment group * $p \leq 0.05$ (n=2).

3.3.4 Validation at the mRNA transcript level by RT-qPCR

We further validated the extent of silencing by evaluating the specific mRNA reduction using RT-qPCR after 3 days of treatment. A strong decrease in HSP90B1 mRNA levels, ranging between 80% and 85%, was recorded with ratios 1:5:0, 1:5:0.15 and 1:5:0.3 in comparison to the CsiRNA treatment group, which was consistent with our previous observation with cell viability and wound closure (**Fig 3.4A**). We also, analyzed the mRNA levels of ITGB1, CDC20 and BCL2L12 after treating with HSP90B1 siRNA. A significant decrease between 60% and 80% was observed in ITGB1 and CDC20 mRNA levels respectively, which could indicate a possible linkage with HSP90B1 (**Fig 3.4B**). CsiRNA treatment did not affect the mRNA levels of ITGB1 and CDC20, but BCL2L12 levels were

significantly decreased, which was equivalent to HSP90B1 siRNA treatment levels, so that the latter changes were attributed to non-specific effects of the nanoparticles.

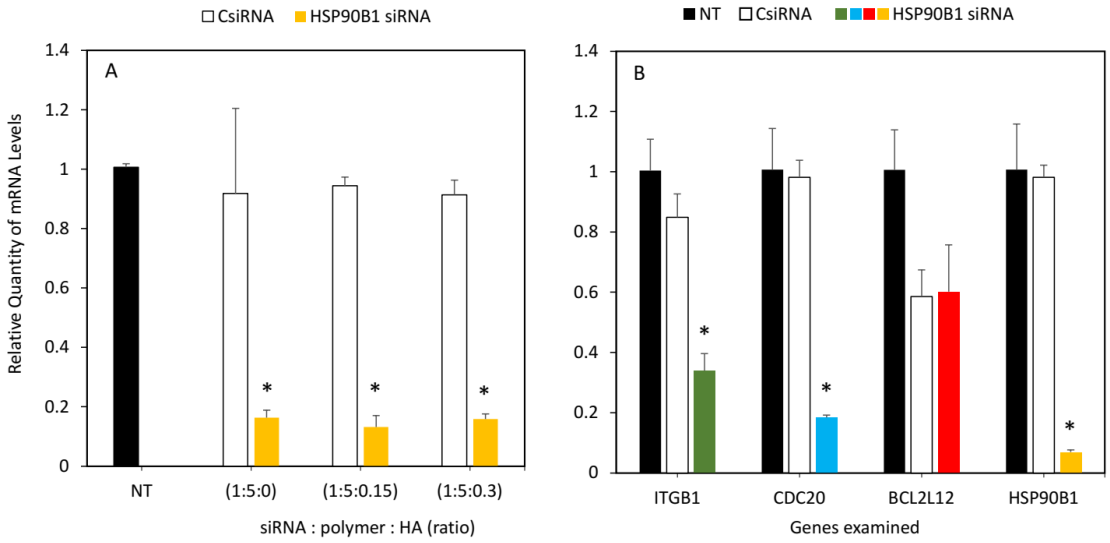

Figure 3.4: Knockdown efficacy at the mRNA level by RT-qPCR. Cells were treated with 60 nM of HSP90B1 siRNA at (**A**) 1:5:0, 1:5:0.15 and 1:5:0.3 siRNA/polymer/HA w/w ratio and (**B**) 1:5 siRNA/polymer w/w ratio for 3 days. Quantity of mRNA transcript levels of the specific gene relative to no treatment (NT; taken as 1) was calculated. Significance in comparison with CsiRNA treatment of individual group * $p \leq 0.05$ (n=2).

3.3.5 Combinational treatment

We had shown the importance of ITGB1 in the migration of breast cancer in a previous report [20]. Since it is also partially down-regulated due to HSP90B1 silencing, we wanted to combine HSP90B1 silencing with ITGB1 silencing to explore the benefit of using both siRNAs on inhibition of migration. We employed equal amounts of ITGB1 and HSP90B1 siRNAs in the treatments, along with the CsiRNA as reference. At 24 hours, 10+10 nM of CsiRNA+HSP90B1 siRNA combination showed equivalent effect to that of ITGB1+HSP90B1 siRNA combination however only the later showed significant

difference compared to CsiRNA+ITGB1. Similarly, with 20+20 nM siRNA combination both treatments showed equivalent levels of inhibition. At 30+30 nM, only ITGB1+HSP90B1 showed significant decrease and at 40+40 nM siRNA combinations, a slight difference was observed but this was not significant (**Fig 3.5A** and **3.6**). After 48 hours, 10+10 nM and 20+20 nM CsiRNA+HSP90B1 siRNA combinations as well as ITGB1+HSP90B1 siRNA combinations showed similar levels of migration, however only 10+10 nM of ITGB1+HSP90B1 could exhibit significant difference. At the higher 30+30 nM and 40+40 nM siRNA combinations of ITGB1+HSP90B1, the inhibition in migration was more significant when compared to the CsiRNA combinations with ITGB1 as well as HSP90B1 siRNAs (**Fig 3.5B** and **3.6**).

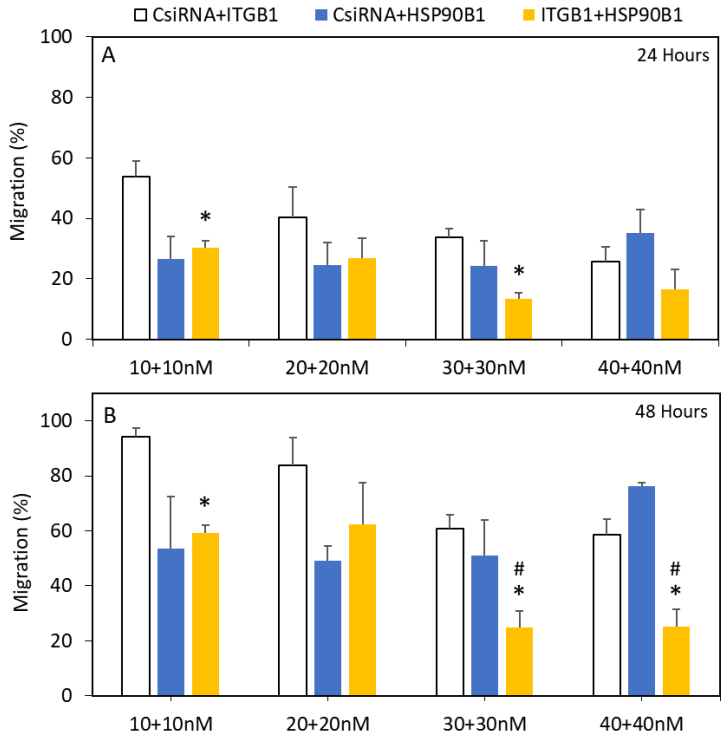

Figure 3.5: Combinational siRNA delivery against ITGB1 and HSP90B1. Polymer/siRNA complexes with CsiRNA+ITGB1, CsiRNA+HSP90B1 and ITGB1+HSP90B1 were given at 10+10 nM, 20+20 nM, 30+30 nM and 40+40 nM each. Inhibition in migration was

assessed by calculating wound closure percentage after (**A**) 24 hours and (**B**) 48 hours. * Significance compared to CsiRNA+ITGB1 treatment and # compared to CsiRNA+HSP90B1 treatment, $p \leq 0.05$ (n=2).

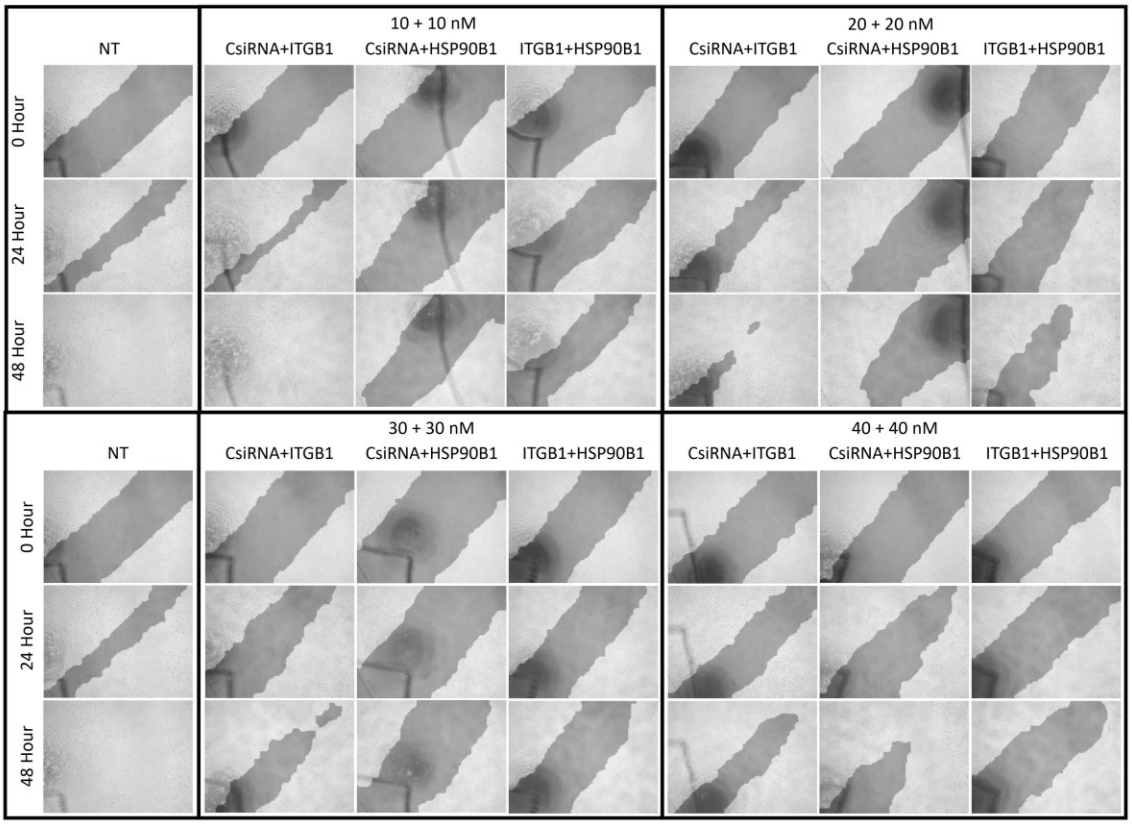

Figure 3.6: Microscopic images of migration inhibition, determined using scratch assay for the combinational siRNA delivery against ITGB1 and HSP90B1. Polymer/siRNA complexes with CsiRNA+ITGB1, CsiRNA+HSP90B1 and ITGB1+HSP90B1 were given at 10+10 nM, 20+20 nM, 30+30 nM and 40+40 nM each. Inhibition in migration was assessed by calculating wound closure percentage after 24 and 48 hours using TScratch software.

3.4 DISCUSSION

The use of PEI for gene therapy has gained considerable momentum in the past few years with several agents currently tested in various stages of clinical trials and in different cancer types [23,24]. Since, high molecular weight PEIs have shown to be toxic to the cells, its low molecular weight counterparts have been modified to deliver the nucleic acid more

efficiently at low toxicity. Low molecular weight PEI such as the 1.2 kDa PEI exhibits poor cellular internalization of its nucleic acid cargo and thereby is not functional for siRNA delivery; however, functionalization with small lipids improves its overall efficacy to deliver nucleic acid cargo [15]. The linoleic acid (LA) modification, for example, improves the binding, cellular uptake as well as intracellular release of intact nucleic acid [25–27]. Previously, we have shown the high cytoplasmic localization of 1.2PEI-LA/siRNA complexes that helped to accomplish a strong knockdown of ITGB1 with specific siRNAs [20]. A similar effect was observed with HSP90B1 silencing using the same delivery agent in this study. The use of HA as additives was previously shown to further improve cellular uptake of the complexes, which was partly due to high levels of CD44 (i.e., HA binding ligand) on the MDA-MB-231 cells, as well as the persistent intracellular availability of siRNA, thereby increasing the overall silencing [28]. Since we used the same polymeric delivery system, we could extrapolate that the complexes carrying HSP90B1 siRNA similarly underwent enhanced delivery, improving the silencing efficiency of the siRNA target. The use of HA can also reduce the ζ-potential of the complexes, facilitating lower disruption of the cell membrane while improving the uptake and intracellular release of the siRNA. Optimizing the right formulation of the siRNA, in terms of the ratio of specific reagents used to make up the nanoparticle, is also crucial as higher ratios can reduce the ζ-potential drastically impairing the delivery [29]. In our hands, siRNA:polymer ratio of 5, along with a trace amount of HA (siRNA:HA ratio of 1:0.15) performed the best in all the assays. This was similar to previous studies where the use of HA was successful in silencing cell cycle protein CDC20, survivin and various

phosphatases such as, PPP1R7, PTPN1, PTPN22, LHPP and PPP1R12A showing the wide applicability of this HA additive formulations. [28,29].

From the un-biased library screen in this study, we shortlisted two genes, CDH9 and HSP90B1, which showed cell death equal to or higher than the reference target, BCL2L12 when targeted with specific siRNAs. A recent report on the role of CDH9 in breast cancer suggested that its silencing resulted in enhanced lung metastasis and was identified as a crucial gene for tumor metastasis, hence its knockdown could be detrimental [30]. On the other hand, HSP90B1 was reported by several studies to be an important gene which is upregulated in various cancers including breast cancers and its over-expression had a strong correlation with poor survival of breast cancer patients [31]. It was interesting that such contrasting targets turned out to be positive hits in our library screens. Needless to say, we chose to pursue further studies with HSP90B1 given its better-established role in breast cancer. HSPs functions as molecular chaperones and promotes various fundamental cellular programs in normal as well as transformed cells. HSPs help in proper folding of the proteins, counteracts stressed-induced protein misfolding, and has been classified based on its molecular weight into six main families [32]. The HSP90 family of proteins consists of five isoforms: two cytoplasmic HSP90α and HSP90β, an endoplasmic reticulum localized glucose regulated protein 94 (HSP90B1), a mitochondrial tumor necrosis factor receptor-associated protein 1 (TRAP1), and a membrane-associated HSP90N [33]. HSP90B1 has shown to be over-expressed in various cancers, modulating several cellular functions like cell proliferation and metastasis, regulation of various apoptotic molecules that aid or inhibit cell death machinery that makes it a highly critical protein [34]. HSP90B1 overexpression has also been documented in chronic lymphocytic

leukemia (CLL) by next-generation sequencing in patient cells and identified as a potential therapeutic target, especially to address CLL drug resistance [35]. Studies have also shown the correlation between high HSP90B1 expression linked to poor overall survival (OS) as well as disease free survival (DFS) in non-small cell lung cancer (NSCLC) patients [36]. In osteosarcoma, the downregulation of HSP90B1 resulted in tumor growth inhibition *in vitro* and *in vivo* through the PI3K/ Akt/ mTOR pathways [37]. Similarly, inhibition of HSP90 in myeloma, B-cell lymphoma, and hepatocellular carcinoma by PU-H71, a specific HSP90 inhibitor, was shown to be beneficial to reduce the cell growth and induce apoptosis [38–40]. In breast cancer, proteomic analysis of patient samples for relative and absolute quantitation (iTRAQ) method revealed a strong correlation between high expression of HSP90B1 to distant metastasis and low overall survival when compared to samples with low expression who had higher survival [41]. Similar observations were made by other groups where patients with poor survival had high mRNA and protein expression of HSP90B1 [42]. In the brain metastasis of breast cancer, HSP90B1 over-expression helped the cancer cells to survive under hypoglycemic condition when compared to bone and lung metastatic cells. The HSP90B1 overexpression observed in the brain metastatic breast cancer cell line was accompanied by up-regulation of anti-apoptotic genes such as B-cell lymphoma 2 (*BCL-2*), cellular inhibitor of apoptosis protein (*HIAP1*) and X-linked inhibitor of apoptosis protein (*XIAP*) helping in the survival of metastasized cells. In addition, activation of pro-survival autophagy due to HSP90B1 upregulation was also observed in the brain metastatic cells and the ablation of HSP90B1 reduced the viability of brain metastatic cells *in vitro* as well as brain metastasis *in vivo*, increasing the survival of the animals compared to the control groups [43,44]. The over expression of HSP90B1

among breast cancer stem cells in $CD44^{hi}/CD24^{lo}$ phenotype and in oxidative stress-resistant breast cancer cells contributing towards enhanced proliferation and migration further strengthens the importance of HSP90B1 in highly aggressive breast cancer cells as a potential therapeutic target [45,46]. Although, various drugs have been tested *in vivo* and in clinical trials to neutralize HSP90B1 in several other cancer types, none has been introduced for breast cancer treatment due to toxicity and specificity related issues [47–50].

Based on strong outcome by silencing a single integrin target (ITGB1) from our previous report [20], we hypothesized that identifying new integrins could be beneficial to achieve superior inhibition of migration in breast cancers. Hence, from the scratch assay with the library screen, we selected targets that were highly effective as well as being a member of the integrin gene family (ITGA4, ITGA6 and ITGAX). ITGA4 was shown to be hypermethylated in breast cancer tissue samples and acts as an important marker for early diagnosis [51]. It is also the α-subunit in Very Late Antigen-4 (VLA-4/ integrin-α4β1) receptor formed with ITGB1 and binds to fibronectin in the extracellular matrix or to vascular cell adhesion protein-1 (VCAM-1) [52] important for migration. Similarly, ITGA6 was found to be over-expressed in breast cancer and contributes towards the resistance against radiation therapy partially through the activation of PI3K/Akt or MEK/Erk signaling pathways [53]. This α-subunit may associate with ITGB1 or ITGB4 forming VLA-6 (α6β1 or α6β4) and act as a laminin receptor [54,55]. Over-expression of ITGAX was associated with aggressive prostate cancer, but we could identify only one report where a moderate expression in invasive breast cancer tissue sample was reported [56,57]. The siRNA library also contained siRNA against various other integrin-α (ITGA)

genes such as ITGA1, ITGA2, ITGA2B, ITGA3, ITGA5, ITGA7, ITGA8, ITGA9, ITGA10, ITGA11, ITGAD, ITGAE, ITGAL, ITGAM, ITGAV, and ITFG2 (integrin-a FG-GAP repeat containing 2), whose silencing did not manifest any effect on migration as well as cell growth inhibition in this study. Similarly, silencing of other heat shock proteins (HSP) and glycoproteins (GP) like HSP90AA2, HSP90AB1, HSPA1A, HSPA2, HSPD1, GP9 and GPA33 did not alter the growth of breast cancer cells. Since each gene was targeted by a pool of 3 siRNAs in the library format, we can negate the possibility of not silencing these genes, thereby we could possibly say that these genes are of low importance for the migration of MDA-MB-231 cells according to the outcome of this study. We did not observe any correlation between the cell growth and migration inhibition, which shows the heterogenic functionality of these genes that were included in the study.

On the other hand, since the role of HSP90B1 in regulation of cell death and tumor invasion/metastasis has been well studied [33,34,58,59], various chemical inhibitors of HSP90 have been developed and are under exploration for breast cancer treatment, including ganetespib, 17-allylamino-17-demethoxygeldanamycin (17-AAG), PU-H71, AT-533, BJ-B11, DCZ3112 and TAS-116 [38,47–50,58–60]. Though considerable progress has been achieved with these drugs in different stages of clinical trials, some displayed significant side effects and some failed due to non-specific effects in patients, so that there is still an urgent need to develop alternate approaches for targeting HSP90B1 in breast cancer.

Following the identification and literature validation of these four targets (ITGA4, ITGA6, ITGAX and HSP90B1), we used dicer-substrate siRNAs which are longer than the conventional siRNA (27 base pairs vs. 19-21 base pairs, respectively) as the former can

undergo cleavage by the dicer enzyme in the RNA induced silencing complex (RISC) and has been showed previously to improve the silencing efficiency in some cases [20,61]. Silencing HSP90B1 at the mRNA level was possible even without the HA additive (1:5:0), and its effect on cell growth (30% inhibition) was also observed using 60 nM of siRNA, although the difference from CsiRNA treatment was not significant. The 30% growth inhibition after HSP90B1 silencing was comparable to the effect observed in the initial library screen, which did not utilize the HA additive to employ simpler nanoparticle formulations. The addition of HA at the 1:5:0.15 ratio also gave strong mRNA reduction, which was reflected in cell viability as well as in migration. With the 1:5:0.3 ratio, the decrease in mRNA was accompanied by changes in cell growth but the cell migration was unaffected. Although all the three ratios (1:5:0, 1:5:0.15, 1;5:0.3) displayed excellent mRNA reduction, only 1:5:0.15 (0.15 HA) could exhibit the desired comprehensive outcomes. It is possible that a more effective siRNA release was achieved from the complex with this formulation. It is also possible that excess HA in the nanoparticle formulation might have attenuated the effect of HSP90B1 silencing by yet unknown mechanism. There are many factors that can influence the efficacy of the delivery system such as its size, zeta potential, cellular uptake pathway, intracellular localization of the nucleic acid and the type of lipid substitution [20,25,29,62]. In addition, the specificity of siRNA, expression level and turnover rate of the target, cell type and proliferation rate of the cell line used, can also influence the overall results [63–65]. In our study, the use of HA could have reduced the zeta potential of the complexes influencing its interaction with the cell membrane and its cytoplasmic localization, which in turn could have helped in better silencing of HSP90B1. Another important factor might be the high proliferation rate

of MDA-MB-231 cells and the high expression levels of CD44, a primary receptor for HA which could have helped in better cellular uptake.

Previously, RNAi was used to silence HSP90B1, reducing the migration (at 6 and 24 hours) as well as the proliferation of MDA-MB-231 and an oxidative stress resistant MCF-7 cells, which showed similar levels of high HSP90B1 expression. This outcome was achieved using 100 nM siRNA delivered with Dharmafect-1 reagent, a lipid-based delivery agent. In the same study, HSP90B1 expression was shown to be high in tumors compared to normal tissues and, furthermore, it was higher in recurrent tumors from patients who underwent chemotherapy compared to the initial breast tumors. Another study probed the role of HSP90B1 in tumor invasiveness [66] under low glucose conditions, which is a key characteristic of tumor environment. Using the Dharmafect-1 transfection reagent, reduction of HSP90B1 levels lowered the invasive potential of MDA-MB-231 cells by 67% and expression of HSP90B1 was elevated in low glucose conditions in MCF-7 cells. Moreover, the sensitivity towards doxorubicin was increased following HSP90B1 silencing with an increase in caspase-3 activity [66]. We also used the metastatic breast cancer MDA-MB-231 and were able to achieve strong reduction at mRNA levels followed by inhibition in proliferation (cell viability) and migration at 24 and 48 hours, but by employing a lower concentration of siRNA (60 nM). This is advantageous to avoid any undesirable effects of the siRNA treatment.

HSP90B1 is known to regulate the functioning of a wide range of ‘client’ proteins, including Toll-Like Receptors (TLRs) and integrins. Hence, it was not surprising that silencing HSP90B1 reduced the mRNA levels of ITGB1 in this study. In a HSP90B1-knockout mice to study its role in B-cell functioning, the expression of various integrins

such as ITGA4, ITGAL, ITGB7 and ITGB2 were down-regulated but not the ITGB1 [67]. The inhibition of the αI domain (AID), which is a ligand binding domain common among all α-integrin subunits, using TAT-α7 helix peptide reduced the maturation and expressions levels of various integrins such as ITGAL, ITGAM and ITGA4, but not ITGB1. That study showed the crucial role of HSP90B1 in the proper maturation and function of integrins [68]. Our study could note a relationship between the HSP90B1 expression and ITGB1, however further studies are warrantied to confirm these findings. Similarly, the mRNA levels of CDC20, a cell division cycle protein 20 homolog, was downregulated following the knockdown of HSP90B1, which could be probed further and would help to better understand the intracellular consequences of HSP90B1 in breast cancer treatment.

In conclusion, this study has shown that linoleic acid-substituted PEI and an HA additive siRNA complex was a viable method to effectively deliver the dicer-substrate siRNA and silence HSP90B1 expression. This reduced the proliferation and migration of the metastatic breast cancers *in vitro*. Given the wide spectrum of HSP90B1 function in breast cancer invasiveness, aggressiveness, brain metastasis and resistance to drug treatment, our method is a promising alternative to chemical inhibitors for effective silencing of this important target. Further studies on the relation between HSP90B1 and various other integrins as well as cell cycle proteins could help to better understand the role of HSP90B1 down-regulation in cellular outcomes. In addition, the effect of HSP90B1 silencing on the sensitivity of various anti-cancer drugs in breast cancer treatment could be a crucial information with high clinical translation potential.

3.5 ACKNOWLEDGMENTS

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4. THERAPEUTIC POTENTIAL OF INTEGRIN-β1 SILENCING IN DRUG RESISTANT CHRONIC MYELOID LEUKEMIA IMPLEMENTED BY POLYMERIC siRNA DELIVERY

4.1 INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by uncontrolled expansion of a select population of pluripotent hematopoietic progenitor cells at the expense of normal hematopoietic progenitor cells, resulting in abnormally high accumulation of ill-formed myeloid cells [1]. The hallmark of CML is the reciprocal chromosomal translocation of the chromosome 9 having *ABL* gene (a human homologue of *v-abl* oncogene carried by Abelson murine leukemia virus) and chromosome 22 that breaks in a specific breakpoint cluster region (BCR) and results in the formation of fusion oncogene *BCR-ABL.* The encoded protein has enhanced tyrosine kinase activity and stimulates multiple undesirable events, including uncontrolled proliferation, growth factor independence, decreased attachment to stroma cells and extracellular matrix along with reduced apoptosis [1,2]. The disease progression in CML can develop in three phases when left untreated; (i) the initial *chronic phase* that last from 3 to 6 years and where most patients are diagnosed, (ii) the subsequent *accelerated phase* (6-9 months), and (iii) the terminal *blast crisis phase* that lasts 3 to 6 months [3]. The presence of *BCR-ABL* fusion protein in all CML patients make it a unique, and highly viable therapeutic target. This fusion oncogene has been effectively controlled by the tyrosine kinase inhibitor (TKI) imatinib mesylate (IM), which was approved as front-line therapy for CML. The IM binds to the amino acids present at the ATP binding site of BCR-ABL tyrosine kinase protein and stabilizes it in an inactive form. This eventually "switches-off" the downstream signaling pathways leading to leukemogenesis. The IM has become the front line drug to treat newly diagnosed CML and aims to maintain remission, prevent relapse and disease progression to the accelerated phase or blast crisis [4–6]. However, after initial IM

treatment, some patients become unresponsive to the drug due to point mutations at the IM-binding site of *BCR-ABL* and this led to development of second generation (such as dasatinib, nilotinib and bosutinib) and third generation TKIs (ponatinib) [7–9]. In addition to mutagenic response to IM, certain populations of CML cells become unresponsive to TKI treatment due to their direct interaction and binding with the bone marrow (BM) and/or extracellular matrix (ECM) proteins through cell surface molecules [10–12].

Integrins are key cell-surface molecules that can regulate various intracellular signaling pathways such as proliferation, migration and apoptosis of the cells [13]. There are 24 different integrin receptors that are formed by the dimerization of 18 α- and 8 β-integrin subunits and their expression vary significantly from cell to cell [14]. The binding of CML cells to the ECM protein fibronectin through cell surface integrins was shown to induce drug resistance and provide protection from apoptosis by the activation of tyrosine kinases [13,15]. *BCR-ABL* expression was shown to alter Integrin-β1 (ITGB1) functioning in different studies; it leads to integrin-β1 clustering, cytoskeleton remodelling and increased binding to fibronectin and stromal cells [16,17]. The direct interaction of CML cells with stromal microenvironment play an important role in the drug resistance in CML patients and was demonstrated by the interaction of the CML model K562 cells with stromal cells which provided protection against apoptosis induced by IM treatment. The binding to bone ECM via integrins could activate various intracellular signaling pathways altering the expression of multiple genes as well as the secretion of various growth factors from the stromal environment which could confer drug resistance and act as a sanctuary for minimal residual disease (MRD) [18,19]. In all these studies, the binding of CML cells

was the key in providing resistance to TKI treatment and, if this binding event could be interfered, the sensitivity of CML cells to the drugs could be improved.

