Lipopolymer Mediated SiRNA Therapy in Acute Lymphoblastic Leukemia

by

Mahsa Mohseni

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ABSTRACT

The nuclear transcription factor, Signal Transducer and Activator of Transcription 5 (STAT5) is one of the key drivers of Acute Lymphoblastic Leukemia (ALL), the most common childhood cancer, that promotes initiation, maintenance and progression to more aggressive stages of the malignancy by regulating the expression of oncogenes resulting in the aberrant proliferation of leukemic cells. Current therapeutic approaches for ALL including multi-agent chemotherapy and hematopoietic stem cell transplantation (HSCT), are associated with certain limitations, such as development of drug resistance leading to high rate of relapse. Therefore, it is outmost of importance to develop more effective and specific therapies for ALL including targeted therapeutic strategies. Small interfering RNA (siRNA) represents a promising tool to specifically target and inhibit the expression of genes that are involved in regulating fundamental cellular activities such as cell proliferation and migration. However, the properties of siRNA molecules including their biological instability, negative charge and large molecular weight prevent cellular delivery. Hence, potent carriers that can overcome extracellular and intracellular delivery hurdles of siRNA molecules and are effective in transfection of difficult-to-transfect cells, such as suspension ALL cells, are warranted for the progression of siRNA-based therapies towards clinical applications. Cationic polymers are one of the promising non-viral delivery systems for safe siRNA delivery due to their ability to bind and neutralize the anionic charges of siRNA molecules and package them into nano-scale complexes. In this thesis, we evaluated the potential of using lipid-modified polyethylenimines (PEIs) of low-molecular weights (0.6, 1.2, 2.0 kDa) in *in vitro* ALL models and patient-derived ALL cells and investigated the efficacy of lipopolymers for siRNA transfection with regard to cytotoxicity, siRNA uptake, gene silencing and biological effects (i.e., cell growth,

and colony formation). The efficacy of siRNA transfection of lipid-substituted PEIs was explored in B-ALL RS4;11 and SUP-B15 cell lines grown in suspension and in ALL primary cells. The hypothesis of this thesis work was that lipid-modified PEIs would be able to effectively deliver siRNA molecules to ALL cells leading to the inhibition of target gene to achieve functional outcomes that could be potentially applied as an alternative therapeutic approach for ALL. Among the library of modified PEIs, linoleic acid (LA) and lauric acid (Lau) substituted PEIs (PEI-LA and PEI-Lau) have proven to be highly effective in delivering siRNAs to ALL cells, and higher uptake of siRNA/polymer complexes was observed compared to other polymer groups. In addition, STAT5A siRNA transfection by effective polymeric delivery systems caused a significant decrease in STAT5A mRNA levels in RS4;11 and SUP-B15 cells and some ALL primary cells which consequently led to marked increase in cell growth inhibition and decrease in colony formation ability *in vitro* compared to control groups. As promising outcomes were obtained in ALL cell lines and also primary cells by siRNA-mediated STAT5A silencing, we then explored the therapeutic effects of combining STAT5A siRNA and currently used chemotherapeutics for ALL including dexamethasone, doxorubicine, vincristine, and also TKIs such as nilotinib and dasatinib in RS4;11 and SUP-B15 cells. However, the results indicated that no synergistic effect was observed when cells treated with the combination of STAT5A siRNA and chemotherapeutics in comparison with individual siRNA or drug groups. Overall, the findings from this study showed the potential of siRNA-mediated STAT5A silencing as an individual therapy and are encouraging for the future design of non-viral delivery system with clinical translation capabilities for the treatment of ALL. This thesis work suggests opportunities for polymeric delivery systems that could be beneficial to inhibit other target genes in ALL and other leukemias (i.e., chronic and acute myeloid leukemias) for therapeutic purposes.

Preface

Parts of this thesis have previously been published, as described below. All chapters presented here are conceptualized, researched and written by me with the involvement of supervisory authors, Prof. Joseph Brandwein and Prof. Hasan Uludağ. Specific contribution of other authors in each chapter are acknowledged and outlined below. Additional acknowledgements are listed at the end of respective chapter. The studies with human cells were conducted under the approval of the University of Alberta Research Ethics Board.

Chapter 1 highlights the importance of the research work and contains portions of published work as a literature review (**Mahsa Mohseni**, Hasan Uludağ, Joseph M Brandwein. Advances in biology of acute lymphoblastic leukemia (ALL) and therapeutic implications. American Journal of Blood Research. 2018 Dec; 8(4), 29–56.) that highlights latest advances in the understanding of ALL biology including the identification of prognostic factors and putative therapeutic targets and summarizes recent studies evaluating the current status of, and ongoing progress in, the development of potential novel therapeutic strategies for ALL based on the understanding of disease biology. As the lead author of this publication, I was responsible for concept formation, conducting the literature review and writing the manuscript. The other section of this chapter focuses on STAT5 nuclear transcription factor as a potential therapeutic target for siRNA therapy following by describing different kinds of delivery systems applied for siRNA therapy.

Chapter 2 is a version of a manuscript submitted to the journal PLOS One journal as (**Mahsa Mohseni**, Cezary Kucharski, Remant Bahadur KC, Xiaoyan Jiang, Hasan Uludağ, Joseph Brandwein. Therapeutic Delivery of siRNA with Polymeric Carriers to Down-regulate STAT5A Expression in High-Risk B-cell Acute Lymphoblastic Leukemia (B-ALL). Submitted to PLOS

One journal on Sep 14, 2020, under review) that focuses on the therapeutic potential of STAT5A inhibition in B-ALL cell lines and ALL patient-derived cells by screening a library of lipid-substituted polymeric nanoparticles to determine the most effective polymeric carriers for STAT5A siRNA delivery. The frozen ALL patient cells were obtained from the biobank at the University of Alberta Hospital (Edmonton, AB, Canada) with the approval of the institutional Health Research Ethics Board. Patient samples with specific genetic abnormalities were selected according to the World Health Organization guidelines for categorizing the ALL subtypes. As the primary author, I designed and performed experiments, collected and analyzed data, and wrote the manuscript. Drs. Brandwein and Uludağ provided guidance with leukemia expertise. Lipid polymer used in these studies were synthesized by R. KC. C. Kucharski provided technical help with cell culture and also harvesting of primary ALL cells and qRT-PCR studies.