In this study, we hypothesize that the knockdown of a specific integrin molecule (integrin-β1) present on CML cells will improve the sensitivity of the cells to the TKI treatment. For this purpose, we used the well-established CML model K562 cells *in vitro*. This integrin knockdown was achieved using the siRNA approach, which has the advantage to target the specific genes of interest without altering other related genes. The effectiveness of siRNA-mediated silencing, however, depends on the efficiency of the gene delivery vector used. Polyethyleneimine (PEI) polymers are safe, non-viral delivery systems that can complex with siRNA to form nanoparticle complexes suitable for cell uptake. Lipid modification of PEI was shown to improve the interaction of cationic PEI/siRNA complexes with the anionic cell membrane. For this purpose, lipid-modified 1.2 kDa PEI (1.2PEI) polymers were utilized in this study to deliver dicer-substrate siRNA that helps to better incorporate into the RISC complex and provides higher knockdown as observed in our previous studies [20,21]. Here, we show that; (i) K562 CML cells can bind to fibronectin, (ii) integrin-β1 is highly expressed by K562 cells compared to other integrins, (iii) successful knockdown of integrin-β1 can reduce its binding of K562 cells towards fibronectin and human bone marrow stromal cells (hBMSC) and (iv) silencing of integrin-β1 can also help in the detachment of the leukemic cells attached to the stroma cells which can be targeted by TKIs. We also demonstrate that K562 cells can become resistant to BCR-ABL siRNA treatment when cultured on fibronectin and the combinational knockdown of integrin-β1 with BCR-ABL improves the sensitivity to siRNA treatment. If these results could be translated to a preclinical model, the detachment

of leukemic cells from the bone marrow microenvironment would aid in the TKI mediated killing.

4.2 MATERIALS and METHODS

4.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM) with D-glucose, L-glutamine and sodium pyruvate, Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine and HEPES, DMEM/F12 with L-glutamine and HEPES, Hank's Balanced Salt Solution (HBSS), Penicillin-Streptomycin (10,000 U/mL), UltraPure DNase/RNase-free dH_2O, MEM Non-essential amino acids, GlutaMAX-I, TRIzol Reagent, chloroform, acetic acid, Lipofectamine^{TM} 2000 and Lipofectamine^{TM} RNAiMAX were purchased from Thermo Fisher Scientific (Ottawa, Canada). Fibronectin, formaldehyde solution, methyl cellulose, thiazolyl blue tetrazolium bromide (MTT) and the primers used for various integrin gene expression analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethyleneimine of 1200 Da (1.2 PEI) was from Polysciences (Warrington, Pennsylvania). Fetal bovine serum (FBS) was from VMR Life Science Seradigm (Mississauga, Ontario). Phycoerythrin (PE)-labeled mouse anti-human CD29 (integrin-β1) was from BD Biosciences (Oakville, Canada). Dicer substrate siRNA (ITGB1 sense: 5'-AGUUAACAGUGAAGACAUGGAUGCT-3', antisense: 5'-AGCAUCCAUGUCUUCACUGUUAACUUC-3') and 6-carboxyfluorescein (FAM) labelled scrambled siRNA were from IDT (Coralville, USA). Crystal violet from Allied Chemical. K562 cells and MDA-MB-231 cells were authenticated by genetic sequencing at the Centre for Applied Genomics, Toronto, Canada.

4.2.2 Cell models and culture

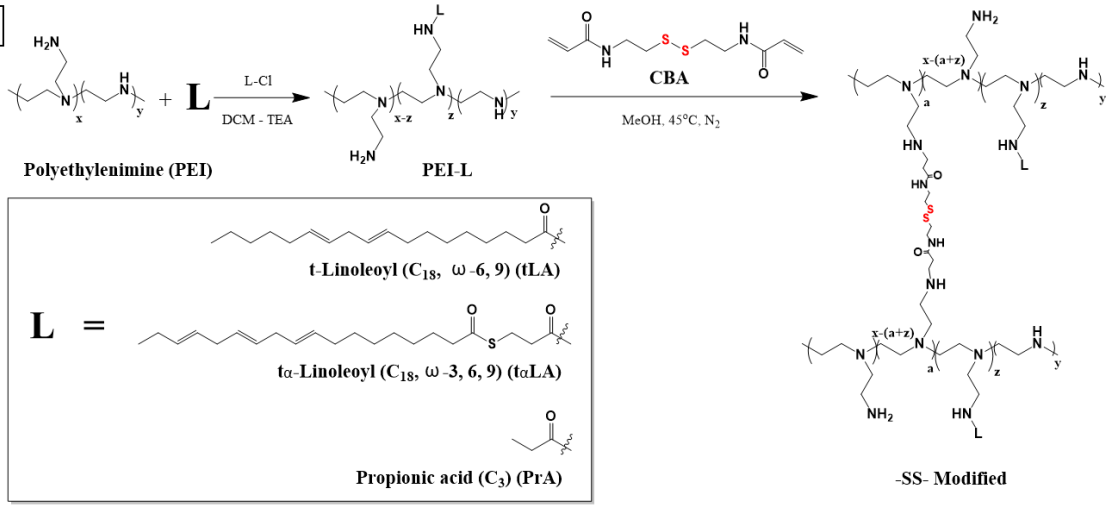
The *BCR-ABL* positive K562-WT (wild type) cell line established from a CML patient in blast crisis was purchased from the American Type Culture Collection (Virginia, USA) and K562 cells expressing green fluorescent protein (GFP) were maintained in RPMI medium containing 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2. Spent medium was removed every third day by centrifugation at 500 rpm (5 min) and diluted 10 times in 20 mL of fresh medium for cell expansion. MDA-MB-231 breast cancer cell line was kindly provided by Dr. Judith Hugh (Faculty of Medicine and Dentistry, University of Alberta, Edmonton) and were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C and 5% CO_2. After reaching 80% confluency, MDA-MB-231 cells were passaged using 0.05% Trypsin/EDTA at 37°C for 2 min and complete DMEM was used to collect the cells followed by centrifugation at 600 rpm (5 min). MDA-MB-231 cells were seeded 24 hours prior to the treatment. Human bone marrow stromal cells (hBMSCs) were isolated from patients (between 25-50 years of age) based on a procedure [22] approved by the Research Ethics Board of University of Alberta. These hBMSC cells were maintained in DMEM/F12 with 12% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1% MEM Non-essential amino acids and 0.1% GlutaMAX-I at 37°C and 5% CO_2.

4.2.3 Preparation of polymers and polymer/siRNA complexes

A library of lipid-modified 1.2PEI polymers was synthesized through amide and thioester linkages using linoleic acid (LA), α-linoleic acid (αLA) and propionic acid (PrA) based on previously published protocols [23–26]. A redox-sensitive polymer library was

also prepared through disulfide cross-linking (-SS-) of lipid-modified PEIs via condensation reaction using cystamine bisacrylamide (CBA) as the cross-linker [24] (Scheme 1A). In all cases, the degree of lipid substitution on the polymers was determined through ^{1}H-NMR spectroscopy as described earlier [23-26]. Scheme 1B summarizes the polymers explored in this study.

A



B

S.No	Unmodified	-SS- Modified
1	1.2PEI	1.2PEI-ss
2	1.2PEI-tLA2	1.2PEI-tLA2-ss
3	1.2PEI-tLA6	1.2PEI-tLA6-ss
4	1.2PEI-tαLA2	1.2PEI-tαLA2-ss
5	1.2PEI-tαLA6	1.2PEI-tαLA6-ss
6	1.2PEI-PrA1	1.2PEI-PrA1-ss

Figure 4.1: (**A**) Schematic representation of polymer synthesis by grafting lipids *via N*-acylation followed by disulfide cross-linking (-SS- Modified). Lipid grafted PEI polymers were reacted with cystamine bisacrylamide (CBA) at 45ºC in methanol under N_2 environment to prepare the disulfide cross-linked polymers. (**B**) List of polymers explored in this study, the lipid substituted polymers (unmodified) and its corresponding disulfide cross-linked polymers (-SS- Modified).

The polymer/siRNA complexes (typically at 9:1, w/w) were prepared in serum-free media (RPMI for K562 and DMEM for MDA-MB-231 cells) by incubating the desired polymer with siRNA for 30 min at room temperature. Lipofectamine^{TM} 2000/siRNA complexes (2:1, w/w) (for Fig 4.6, and Fig 4.S1) and Lipofectamine^{TM} RNAiMAX/siRNA (5:1, w/w) (for Fig 4.4, 4.5 and 4.S2) were prepared according to the optimized protocol provided by the manufacturer.

4.2.4 Fibronectin binding assay

The fibronectin binding assay was carried out based on a previously published protocol [27] with minor modifications. Briefly, 40 µL of 25 µg/mL concentration of fibronectin was coated onto 96-well flat bottom plates at room temperature for 1 hour. Viable cells were counted using trypan blue dye exclusion assay and equal number of cells were added in triplicates to the fibronectin coated plates. Following 1 hour of incubation at 37°C, plates were inverted and further incubated for 3, 7, 19 and 23 hours at 37°C. Cells were fixed with 3.7% formaldehyde followed by staining with 0.1% crystal violet (in water) for 30 min and washed twice with HBSS to remove excess dye. The cell-associated dye was solubilized with 10% acetic acid and the absorbances were measured at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments). Microscopic images were also captured at different time points. For the integrin-β1 silencing studies, the cells were treated with desired concentrations (see Figure legends) of polymer/siRNA complexes for 3 days, following which K562 cells were collected, centrifuged at 1400 rpm for 5 min and resuspended in 100 µl of HBSS. Cells were stained with DiI dye for 20 min at room temperature and excess dye was removed by HBSS wash (twice). Cells were then

re-suspended in desired volume of fresh complete medium (10% FBS) and fibronectin binding was carried out for 4 hours. The non-adherent cells were removed by washing and the fluorescence was recorded at excitation of 536 nm and emission of 607 nm using Fluoroskan Ascent plate reader (Thermolab Systems).

4.2.5 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis

The expression levels of integrins α2b (ITGA2B), α3 (ITGA3), α5 (ITGA5), α10 (ITGA10), αE (ITGAE), αV (ITGAV), β1 (ITGB1), β3 (ITGB3) and β5 (ITGB5) in K562 cell line was performed by qRT-PCR. Briefly, total RNA was extracted using Trizol reagent based on the manufacturer's instruction and 2 µg of total RNA was reverse transcribed to synthesize cDNA by using 2 µL of mix-1 containing 0.5 µL random hexamer primer, 1 µL (10mM) dNTP's, 0.5 µL Oligo (dT) which was heated at 65 °C for 5 min. Then, 8 µL of mix-2 containing 4 µL synthesis buffer (5x), 2 µL DTT (0.1M), 1 µL RNase out and 1 µL M-MLV RT enzyme were added and incubated at 25 °C for 10 min, 37 °C for 50 min and 70 °C for 15 min. RT-PCR was carried out on a StepOnePlus (Applied Biosystems) RT-PCR system with human β-actin as endogenous housekeeping gene using the primers which were screened with the help of IDT (Integrated DNA Technologies) PrimerQuest Tool and NCBI Primer-BLAST are shown below:

Gene	Forward	Reverse
ITGA2B	CACGCATGGTTCAACGTGTC	CTGTGTCCACACCTGAGCTT
ITGA3	GCGCAAGGAGTGGGACTTAT	CTGCATCGTGTACCCAATATAGA
ITGA5	TGCCGAGTTCACCAAGACTG	TGCAATCTGCTCCTGAGTGG
ITGA10	TGGGTGTACCTAGGCAGTAT	ACTCTCTCTCCTGTCTGCTT
ITGAE	GCCTCCCTCTCCAAACATTCA	CTGAAACATGAGCAGATGACCTC
ITGAV	GTTGGGAGATTAGACAGAGGAAAG	GCAGACGACTTCAGAGAATAGG
ITGB1	GCCTGTTAGACATGACTGATGA	TTTCCCATGGCCTTTGTAGAT
ITGB3	CATCACCATCCACGACCGAA	GGTTGTTGGCTGTGTCCCAT
ITGB5	GATGACACCACAGGAGATTGC	GGTAGTACAGGTCCACAGGA
β-actin	GCGAGAAGATGACCCAGAT	CCAGTGGTACGGCCAGA
GAPDH	TCACTGTTCTCTCCCTCCGC	TACGACCAAATCCGTTGACTCC

10 µL of reaction mixture containing 5 µL master mix SYBR Green, 2 µL of 10 × 10^{-6} m primers, and 3 µL of 5 ng μL^{-1} cDNA template was added in triplicates to the MicroAmp Fast Optical 96-well reaction plate. The reaction mixtures were heated at 95 °C for 10 min before proceeding through 40 cycles of the denaturation step, 95 °C for 15 s, and annealing/elongation step, 60 °C for 1 min.

The integrin-β1 mRNA levels following polymer/siRNA treatment in K562 and MDA-MB-231 cells were analyzed using the described RT-PCR protocol at desired time points (days 1, 3 and 6). Briefly, 80,000 K562 cells per well in 800 ul of media (24 well plate) were treated with complexes for 1 day and cells were harvested. A similar setup was carried out for day 3 timepoint, following which 600 ul of cell suspension was used for RNA extraction and the remaining 200 ul was added to a new plate with an additional 600 ul of fresh media which was further incubated for 3 more days (day 6 time point) before harvesting. For MDA-MB-231 cells, 80,000 cells per well (6 well plate) were seeded 24-hour prior to treatment with three different setups for day 1, 3 and 6. For day 6 time point, media was changed on day 3 following which cells were harvested for RT-PCR analysis. ΔC_T, $\Delta\Delta C_T$ and Relative Quantity (RQ) of mRNA were calculated with respect to the housekeeping gene, while the no treatment (NT) group served as the reference group.

4.2.6 siRNA uptake

The siRNA delivery efficiency was studied using FAM-labeled control (scrambled) siRNA in both cell lines. MDA-MB-231cells were allowed to attach and grow for 24 hours, after which polymer/FAM-siRNA complexes prepared at a weight/weight ratio of 9:1 was added to the cells at 60 nM, with unlabeled control siRNA serving as a negative control.

K562 cells were collected and centrifuged at 1400 rpm for 5 min whereas MDA-MB-231 cells were centrifuged following trypsinization and collected in complete medium. Both the cell lines were washed with HBSS and fixed with 3.7% formaldehyde. The uptake was quantified using BD Accuri^{TM} C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA). The uptake of FAM-siRNA was determined and expressed as mean siRNA levels per cell (in arbitrary fluorescent units) and as percentage of cell population positive for FAM-labeled siRNA with cells carrying unlabeled siRNA complexes designated as 1% positive population.

4.2.7 Cell surface integrin-β1 analysis by flow cytometry

K562 cells were added to the wells along with polymer/siRNA complexes whereas MDA-MB-231 cells were seeded 24 hours prior to the treatments. Both cell lines were treated with the polymer/siRNA complexes for 3 days, following which K562 cells were collected by centrifugation, where as MDA-MB-231 cells were collected following trypsinization. The cells were washed with HBSS, followed by 1 hour of staining with PE-labeled Integrin-β1 antibody at room temperature according to the manufacturer's protocol. Cells were fixed with 2% formaldehyde prior to analysis with BD Accuri^{TM} C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA). The extent of integrin-β1 expression was calculated as a percentage of mean fluorescence intensity relative to no treatment samples.

4.2.8 hBMSC binding assay

The K562 cells were treated in 48 well plates with the desired polymer/siRNA complexes and harvested after 3 days of treatment. hBMSC were seeded in 96-well flat

bottom plates and were maintained for 2-3 days to reach confluency [28]. The treated K562 cells were counted using the trypan blue exclusion assay and equal number of cells were stained with DiI dye (carbocyanine dye) for 20 min, after which they were washed twice with the HBSS. These cells were added to the confluent hBMSC monolayers in 96-well plates and incubated for 1 hour at 37 °C. The plates were then placed upside down and further incubated for 3 hours at 37 °C. The non-adherent cells were removed by washing and the fluorescence was recorded at excitation of 536 nm and emission of 607 nm using Fluoroskan Ascent plate reader (Thermolab systems).

4.2.9 K562 detachment assay

hBMSCs from different donors were seeded on a 6-well plate and was grown for 24 hours till they reached 60% confluency, following which K562-GFP cells were seeded on this monolayer and allowed to attach for additional 24 hours. The unattached/floating K562-GFP cells were removed, and fresh complete media was added followed by complex treatment for 3 days. Unattached/floating cells were once again removed, followed by the addition of fresh media, and further incubated for 2 days. Finally, K562-GFP cells were removed from the hBMSC monolayer and washed with HBSS to attain complete collection of the cells and the GFP fluorescence was recorded at excitation of 485 nm and emission of 510 nm using Fluoroskan Ascent plate reader (Thermolab systems). Percentage of detachment was calculated relative to non-treatment group. The cells that remained attached to the monolayer was collected by trypsinization and analyzed for GFP fluorescence by BD Accuri^{TM} C6 Plus Flow Cytometer (BD Biosciences, San Jose, USA).

4.2.10 Cell viability analyzed by MTT and trypan blue exclusion assay

To develop a K562 cell line resistant to Dasatinib (DA) treatment due to hBMSC binding, we cultured K562-GFP cells on a monolayer of hBMSC for 1, 3 and 5 days and the unattached cells were removed followed by DA addition at 5 nM and 10 nM. DA treatment which was allowed for 4 days under each treatment group separately. Dead cells percentage was calculated by staining the cells with 0.0002% trypan blue (final concentration) and analyzed by flow cytometry. Cell population positive to GFP as well as trypan blue dye was calculated for this purpose.

K562-WT cells were treated with complexes having 60nM of siRNA for 2 days followed by Imatinib addition at 300 nM, 500 nM and 1000 nM. Cells were further incubated for an additional 3 days after which cell viability was accessed by MTT assay. Briefly, 1mg/ml final concentration of MTT was added to the cells and incubated at 37 °C for 2 hours. Cells were collected by centrifugation at 1400 rpm for 5 minutes, supernatant was removed and the pellet containing the crystals was dissolved in DMSO by vortexing and absorbance was recorded at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments).

4.2.11 Methylcellulose colony-formation unit (CFU) assay:

The K562 cells were allowed to attach and grow on fibronectin-coated plates for 2 days, following which they were treated with polymer/siRNA complexes for 3 days. Cells were collected by HBSS washing, counted using trypan blue dye exclusion assay and equal number of viable cells were seeded with 400 µL of Methylcellulose and allowed to form

colonies at 37 °C. Colony counts were assessed using a light microscope on day 7 and day 14.

4.2.12 Statistical analysis:

All results are summarized as mean ± standard deviation and unpaired Student's *t*-test was used to assess the statistical differences between the group means with p-value < 0.05 considered as statistically significant. Where specified, the number of independent experiments used to generate the data (n) are indicated. Pearson's correlation coefficient was calculated where indicated.

4.3 RESULTS

4.3.1 K562 cells can effectively bind to fibronectin at relatively short duration

To assess the fibronectin binding ability of K562 cells, the cells were seeded on fibronectin coated wells and the bound cells were quantified at different time points (4h, 8h, 20h and 24h). The cells retained in wells without fibronectin (WO-FN; **Fig 4.2A**) was minimal which remained at similar levels for all the time points. The corresponding microscopic images confirmed this result (**Fig 4.2B**). In contrast, the wells with fibronectin coating (W-FN; **Fig 4.2A**) showed large number of cells binding to fibronectin which was observed as early as 4h. The attachment was not also altered with increase in time. The corresponding microscopic images (**Fig 4.2B**) provided a confirmation on the fibronectin binding ability of the K562 cells, which suggest the presence of cell surface receptors (presumably integrins) that could help in this binding.

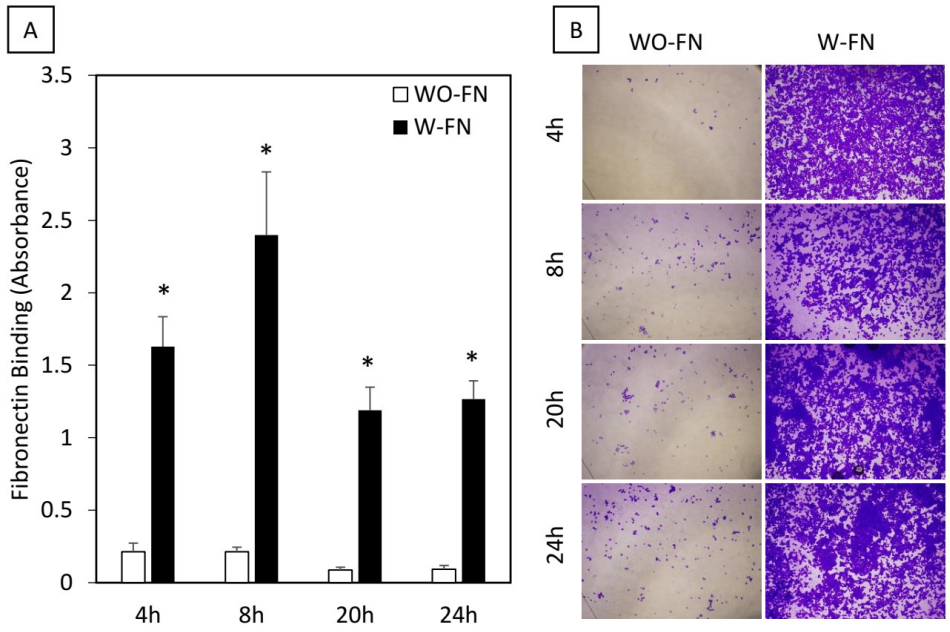


Figure 4.2: Fibronectin binding ability of K562 cells at different time points. (**A**) The number of cells bound to fibronectin as given by relative absorbance units and (**B**) the corresponding microscopic images. The results are summarized for without fibronectin (WO-FN: white bars) and with fibronectin (W-FN: black bars) coated wells. Significance compared to WO-FN at each time point (* $p \leq 0.05$, n=2).

4.3.2 Integrin-β1 (ITGB1) is highly expressed in K562 cells

Following the confirmation of fibronectin binding, we investigated the presence of potential integrins that could play a role in this fibronectin binding process. Hence, we assessed the levels of mRNA transcripts of various integrins in the K562 cells. The RQ of mRNA levels were calculated with respect to β-actin housekeeping gene. Integrin-β1 was highly expressed when compared to other integrin genes, followed by integrin-α5 and integrin-α_V (**Fig 4.3**). All other genes such as ITGA2B, ITGA3, ITGA10, ITGAE, ITGB3 and ITGB5 had very low or no expression (**Fig 4.3**). Given the well-established role of integrin-β1 in fibronectin binding [29–33], this analysis suggests that integrin-β1 could be primarily responsible for the fibronectin binding ability of the K562 cells as it is highly

expressed. A similar level of Integrin-β1 was also observed when K562 cells were cultured in contact with hBMSC (**Fig 4.3**, insert)

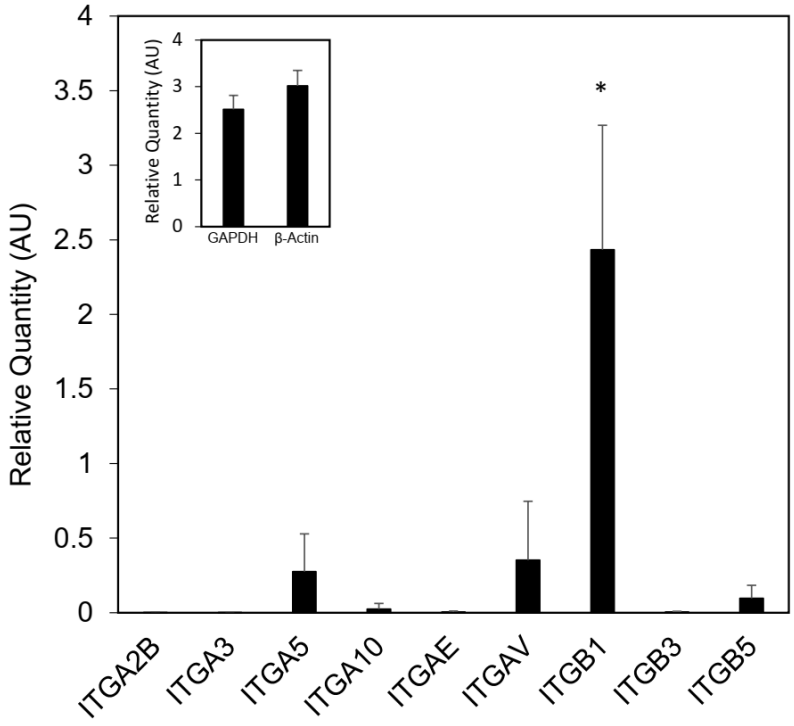


Figure 4.3: Relative quantity of integrin expression in K562 cells with respect to β-actin, evaluated by RT-PCR. The mRNA levels of integrin-β1 (ITGB1) were significantly higher than all other integrins included in this study as denoted by * $p \leq 0.05$ (n=3). The expression of integrin-β1 was also high when cultured on hBMSC with GAPDH or β-actin house keeping genes (insert).

4.3.3 Successful delivery of siRNA into K562 Cells

The high expression of fibronectin-binding integrin-β1 makes it a viable target for siRNA-mediated knockdown, which would help to reduce the ECM binding ability of K562 cells. As the siRNA delivery is dependent on the efficiency of gene delivery vehicle, we used several lipid-modified PEIs in this study to identify optimal delivery system. The 1.2PEIs that had linoleic acid, α-linoleic acid, propionic acid substitutions and their respective redox-sensitive disulfide cross-linked (-ss-) polymers were used for siRNA

delivery. MDA-MB-231 cells were used as reference cell phenotype due to the high expression of integrin-β1 on their cell surface as shown in our previous studies [34]. The widely used commercial reagent Lipofectamine, and the in-house prepared polymer 1.2PEI-αLA4 which exhibited high siRNA uptake in MDA-MB-231 cells as well as in K562 cells from previous studies [35,36] were used as reference carriers. Among the investigated polymers, 1.2PEI-tLA6, 1.2PEI-tαLA6, 1.2PEI-tαLA6-ss exhibited the highest siRNA uptake as shown by the mean fluorescence intensity in K562 cells (**Fig 4.4A-i**). The same polymers showed high siRNA uptake in MDA-MB-231 cells as well, in addition to 1.2PEI-tLA2 and 1.2PEI-tαLA2 (**Fig 4.4A-ii**). The correlation in the siRNA delivery between the K562 and MDA-MB-231 was calculated ($r^2 = 0.7118$) and found to be significant ($p < 0.0001$). The three polymers 1.2PEI-tαLA6-ss, 1.2PEI-tLA6 and 1.2PEI-tαLA6 (indicated by A, B and C in **Fig 4.4B**) worked best in both cell types.

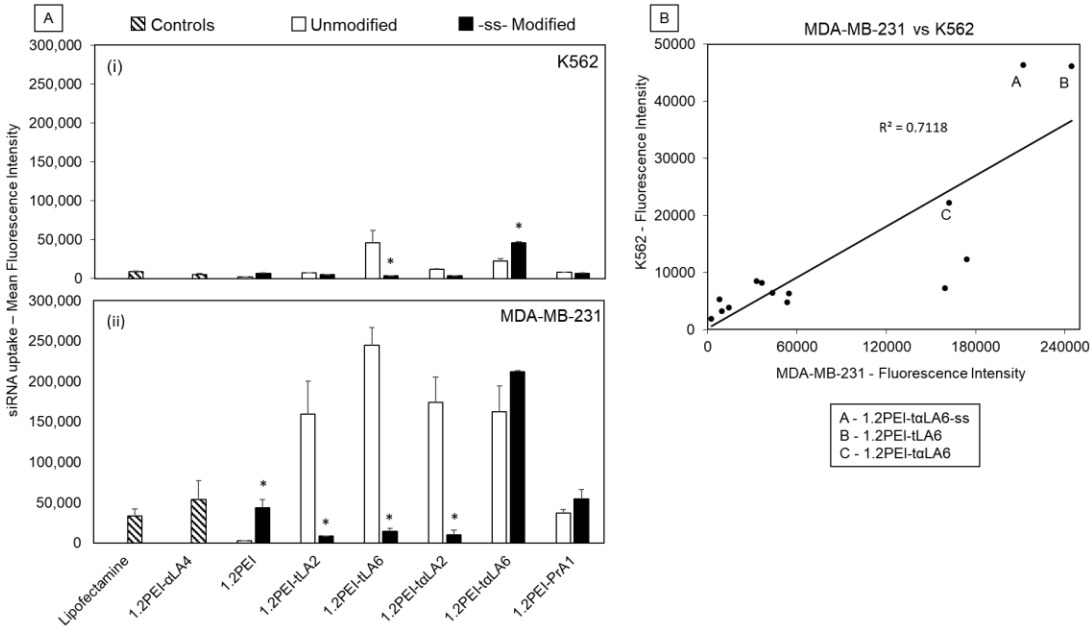


Figure 4.4: The uptake of FAM-labelled siRNA after 24 hours of treatment. (**A**) Mean fluorescence intensity of siRNA relative to the no treatment cells in (i) K562 and (ii) MDA-MB-231 cell lines. Control polymers (striped bars), non-disulfide crosslinked polymers (white bars) and -ss- modified polymers (black bars) with significance compared to the

unmodified (non-disulfide) counterparts * $p \leq 0.05$ and (**B**) The correlation of mean fluorescence between the two cell lines was calculated by Pearson's correlation coefficient ($r = 0.8437$ with $p < 0.0001$).

4.3.4 Significant knockdown of cell surface integrin-β1

The knockdown of integrin-β1 was performed with 1.2PEI-tαLA6 and redox-sensitive -SS- cross-linked derivatives as they may yield better siRNA release inside the cell, resulting in better knockdown [24]. Scrambled siRNA (CsiRNA) was used as control in this experiment. Following 3 days of treatment with polymer/siRNA complex, cells were stained with PE-integrin-β1 antibody and analyzed by flow cytometry. We assessed the cell surface integrin-β1 levels in K562 and MDA-MB-231 cells (**Fig 4.5A**) and observed a drastic difference. K562 cells had readily detectable expression of integrin-β1, but this amount was lower than the levels found in MDA-MB-231 cells. In K562 cells, the polymers 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss showed significant knockdown of the cell surface integrin-β1 (**Fig 4.5B-i**). These polymers also had high siRNA uptake in K562 cells (see **Fig 4.5A-i**) and they also exhibited strong silencing in MDA-MB-231 cells (**Fig 4.5B-ii**). The observed knockdown was achieved with a single treatment of polymer/siRNA complex having 60 nM siRNA concentration.

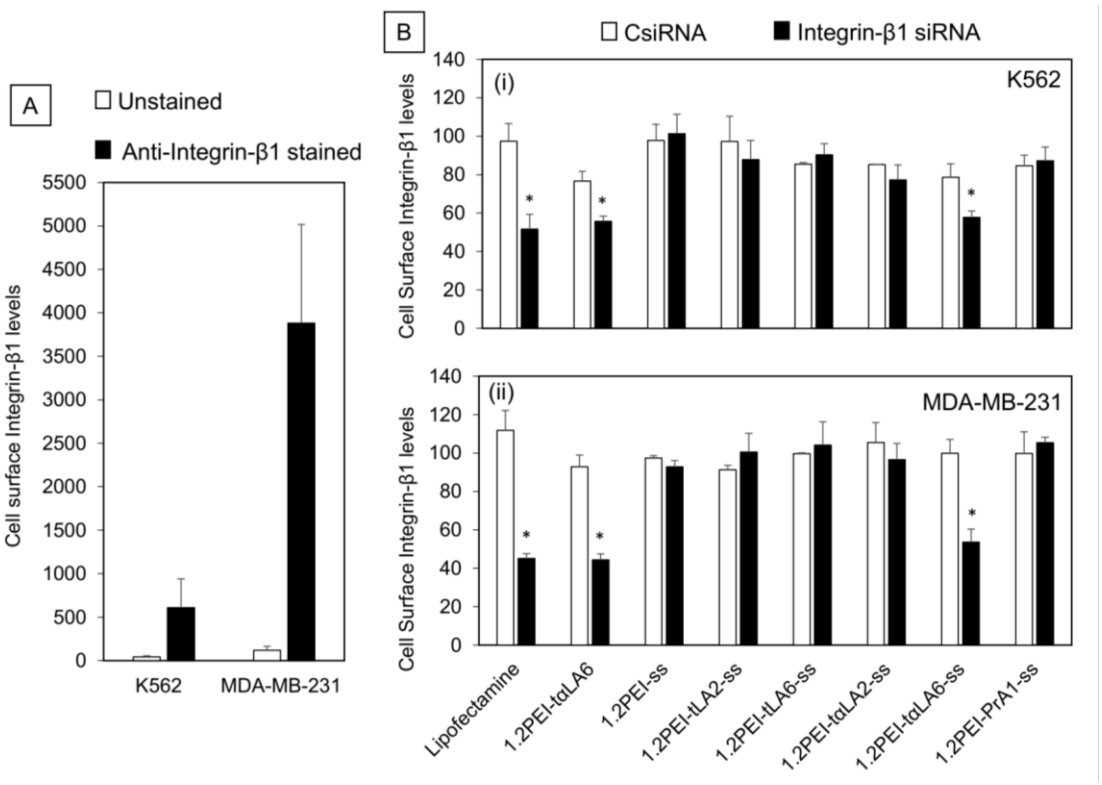


Figure 4.5: (**A**) Cell surface integrin-β1 levels in K562 and MDA-MB-231 cells. Cells without antibody staining are denoted by white bars, and cells stained with PE-integrin-β1 antibody are denoted by black bars. (**B**) Integrin-β1 silencing with siRNA delivery, as evaluated by cell surface immunostaining and flow cytometry. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment cells) were measured after 3 days of siRNA treatment in (B-i) K562 and (B-ii) MDA-MB-231 cells. Control siRNA (scrambled; CsiRNA) is shown as white bars while integrin-β1 siRNA in black bars. A polymer/siRNA (weight/weight) ratio of 9:1 with 60 nM of siRNA concentration was used. Significant reduction compared to CsiRNA treatment with each polymer, * $p \leq 0.05$ (n=2).

We additionally evaluated the effect of polymer/siRNA complex on integrin-β1 mRNA levels at different time points, starting from day 1 to day 6 after a single treatment. In K562 cells (**Fig 4.6A**), the mRNA levels can be reduced significantly at day 1 and day 3, but the silencing effect was not significant at day 6 for 1.2PEI-tαLA6-ss, but the knockdown was still present for the Lipofectamine. The knockdown efficiency was higher

(at the same dose of siRNA) and more prolonged from day 1 to day 6 in MDA-MB-231 cells (**Fig 4.6B**), which was also observed in our previous study.

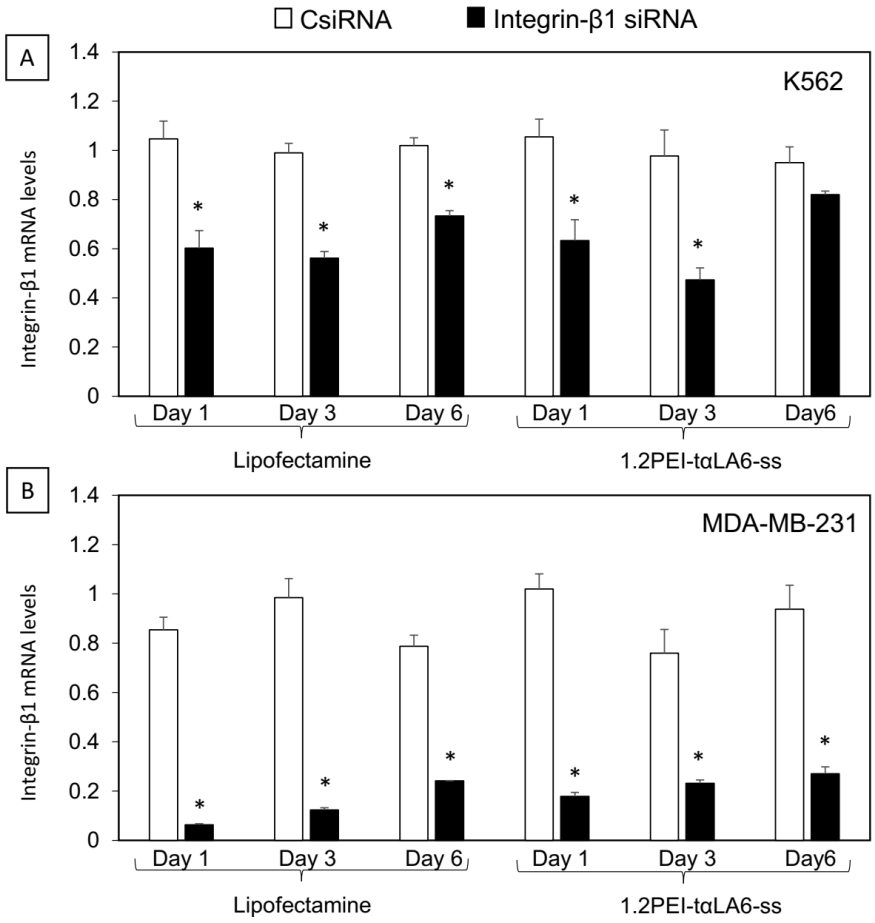


Figure 4.6: Integrin-β1 mRNA levels after siRNA treatment. The mRNA levels of integrin-β1 after 3 days of siRNA treatment with the 1.2PEI-tαLA6-ss was quantified through qRT-PCR (values are plotted relative to no treatment group) in (**A**) K562 and (**B**) MDA-MB-231 cells. Significant difference compared to the CsiRNA treatment of each group, * $p \leq 0.05$ (n=2). CsiRNA: Control scrambled siRNA.

4.3.5 Knockdown of integrin-β1 reduces binding and helps in detachment of K562 cells

Following the successful knockdown of both cell surface protein and mRNA levels of integrin-β1, the functional outcome of this effect was studied by assessing K562 binding to fibronectin and hBMSC. K562 was shown to bind to hBMSC, which contributes towards

the unresponsiveness to drug treatment in the late stage CML and could eventually lead to disease relapse [37–39]. The integrin-β1 reduction helped to significantly decrease its binding ability towards fibronectin and hBMSC when treated with 1.2PEI-tαLA6 polymer (**Fig 4.7A, B**). Similarly, the silencing of integrin-β1 was able to detach the K562 cells from hBMSC monolayer (**Fig 4.7C**).

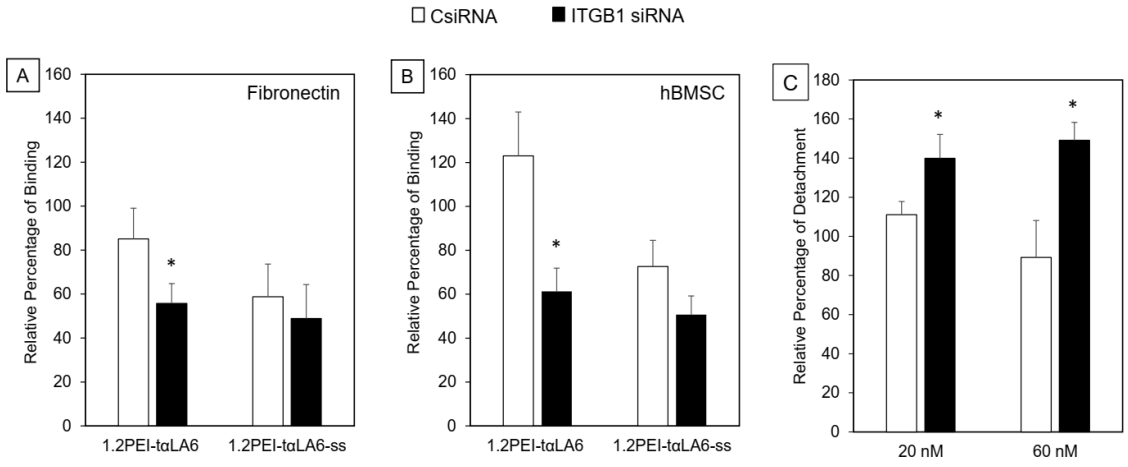


Figure 4.7: Effect of integrin-β1 silencing on the binding ability of K562 cells. (**A**) Binding on fibronectin-coated surfaces: Percentage of K562 cells binding to fibronectin after 3 days of treatment with siRNA using the polymers 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss (n=4). (**B**) Binding towards hBMSCs: Percentage of K562 cells binding to human bone marrow stromal cells (hBMSCs) after 3 days of siRNA treatment. (**C**) Detachment of K562-GFP cells from hBMSC monolayer following 3 days of complex treatment with two different siRNA concentrations and 5 days of attachment. Values are plotted as percentage of cells detached relative to no treatment group (taken as 100%). Significance in comparison to the CsiRNA treatment, * $p \leq 0.05$ (n=3.) CsiRNA: Control scrambled siRNA.

After assessing the detachment of cells from hBMSC monolayer, we also analyzed the cells that remained attached to the monolayers. Separate sources of BMSCs were used in this study. In hBMSC-1, ITGB1 silencing helped to detach most of the K562 cells at 60 nM siRNA treatment. However, with hBMSC-2, no reduction was observed using 60 nM of siRNA concentration (**Fig 4.8A**). Using different sources of hBMSC and with different

concentrations of siRNA (20, 40, 60 and 80 nM), we could observe that hBMSC-3 responded to ITGB1 silencing by decreasing the percentage of attached cells, unlike the K562 cells on hBMSC-4 (**Fig 4.8B**).

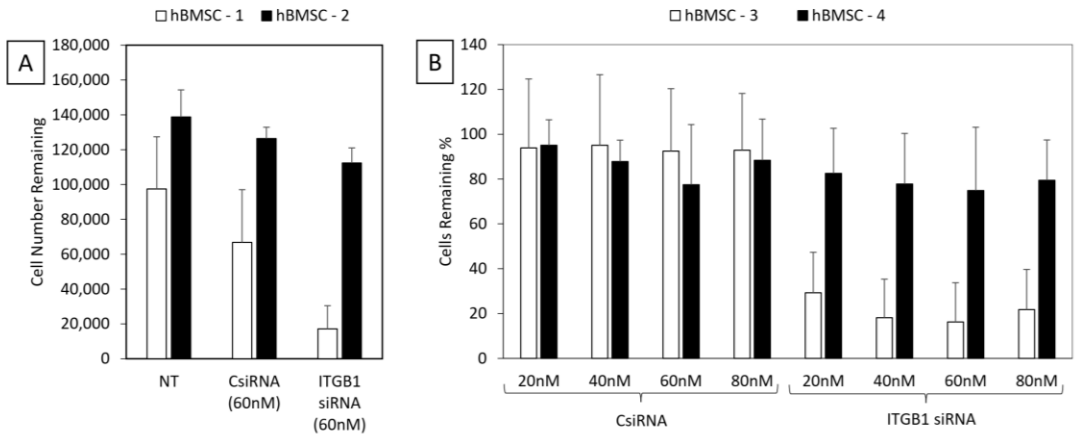


Figure 4.8: Amount of K562 cells remained attached on hBMSC. Integrin-β1 silencing and evaluation of the amount of K562 cells that remained attached to hBMSC from different patient samples. (**A**) Absolute cell number of K562 cells remaining on hBMSC and (**B**) percentage of K562 cells still attached on hBMSC following siRNA treatment at different concentrations analyzed using different hBMSC samples were calculated relative to the attached cells in no treatment group.

4.3.6 Combinational knockdown of integrin-β1 and BCR-ABL to reverse resistance acquired by fibronectin binding

The colony formation by K562 cells that were cultured without fibronectin (WO-FN; white bars) showed a significant drop in the number of colonies when treated with BCR-ABL siRNA/polymer complexes (60 nM siRNA) (**Fig 4.9A, B**). A drop in the colony forming ability of K562 cells on fibronectin (W-FN; black bars) was also seen when the cells were treated with BCR-ABL siRNA, but the decrease in this case was less compared to cells grown in the absence of fibronectin (at both day 7 and day 14). Treatment with integrin-β1 siRNA alone, did not reduce the colony counts at both time points. A

combination of BCR-ABL (30 nM) and integrin-β1 (30 nM) siRNA/polymer complex resulted in colony counts similar to BCR-ABL siRNA/polymer complex treatment alone. Whereas an increase in the integrin-β1 siRNA concentration to 60 nM along with BCR-ABL siRNA of 30 nM resulted in significant reduction of colony counts than the BCR-ABL siRNA/polymer complex treatment alone at day 7 (**Fig 4.9A)**. Therefore, reduced responsiveness to BCR-ABL siRNA treatment observed due to fibronectin binding can be overcome by a combinational knockdown of integrin-β1 in K562 cells (**Fig 4.9A, B**).

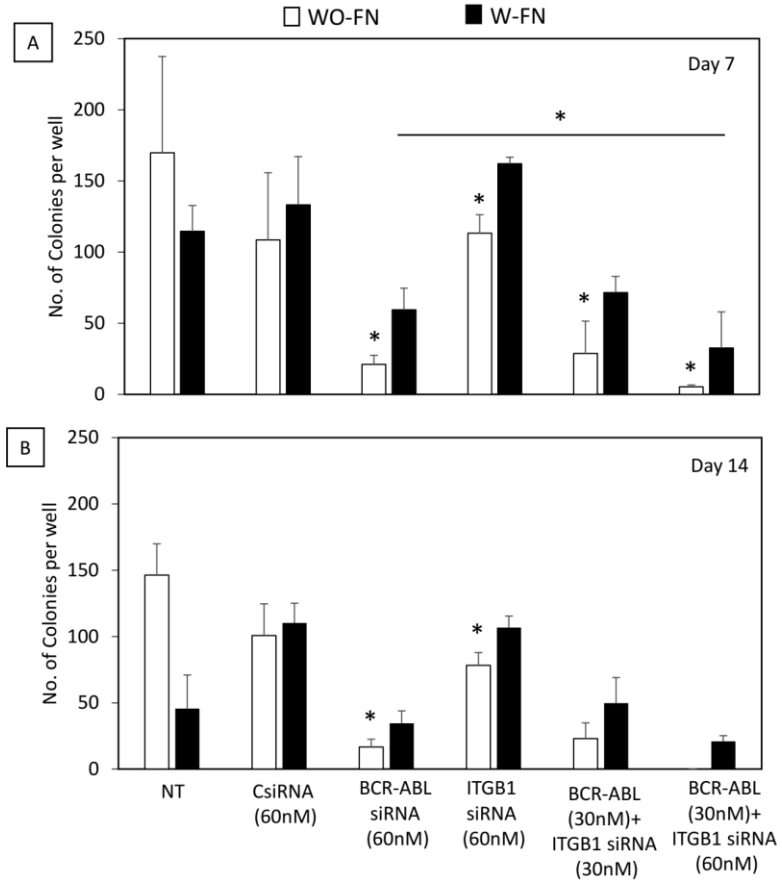


Figure 4.9: Combinational siRNA treatment and its effect on the number of colony forming K562 cells. Cells cultured without fibronectin (WO-FN) are represented by white bars and with fibronectin (W-FN) are represented by black bars. Following treatment, cells were seeded in a semisolid methylcellulose media and colony counts were calculated at (**A**) Day 7 and (**B**) Day 14. Significance between WO-FN and W-FN colony counts *$p \leq 0.05$ (n=2). NT – no treatment cells (without any treatment).

4.4 DISCUSSION

The development of drug resistance in CML was considered to be primarily due to point mutations on the BCR-ABL domain, which alters the binding ability of TKIs. Recent studies have also shown BM microenvironment to play an important role in binding of CML cells and contributing to the observed drug resistance [19,37]. The binding of K562 cells with fibronectin, which is a key molecule that regulates the adhesion process in BM niche via cell surface integrin-β1, conferred resistance to IM treatment in the *in vitro* studies [29,40]. In our initial study to confirm the fibronectin binding ability of CML cells, we could observe a sharp increase in the number of cells binding to fibronectin within a very short period of 4 hours. The sustained binding to fibronectin (from 4 to 24h) could also signify that these cells can stay attached for longer periods of time. Earlier evidence have also shown the fibronectin binding ability of *BCR-ABL* positive cells, providing protection against IM-induced cell death and a decrease in the proliferation which could be a possible survival mechanism to evade drug treatment [13,29]. The binding of CML cells with the aid of integrins could activate various intracellular signaling pathways altering the expression of several genes such as P-gp, Mcl-1, Bcl-xl, and survivin, all of which are involved in IM resistance in CML cells [19]. In addition, the combined *BCR-ABL/*fibronectin signaling can induce morphological changes (cytoskeletal function) in leukemic cells leading to integrin clustering and increased cell adhesion which could also contribute to enhanced resistance to TKI treatment [17,41]. Hence, binding to fibronectin may lead to multiple changes in the CML cells, which necessitates the identification of key integrins involved in this process. Thus, PCR analysis to identify the potential integrin(s) responsible for fibronectin binding were conducted and the results revealed high expression

of integrin-β1 in K562 CML cells. Compelling evidence of integrin-β1 as a key molecule that aids in the binding of CML cells to BM/fibronectin, transform them into a viable target to overcome drug resistance. The fact that integrin-β1 mediated pathway was shown to mediate fibronectin binding by various studies [30–33,36], regulate degradation of Bim pro-apoptotic protein levels [29], prevent apoptosis in *BCR-ABL* positive cells [13], all contributed to our focussing on integrin-β1 as a therapeutic target.

Although integrin-β1 was identified as a key receptor for BM attachment of CML cells, the role of other components of BM microenvironment in drug resistance was also explored by others. The cytokines produced in BM was shown to aid in the survival of *BCR-ABL* positive leukemic cells even in the presence of TKIs and this resistance was reversed, when leukemic cells were isolated from BM and exposed to IM [42]. In addition, culturing of K562 cells in bone marrow stroma-derived conditioned medium resulted in upregulation of STAT3 and increased levels of its target genes Bcl-xl, Mcl-1 and survivin which eventually leads to IM resistance [43]. Hence, the signals received from the BM initiates various downstream signaling pathways which provides *BCR-ABL* independent survival, conferring resistance to TKIs. Targeting of downstream signaling pathways such as PI3K-Akt-mTOR pathway which is shown to be activated in CML cells after IM treatment helps in the survival and resistance, and targeting this pathway resulted in growth inhibition of CML cells [44,45]. Other intracellular pathways such as ERK1/2 and SMAD have also shown to sustain resistance to IM induced apoptosis when CML cells were cultured with the bone marrow stroma cells [37]. All these studies involved direct growth and interaction of CML cells with BM microenvironment or use of bone marrow stroma-conditioned medium, which the CML cells in contact with BM, in turn activates various

signaling pathways for drug resistance. Thus, we speculate that the disruption of the interaction/binding of CML to BM will have therapeutic outcome for overcoming drug resistance.

The other significant integrins expressed in K562 were integrin-α5 and integrin-α_V (**Fig 4.3**), which suggest the possible heterodimerization with integrin-β1 on cell surfaces. An earlier study on cell adhesion-mediated drug resistance in CML unveiled that the binding of K562 cells to fibronectin was via integrin-$\alpha_5\beta_1$ and this contributed to the resistance towards DNA damaging agents such as melphalan, miozantrone and γ-irradiation [46,38]. The knockdown of the single, highly expressed integrin-β1 will reduce its availability to heterodimerize with the other α-subunits. One might additionally have to target other integrins such as integrin-α_5 for increased potency to reduce fibronectin/hBMSC adhesion and this will be explored in our future studies.

For the successful delivery of siRNA into K562 cells, we used lipid-modified 1.2 kDa PEI polymers as they exhibit inherent buffering capacity that helps in endosomal release of the nucleic acid via "proton-sponge" effect [47]. Multiple lipid-modified PEIs and corresponding disulfide (-ss- modified) linked counterparts were screened for this purpose. Lipid substitution on low molecular weight PEIs enhances their cellular binding and permeability across the plasma membrane, whereas the disulfide cross-linked polymers can provide better release of nucleic acid inside the cell due to redox sensitivity of the disulfide linkages [24,48]. In this study, we could observe a significant increase in siRNA uptake after disulfide crosslinking of 1.2PEI-tαLA6 polymer, whereas opposite effect was observed with 1.2PEI-tLA6 polymer (decreased siRNA uptake) (**Fig 4.4A-i**). In attachment- dependent breast cancer cells MDA-MB-231, disulfide crosslinking had

positive effect only with 1.2PEI, whereas detrimental effects were observed with 1.2PEI-tLA2, tLA6 and tαLA2 polymers (**Fig 4.4A-ii**). With 1.2PEI-tαLA6 and PrA1, the siRNA uptake was unaltered after disulfide crosslinking (**Fig 4.4A-ii**). Previous studies with disulfide cross-linked polymers showed comparatively similar results, were the transfection efficiency of 2 kDa PEI was improved post disulfide cross-link, whereas palmitoyl chloride modified PEI did not improve its transfection efficiency [24]. Similar observation were made with disulfide cross-linked 800 Da PEI; the transfection efficiency was improved with disulfide cross-linking and cytotoxicity lowered [48,49]. Based on the comparative siRNA delivery in MDA-MB-231 and K562 cells, 3 polymers (1.2PEI-tαLA6-ss, 1.2PEI-tαLA6 and 1.2PEI-tLA6) could provide high siRNA delivery in both cell lines. The other polymers that did not exhibit high siRNA uptake in K562 cells were not effective in MDA-MB-231 cells as well.

The knockdown of integrin-β1 was performed with the disulfide crosslinked polymers, as they were shown to improve the transfection efficiency and exhibit better intracellular release of the nucleic acid in other studies [48,50]. In K562 cells, the significant knockdown observed with the disulfide cross-linked 1.2PEI-tαLA6-ss polymer was comparable to its counterpart 1.2PEI-tαLA6 (**Fig 4.5B-i**). Similar results were observed with MDA-MB-231 cells which associates with the siRNA uptake of these 2 polymers. In both the cell types, the knockdown efficiency was comparable to that of the commercial transfection reagent Lipofectamine RNAiMAX/2000. As the aim of this study was to evaluate the therapeutic potential of integrin-β1 to overcome drug resistance in CML, we did not explore the various physicochemical properties of the polymers used in this study. To evaluate the decrease in integrin-β1 mRNA levels, we used 1.2PEI-tαLA6-

ss, as its cell surface knockdown efficiency was similar to its unmodified counterpart (1.2PEI-tαLA6). The observed reduction in integrin-β1 mRNA levels (**Fig 5.6A**) correlated with cell surface knockdown (**Fig 4.5B-i**) as well as high siRNA uptake (**Fig 4.4A-i**) with 1.2PEI-tαLA6-ss polymer. A similar outcome was observed with the MDA-MB-231 albeit at much higher extent of silencing (**Fig 4.6B**). This difference could be explained by the high levels of integrin-β1 in MDA-MB-231 when compared to the moderate/low levels in K562 cells (**Fig 4.5A**) as well as ~5-fold increased siRNA delivery in the breast cancer cells. An siRNA concentration of 60 nM was found to be sufficient to perform integrin-β1 knockdown and further increase of siRNA concentration did not affect the outcome at the cell surface levels when treated with the control transfection agent (**Fig 4.S1**). In order to study the applicability of 1.2PEI-tαLA6-ss polymers for other targets, we delivered survivin siRNA and observed significant as well as comparable knockdown to that of the control transfection agent (**Fig 4.S2**), which highlights the high efficacy of this polymer irrespective of the target.

The main objective of this study was to examine the possible reduction of K562 cell binding to hBMSC by integrin-β1 knockdown and improvement of sensitivity to drug treatment. A single treatment with 60 nM of siRNA targeting integrin-β1 was able to reduce the hBMSC binding of K562 CML cells (**Fig 4.7B**) as well as the detachment of hBMSC-attached K562 cells (**Fig 4.7C**). Significant reduction was observed only with 1.2PEI-tαLA6 polymer treatment and not by 1.2PEI-tαLA6-ss polymer which could be explained by the reduced binding observed even with control siRNA (CsiRNA) treated cells, indicating a non-specific effect caused by disulfide cross-linked polymer. Furthermore, the

detachment of K562 cells from hBMSC was confirmed by the reduced amount of K562 cells that remained attached to hBMSC following integrin-β1 knockdown (**Fig 4.8A, B**).

We cultured the K562 CML cells on fibronectin, followed by treatment with polymer/siRNA complexes in order to perform colony formation cell assays. This is a widely used method to study the proliferation and differentiation of hematopoietic cells by looking into the colony counts, which is depended on the ability of K562 cells to form colonies when seeded in a semisolid medium (methylcellulose) [51]. As expected, the knockdown of *BCR-ABL* drastically reduced the colony counts in the absence of fibronectin. However, the presence of fibronectin substrate for cell attachment provided resistance to BCR-ABL siRNA treatment which was observed at day 7 as well as day 14 (**Fig 4.9**). Treatment with integrin-β1 siRNA alone, did not affect the colony formation of K562 cells but the presence of fibronectin increased the colony counts which is due to the favorable growth conditions provided by fibronectin [13,16,29,52]. The significant decrease in the colony counts while using a combinational treatment of BCR-ABL (30 nM) with integrin-β1 siRNA (60nM) highlighted the therapeutic potential of integrin-β1 silencing. But this effect was not observed at day 14, when the colony counts for some groups started to decline. The drastic decline in the colony counts at day 14 of no treatment (NT) when cultured on fibronectin is an interesting observation which could be due to proliferation inhibition due to its binding to fibronectin as reported earlier [53].

Due to the ability of leukemic cells to evade TKIs by residing in the bone marrow niche, we aimed to induce TKI resistance by growing K562 cells on hBMSC for different time points and exposing them to the DA (5 and 10 nM). Furthermore, we also examined the effect on cell viability with a combinational treatment of IM at 3 different concentration

and integrin-β1 silencing. However, we were unable to show that binding to hBMSC would make the K562 cells less effective to TKI treatment in our hands and no difference in cell viability between IM alone, CsiRNA and integrin-β1 treatment was observed (**Fig 4.S3**).

In conclusion, we showed that fibronectin could enhance the binding ability of K562 CML cells in this study. Since this interaction is generally mediated by the presence of integrin-β1, its successful knockdown with the aid of polymeric siRNA delivery helped to decrease it binding to fibronectin, as well as hBMSC, and the detachment of leukemic cells from the stromal layer. The combinational silencing of BCR-ABL and integrin-β1 was found to reduce colony formation especially in the presence of fibronectin. All these outcomes should be further tested in patient cells as well as animal studies to validate the therapeutic potential of integrin-β1 and the polymeric systems to address drug resistance in CML. If the reported results could be successfully translated to these models, combinational silencing of Bcr-Abl and integrin-β1 could be a viable treatment for drug resistant CML clones.

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5. *In vitro* SAFETY STUDIES OF LIPID-SUBSTITUTED LOW MOLECULAR WEIGHT PEIs USED IN GENE DELIVERY SYSTEMS

5.1 INTRODUCTION

Non-viral gene delivery systems have proved to be promising for treatment of various cancers and other disease conditions with advancements in various stages of clinical trials [1]. The ability of these delivery systems to express therapeutic proteins using plasmid DNA, thereby restoring the defective protein, or to knockdown aberrant proteins using RNA interference (RNAi) with short interfering RNA (siRNA) enables researchers to customize interventions according to the requirements of the disease. The success of such delivery systems depends on various factors such as their cationic charge affecting the binding and complexation with the nucleic acids, their ability to protect against nuclease degradation, the uptake efficiency and the localized delivery inside the cell, the intracellular dissociation ability, and the properties to escape endosomal compartment. Most importantly, the ability to remain 'stealth' without causing any adverse reactions in a biological system is of paramount importance irrespective of the application [2–4]. Although viral gene delivery vectors have been traditionally more effective, they carry a high risk of eliciting mutational insertions, carcinogenesis, and immune stimulation [5], which are worrisome for their clinical use.

Recent clinical trials are increasing involving non-viral delivery systems. As representative examples, clinical studies involving a PEG (polyethyleneglycol)-PEI (polyethyleneimine)-cholesterol lipopolymer carrying a plasmid DNA encoding IL-12 gene for ovarian epithelial cancer and fallopian tube cancer treatment in Phase 2 (NCT01118052), a liposomal DOPC (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) delivering EphA2 siRNA for solid tumor in Phase 1 (NCT01591356), a linear PEI-DNA complex (CYL-02) for metastatic pancreatic cancer in Phase 1 (NCT01274455) and for

advanced solid tumors (MK-4621/JetPEI^{TM}) in Phase 1 (NCT03739138). The PEI polymers have been explored extensively since their inception [6] but there are very few reports on the safety of PEI systems and some of which do not provide a direct remark. Although, one of the main advantages of this systems is its inability to cause any major immune response, a proper safety study is of utmost importance for any therapeutic compound during its pre-clinical stages. In a study exploring the use of PEI-DNA delivery as a vaccine, a plasmid DNA expressing human immunodeficiency virus (HIV)-gp120 antigen was successfully administered to enhance IFN-γ secretion from gp120-specific CD8+ T cells. A linear PEI of 22 kDa was employed in this study, although the safety of PEI was not explored, the control group having a PEI solution without the DNA when injected intravenously, did not cause IFN-γ secretion [7]. Another study utilizing the 22 kDa linear PEI/siRNA system successfully downregulated human epidermal growth factor receptor in SPC-A1 (a human lung adenocarcinoma cell line) xenografts in mice, with no significant upregulation of IFN-α and TNF-α levels in the serum [8]. A 2006 study by Kawakami *et al*. on cytokine production by both linear and branched PEI/pDNA in mice employed 10, 25 and 70 kDa branched PEIs and a 25 kDa linear PEI. The results showed low levels of TNF-α, IFN-γ and IL-12 induction after intravenous administration [9]. These studies provide contrasting information on the effect of PEI on cytokine stimulation so that additional studies are warranted to illuminate this issue.

Low molecular weight (LMW) PEIs (0.6, 1.2 and 2 kDa) have gained recent attention due to their low toxicity, however they display poor delivery efficiency [10,11]. This has been successfully addressed by modifying the PEI with small lipids, thereby drastically improving the internalization of nucleic acids to the cells [12–15]. Delivery of

siRNA, pDNA, and mRNA has been accomplished using the lipid modified PEIs in various cell types [16–18]. We have successfully used these systems in the treatment of various cancers such as chronic myeloid leukemia (CML) by silencing BCR-ABL expression [19], in acute myeloid leukemia (AML) by the knockdown of the chemokine receptor CXCR4 [20], in breast cancer by targeting a wide variety of proteins such as cell cycle proteins CDC20 and TTK [18], phosphatases like PPP1R7, PTPN1, PTPN22, LHPP, PPP1R12A and DUPD [21], apoptosis related proteins like BCL2L12 and SOD1 [22], and cell membrane proteins like P-gp and ITGB1 [23,24]. Their application in bone regeneration has also been demonstrated by expressing bone-inducing proteins such as basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) [25]. However, immune stimulation studies for LMW PEIs have not been reported to-date and there is still a paramount need to extensively discern its toxicity and immune response.

Any administered agent will first encounter the red blood cells (RBCs) in circulation whose unintended lysis will be an important concern for any polymeric (multivalent) agent that could lead to severe side-effects [26,27]. The hemolysis-induced release of hemoglobin could become a redox active protein thereby resulting in renal and vascular dysfunction [28]. The binding of the delivery agents with the released hemoglobin can also lead to enhanced elimination of the therapeutic agent [29]. The encounter of the therapeutic agents with immune system could also elicit the secretion of a range of pro-inflammatory cytokines. Among the FDA recommended cytokines to probe immune response are TNF-α, IL-6 and IFN-γ [30]. The pro-inflammatory cytokine, TNF-α can be secreted by activated macrophages, T-cells, and natural killer cells (NK) and the upregulation of TNF-α is shown to cause severe hepatic toxicity. The increased serum

TNF-α levels in various cancers such as leukemia, ovarian cancer and prostate cancer has also been linked to their malignant progression [31–34]. Following the administration of lipopolysaccharide, the increase in TNF-α levels resulted in severe liver toxicity [35,36]. The induction of TNF-α can cause changes to blood coagulation, promote oxidative stress and, interfere with micro-macrovascular circulation [37]. Hence, attempts to neutralize TNF-α using TNF-α receptor1-IgG (TNFR1-Ig) helped to increase the transgene expression and lower production of other proinflammatory cytokines for adenovirus-mediated gene delivery [38]. Upregulation of serum IL-6 in leukemia, ovarian cancer, prostate cancer and breast cancer, was also documented promoting cancer progression, resulting in poor survival and aiding in drug resistance [31–33,39]. Due to the significant role of IL-6 in cancer progression, blocking of IL-6 for cancer treatment is considered a promising therapeutic strategy [39,40]. Lipopolysaccharide induced high IL-6 levels was shown to drastically affect neuronal and cognitive functions through STAT3 activation in brain [41,42]. IL-6 upregulation can affect muscle homeostasis leading to increased tissue degeneration in mice by accumulation of reactive oxygen species in skeletal muscle and similar to TNF-α, IL-6 also plays a vital role in liver toxicity [43,44]. Shielding of lipid nanoparticles by PEG reduced stimulation of both TNF-α and IL-6 *in vitro* and *in vivo* studies [45]. IFN-γ is another major cytokine that is widely produced by the NK cells and believed to be a potent regulator of immune response [46–48]. IFN-γ was demonstrated to directly influence the expression of TNF-α during lipopolysaccharide treatment, contributing to the afore mentioned functions of TNF-α [49]. Recent studies have documented the tumor promoting property of IFN-γ especially at low levels [50,51]. High expression of IFN-γ can inhibit the process of erythropoiesis, hinder hematopoietic

progenitor precursor formation, and play a major role in autoimmune disorders in addition to its contribution toward graft rejection [52,53]. These observations highlight the detrimental effects of upregulated levels of these cytokines and the need to assess them to understand the immune response and toxicity associated with any therapeutic agent.

In this study, we evaluated the toxicity of polymer/DNA complexes with T-lymphocyte model Jurkat cells and peripheral blood mononuclear cells (PBMCs) isolated from fresh human buffy coats. The hemolysis potential of LMW PEIs with lipid modifications has been studied. We used the most successful polymers which has been well studied and reported for their high efficiency in different cancer types and disease conditions. Hemolysis potential was studied for polymers on their own without the nucleic acid to investigate the role of individual lipid molecules used for substitution on PEI. A dose response for hemolysis was established in different patient samples as well as a function of complex formulation (i.e., weight/weight ratios of polymer to DNA and the presence/absence of polymeric additives [54,55]) For the first time, the immune stimulatory behavior of the modified polymers themselves, and as polymer/DNA complexes has been studied in detail among nine different sources of PBMCs. The protein concentration as well as mRNA levels of TNF-α, IL-6 and IFN-γ were documented after 24 hours of exposure to the investigated agents. In addition, a PEI library containing a much wider range of lipids substitutions was used to better understand its influence in upregulating the expression of these three cytokines at the mRNA level.

5.2 MATERIALS & METHODS

5.2.1 Materials

The PEI of 0.6 kDa (0.6PEI), 1.2 kDa (1.2PEI) and 2.0 kDa (2PEI) were purchased from Polysciences (Warrington, Pennsylvania) while 25 kDa PEI (PEI25) was from Sigma-Aldrich (St. Louis, MO). Alpha linolenoyl chloride (αLA), cholesteryl chloroformate (Chol), linolenoyl chloride (LA), lauroyl chloride (Lau), propionyl chloride (PrA), caproyl chloride (CAA), capryloyl chloride (CA), palmitoyl chloride (PA), stearoyl chloride (St), thiazolyl blue tetrazolium bromide (MTT), anhydrous dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), chloroform and polyacrylic acid (PAA) 2 kDa were purchased from Sigma-Aldrich. Roswell Park Memorial Institute (RPMI) 1640 Medium with L-glutamine and HEPES, Hank's Balanced Salt Solution (HBSS), penicillin, streptomycin, UltraPure DNase/RNase-free dH_2O, TRIzol reagent, chloroform, acetic acid, Lipofectamine^{TM} 2000 were procured from Fisher Scientific (Ottawa, Canada). Fetal bovine serum (FBS) was from VWR Life Science Seradigm (Mississauga, Ontario). Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (IO) were from STEMCELL Technologies (Vancouver, Canada). TNF-α, IL-6 and IFN-γ primers used for RT-qPCR were from Integrated DNA Technologies (Coralville, USA). Human TNF-α, IL-6 and IFN-γ Duo Set ELISA kits were procured from R&D Systems (Minneapolis, MN). The gWIZ-GFP plasmid DNA was obtained from Aldevron (Fargo, ND).

5.2.2 Polymer Synthesis & Complex Preparation

Lipid substitution of PEIs were prepared by N-acylation with each polymer having: propionic acid (PrA, C3), caproic acid (CAA; C6), caprylic acid (CA; C8), lauric acid (Lau;

C12), palmitic acid (PA; C16), linoleic acid (LA; C18), stearic acid (St; C18) and cholesterol (Chol) substituents using previously published protocols [55]. Briefly, the lipid and PEI were dissolved separately in ice-cold chloroform for 30 min. Triethylamine (TEA) was added to the PEI solution followed by drop-wise addition of the lipid solution while stirring on ice-bath for overnight. The mixture was precipitated using ice-cold diethyl ether and dried under vacuum for 2 days. LA and tαLA were also linked to PEI by thioester bonds forming tLA and tαLA, as described in [55]. For this purpose, the lipid and mercaptopropionic acid (MPA) were dissolved in trifluoracetic acid separately and MPA solution was added dropwise to the lipid solution under stirring for 3 h at room temperature (RT). The carboxyl end-capped lipid solution was collected by ice-cold hexane and vacuum dried. PEI grafted with this tLA or tαLA was prepared by EDC/NHS activation, were EDC and the lipid dissolved in chloroform were mixed together under stirring for 1 h at RT. NHS methanol solution was added drop-wise to the above solution for another 1 h before adding to PEI solution in chloroform overnight. The final product was precipitated using ice cold diethyl ether and dried under vacuum for 48 h. 0.6, 1.2 and 2.0 kDa PEI were used for this purpose. In addition, redox-sensitive polymers (-SS-) were synthesized by condensation reaction using cystamine bisacrylamide (CBA) as the cross-linker. The degree of lipid substitution on the modified PEIs were determined by ^{1}H-NMR spectroscopy (**Table 5.1**). Complexes were prepared at different weight ratios of the polymer to pDNA and PAA by adding the calculated volumes of polymer solution to the solution containing a mixture of nucleic acid and PAA in serum-free media or 0.9% NaCl solution and incubated for 30 min at RT before usage. Lipofectamine^{TM} 2000 was used according to the manufacturer protocol.

Polymer Name	Molecular Weight	Lipid Substituent	No. of Substituent
1.2PEI-tαLA6	1.2kDa PEI	α-Linoleic acid (C18)	2.7
1.2PEI-Chol3	1.2kDa PEI	Cholesterol	2.2
1.2PEI-LA6	1.2kDa PEI	Linolenic (C18)	2.55
0.6PEI-Lau4	0.6kDa PEI	Lauric acid (C12)	2.75
1.2PEI-Lau8	1.2kDa PEI	Lauric acid (C12)	3.6
1.2PEI-PrA1	1.2kDa PEI	Propionic acid (C3)	0.76
1.2PEI-tαLA6-ss	1.2kDa PEI	α-Linolenic acid with disulfide linkage	2.55

Table 5.1: A summary of the well-studied lipid-modified PEI delivery systems used in the study with the molecular weight of the base polymer, the type of lipid substituent and the number of substituents.

5.2.3 Cell Models

The human T-lymphocyte cell line Jurkat was cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin at 37°C under 5% CO_2. Cells were grown in T75 flasks and every two to three days, the cells were centrifuged at 1200 rpm for 5 min to remove spent medium before diluting it 10 times in 20 mL of fresh medium.

To obtain peripheral blood mononuclear cells (PBMC), buffy coats from Canadian Blood Services were diluted with equal volume of HBSS. 15 mL of Ficoll-Paque Plus was added to a 50 mL tube and 20 mL of the diluted blood was carefully layered over it. After centrifugation at 400g for 30-40 min at room temperature, upper plasma layer was removed without disturbing the interface lymphocyte layer. The lymphocytes were collected in a new tube and fresh HBSS (x3 volume) was added before centrifugation at 100g for 10 min. The supernatant was removed, and the cells were suspended and centrifuged in HBSS again before culturing the PBMC in RPMI 1640 containing 10% FBS, 100 IU ml^{-1}

penicillin/streptomycin and 40 IU ml $^{-1}$ IL-2 at 37°C with 5% CO_2. The medium was changed every 3-4 days without splitting the cells.

Red blood cells (RBC) were obtained during the isolation of PBMC using the Ficoll-Paque method above. The bottom layer containing the RBC were collected separately and stored at 4°C (the FDA recommended storage temperature for RBC is between 1°C and 6°C [56]) until further use. Further RBC were suspended in three times the volume of HBSS and centrifuged at 500g for 10 min for hemolysis assay.

5.2.4 Cellular toxicity by MTT assay

Jurkat and PBMC cells were treated with 1 μg/mL of gWIZ-GFP complexes at 0, 0.5, 1, 2, 4, 6, 8, and 10 weight/weight (w/w) ratios of polymer to pDNA for 3 days and the viable cells were quantitated using the MTT assay. With PAA additive complex treatments, 1 μg/mL of gWIZ-GFP complexes at polymer:pDNA ratios of 5, 7.5 and 10 was used for cell treatments. The complexes had either no PAA or PAA was added to pDNA solution for complex formation at the w/w ratio of 1:1 (pDNA:PAA). After incubation with complexes, MTT at 1 mg/mL of final concentration was added to the cells and incubated for 37°C for 2-3 hours following which the cells were centrifuged at 2000 rpm for 10 min and the supernatant was removed. The crystal pellets were dissolved in DMSO and absorbance was recorded at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments).

5.2.5 Hemolysis assay

RBC isolated from the buffy coat was washed three times with HBSS and diluted 10 times in HBSS for use in the assay. Briefly, 100 µL of polymer solutions at 2.5, 5, 7.5, 10, 15, 20, 50, 100, 250, 500 µg/mL of final concentration were added to a 'U' bottom 96-well plate and 100 µL of diluted RBC in HBSS was added to the wells and incubated at 37 °C for 30 min. Complexes prepared at ratios 5, 7.5 and 10 (polymer:pDNA) without and with PAA additive (1:1 pDNA:PAA ratio) using gWIZ-GFP pDNA were used at final pDNA concentration of 1 µg/mL. The plate was then centrifuged at 500g for 10 min and 100 µL of supernatant was removed carefully without disturbing the intact RBC pellet. The absorbance of heme was recorded at 570 nm using EL_x800 Universal Microplate reader (Bio-Tek Instruments). As additional controls, RBC was incubated in water, saline or 0.1% Triton-X solution during the assay.

5.2.6 Cytokine quantification by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The mRNA levels of TNF-α, IL-6 and IFN-γ following 24 h of treatment was quantitated by RT-qPCR. Following complex treatments, Jurkat or PBMC cells were collected, and total RNA was extracted by TRIzol following which cDNA was synthesized using SensiFast™ kit (Bioline Meridian Bioscience) according to the manufacturer's protocol. PCR was carried out on a StepOnePlus™ Real-Time PCR system with β-actin as housekeeping gene; forward: 5'-GCGAGAAGATGACCCAGAT-3', reverse: 5'-CCAGTGGTACGGCCAGA-3' with TNF-α; forward: 5'-CCAGGGACCTCTCTCTAATCA-3' reverse: 5'-TCAGCTTGAGGGTTTGCTAC-3',

IL-6; forward: 5'-CCTAGAGTACCTCCAGAACAGA-3' reverse: 5'-CAGGAACTGGATCAGGACTTT-3', IFN-γ; forward: 5'-AGTGATGGCTGAACTGTCGC-3' reverse: 5'-CTGGGATGCTCTTCGACCTC-3'. Briefly, 10 µl of reaction mixture containing 5 µl of SYBR Green master mix, 2 µl of 10 µM primers and 3 µl of 5 ng/µl cDNA was used. The reaction was setup at 95°C for 10min before advancing to 40 cycles of denaturation at 95°C for 15s and annealing/elongation at 65°C for 30s on a MicroAmp Fast Optical 96-well plate. ΔC_T, $\Delta\Delta C_T$ and Relative Quantity (RQ) of mRNA for TNF-α, IL-6 and IFN-γ were calculated with β-actin as reference relative to no-treatment.

5.2.7 Cytokine quantification by enzyme-linked immunosorbent assay (ELISA)

Jurkat and PBMC cells were treated with the desired complexes for 24 h, following which the supernatants were collected after centrifuging the cells at 2000 rpm for 10 min. The quantity of TNF-α, IL-6 and IFN-γ proteins secreted in the supernatant was quantitated using the validated human Duo Set ELISA kits from R&D Systems according to the protocol provided by the manufacturers.

5.2.8 Statistical analysis

Data were summarized as mean with standard deviation and statistical analysis were performed using unpaired *t* test between two group means. A p-value < 0.05 was considered as statistically significant indicated by (*) for reduction and (#) for upregulation. Each RBC and PBMC used are from distinct healthy blood donors as indicated by suffix number.

5.3 RESULTS

5.3.1 Effect of LMW PEI complexes on cytotoxicity

The lipid-modified PEIs used in this study were previously analyzed for their toxicity using a variety of cancerous and non-cancerous cells. Irrespective of the delivery agent, once injected into animal models or into humans in clinical studies, the main cell type that immediately encounter these nanoparticles are the cells in circulation, the mononuclear cells and the RBC. Hence, we initially used human T-lymphocyte Jurkat cells as well as PBMCs isolated from different patients to assess direct toxicity effects of the complexes to better understand the effect of different lipid substituents and other physicochemical parameters on the toxicity of the complexes. First, we studied the toxicity of the complexes prepared with gWIZ-GFP pDNA at a range of polymer to pDNA weight ratios (0.5, 1, 2, 4, 6, 8, and 10 w/w). Following 3 days of treatment, the percent toxicity was calculated using the MTT assay relative to the no treatment group. In Jurkat cells, ~80% or higher percentage of cells remained viable with 1.2PEI-tαLA6 and 0.6PEI-Lau4 at all the ratios along with the control agents PEI25 and Lipofectamine 2000 (**Fig 5.1**). Other polymers also showed ~80% viability at most ratios except at isolated points for some polymers; 1.2PEI-Chol3 (except at ratio 4), 1.2PEI-LA6 (except at ratio 10 with significant drop in viability), and 1.2PEI-Lau8 (except at ratios 8 and 10). However, with 1.2PEI-PrA1, toxicity increased in a polymer dose dependent (weight/weight ratio) manner, and we could observe that only 70%, 55% and 37% cells remained viable at ratios 6, 8 and 10, respectively (**Fig 5.1**).

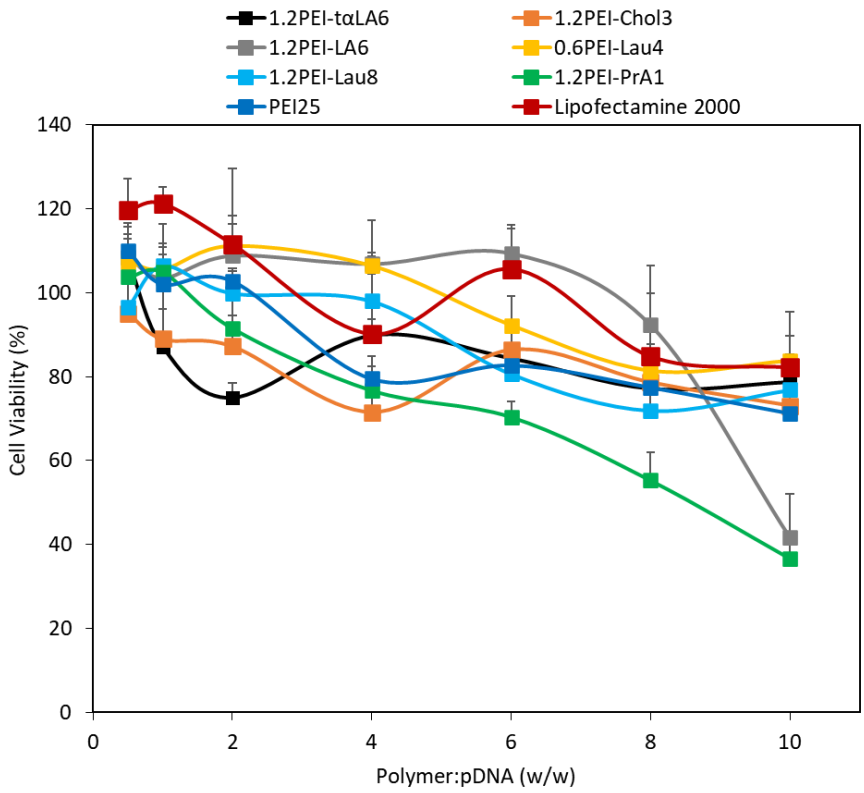

Figure 5.1: Viability of Jurkat cells exposed to complexes at different weight/weight (w/w) ratios of polymer to pDNA following 3 days of treatment. The cell viability was assessed by the MTT assay and percent viability was calculated relative to the viability of no treatment group (taken as 100%).

Three separate donor sources of PBMCs (PBMC-1, PBMC-2 and PBMC-3) were next used for toxicity assessment. In PBMC-1, 1.2PEI-tαLA6 and 1.2PEI-Chol3 did not exhibit any toxicity and with the rest of polymers, we could observe >85% viability in most of the treatment groups except 1.2PEI-LA6 that showed ~65% at ratios 8 and 10 and 1.2PEI-PrA1 that displayed ~85% at ratio 2 and lower viabilities of ~60% at ratios 4 to 10 (**Fig 5.2A**).

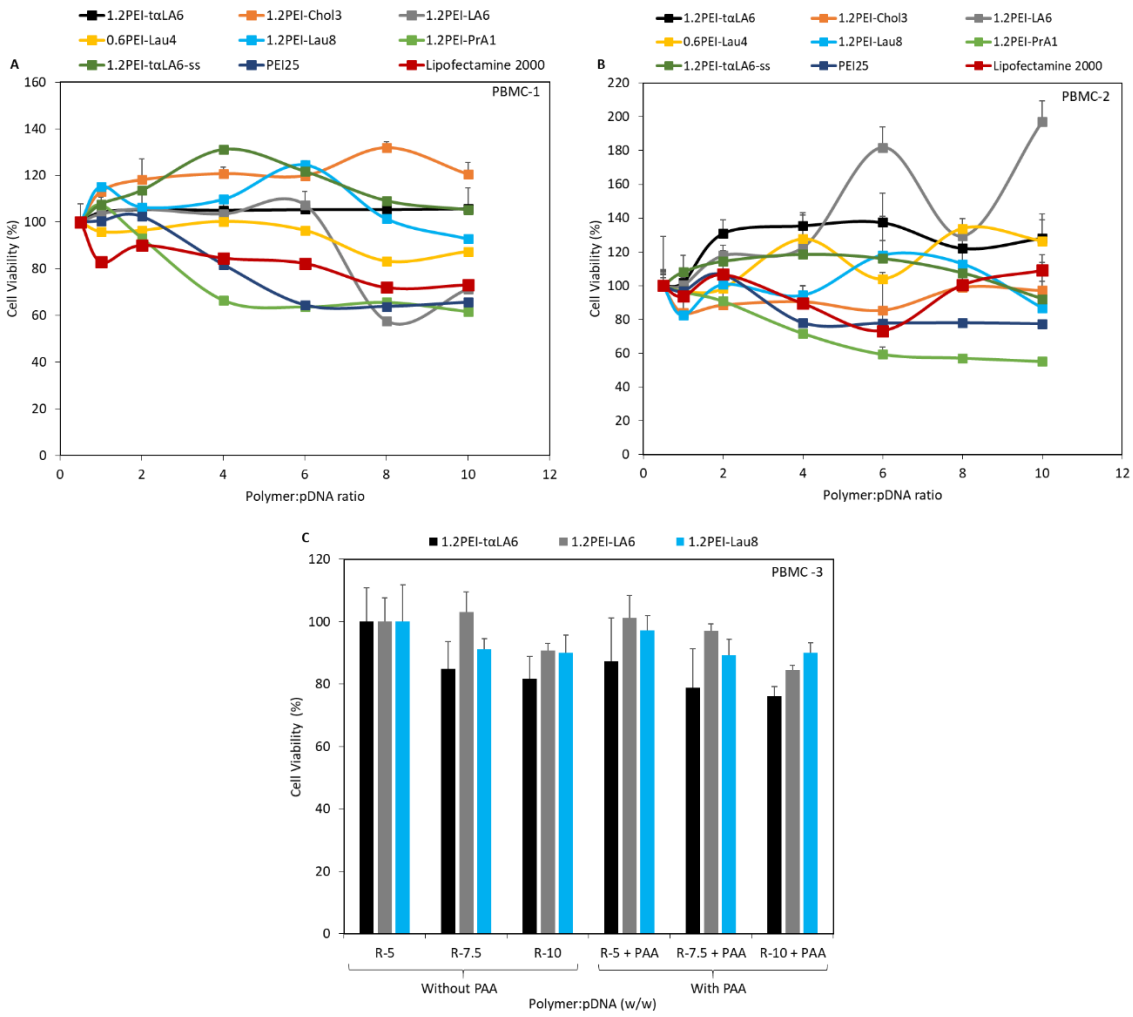

Figure 5.2: Cell viability of complexes at different weight/weight (w/w) ratios of polymer to pDNA following 3 days of treatment assessed by MTT assay, normalized to the lowest ratios for each polymer in (**A**) PBMC-1 and (**B**) PBMC-2. (**C**) Influence of PAA as additive on the *in vitro* toxicity; for selective polymers as model delivery agents, cells were treated without PAA and with PAA at pDNA to PAA weight/weight ratio of 1:1 and evaluated by MTT assay in PBMC-3.

With PBMC-2, 1.2PEI-tαLA6, 1.2PEI-Chol3, 0.6PEI-Lau4, 1.2PEI-Lau8 and 1.2PEI-tαLA6-ss showed >80% viability at all ratios tested (**Fig 5.2B**). Surprisingly, 1.2PEI-LA6 showed viability beyond 100% (indicating some degree of metabolic activation of the cells) but remained non-toxic irrespective of the ratio assessed. The 1.2PEI-PrA1, exhibited a dose dependent increase in toxicity similar to PBMC-1 results

(**Fig 5.2A** and **5.2B**). The controls, PEI25 showed ~60% cell viability in PBMC-1 and ~75% in PBMC-2 at high ratios whereas Lipofectamine 2000^{TM} showed 70-80% or higher viability.

To assess the impact of PAA additive on PBMC toxicity, we selected 3 polymers (1.2PEI-tαLA6, 1.2PEI-LA6 and 1.2PEI-Lau8) and treated PBMC-3 with complexes prepared at ratios 5, 7.5 and 10 (polymer:pDNA ratio) without and with PAA (**Fig 5.2C**). We did not observe any difference between the toxicity exerted by the PAA complexes and complexes without PAA. The 1.2PEI-LA6 showed ~85% cell viability at R:10 and was less toxic at lower ratios. The presence or absence of PAA did not influence the cell viability. The 1.2PEI-tαLA6 and 1.2PEI-Lau8 also showed similar viability of ~80% or higher. (**Fig 5.2C**).

5.3.2 Effect of PEI complexes on RBC hemolysis

The RBC lysis was next investigated to better understand the lytic activity of complexes towards cell membrane. RBCs from 2 different patients were used to study the hemolysis of the bare polymers at different concentrations as well as polymer/pDNA complexes at different weigh ratios and with/without the PAA as additive. With RBC-1, the polymers were diluted in water and the heme (absorbance) released with the positive control Triton X-100 solution was taken as 100% hemolysis. The complexes were tested in the practical concentration range used for cell modifications (pDNA conc of 1 μg/mL with polymer:pDNA ratios of 5 to 10). The overall hemolysis percentage ranged between 0 and 10% except 1.2PEI-LA6 and the control (water) showed 8% hemolysis by itself, hence water could have contributed to the observed lysis irrespective of the delivery agent.

The inclusion of PAA as an additive helped to reduce the hemolysis observed at ratio 7.5 with 1.2PEI-LA6 (representing the most hemolytic complex), from 31% without PAA to 19% with PAA ($p<0.05$) (**Fig 5.3A**). With bare polymers, the concentrations used were gradually increased in excess of practical concentrations used for cell treatments (up to 500 μg/mL). It was clear that all polymers displayed a dose-dependent increase in hemolytic activity; 1.2PEI-Chol3, PEI25 and Lipofectamine 2000^{TM} displayed ≤5% hemolysis with concentration as low as 5 μg/mL and 1.2PEI-Chol3, PEI25 showed >5% lysis only at 20 μg/mL. The highest hemolysis was seen with 1.2PEI-LA6 (**Fig 5.3A** inset).

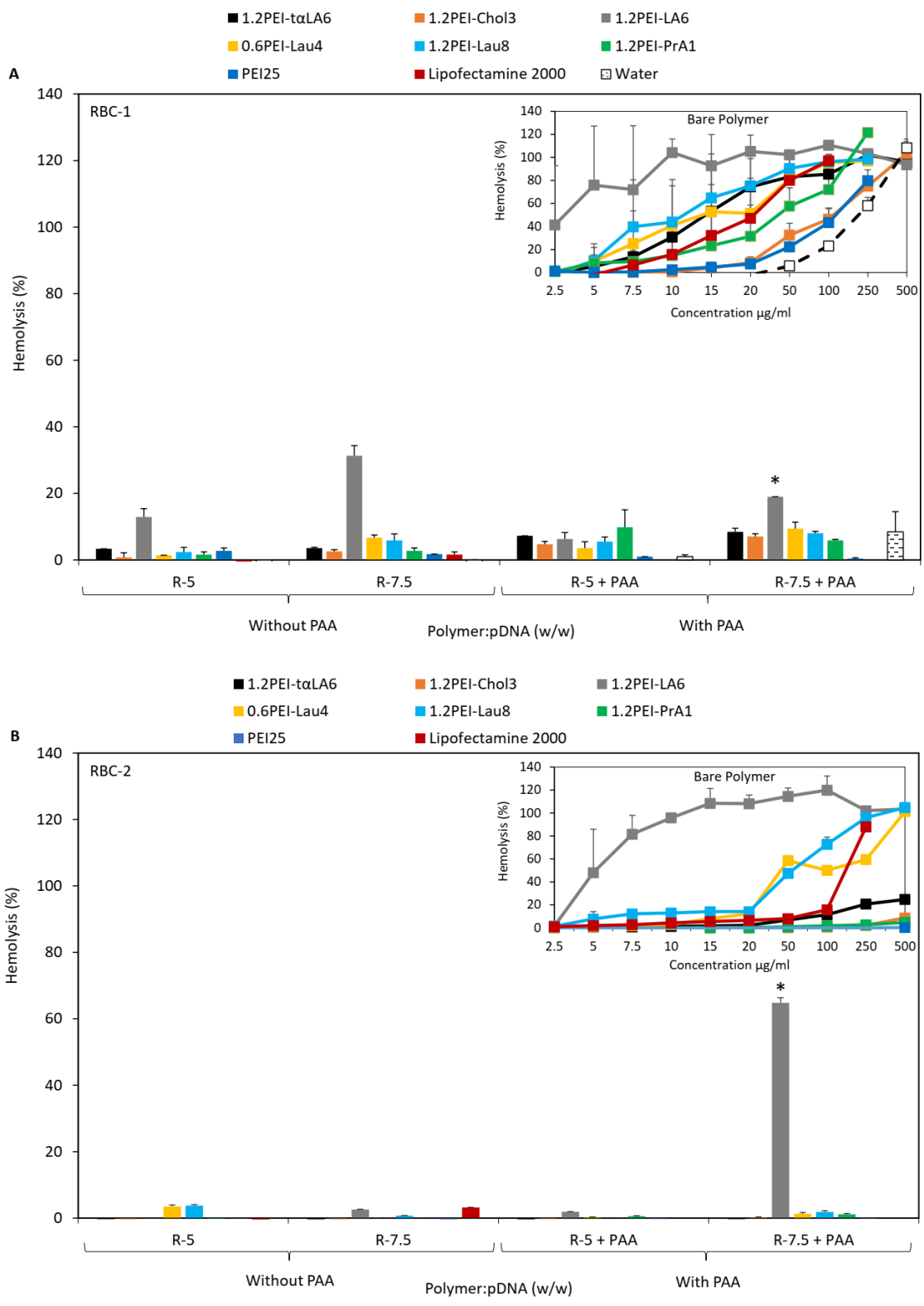

Figure 5.3: Hemolysis by polymer/pDNA complexes at weight/weight ratios of R:5 and R:7.5 without and with PAA as additive, evaluated by incubating for 30 minutes with

diluted RBC from 2 sources, (**A**) RBC-1 and (**B**) RBC-2. Inset represents the hemolysis of bare polymer without pDNA for different agents at varying concentrations. The percentage of hemolysis was calculated relative to heme absorbance recorded with the treatment of Triton X - 100 solution taken as 100% hemolysis. The significant difference in hemolysis with PAA has been denoted by * with $p \leq 0.05$ compared to without PAA of the respective group.

With RBC-2, the polymers were diluted in saline (0.15 M NaCl) solution. With the pDNA complexes, the highest hemolysis recorded in the absence of PAA was 4%, and similar outcomes were obtained with the addition of PAA in complexes. The only exception was the complexes prepared with 1.2PEI-LA6, where significant hemolysis was (65%) was seen with PAA complexes at ratio 7.5. in line with results in RBC-1 (where complexes were diluted in water). Interestingly, the inclusion of PAA caused hemolysis in RBC-2, which contrasted with the observations with RBC-1 (compare **Fig 5.3A** and **Fig 5.3B**; note again that the solvent was different between the 2 tests). For the bare polymers (**Fig 5.3B** inset) the results showed that 1.2PEI-tαLA6 remained non-toxic (i.e., <5% hemolysis) up to 50 µg/mL (7% at this concentration), 1.2PEI-tαLA6-ss up to 5 µg/mL (~7%), 1.2PEI-Lau8, 0.6PEI-Lau4 and Lipofectamine 2000^{TM} up to 15 µg/mL (~8%) and 1.2PEI-Chol3 and 1.2PEI-PrA1 up to 500 µg/mL (~8%). The PEI25 was apparently non-toxic in this assay. The only polymer with high hemolysis (e.g., 50% at concentrations as low as 5 µg/mL) was again 1.2PEI-LA6, as in RBC-1.

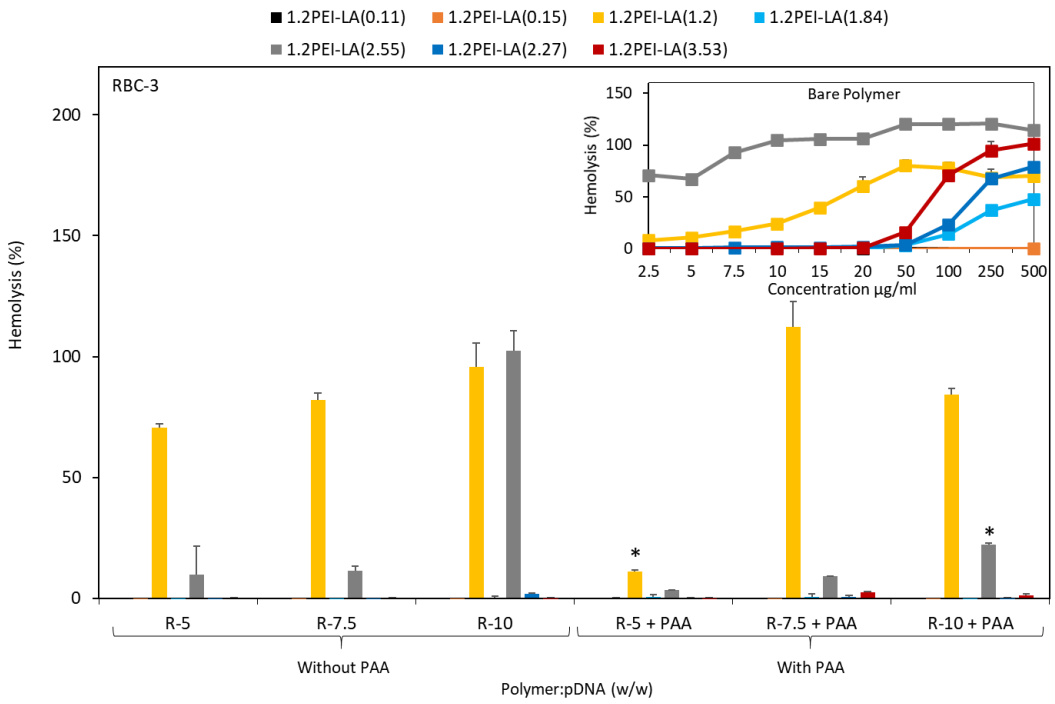

Figure 5.4: Hemolysis by LA-substituted polymers having different levels of substitution in the presence of polymer/pDNA complexes at weight/weight ratios of R:5, R:7.5 and R:10 without and with PAA as additive in RBC-3 evaluated by incubating for 30 minutes with diluted RBCs. Inset represents the hemolysis of bare polymers at varying concentrations without pDNA. Hemolysis % was calculated relative to Triton X - 100 treatment taken as 100% lysis. The significant decrease in hemolysis with PAA has been denoted by (*) with $p \leq 0.05$ compared to without PAA of the respective group. The level of LA substitution in the modified PEIs are indicated in the brackets of the legend.

Since 1.2PEI-LA6 on its own displayed relatively high hemolysis in all RBC samples, we employed 1.2PEI polymers with different levels of LA substitution to evaluate if the observed effect is solely due to the presence of LA or whether this batch of polymer has exceptional features. Using a new source of RBC (RBC-3) and saline as solvent, low LA-substituted polymers 1.2PEI-LA(0.11) and 1.2PEI-LA(0.15) was non-lytic irrespective of the concentration used, whereas 1.2PEI-LA(1.2) and 1.2PEI-LA(2.55) caused >5% hemolysis even at the lowest 2.5 µg/mL concentration. 1.2PEI-LA(1.84), 1.2PEI-LA(2.27) and 1.2PEI-LA(3.53) remained non-lytic up to 20-50 µg/mL (**Fig 5.4** inset). With

polymer/pDNA complexes without PAA, the 1.2PEI-LA(1.2) displayed high hemolysis at all ratios while the addition of PAA reduced the hemolysis. 1.2PEI-LA(2.55) was lytic at ratio 10 and PAA decreased its effect drastically (**Fig 5.4**). Therefore, the lytic activity appeared to be related to PEI polymers substituted with medium levels of LA.

5.3.3 Comparison of TNF-α, IL-6, and IFN-γ mRNA levels in complex treated Jurkat and PBMC

To evaluate pro-inflammatory cytokine production, we measured the FDA recommended cytokines TNF-α, IL-6 and IFN-γ [30]. We first evaluated the secretion of these cytokines at the transcript level in Jurkat T-cells using RT-qPCR after 24 h of treatment with 1.2PEI-tαLA6/pDNA complexes since 1.2PEI-tαLA6 was a leading carrier for pDNA delivery compared to other polymers from previous observations (**Fig 5.5**). In order to evaluate the impact of pDNA concentration as well as polymer:pDNA (weight/weight) ratio, we used 0.5, 1 and 2 μg of pDNA at ratios 5 and 10, without and with PAA as additive. PMA/IO combination was used as positive control for cytokine stimulation at 3 concentrations. The relative quantity of TNF-α was unchanged for all the treatment groups, with positive controls exhibiting maximum stimulation (~460-fold) at 10 ng/0.5 μg of PMA/IO. The only exception was the complexes at ratio 5 and 1 μg/mL pDNA, where the TNF-α mRNA levels were upregulated (~60-fold). The relative mRNA levels of IL-6 increased in a dose dependent manner with the positive controls, however the levels remained unchanged for most of the complex treatment groups except for ratio 5 with 0.5 and 1 μg/mL pDNA again. Similarly, IFN-γ mRNA levels were increased with PMA/IO treatment and for complexes at polymer:pDNA ratio of 5 with 0.5 and 1 μg/mL

pDNA concentrations. All other complex formulations showed negligible change in IFN-γ expression (**Fig 5.5**).

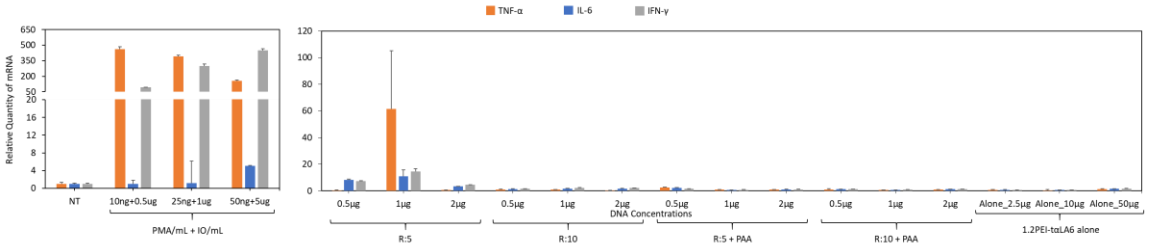

Figure 5.5: Relative quantity of cytokine mRNA levels assessed after 24 hours of treatment of Jurkat cells with positive controls (PMA/IO), and 1.2PEI-tαLA6/pDNA complexes at ratios R:5 and R:10 without and with PAA. The concentrations of the complexes were 0.5, 1 and 2 µg/mL (pDNA concentrations) along with 1.2PEI-tαLA6 polymer alone at 2.5, 10 and 50 µg/mL concentration.

Next, cytokine stimulation was determined in PBMCs based on mRNA and protein quantification. The highest TNF-α mRNA levels in PBMC-3 (~19-fold) were observed with the positive control 50 ng/mL and 5 µg/mL of PMA/IO, which was in line with the protein concentrations in the supernatant (1150 to 1650 pg/mL; **Fig 5.6A**). All other groups showed a lower TNF-α mRNA increase (~4-fold), which was constant regardless of the ratio and pDNA concentration of complexes. The TNF-α protein concentrations in supernatants were also >10-fold decreased compared to positive PMA/IO controls (ranging from 40 to 100 pg/mL in treatment groups while the no treatment had 15 pg/mL). A significant reduction in TNF-α protein amount was observed in the presence of PAA at three treatment groups, R:5 with 2 µg/mL pDNA, R:10 with 1 and 2 µg/mL pDNA.

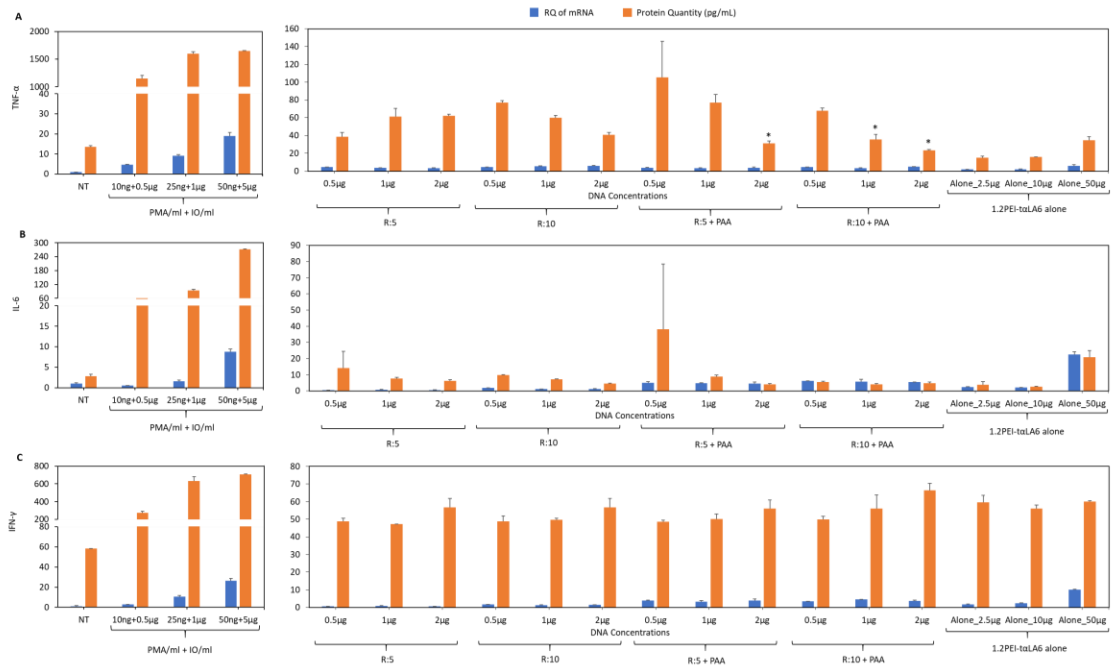

Figure 5.6: Cytokine levels assessed after 24 hours of treatment in PBMC-3 with positive controls (PMA/IO), 1.2PEI-tαLA6/pDNA complexes at ratios R:5 and R:10 without and with PAA at 0.5, 1 and 2 µg/mL pDNA concentrations along with 1.2PEI-tαLA6 alone at 2.5, 10 and 50 µg/mL. The relative quantity (RQ) of mRNA was quantified by the RT-qPCR (relative to no treatment cells) and protein quantity by specific ELISAs. (**A**) TNF-α, (**B**) IL-6 and (**C**) IFN-γ. Significance compared to the respective treatment groups in the absence of PAA, * $p \leq 0.05$.

The relative levels of IL-6 (both mRNA and protein) increased in a dose dependent manner with the positive controls resulting in ~10-fold stimulation at mRNA level and a protein concentration ranging between 20-270 pg/mL (**Fig 5.6B**). Negligible change (relative quantity of 2 to 6-fold for IL-6) was seen with the PAA additive groups in mRNA levels which was also true with IL-6 protein amounts. Among the complexes, only at R:5 with PAA having 0.5 µg/mL pDNA showed upregulation. Treatment with 1.2PEI-tαLA6 alone at highest concentration of 50 µg/mL caused a clear increase (~22-fold) in IL-6 mRNA and protein levels (**Fig 5.6B**). Similar to other cytokines, the IFN-γ mRNA levels increased with PMA/IO concentrations at both mRNA and protein levels (**Fig 5.6C**). All

other complex formulations showed negligible change in INF-γ levels in PBMC. The 1.2PEI-tαLA6 alone at 50 μg/mL caused an increase in IFN-γ mRNA levels (~10-fold), which was also previously observed with IL-6 expression, but this was not reflected in the IFN-γ protein amounts. The PAA addition in complexes did not give any obvious changes in IFN-γ expression (**Fig 5.6C**).

5.3.4 Impact of pDNA concentration, ratio (polymer to pDNA) and the additive on cytokine secretion

Since variations in cytokine response among donors are known [57,58], we then investigated the cellular upregulation (mRNA levels) and secretion (protein concentration in supernatants) of the cytokines in 3 additional PBMC samples (PBMC-4 to PBMC-6) using similar complexes. TNF-α mRNA levels were strongly upregulated with the positive control, although to a lesser extent in PBMC-5 and 6 (**Fig 5.7A**) and a similar observation of weak TNF-α secretion was seen in PBMC-6 in which 76 pg/mL was the highest compared to 400-1600 pg/mL TNF-α in other PBMCs (**Fig 5.8A**). Untreated PBMCs gave ~15 pg/mL TNF-α (**Fig 5.8A**). In PBMC-5 and PBMC-6 the complexes gave TNF-α concentration ranging between 10 to 40 pg/mL while the PBMC-4 was more sensitive to the complexes, exhibiting 40 to 1100 pg/ml of TNF-α in the supernatant (**Fig 5.8A**). The mRNA results were also in line with the TNF-α protein secretion (**Fig 5.7A**). The inclusion of PAA had contrasting results with the complexes at the 2 weight ratios used. The mRNA TNF-α levels were upregulated at ratio 5 with complexes including PAA among 4 groups and at ratio 10 with 2 groups (**Fig 5.7A**). Based on secreted TNF-α protein levels, 4 groups exhibited an increase in TNF-α secretion at R:5/0.5 μg/mL with PAA, (PBMC-4/-5/-6) and

R:5/1 µg/mL (PBMC-4). At ratio 10 with PAA, 8 out of 9 groups showed a decrease in TNF-α levels at 0.5 µg/mL (PBMC-4/-5), at 1 µg/mL and 2 µg/mL in all PBMCs. The 1.2PEI-tαLA6 polymer up to 50 µg/mL concentration did not affect TNF-α secretion (**Fig 5.8A**), although the mRNA levels were upregulated in PBMC-4 (**Fig 5.7A**).

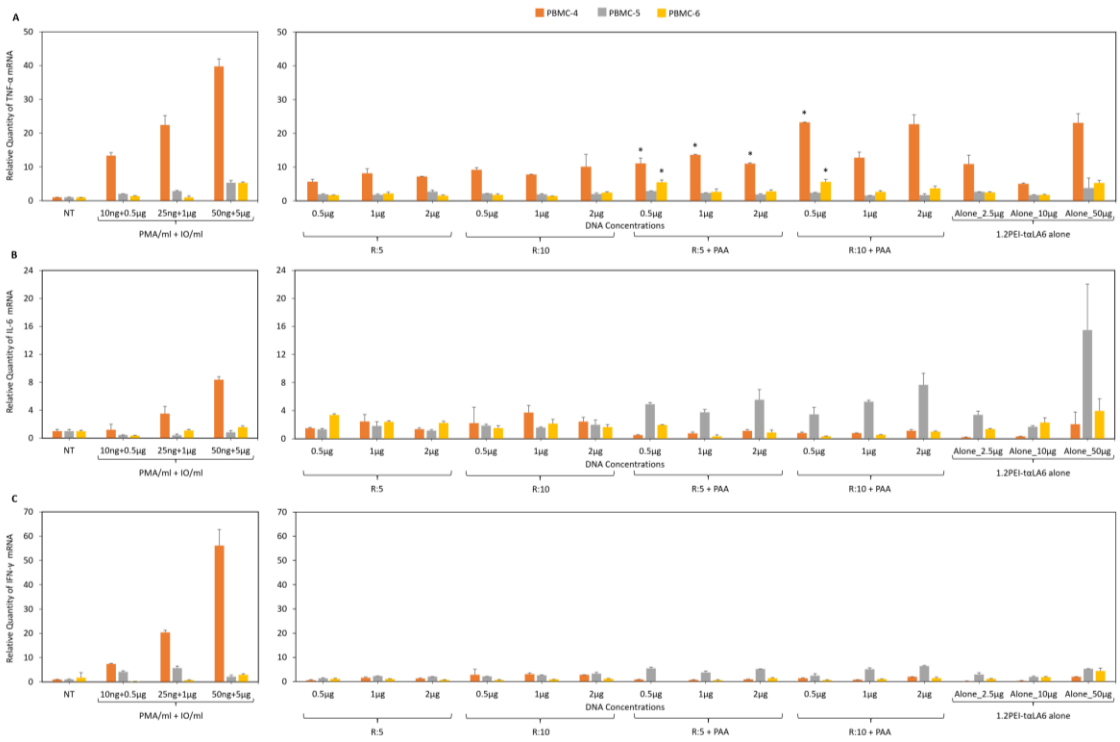

Figure 5.7: Cytokine mRNA levels assessed by RT-qPCR (relative to no treatment) after 24 hours of treatment in three different PBMCs (4-6) with positive controls PMA/IO; (**A**) TNF-α, (**B**) IL-6 and (**C**) IFN-γ. The 1.2PEI-tαLA6/pDNA complexes were at ratios R:5 and R:10 without and with PAA and 0.5, 1 and 2 µg/mL of pDNA concentrations. The 1.2PEI-tαLA6 alone was used at 2.5, 10 and 50 µg/mL concentration. Significance compared to the respective treatment groups in the absence of PAA, * $p \leq 0.05$.

Stimulation with PMA/IO resulted in ~8-fold increase in IL-6 mRNA levels with PBMC-4 but not in PBMC-5 and -6 (**Fig 5.7B**). Similarly, 10-30 pg/mL of IL-6 secretion was detected in PBMC- 4, however PBMC-5 and 6 showed very minimal increase in IL-6 secretion (**Fig 5.8B**). With IL-6 protein secretion, except for complexes at ratio 5 at 0.5

µg/mL pDNA, we did not record any stimulation with 1.2PEI-tαLA6/pDNA complex groups (**Fig 5.8B**). In PBMC-5 with PAA-bearing complexes, we could detect a slight increase in IL-6 mRNA levels (**Fig 5.7B**) but the use of PAA decreased the IL-6 secretion for complexes at R:5/0.5 µg/mL, R:5/2 µg/mL and for complexes at R:10/0.5 µg/mL in PBMC-4 (**Fig 5.8B**). There was no increase in IL-6 secretion in PBMC-5 and -6 (**Fig 5.8B**).

For the IFN-γ secretion, the complex exposure resulted in no stimulation (with both mRNA and protein) in all groups compared to untreated cells, which gave IFN-γ concentrations between 30-60 pg/mL (**Fig 5.8C**). Treatment with the positive controls PMA/IO resulted in high IFN-γ levels for PBMC-4 and -5 (30-700 pg/mL), but not for PBMC-6 (**Fig 5.8C**). Similarly, the mRNA IFN-γ levels were upregulated in PBMC-4 (10 to 60-fold) and to lesser extent in PBMC-5 (~5-fold) but not in PBMC-6 (**Fig 5.7C**).

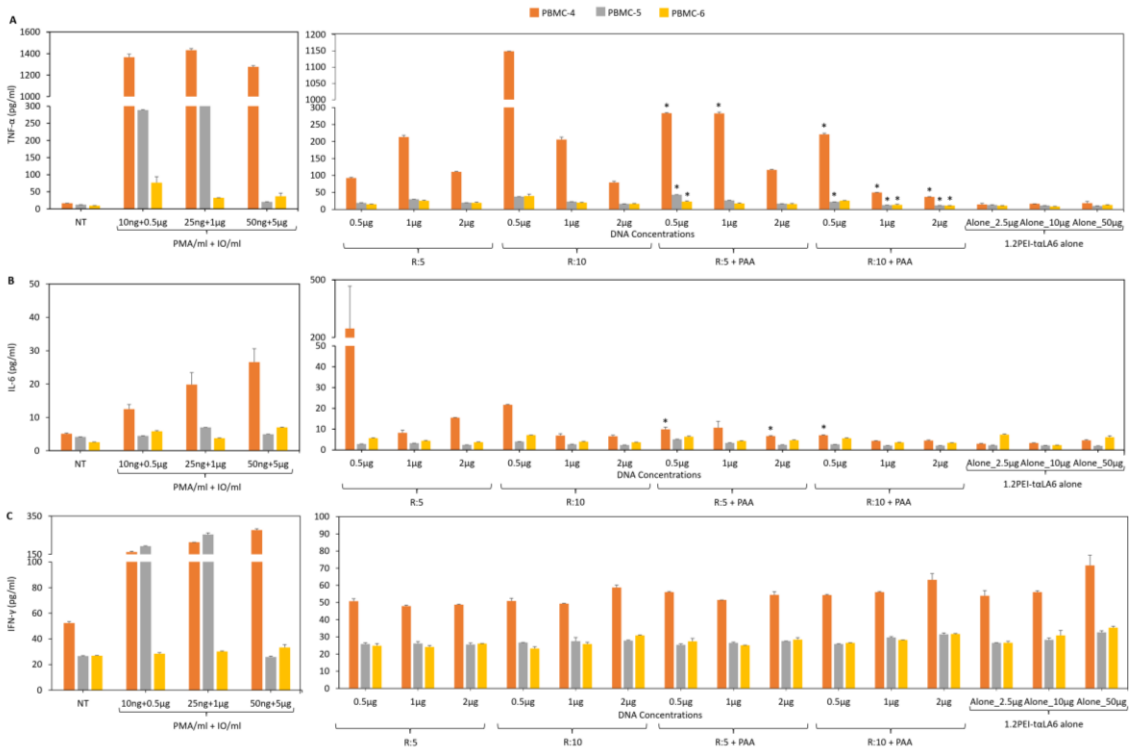

Figure 5.8: Cytokine protein levels in culture supernatants assessed by ELISA after 24 hours of treatment in three different PBMCs (4-6) with positive controls PMA/IO: (**A**) TNF-α, (**B**) IL-6 and (**C**) IFN-γ. The 1.2PEI-tαLA6/pDNA complexes were at ratios R:5

and R:10 without/with PAA and 0.5, 1 and 2 μg/mL of pDNA concentrations. The 1.2PEI-tαLA6 alone was used at 2.5, 10 and 50 μg/mL concentration. The significant difference with PAA has been denoted by (*) with $p \leq 0.05$ in comparison to the respective treatments in the absence of PAA.

The impact of complexes carrying siRNA instead of pDNA on cytokine stimulation was also explored using RT-qPCR for 1.2PEI-tαLA6, 1.2PEI-Lau8 and 1.2PEI-LA6 polymers using a control siRNA (CsiRNA) and blimp siRNA in PBMC-5 and PBMC-6. TNF-α mRNA levels were unchanged for all the polymer/siRNA treatment groups when compared to no treatment (**Fig 5.S1A**). However, with IL-6 mRNA levels, we could observe an increase with 1.2PEI-LA6 complex treatment in both CsiRNA as well as blimp siRNA in both PBMCs and to a lesser extent in 1.2PEI-Lau8 (**Fig 5.S1B**). Similar to TNF-α results, the IFN-γ mRNA levels were not altered by the complex treatment using the three polymers (**Fig 5.S1C**).

5.3.5 Impact of different PEI carriers on cytokine secretion

We next investigated the influence of different polymers on cytokine secretion at polymer:pDNA ratios 5 and 10, without/with PAA as additive, along with polymers alone in PBMC-7 and 8. Treatment with PMA/IO resulted in dose-dependent stimulation of all three cytokines (TNF-α: 200 to 950 pg/mL, IL-6: 2 to 25 pg/mL, IFN-γ: 200 to 700 pg/mL) for both PBMCs (**Fig 5.9Ai, ii** and **iii**). 1.2PEI-Chol3 showed minimal TNF-α increase in PBMC-7 (~10 pg/ml) but not in PBMC-8 (**Fig 5.9B-i**). The IL-6 and IFN-γ protein levels were unchanged compared to no treatment and the use of PAA did not alter the outcome (**Fig 5.9B-ii** and **iii**).

The 1.2PEI-LA6 complexes caused a slight increase in TNF-α levels with PBMC-7 (~50 pg/mL) but not in PBMC-8 similar to the 1.2PEI-Chol3 complexes (**Fig 5.9C-i**). No major change was observed in IL-6 and IFN-γ levels and addition of PAA to complexes did not affect the cytokine secretion (**Fig 5.9C-ii** and **iii**).

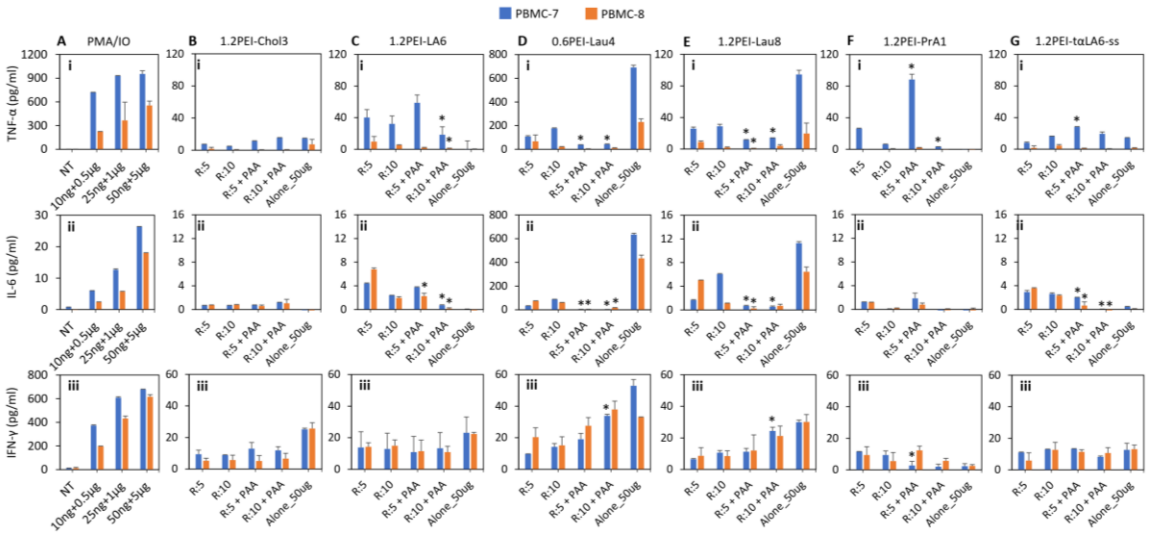

Figure 5.9: Cytokine protein levels assessed by ELISA after 24 hours of treatment in two different PBMCs (7 and 8) with positive controls PMA/IO: **A**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ. Polymer/pDNA complexes at R:5 and R:10 without and with PAA using 1μg of pDNA. 1.2PEI-Chol3: **B**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 1.2PEI-LA6: **C**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 0.6PEI-Lau4: **D**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 1.2PEI-Lau8: **E**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 1.2PEI-PrA1: **F**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ and 1.2PEI-tαLA6-ss: **G**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ. The significant difference with PAA has been denoted by (*) compared to the respective treatments in the absence of PAA.

With 0.6PEI-Lau4 complexes, we observed a sharp increase in TNF-α secretion, but PAA addition decreased the TNF-α secretion from ~150 to ~40 pg/mL in PBMC-7 and from ~40 to ~5 pg/mL in PBMC-8. The 0.6PEI-Lau4 alone at 50 μg/mL resulted in ~700 pg/mL and ~200 pg/mL of TNF-α levels in PBMC-7 and PBMC-8, respectively (**Fig 5.9D-i**). Similar observations were made in IL-6 secretion where PAA addition to complexes reduced secretion by 20-fold and 7-fold in PBMC-7 and PBMC-8, respectively. The

0.6PEI-Lau4 polymer alone (50 μg/mL) resulted in high IL-6 levels of ~600 and ~400 pg/mL in PBMC-7 and PBMC-8, respectively (**Fig 5.9D-ii**). However, no major change was observed with IFN-γ compared to no treatment, except high concentration of 0.6PEI-Lau4 alone in both PBMCs (**Fig 5.9D-iii**).

The 1.2PEI-Lau8 complexes caused a slight increase in TNF-α levels in PBMC-7 of ~25 pg/mL but this was decreased to ~10 pg/mL with PAA, while the polymer alone gave ~90 pg/mL TNF-α. In PBMC-8, 1.2PEI-Lau8 complexes did not result in any considerable change except the polymer alone treatment (~20 pg/mL TNF-α; **Fig 5.9E-i**). The protein levels of IL-6 and IFN-γ were minimally altered, and 1.2PEI-Lau8 polymer alone (50 μg/mL) was the only group that caused a change in these protein in both PBMCs (**Fig 5.9E-ii** and **iii**).

With 1.2PEI-PrA1 complexes, only R:5 complexes with PAA caused a strong increase in TNF-α levels (80 pg/mL) in PBMC-7 (**Fig 5.9F-i**). Other treatment groups did not result in increasing TNF-α, IL-6 and IFN-γ secretion in the PBMCs (**Fig 5.9F-i, ii** and **iii**).

The 1.2PEI-tαLA6-ss complexes stimulated TNF-α secretion in PBMC-7 and not in PBMC-8 (**Fig 5.9G-i**) and IL-6 as well as IFN-γ protein levels were mostly unchanged with this polymer (**Fig 5.9G-ii** and **iii**).

5.3.6 Impact of different lipid substituents on cytokine transcript levels

The studies above were conducted using PEI base polymers with a single level of lipid substitution. We further investigated the role of different lipid substituents by using 1.2PEI polymers substituted with different levels of propionic acid (PrA), caprylic acid

(CA), palmitic acid (PA), lauric acid (Lau), stearic acid (St), cholesterol (Chol), linoleic acid (LA), α–linoleic acid (αLA), and disulfide-linked PEIs. The PMA/IO treatment showed an increase in all 3 cytokine mRNA levels, but dose dependent increase was evident with TNF-α only (**Fig 5.10A**).

The complexes with PrA-substituted polymers (1.2PEI-PrA1, 1.2PEI-PrA6 and 1.2PEI-PrA8) did not exhibit any change in cytokine mRNA levels compared to no treatment. Difference in PrA substitution had no influence in all three cytokine mRNA levels (**Fig 5.10B**).

With CA, 1.2PEI-CA4(1.87) increased IL-6 levels and the other CA substituted polymers did not result in increased cytokine mRNA levels and no association between different levels of CA substitutions and cytokine stimulation was observed (**Fig 5.10C**).

The complexes from PA polymers did not alter any of the cytokine levels (**Fig 5.10D**). A similar observation was noted with Lau substituted polymers except 1.2PEI-Lau4(2.1) for IL-6 mRNA levels and to a lesser extent with IFN-γ levels (**Fig 5.10E**). Though there was slight elevation in IFN-γ mRNA levels with St-substituted PEIs, no significant observations were detected (**Fig 5.10F**), similarly, the IL-6 and TNF-α mRNA levels were not altered among the different batches of St-substitutions (**Fig 5.10E**).

The complexes with different cholesterol substitutions did not generate cytokine stimulation (**Fig 5.10G**).

LA-substituted PEIs did not exhibit any change in cytokine mRNA levels (**Fig 5.10H**), However with αLA, IL-6 and IFN-γ levels were upregulated at lower substitution (1.2PEI-αLA1(0.75)) (**Fig 5.10I**) and also with thiol-linked PEI (1.2PEI-tαLA2(0.8)). Other batches did not display any upregulation of the cytokines (**Fig 5.10I**).

With the disulfide-linked 2PEIs, complexes from PrA substitution increased the mRNA levels of IL-6 and IFN-γ and 2PEI-PrA1-ss showed the highest stimulation whereas TNF-α expression was unaltered (**Fig 5.10J**). A similar observation was recorded with CA-substituted PEIs, 2PEI-CAA4-ss(1.46) and 2PEI-CAA6-ss(2.15), where IL-6 and IFN-γ expression was upregulated, but not TNF-α (**Fig 5.10K**). Complexes from Lau-substituted disulfide polymers did not alter the cytokine levels and differences in Lau substitution did not cause any change (**Fig 5.10L**). St-substitution (2PEI-St6-ss(2.32)) caused TNF-α upregulation; however no other significant observations were made with this polymer (**Fig 5.10M**). Complexes with disulfide-linked tLA and tαLA substituted PEIs showed high IL-6 and IFN-γ upregulation, but not TNF-α (**Fig 5.10N and O**). 1.2PEI-tαLA2-ss(0.8) showed the highest stimulation among the disulfide polymers tested in this study (**Fig 5.10O**). We explored correlations between different lipid levels and cytokine stimulation but did not observe any significant relationships (**Fig 5.S2**).

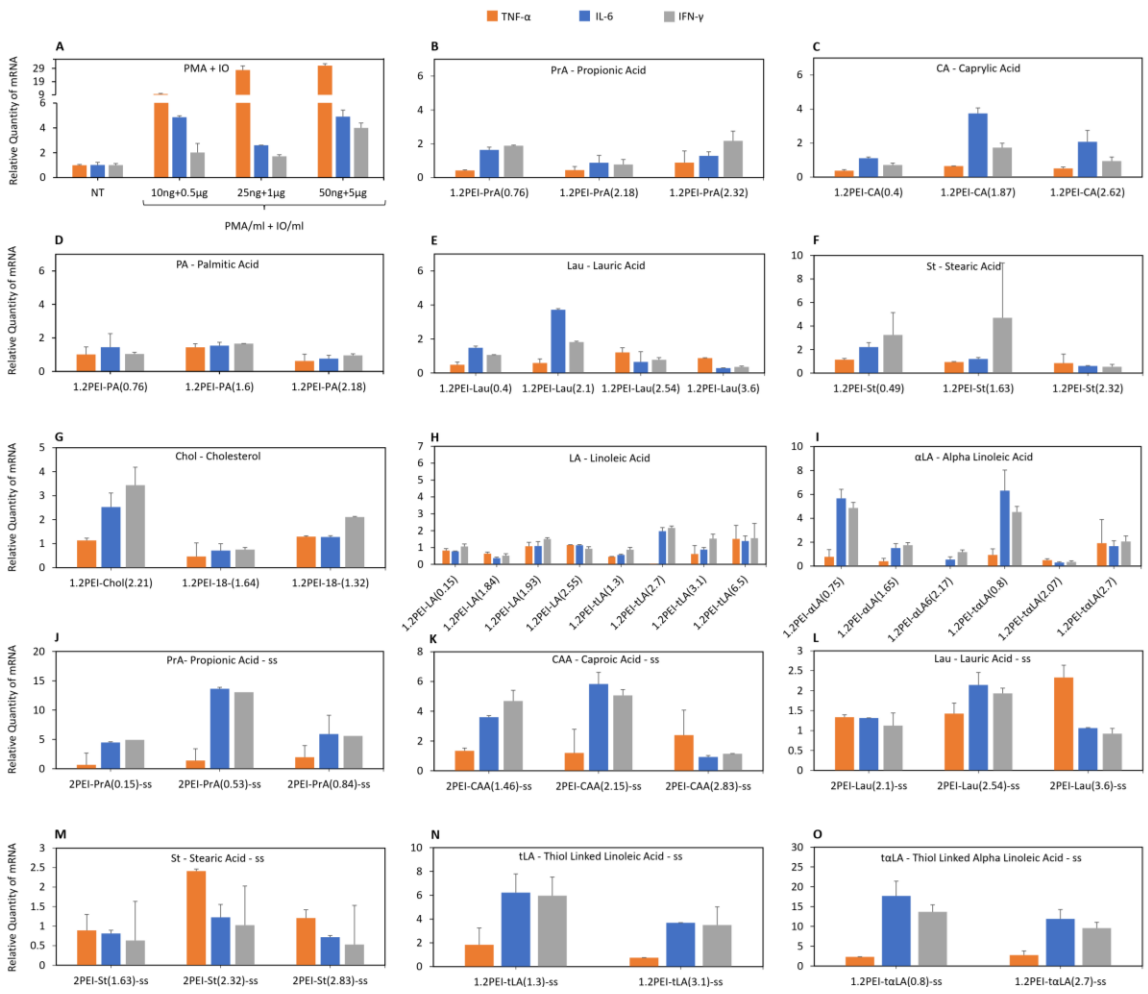

Figure 5.10: The mRNA levels of TNF-α, IL-6 and IFN-γ assessed after 24 hours of treatment in Jurkat cell line (relative to no treatment cells) using PEIs substituted with different lipids, complexed with 1µg of pDNA at R:5. (**A**) positive controls PMA/IO, (**B**) PrA-Propionic acid, (**C**) CA-Caprylic acid, (**D**) PA-Palmitic acid, (**E**) Lau-Lauric acid, (**F**) St-Stearic acid, (**G**) Chol-Cholesterol, (**H**) LA-Linoleic acid, (**I**) αLA-alpha linoleic acid and (**J**) PrA-Propionic acid-ss, (K) CAA-Caproic acid-ss, (**L**) Lau-Lauric acid-ss, (**M**) St-Stearic acid-ss, (**N**) tLA-thiol linked linoleic acid-ss, and (**O**) tαLA-thiol linked alpha linoleic acid-ss.

5.4 DISCUSSION

The success of a gene delivery vehicle is not only reflected by their protein expression or gene knockdown efficiency, but also by their ability to remain 'stealth' and elicit no or minimal immune response in a host. In this study, we explored the safety of

lipid-modified LMW PEIs that have been reported in several previous studies from our group. A central polymer explored in this study was 1.2PEI-tαLA6, whose efficacy was previously shown for delivery of siRNA and pDNA to induce apoptosis in breast cancer cell models in both *in vitro* and *in vivo* [13]. Toxicity studies with 1.2PEI-tαLA6 complexed with CsiRNA or CsiRNA/pDNA combination in a range of breast cancer cells (MDA-MB-231, MDA-MB-436, MCF-7 and SUM 149) showed minimal toxic effect on cells (i.e., 80-100% viability). The viability of non-cancerous cells such as hBMSC, HUVEC and MCF-10A were also unaltered by this vehicle [13,24,59]. Toxicity results here with 1.2PEI-tαLA6 in Jurkat cells and in different PBMCs at a wide range of polymer:pDNA ratios revealed a similar outcome. From prior reports, the cholesterol substituted PEI showed effective BCR-ABL silencing in CML K562 and drug-resistant K562-DR cells, as well as STAT and JAK silencing in the breast cancer cells [60]. This delivery system did not alter the cell viability with CsiRNA complexes [14,61] and similar results were seen here with Jurkat T-cells and PBMCs. The LA-substituted 1.2PEI-LA6 has been also explored for breast cancer treatment and its success in silencing several genes was shown in both *in vitro* and *in vivo* studies. Toxicity exerted by 1.2PEI-LA6 complexes carrying CsiRNA remained low (>80% viability) in breast cancer cells and a broad range of non-cancerous cells [18,21,24,54]. In the current studies with Jurkat T-cells and PBMC-1, we observed ~50% and ~30% reduction in the viable cell percentage (toxicity) at the highest ratio of 10, which was higher than the ratios used before. One of the key reasons for the observed toxicity could be attributed to the presence of free 1.2PEI-LA6 in the complexes, especially at high ratios. The free LA at very high concentrations (~100 μM) has been shown to decrease the viability of Jurkat cells, although the LA present here is

conjugated to the 1.2PEI (2.55 LA/PEI) and is expected to be present at low concentration (~ 20 μM) when compared to the free linoleic acid used in that study [62]. An in-depth study on the toxic effect of the free lipids which are employed in PEI lipid-substitution could provide more information on the safety of our lipid conjugates. The Lau substituted PEIs, 0.6PEI-Lau4 and 1.2PEI-Lau8, are relatively novel polymers which has shown promising silencing efficacy in MDA-MB-231 and MDA-MB-468 breast cancer cells [60]. The cytotoxicity of 1.2PEI-Lau8 was previously shown to be low (with CsiRNA) and this study also did not exhibit any toxicity in Jurkat T-cells and PBMCs with Lau substituted PEIs. The next polymer explored for toxicity was 1.2PEI-PrA1 with PrA substitution, which were successfully used for pDNA (GFP) delivery in breast cancer cells. The toxicity studies revealed relative cell viability of 80% until R:10, thereafter a toxic effect was noted at R:15 and R:20 with 60% viable cells with MDA-MB-231. However, MCF-7 cells were insensitive even at the highest ratio of R:20 [15]. Similarly, toxicity studies in K562 cells with siRNA delivery also showed 80% viability up to R:20, after which the viability declined to 30% at R:40 [63]. In Jurkat and PBMCs, we observed a consistent effect of 1.2PEI-PrA1/pDNA complexes with an increase in toxicity corresponding to an increase in polymer:pDNA ratios. The high surface charge of 1.2PEI-PrA1/pDNA complexes was shown to correlate with its higher efficacy in breast cancer cell lines along with better localization near the nuclear membrane [15]. Similarly, this observed effect on T-lymphocyte toxicity could be attributed to their high surface charge leading to higher interaction on the cell membrane with an increase in polymer ratio. The other delivery system tested, 1.2PEI-tαLA6-ss bearing disulfide linkage, is a novel polymer that showed no toxicity in PBMCs. The inclusion of PAA as an additive was shown to improve the

nucleic acid delivery in addition to reducing the surface charge of the LA-substituted polymers [54]. We did not see any change in the toxicity of complexes carrying PAA at all three ratios (R:5, R:7.5 and R:10) in PBMC-3.

Understanding the hemolytic ability of any new therapeutic agent is crucial before proceeding to clinical evaluation. The acceptable level of hemolysis is considered 5% for any new agent [64]. The hemolytic behavior of bare polymers and polymer/pDNA complexes could be partly due to the surface charge; cationic species have been demonstrated to show high hemolysis unlike the anionic species [27]. The free primary amines was also a causative factor for hemolysis in PEI-like polycations such as polyamidoamine and polylysine [65,66]. Among the polymer systems evaluated in this study, the 1.2PEI-LA6 consistently caused high hemolysis in its bare state but the complexes remained non-toxic at physiologically relevant concentrations and ratios (R:5 and R:7.5). We also used LA-substituted PEIs with different lipid substitution levels to better reveal its impact on hemolysis. The PEIs with LA substitutions of 2.6 and 1.2 were most lytic suggesting a critical range of substitutions to be the key for its hemolytic activity [65,66]. It is also possible that details of individual polymer preparation conditions might be also important for final hemolysis activity, but this issue was not explored here.

Variations in the source of RBC could be a key reason for differences in the hemolysis observed among different RBC samples. Any difference in the storage conditions of the RBC could have caused a change in the integrity of RBC, as some patient samples required more than three PBS wash to completely remove the free hemoglobin in this study [67]. Due to these reasons some RBC might be more sensitive to treatment. The cells were available to us by overnight shipping so that the transport conditions might be

an additional factor that might have affected RBC stability among different sources. Irrespective of the RBC source, we observed that the PAA additive was able to shield the hemolytic effect of the polymer/pDNA complexes (most evident in RBCs treated with saline-dissolved samples). Previous studies have reported on PEG, tripeptide lipid-sucrose laurate (L-S) and mucin to reduce or neutralize the hemolytic property of nanoparticles from chitosan, lipids and polylactic-co-glycolic acid (PLGA) [26,45,68,69]. The PAA could be also explored as an additional agent to reduce hemolysis with other delivery agents. The PAA was shown to reduce the surface charge of complexes from ~40 mV (without PAA) to ~10 mV (with PAA) in siRNA complexes [54]. Thus, the beneficial effect of PAA on the hemolysis could be due to a decrease in the surface charge leading to lowered interactions with the RBC membrane [27].

The cytokine response to nanoparticle therapeutics is critical as it can lead to life threatening organ failures [70]. To decode the cytokine response to LMW PEIs, cellular mRNA levels and protein concentration in culture supernatants were assessed in Jurkat and PBMC cells, with good agreement. Upregulation of TNF-α, IL-6 and IFN-γ expression was evident with R:5 complexes of 1.2PEI-tαLA6 in Jurkat cells, but not in PBMCs. We observed that Jurkat cells were generally more sensitive even with the positive controls PMA/IO treatment in comparison to PBMC. In PBMC-3/-5/-6, the polymer alone at excessive (50 μg/mL) doses showed an increase of IL-6 expression along with TNF-α expression in PBMC-4, and such information is valuable while performing *in vivo* and future clinical studies. An increase in pDNA concentration led to a general decrease in the TNF-α response (except with R:5), which was consistent across different PBMC samples and whether PAA was incorporated in the complexes or not. Increasing the pDNA dose

with linear PEI was shown to increase the TNF-α protein levels in mice serum [9] as well as with liposomal delivery systems [71]. The observed decrease in response could be due to a change in their surface charge or the size of the complexes as these can be influenced with different pDNA concentrations in culture supernatant. However, with IL-6 secretion, only R:5 with PAA showed a decrease from 21 pg/mL to 6 pg/mL in PBMC-4, and with IFN-γ we did not observe such a decrease in their protein levels with increase in pDNA concentration. Such a decrease in cytokine secretion with increase in pDNA concentration was not documented in their mRNA levels. We also observed that PBMC-4 showed relatively high sensitivity to our treatments (based on both mRNA and protein assessment), which re-emphasized the need to investigate *in vitro* responses in multiple samples due to variations in the source of PBMC. With polymer/siRNA complexes, 1.2PEI-LA6 polymer which previously showed hemolysis, was the only system to upregulate the IL-6 mRNA levels.

The inclusion of PAA in complexes, especially at R:10, showed a significant decrease in TNF-α protein secretion, whereas we observed an opposite effect at R:5 (denoted by #), which was also noted at the mRNA levels. The addition of PAA decreases the surface charge of the complexes and may have contributed to the reduction of TNF-α. The levels of IL-6 were widely undisturbed except at R:5 in PBMC-4 with some treatment groups having PAA a decrease in IL-6 levels were observed and IFN-γ was not upregulated by these delivery systems. In 1.2PEI-LA6, 0.6PEI-Lau4 and 1.2PEI-Lau8 complexes, the inclusion of PAA reduced TNF-α as well as IL-6 secretion.

The 1.2PEI-Chol3 did not cause cytokine stimulation, similar to its minimal effect on cell toxicity and hemolysis. The 1.2PEI-LA6 complexes, which was hemolytic in some

treatment group, exhibited TNF-α upregulated in PBMC-7 alone whereas other cytokines were not altered. Similarly, with other polymer/pDNA complexes, 1.2PEI-Chol3, 0.6PEI-Lau4, 1.2PEI-Lau8, 1.2PEI-PrA1 and 1.2PEI-tαLA6-ss, upregulation of TNF-α can be observed to varying extents only with PBMC-7 and not with PBMC-8. Among the delivery systems, 0.6PEI-Lau4 (complex as well as polymer alone) with 2.8 Lau/0.6PEI was the only polymer that stimulated TNF-α and IL-6 to a high extent. In contrast, 1.2PEI-Lau8 with 3.6 Lau/1.2PEI did not cause any major cytokine response, hence the observed effect may not be due to the presence of Lau. Different PEIs (0.6, 1.2 and 2.0 kDa branched PEI and 25 kDa linear PEI) without any lipid substitution did not cause immune stimulation in our hands (**Fig 5.S3**), confirming the safety of the base polymers. The final outcomes of the cytokine secretion from different PBMCs have been summarized as heat map for a better understanding and comparison (**Fig 5.11**).

Figure 5.11: Heat map showing the stimulation of three cytokines in seven different PBMCs following its treatment with controls and various PEI delivery agents at two different ratios with PAA as additive. Cytokine secretion was quantitated by ELISA and has been categorized based on the protein amount (pg/mL) with separate legends for each cytokine. P - PBMC, NT - no treatment, n/a – data not available.

One of the main reasons for low cytokine response could be the low amount of lipids being grafted on PEIs and the small quantity of the polymer used for complexation with pDNA or siRNA. The lipids employed in this study are relatively simple lipids and

are widely used for treatment of various conditions; e.g., PrA was shown to reduce obesity-associated inflammation by decreasing TNF-α levels [72]. CA could increase mitochondrial respiration and improve inflammatory condition like sepsis [73]. PA and St at high concentrations can induce production of proinflammatory cytokines [74,75]; however, we did not detect any such upregulation presumably the amount of grafted lipids on PEI is low. Similarly LA was demonstrated to exhibit anti-cancer as well as anti-inflammatory properties [76,77]. The disulfide-linked polymers have an added feature of being reduced in the intracellular space when they encounter a highly reductive environment [78]. Some of these polymers showed elevated cytokine levels, which could be attributed to their larger size or disulfide moiety used in the polymers.

Comparison of TNF-α levels between lipid/pDNA complexes, PEI/pDNA complexes of linear PEI 25 kDa, and branched PEIs (10, 25 and 70 kDa) showcased ~40 pg/mL of TNF-α for all the PEIs irrespective of their molecular weight emphasizing the overall safety of PEIs *in vivo* [9]. We employed 0.6 to 2.0 kDa PEIs in our study and have shown minimal response to PEI/pDNA complexes, where TNF-α levels ranged between 6 pg/mL to 230 pg/mL (overall) between different ratios, polymers and PBMCs while the baseline levels (i.e., no treatment) were ~10 pg/mL. Complexes with PEI 25 kDa have also been successfully delivered as aerosols instead of the systemic route, whose TNF-α response was of ~5 pg/mL in the lungs and 20 pg/mL in the serum. Whereas the i.v administration of the same complexes resulted in 60 pg/ml of lung TNF-α levels and 100 pg/ml of serum TNF-α which are minimal further pointing the safety of PEIs and their wide application [79] (**Table 5.2**). In a previous study using *in vivo*-jetPEI^{TM} (linear PEI)/pDNA and DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane)/pDNA complexes, TNF-α

production was <4 pg/mL but IFN-γ levels were drastically upregulated reaching ~8000 pg/mL for both systems and IL-6 concentration was ~300 pg/mL when evaluated in mice serum. However, the increase in IFN-γ was shown to be due to the presence of DNA CpG motifs as same delivery systems with siRNA did not cause cytokine stimulation [80]. Both delivery agents (PEI and DOTAP) showed IFN-γ upregulation, and a similar stimulation of IFN-γ (~10,000 pg/mL) in serum was documented by another group using linear PEI/pDNA complex [9]. The treatment with pDNA alone, did not upregulate any of the cytokines assessed in our hands (**Fig 5.S4**), which indicates the observed upregulation in certain groups to be mainly due to the polymer. Similarly, IL-6 upregulation was mostly reported to be negligible with PEI systems both *in vitro* and *in vivo* [81,82], but some IL-6 production (~400 pg/mL with *in vivo*-jetPEI^{TM} linear PEI [80] and ~150 pg/mL with branched 25 kDa PEI) could also be observed in the literature [83]. It was interesting to note that the branched 25 kDa PEI with stearic acid lipid substitution also produced similar amounts of IL-6 (~200 pg/mL) to that without substitution with siRNA complexes [84]. In our study with LMW PEIs having different lipid substitutions, the overall IL-6 levels were ~10 pg/mL, while no treatment had ~3 pg/mL suggesting minimal stimulation of LMW PEIs compared to their high MW counterparts.

Cytokine	Baseline concen-tration	Positive control	PEI	Concentration post-treatment	Route of adminis-tration	Therapy	Safe Yes/No	Reference
TNF-α (serum)	~25 pg/mL	~300 pg/mL	L-22 kDa	~25 pg/mL	i.p.	Non-small-cell lung cancer	Yes	[8]
TNF-α (serum)	~15 pg/mL	~320 pg/mL	B-10 kDa	~30 pg/mL	i.v.	Cytokine response	Yes	[9]
TNF-α (serum)	~20 pg/mL	~320 pg/mL	B-25 kDa	~30 pg/mL	i.v.	Cytokine response	Yes	[9]
TNF-α (serum)	~5 pg/mL	~320 pg/mL	B-70 kDa	~60 pg/mL	i.v.	Cytokine response	Yes	[9]
TNF-α (serum)	~5 pg/mL	~320 pg/mL	L-25 kDa	~40 pg/mL	i.v.	Cytokine response	Yes	[9]
TNF-α (serum)	~10 pg/mL	n/a	L-25 kDa	~100 pg/mL	i.v.	Pulmonary gene therapy	Yes	[79]
TNF-α (lung)	~5 pg/mL	n/a	L-25 kDa	~60 pg/mL	i.v.	Pulmonary gene therapy	Yes	[79]
TNF-α (serum)	~10 pg/mL	n/a	L-25 kDa	~20 pg/mL	Aer	Pulmonary gene therapy	Yes	[79]
TNF-α (lung)	~5 pg/mL	n/a	L-25 kDa	~5 pg/mL	Aer	Pulmonary gene therapy	Yes	[79]
TNF-α (serum)	<4 pg/mL	~5000 pg/mL	L-25 kDa	<4 pg/mL	i.v.	Cytokine response	Yes	[80]
IL-6 (serum)	~100 pg/mL	~60000 pg/mL	L-25 kDa	~300 pg/mL	i.v.	Cytokine response	Yes	[80]
IL-6 (supernatant)	n/a	n/a	B-25 kDa	2.5-fold	*In vitro*	Cytokine response	Yes	[81]
IL-6 (serum)	~50 pg/mL	~2000 pg/mL	n/a	~50 pg/mL	i.v.	Cytokine response	Yes	[82]
IL-6 (supernatant)	~50 pg/mL	n/a	L-25 kDa	~150 pg/mL	*In vitro*	Cancer vaccination	Yes	[83]
IL-6 (supernatant)	~200 pg/mL	n/a	B-25 kDa	~400 pg/mL	*In vitro*	Melanoma gene therapy	Yes	[84]
IFN-γ (serum)	~5 pg/mL	n/a	L-25 kDa	~10000 pg/mL	i.v.	Cytokine response	Yes	[9]
IFN-γ (serum)	~4pg/mL	n/a	L-25 kDa	~8000 pg/mL	i.v.	Cytokine response	Yes	[80]

Table 5.2: Summary of previous reports on the secretion of TNF-α, IL-6, and IFN-γ by *in vitro* or *in vivo* evaluation. The baseline concentration, positive control concentration, PEI (MW of branched or linear used) used, cytokine concentration post-treatment, the route of administration, the type of therapy or study and the safety statement mentioned are

included. i.p. – intraperitoneal, i.v. – intravenous, Aer - aerosol delivery, B – branched and L – linear.

In conclusion, the lipid-substituted LMW PEIs evaluated in this study were found to be generally safe towards the blood components at appropriate polymer to pDNA/siRNA ratios and concentrations. The complexes prepared with 1.2PEI-tαLA6, 1.2PEI-Chol3, and 1.2PEI-Lau8 were consistently safe in their toxicity, hemolysis, and immune stimulation outcomes. The 1.2PEI-LA6 and 1.2PEI-PrA1 showed marginal toxicity whereas the former (1.2PEI-LA6) displayed hemolysis additionally, only at high ratios but did not elicit an obvious cytokine response. 0.6PEI-Lau4 on the other hand was the only polymer to show an immune stimulation compared to all the PEIs investigated. Among the disulfide cross-linked PEIs, 1.2PEI-tαLA6-ss was safe in toxicity and cytokine protein response. However, the mRNA levels with 1.2PEI-tαLA6ss were upregulated along with 2PEI-PrA1-ss and 1.2PEI-tαLA2-ss. The addition of PAA reduced the RBC hemolysis of the complexes as well as the immune stimulatory effect to a reasonable extent. Nevertheless, LMW PEI with lipid modifications could be explored further for *in vivo* immune response studies and act as a key alternative for HMW PEI where mixed findings on their cytokine stimulation have been reported. The lipids of varying lengths used for PEI substitution did not show any relation with cytokine response although the different amount of substitution with LA polymers showed the importance of substitution level in hemolysis. As these polymers are being explored for a wide range of disease conditions, it would be interesting to further explore and understand the rationale involved for the polymers that exhibited some cytokine response.

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6. OVERALL CONCLUSIONS, DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS

6.1 OVERALL CONCLUSIONS AND DISCUSSION

RNAi is a promising technology for cancer treatment given its feasibility to customize and target the overexpressed genes aiding in cancer progression and drug resistance. This dissertation primarily focused on using RNAi technology to address breast cancer metastasis by targeting an overexpressed cell adhesion molecule (CAM), integrin-β1 (ITGB1) and an intracellular molecule, HSP90B1 and address CML drug resistance by silencing the upregulated integrin-β1. The successful delivery of siRNA and the therapeutic outcome of silencing in each cancer was provided by lipid-modified low molecular weight PEI gene delivery systems, whose safety was the secondary focus of this thesis as they are crucial while heading into physiological applications. In **Chapter 1**, we summarized the success of siRNA-based therapies which was evident by the various clinical trials in different types of cancers highlighting its adaptability. A comprehensive understanding on the properties of PEI systems, the steps taken to modify the LMW PEI such as lipid grafting, inclusion of thioester linkage and disulfide cross-linking to achieve optimal gene delivery and addition of small anionic polymers like HA, PAA and DS to further improve their efficacy and the current status of its toxicity as well as immune response studies were provided. We further established the urgent need to address (i) breast cancer metastasis and the important role of cell adhesion molecules, particularly integrin-β1 and the impact of HSP90B1 overexpression in disease progression, and to overcome (ii) CML drug resistance that are independent of BCR-ABL signaling, particularly the resistance provided by bone marrow stromal environment through integrin-β1 mediated binding of leukemic cells.

As the overexpression of integrin-β1 was of paramount importance for breast cancer metastasis based on literature proof, in **Chapter 2**, we targeted its expression using three polymers: 1.2PEI-LA (1.2PEI-LA6, linoleic acid), 1.2PEI-Lau (1.2PEI-Lau8, lauric acid), and 1.2PEI-tαLA (1.2PEI-tαLA6, alpha linoleic acid) in MDA-MB-231 breast cancer cells. The highest siRNA uptake was observed with 1.2PEI-LA6 with better cytoplasmic localization which is advantageous for siRNA mediated mRNA degradation of the target. The surface and mRNA integrin-β1 levels were strongly reduced by 50% and 90% respectively with a single treatment of 40 nM siRNA which was prolonged up to 9 days. As the major step in metastasis is the ability of breast cancer cells to attach at the distant site following migration, integrin-β1 silencing significantly reduced the percentage of cells binding to FN (65%) and hBMSC (20%) following the treatment with 1.2PEI-LA6/siRNA complexes. Similar outcomes were achieved in their migration, as integrin-β1 silencing established 85% and 50% inhibition in migration compared to no-treatment and CsiRNA respectively, using two different cell migration techniques (scratch assay and transwell migration assay). This study highlighted the importance of integrin-β1 in breast cancer migration and the therapeutic effectiveness of 1.2PEI-LA6 polymer for siRNA delivery. Very minimal impact was observed in the viability of cancer cells following integrin-β1 silencing which signifies the strong link between integrin-β1 expression and migration rather on cancer cell viability as reported previously [1]. Given the various downstream signaling pathways activated by integrin-β1, the functional outcome of siRNA treatment could have downregulated the expression of FAK, ERK, MAPK and JNK expression which in turn could have contributed to the migration inhibition of the cancer cells [2].

To further address breast cancer metastasis, we intended to identify additional CAMs in the model cell line MDA-MB-231 by screening a siRNA library targeting various cell adhesion and cytoskeleton genes (496 genes) in **Chapter 3**. siRNA delivery was carried out by the successful 1.2PEI-LA6 polymer with migration studies by scratch assay and cell viability by MTT assay. The siRNA library constituted various CAMs like cadherins (CDH), protocadherins (PCDH), selectins and all the α- integrins along with some heat shock family of proteins, and other cytoskeletal genes. Initial findings revealed a significant reduction in migration following integrin-α4 (ITGA4), integrin-α6 (ITGA6) and integrin-αX (ITGAX) silencing and growth inhibition by CDH9 and HSP90B1. For further target validation studies, we successfully employed HA as additives during PEI/siRNA complex preparation having shown its benefit in higher silencing of CDC20 in a previous study [3]. A weight/weight ratio of 1:5:0.15 i.e., siRNA:PEI:HA was established as the optimal formulation to inhibit growth and migration with HSP90B1 silencing. The other targets (ITGA4, ITGA6 and ITGAX) failed in validation studies whereas CDH9 expression was reported to be essential to inhibit breast cancer progression [4], hence they were not pursued further. Increase in HA or polymer concentration hampered the efficacy, highlighting the need for careful optimization of siRNA/PEI/HA formulation. The high performance of HA additive complexes was attributed to enhanced dissociation, increased cytoplasmic availability along with the high presence of CD44 receptors in MDA-MB-231 cells [5]. Similar to the strong (90%) silencing of integrin-β1, we obtained equivalent reduction of HSP90B1 (~80%) mRNA levels following HSP90B1 siRNA treatment with 1.2PEI-LA6 polymer. Interestingly, treatment with HSP90B1 siRNA also decreased the expression of integrin-β1 and CDC20 at the mRNA level which can be explained by the

presence of various client proteins under HSP90B1 which includes integrins [6]. Furthermore, combinational siRNA delivery against integrin-β1 and HSP90B1 was established by significant reduction in the migration of breast cancer cells showing the benefit of silencing an upregulated CAM and an intracellular molecule for breast cancer treatment.

To address the BCR-ABL independent resistance observed at the bone marrow niche by integrin-β1 mediated binding of chronic myeloid leukemia cells, siRNA against integrin-β1 was successfully delivered using PEI delivery systems as shown in **Chapter 4.** Screening of a PEI library containing unmodified and disulfide (-SS-) cross-linked lipid substituted delivery agents were established in K562 CML cells. The presence of -SS- link would help to achieve enhanced intracellular release due to the reducing environment inside the cell by glutathione as they are higher in the intracellular compartment in comparison to the extracellular space, however we did not observe any benefit of -SS-linked systems in our study. This could be possibly due to the higher size caused by lipid modification in 1.2 kDa PEI, in contrast to 0.8 Da PEI which were used for -SS- modification that showed beneficial effect in a different study [7]. FN binding studies showed strong attachment of K562 cells which confirmed the presence of CAMs and further evaluation established integrin-β1 to be upregulated and possible reason for its binding to bone stromal cells. In this study, cell surface integrin-β1 were detected in K562 cells but were much lower in comparison to MDA-MB-231 cells and integrin-β1 silencing was attained at surface and mRNA levels. Silencing was established by 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss polymers. The functional benefit of integrin-β1 silencing was evident by the decreased binding and increased detachment of K562 cells to/from hBMSC which

would benefit in the easy access and targeting by tyrosine kinase inhibitors. Moreover, silencing of integrin-β1 along with BCR-ABL was beneficial to reduce the proliferation of K562 cells which was assessed by the number of colonies formed. Hence the therapeutic outcome of silencing specific genes using PEI delivery systems in two types of cancers, breast cancer as a model for solid and CML as a model for liquid cancer was established.

In **Chapter 5**, the toxicity and cytokine stimulation by various PEI-based delivery systems including 1.2PEI-LA6, 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss that were used for breast cancer and CML studies were demonstrated *in vitro*. From the leading 7 polymers used in this study, 1.2PEI-PrA1 (propionic acid) was the only polymer that showed dose dependent (polymer:pDNA - weight/weight ratio) increase in Jurkat and PBMC toxicity. We demonstrated the importance of polymer to nucleic acid (pDNA) ratio on the toxicity observed in T-lymphocytes. The extent of human red blood cell lysis by the polyplexes (polymer:pDNA) and polymer alone revealed that some linoleic acid substitutions caused hemolysis, however all other polymers displayed safe levels of lysis. Free linoleic acid is known to be toxic at very high concentrations, but in our study linoleic acid was conjugated to PEI and not free, however further studies are required to decipher the reason for the toxicity. We also demonstrated the lowered toxicity in polyacrylic acid additive treatments, possibly due to the decrease in surface charge reducing the lysis activity on the cell membrane [3,8]. Similarly, cytokine response was demonstrated to be lowered in certain treatment groups with additive complex treatments. The TNF-α, IL-6 and IFN-γ cytokine stimulation by 1.2PEI-LA6, 1.2PEI-tαLA6 and 1.2PEI-tαLA6-ss was negligible in comparison to the positive controls PMA/IO in two different PBMCs. Among the 7 PEI delivery agents studied, only 0.6PEI-Lau4 exhibited some stimulation in all three

cytokines, however the upregulation remained minimal when compared to the positive controls. We also showed that the nucleic acid concentration as well as the ratio does not affect the cytokine secretion from studies with 4 different PBMCs. PBMC-4 appeared to be highly sensitive for cytokine secretion for all the treatments including controls, which can be explained by the inherent variations in cytokine production among humans [9]. Furthermore, the difference in the type of lipid substitution and the amount of substituent did not influence the cytokine secretion. However, some of the disulfide cross-linked thio-ester alpha linoleic polymers showed an increase in cytokine mRNA levels, especially IL-6 and IFN-γ, but no upregulation was recorded in the protein levels with 1.2PEI-tαLA6-ss polymer. Altogether, the safety of the lipid substituted LMW-PEI gene delivery agents used for breast cancer and CML treatment were demonstrated with an emphasize on the careful optimization of polymer:nucleic acid ratio.

6.2 LIMITATIONS

In the intracellular localization studies of polymer/siRNA complexes using confocal microscopy with MDA-MB-231 cells in **Chapter 2,** use of a cytoplasm staining dye could have provided a better visualization on the exact location of the complexes by their co-localization with the cytoplasm. Also, a non-specific PE-labeled antibody as a control while evaluating the cell surface levels of integrin-β1 could have been used. Similarly, controls to negate the possible interaction between FN and the PEI polymers or the influence of polymers in the FN binding study could have been employed as the binding ability was >100% for CsiRNA treatments. Extended evaluation of migration (48 hours) in scratch assay could have been performed in addition to 24 hours. Other TNBC cell lines

like BT20 and MDA-MB-435 could have been employed to confirm the therapeutic benefit of integrin-β1 silencing for breast cancer treatment.

For the library screening studies in **Chapter 3**, specialized 96 well plate-setup that helps to attain uniform wounds in all the wells could have been utilized to avoid well to well variation, as wound/scratches were created manually for all the wells. Additionally, a rapid microscopic imaging system to capture the wounds in the 96 well plate could have helped to acquire the migration information for a longer time point like 48 hours (as migration was calculated only for 24 hours). Use of pooled siRNAs targeting various regions of the target gene could have also been used in the validation studies. ITGA4, ITGA6 and ITGAX silencing showed migration inhibition in the library screening (which had 3 different siRNAs pooled together for each gene) but failed to elicit a therapeutic outcome in the validation studies where a single dicer substrate siRNA (DsiRNA) was used. However, the DsiRNA for HSP90B1 provided functional outcome and the decrease in HSP90B1 mRNA levels could have been confirmed at the protein level using western blot. The successful inhibition in migration following the combinational knockdown of integrin-β1 + HSP90B1 could have been potentially confirmed by performing an invasion assay using transwell inserts.

In **Chapter 4**, an additional CML cell line, KU812 attained from a 38-year-old male in blast crisis could have helped to validate the effect of integrin-β1 silencing for CML treatment in addition to K562 cell line (attained from a 53-year-old female in blast crisis).

Additional cell viability methods to understand the toxicity of the complexes in **Chapter 5** for Jurkat and PBMC, might have helped to validate the findings. The

immediate use of RBCs following isolation from whole blood could have potentially helped to avoid differences in hemolysis studies caused due to varying storage times of RBC before use. Use of additional PBMCs (~10) to study the cytokine stimulation ability of the 7 lead polymers might have provided a more detailed confirmation of the findings. Evaluation of the cytokine mRNA levels to PEIs having different type of lipid substitutions and varying number of substituents could have been additionally confirmed at the protein level.

6.3 FUTURE DIRECTIONS

6.3.1 Future directions to address breast cancer metastasis

The siRNA delivery agents were able to achieve 90% silencing of integrin-β1 mRNA levels, however at the cell surface we observed 50% inhibition, reflecting the presence of integrin-β1 on the cell membrane despite attaining high mRNA reduction. Therefore, a combination of integrin-β1-blocking antibody or peptide at low concentrations along with siRNA treatment could result in much higher impact in migration and binding to FN and hBMSC [10–13]. Inclusion of additives like HA or PAA can be explored to determine whether they help in increasing much higher silencing of integrin-β1 at the cell surface [3]. In addition, targeting of other β integrins like β3, β4 and β5 can provide additional edge, as their decreased availability can impact the heterodimerization with the wider range of α- subunits [14–16]. Targeting of other CAMs like protocadherins that are strongly linked to migration and invasiveness of breast cancer [17] can be also employed with effective PEI/siRNA delivery systems which has been established in this dissertation. Combinational silencing of integrin-β1 and HSP90B1 showed significant decrease in the

migration of breast cancer cells. Similar strategies of combinational silencing of integrin-β1 with apoptosis related protein (BCL2L12), cell division related protein (CDC20) and phosphatase protein (PPP1R7) can be employed [5,18]. Although the overexpression of integrin-β1 and HSP90B1 in various breast cancer cell lines and breast cancer patient samples have been reported by other research groups, the impact of silencing these targets in normal breast cells would provide stronger evidence on their specificity and help to negate any non-specific toxicity. *Ex vivo* cultured breast cancer patient samples can also be used to validate the therapeutic silencing of integrin-β1 and HSP90B1 [19]. Additionally, 3D cell cultures which mimic the tumor microenvironment could provide a better understanding on the distribution, and efficacy of PEI delivery systems as well as the therapeutic benefit of silencing these targets [20]. *In vivo* evaluation in clinically relevant animal models representing bone or brain metastatic breast cancers, especially given the brain metastasis governance and survival benefit of HSP90B1 expression, silencing in combination with integrin-β1 will be essential to further these findings. The highly metastatic triple negative breast cancer MBA-MB-231 cell line can be used to attain breast cancer xenografts in mice [21]. Furthermore, HSP90B1 silencing by these PEI delivery systems can be employed in combination with HSP90 inhibitors which would help to reduce the dose of the inhibitor and ultimately lower the side effects observed in clinical trials [22].

6.3.2 Future directions to address CML drug resistance

Antibody mediated evaluation of cell surface integrin expression on K562 cells would help to further confirm the findings from mRNA quantification. Though we

established resistance to BCR-ABL siRNA treatment when K562 cells were cultured on FN through colony formation assay, we could not develop BCR-ABL independent-TKI (drug) resistant cell lines. Hence development of CML cells that are dependent on hBMSC attachment for survival would be vital to evaluate the therapeutic outcome of integrin-β1 silencing. Although we established 50% reduction at the cell surface and mRNA levels of integrin-β1, superior PEI delivery agents can be designed to achieve better silencing and additives can be included to enhance the efficiency. Similar to the breast cancer studies reported here, other integrins can also be explored to benefit CML attachment and detachment from hBMSC to aid in TKI treatment [23]. In addition to *in vitro* studies, CML patient samples responding to TKI treatment and those experiencing resistance could be cultured to study integrin-β1 silencing in combination with BCR-ABL and TKI treatment [24]. CML *in vivo* drug resistant models can be used to perform final evaluation of combinational silencing and their benefit to address bone marrow environment induced drug resistance.

6.3.3 Future directions to better understand and engineer safe PEI gene delivery systems

Having shown a strong correlation between surface charge of nanoparticles and RBC lysis [8], additives other than HA or PAA like chondroitin sulfate (CS) can be explored. CS have been successfully used as polyplex coatings to enhance the transfection efficiency for peptide mediated gene delivery [25], hence they can also be explored for PEI delivery systems to increase the efficiency as well as minimize the chance of RBC lysis. Evaluation of polyplex size, surface charge along with the intracellular localization, and uptake pathway of the polyplexes could provide a better understanding on any correlation

to cytokine secretion. Furthermore, the lipids used in PEI substitution could be studied independently for their ability to cause toxicity, and immune response *in vitro* as this information will help in a better understanding of their safety [26]. Studies on the *in vivo* biodistribution, organ toxicity, and immune response of the PEI gene delivery is needed to understand their pharmacokinetics and absolute safety which are inevitable information for their advancement. Other RNAi methods can also be employed for target gene silencing. Regulation of several integrins by specific miRNA unlocks their therapeutic potential, as many targets can be downregulated for cancer treatment [27]. Similar applications can be explored in targeting HSP90 proteins for cancer treatment [28]. The findings of this thesis work provide the safety information of the lipid modified LMW PEI delivery systems and the therapeutic proof of silencing targets for the treatment of two different cancer types. Such PEI/siRNA therapy can also be deployed for other types of cancers.

6.4 REFERENCES

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APPENDIX

A. APPENDIX CHAPTER 2

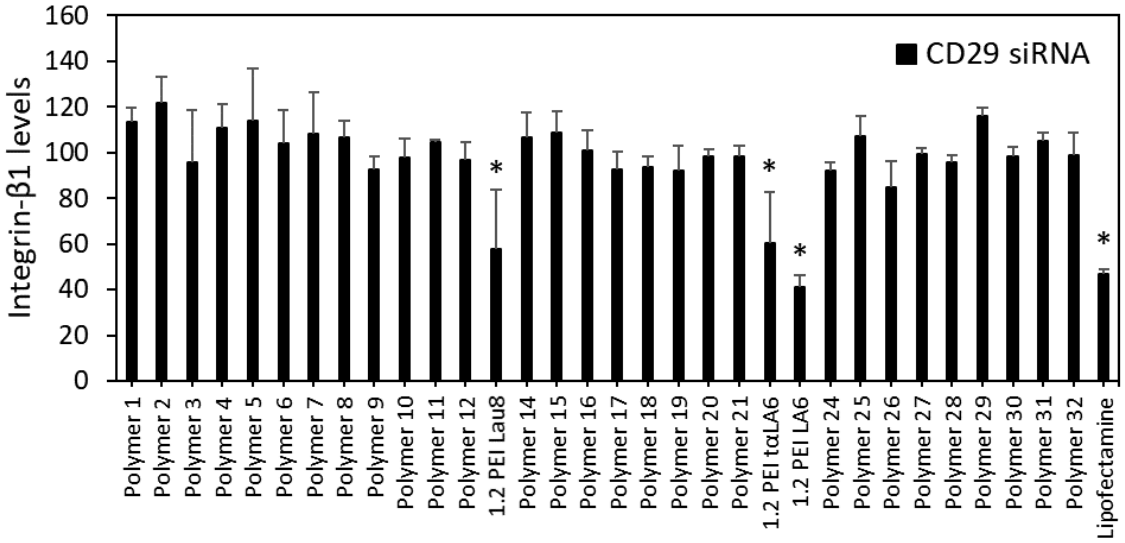

Figure 2.S1: Polymer screening: Integrin-β1 silencing with siRNA delivery after 3 days of treatment. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment group) were measured using labelled antibody against integrin-β1. A polymer:siRNA (weight/weight) ratio of 4 with 40 nM of siRNA concentration was used. Significance compared to the no treatment cells * $p \leq 0.01$, (n=2).

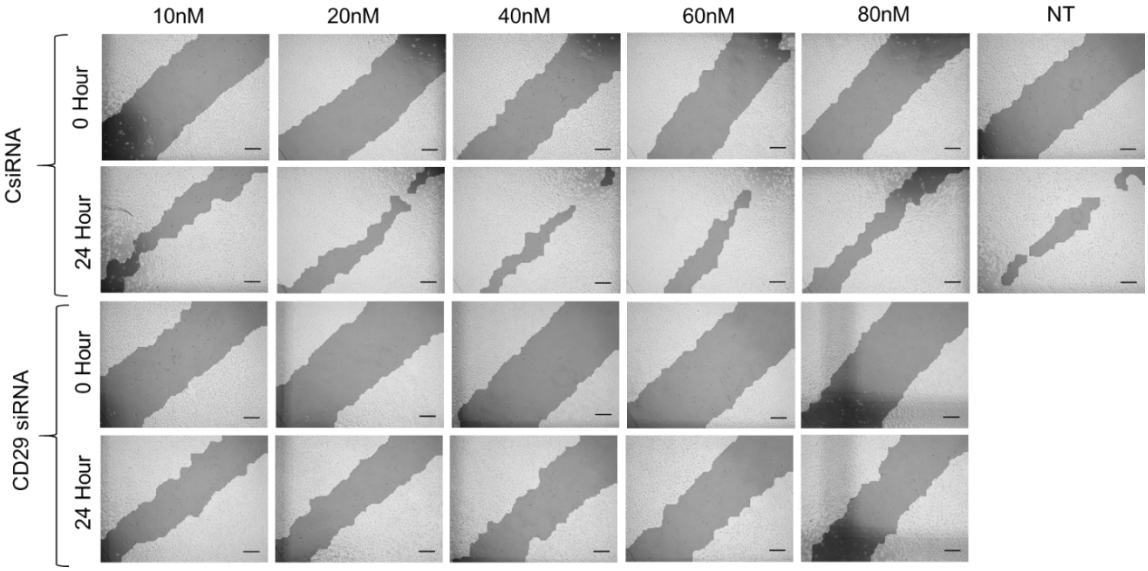

Figure 2.S2: Scratch assay microscopic images: Microscopic images of scratches taken at 0 and 24 hours after treating with 1.2PEI-LA polymer at different siRNA concentrations for 2 days. Scale bar = 200 μm

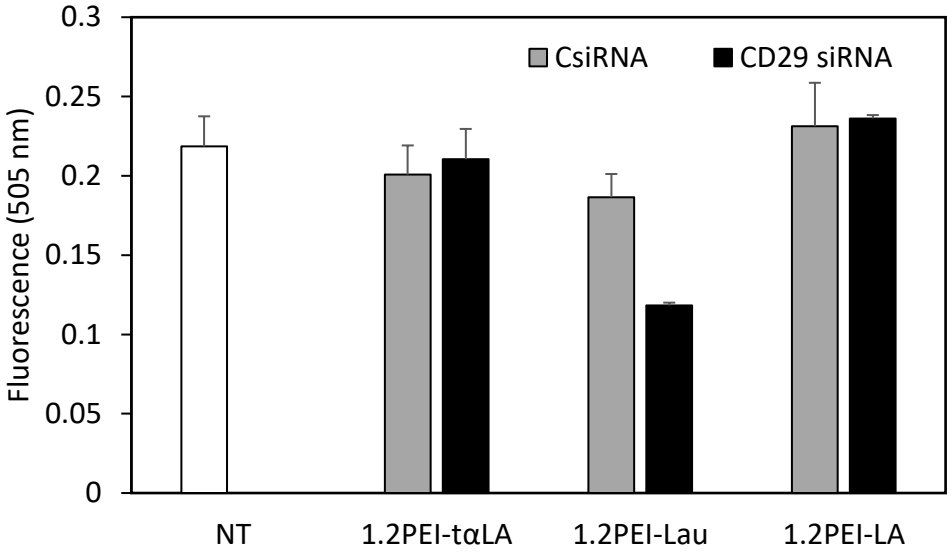

Figure 2.S3: Caspase-3 activity: Caspase-3 activity was measured by a fluorometric assay using the capase-3 substrate Ac-DEVD-AFC after 3 days of treatment with the polymer:siRNA complex. Fluorescence was detected at Ex: 400 nm and Em:505 nm.

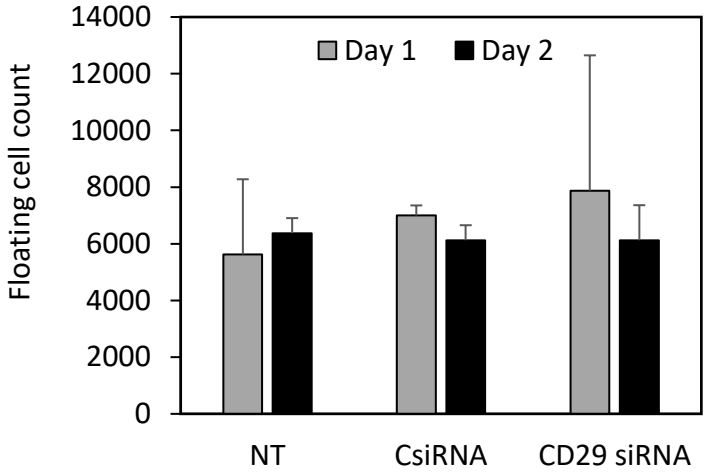

Figure 2.S4: Floating cell number: The number of floating cells were counted after treating the cells with 1.2PEI-LA polymer/siRNA complex for 1 and 2 days.

B. APPENDIX CHAPTER 3

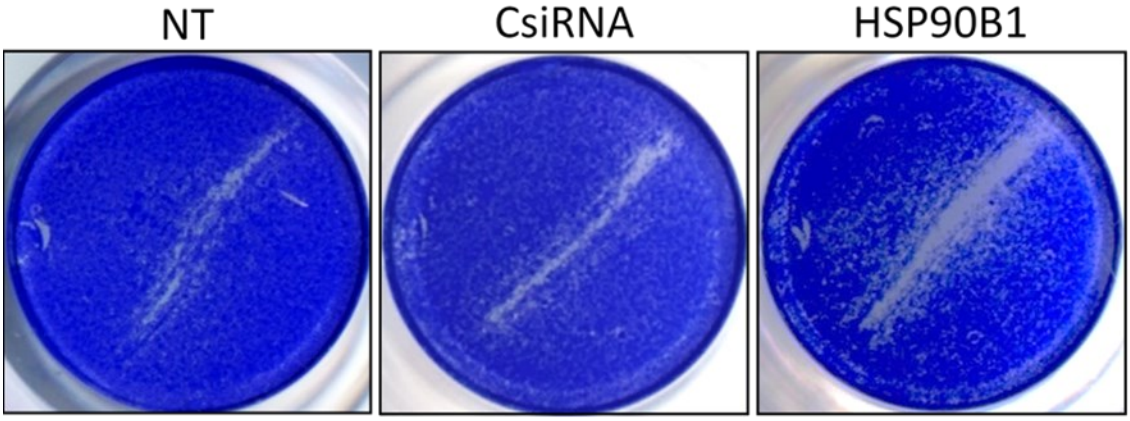

Figure 3.S1: Representative images of NT, CsiRNA and HSP90B1 treatment groups from the siRNA library containing 496 genes, using scratch assay. Migration inhibition was calculated relative to no treatment (NT) 24 hours after scratch formation.

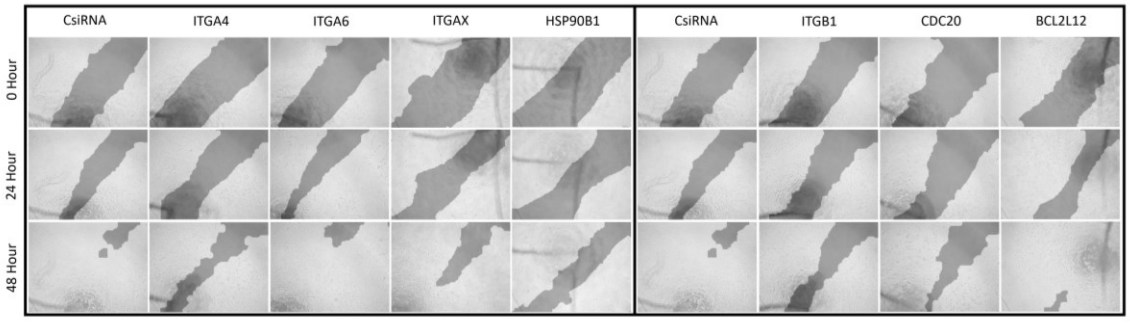

Figure 3.S2: Microscopic images of scratch assay for 1:5:0.15 weight ratios of siRNA:polymer:HA. Effect of silencing ITGA4, ITGA6, ITGAX and HSP90B1 on migration inhibition with CsiRNA as negative control and ITGB1, CDC20 and BCL2L12 as positive controls. Scratch assay was performed after 2 days of siRNA treatment and images of scratches were captured at 0, 24 and 48 hours and the inhibition in migration was calculated using TScratch software.

C. APPENDIX CHAPTER 4

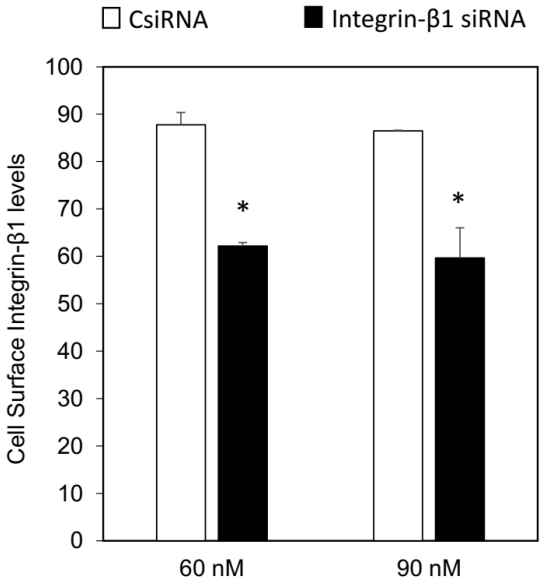

Figure 4.S1: Integrin-β1 cell surface levels with Lipofectamine 2000/ITGB1 siRNA treatment. Integrin-β1 silencing with Lipofectamine 2000/ITGB1 siRNA delivery at 2:1 wt/wt ratio evaluated by flow cytometry at two different siRNA concentrations 60 nM and 90 nM. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no treatment group) were measured after 3 days of siRNA treatment in K562. Control scrambled siRNA - CsiRNA (white bars), ITGB1 siRNA (black bars). Significant difference compared to CsiRNA treatment of each group * $p \leq 0.05$ (n=2).

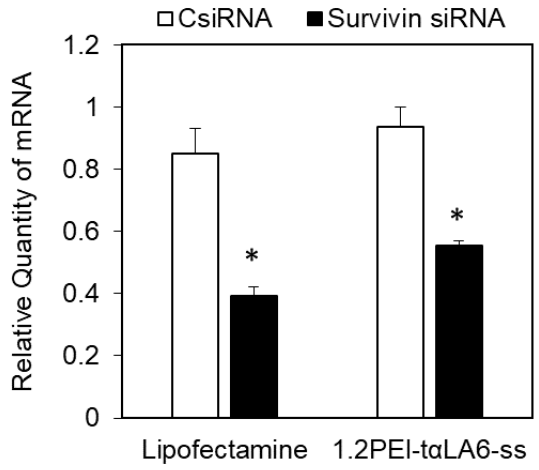

Figure 4.S2: Survivin silencing with 1.2PEI-tαLA6-ss polymer was evaluated by qRT-PCR with 60 nM of siRNA at 9:1 polymer/siRNA wt/wt ratio. Control scrambled siRNA -

CsiRNA (white bars), survivin siRNA (black bars). Significant reduction compared to CsiRNA treatment with each delivery agent *$p \leq 0.05$.

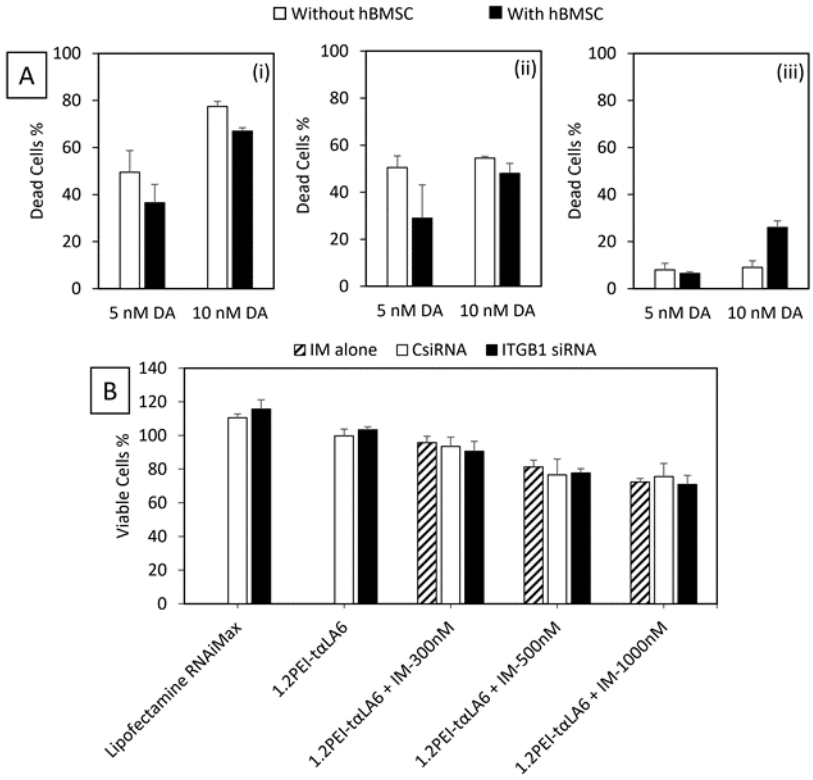

Figure 4.S3: Treatment studies with Dasatinib and Imatinib. (**A**) K562-GFP cells were cultured without and with hBMSC and binding was carried for (i) 1 day, (ii) 3 days, and (iii) 5 days. DA treatment at 5 and 10 nM was accompanied for an additional four days of culture. Dead cell percentage was determined using trypan blue exclusion assay. (**B**) K562-WT cells were treated with polymer complexes of CsiRNA and ITGB1 siRNA alone and in combination with 300, 500 and 1000 nM of IM. Cell viability was measured by MTT assay after 5 days: 2 days of complex treatment and 3 days of IM treatment. Values are plotted relative to no-treatment group.

D. APPENDIX CHAPTER 5

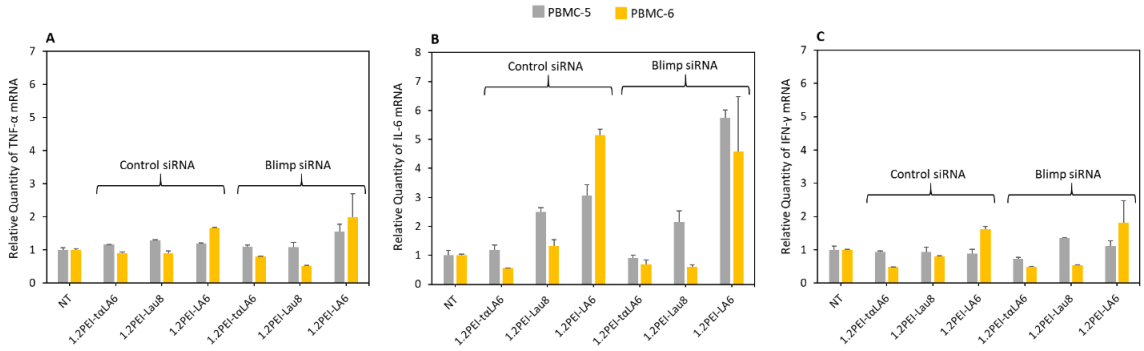

Figure 5.S1: Cytokine mRNA levels with polymer/siRNA complexes at R:6 having 50nM of CsiRNA and blimp siRNA assessed by RT-qPCR after 24 hours of treatment for (**A**) TNF-α, (**B**) IL-6 and (**C**) IFN-γ.

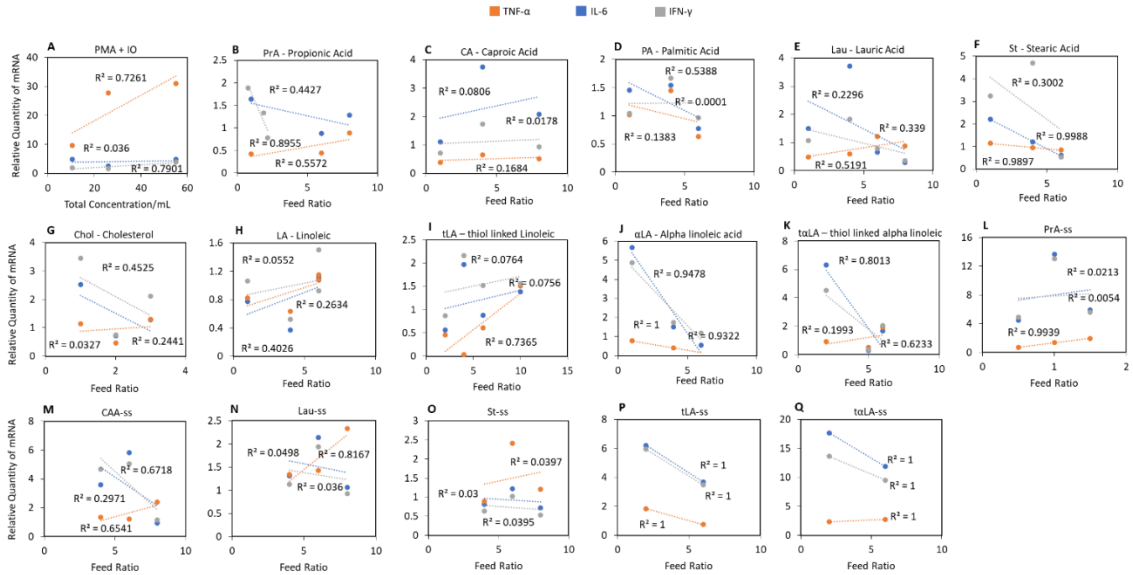

Figure 5.S2: The correlation between different lipids and the batches of lipid substitution. The mRNA levels of TNF-α, IL-6 and IFN-γ assessed after 24 hours of treatment in Jurkat cell line using PEIs substituted with different lipids, complexed with 1µg of pDNA at R:5. (**A**) positive controls PMA/IO, 1.2PEI substituted with (**B**) PrA-Propionic acid, (**C**) CA-Caprylic acid, (**D**) PA-Palmitic acid, (**E**) Lau-Lauric acid, (**F**) St-Stearic acid, (**G**) Chol-Cholesterol, (**H**) LA-Linoleic acid, (**I**) tLA-thiol-linked linoleic acid (**J**) αLA-alpha linoleic acid, (**K**) tαLA-thiol-linked alpha linoleic acid and disulfide cross linked 2PEI substituted with (**L**) PrA-Propionic acid-ss, (**M**) CAA-Caproic acid-ss, (**N**) Lau-Lauric acid-ss, (**O**) St-Stearic acid-ss, (**P**) tLA-thiol linked Linoleic acid-ss, and (**Q**) tαLA-thiol linked alpha linoleic acid-ss.

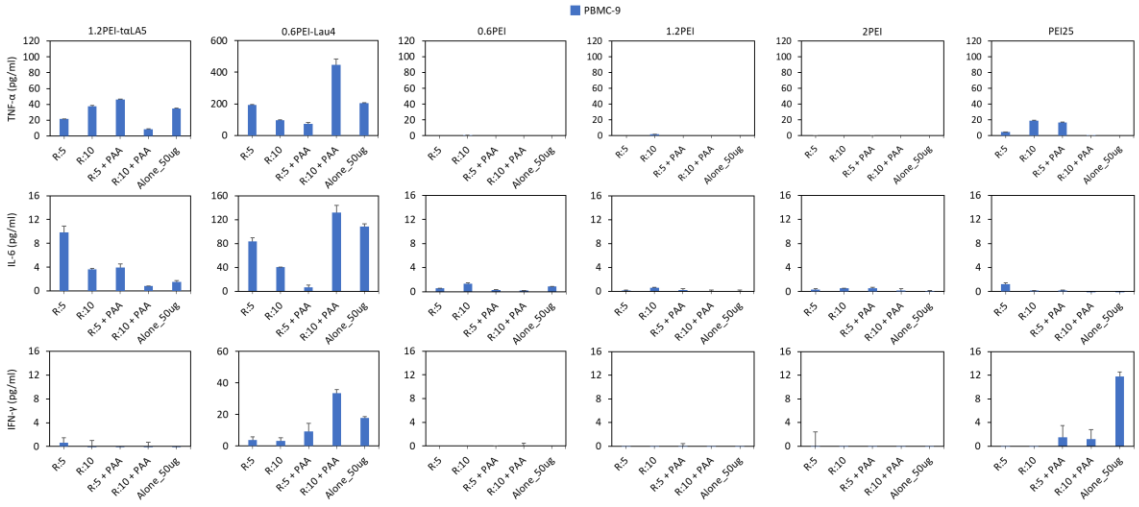

Figure 5.S3: Cytokine protein levels assessed by ELISA after 24 hours of treatment in PBMC-9 with 1.2PEI-tαLA6: **A**- (i) TNF-α, (ii) IL-6, (iii) IFN-γ and 0.6PEI-Lau4: **B**- (i) TNF-α, (ii) IL-6, (iii) IFN-γ as controls. Polymer/pDNA complexes at R:5 and R:10 without and with PAA using 1µg of pDNA were used. Base polymers without lipid modifications were analyzed; 0.6PEI: **C**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 1.2PEI: **D**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ; 2PEI: **E**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ, and PEI25: **F**- (i) TNF-α, (ii) IL-6 and (iii) IFN-γ.

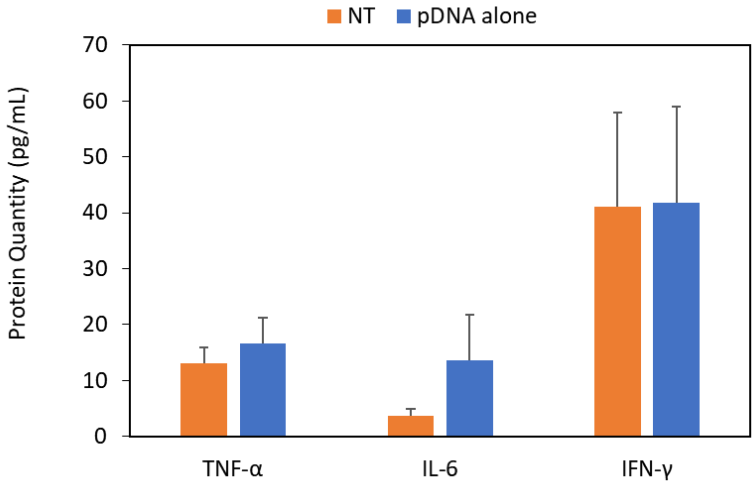

Figure 5.S4: Average cytokine protein stimulation by NT-no treatment and 2 µg of pDNA alone from PBMC-3 to 6 determined following 24 hours of treatment using ELISA.