Chapter 3 contains a short study on the impact of siRNA-mediated STAT5A silencing on the cytotoxic effect of commonly used drugs in ALL treatment including vincristine, dexamethasone, doxorubicine and also TKIs in RS4;11 and SUP-B15 cells. I was responsible for the design and performing the experiments, collection and analysis of the data, and writing this chapter. R. KC synthesized polymers for siRNA delivery and Drs. Brandwein and Uludağ provided help with the concept formation with leukemia expertise and editing the chapter. As the generated data were not promising, we did not further continue this study.

Chapter 4 consists of overall discussion, conclusions and future directions. This chapter mainly derived from the discussion and conclusions of above three chapters and knowledge gained through my PhD studies.

DEDICATION

To My Mom, Dad and Sister for their Support & Unconditional Love.

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Over the course of my graduate studies, I have had the great opportunity to work with many brilliant researchers and amazing colleagues, and I would like to thank them for their valuable inputs to improve my personal as well as professional life.

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LIST OF ABBREVIATIONS

Ab	Antibody
ABL	Abelson murine leukemia viral oncogene homolog 1
ADC	Antibody drug conjugate
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
AGO	Argonaute
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myelogenous Leukemia
BCL2	B-cell lymphoma 2
BCR	B-cell receptor
BCR	Breakpoint cluster region
BCR-ABL	Breakpoint cluster region – Abelson murine leukemia viral oncogene homolog 1
BFU-E	Burst-forming unity erythroid
BM	Bone marrow
BSA	Bovine serum albumin
CA	Caprylic Acid
CD	Cyclodextrin
Cer	Ceramide
CFC	Colony-forming cell
CFU-GEMM	Colony-forming unit granulocyte, erythrocyte, macrophage, megakaryocyte

CFU-GM	Colony-forming unit granulocyte/macrophage
Chol	Cholesterol
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
COX	Cyclooxygenase
CPP	Cell penetrating peptide
CsiRNA	Scrambled Control siRNA
CTCL	Cutaneous T-cell Lymphoma
CXCR	Chemokine receptor
DMA	1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DODAB	Dioctadecyldimethylammonium bromide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOX	Doxorubicin
DsiRNA	Dicer-Substrate siRNA
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
FAM	Carboxy fluorescein

FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
H-NMR	Hydrogen-1 nuclear magnetic resonance spectroscopy
HSC	Hematopoietic Stem Cell
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
JAK	Janus kinase
KIT	Tyrosine-protein kinase
LA	Linoleic acid
LNP	Lipid nanoparticle
LSC	Leukemic stem cells
LSM	Laser scanning microscopy
LT-HSC	Long term - hematopoietic stem cell
LTC-IC	Long-term culture – initiating cells
MGF	Mammary gland factor
mRNA	Messenger RNA

miRNA	Micro RNA
M-MLV	Moloney murine leukemia virus
MMP	Metalloproteases
MN	Mononuclear
MO	Monoolein
MPA	Mercaptopropionic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
NK	Natural killer
NP	Nanoparticle
NOD	Non-obese diabetic
ODN	Oligonucleotides
PA	Palmitic Acid
PB	Peripheral Blood
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene Glycol
PEI	Polyethylenimine
Ph	Philadelphia
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-biphosphate-3-kinase

PLL	Poly-L-Lysine
PTFE	Polytetrafluoroethylene
RGDS	Arginine-Glycine-Asparagine-Serine peptide
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Real time – polymerase chain reaction
SEM	Scanning Electron Microscopy
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMPB	Sulfo-succinimidyl-4-(p-maleimidophenyl) butyrate
SNALP	Stable nucleic acid lipid particle
STAT	Signal transducer and activator of transcription
TAT	Twin-arginine translocation
TLR	Toll-like receptors
SCT	Stem cell transplantation
STAT	Signal transducer and activator of transcription
TFA	Trifluoroacetic acid
TFR	Transferrin receptor

TKI	Tyrosine kinase inhibitor
T315I	Threonine position 315 to isoleucine mutation
VEGFR	Vascular endothelial growth factor receptor
XPS	X-ray photoelectron spectroscopy
aLA	a- Linoleic Acid

SCOPE

The work of this thesis is focused on the non-viral siRNA delivery in acute lymphoblastic leukemia (ALL) cells. Here, I investigated the ability of lipid-modified polymers to deliver siRNA and obtain gene silencing to produce therapeutic effects in ALL models. The chosen target gene was the signal transducer and activator of transcription 5 (STAT5) gene as it is involved in the induction, and maintenance of disease as well as proliferation and survival of ALL cells. The various lipid-modified polymers used throughout this thesis were chosen based on the use of the most effective polymer available at the time of each study, and as new iteration of polymers generated based on the research findings of this thesis work became available. In Chapter 1, a literature review of the most current knowledge of biology of ALL, and the available therapeutic strategies and their limitations were presented. Furthermore, the RNA interference (RNAi) mechanistic process and a current state of the art of siRNA delivery systems and their therapeutic outcome in ALL models were described. This review highlighted the potential of RNAi in ALL and demonstrated the challenges needed to be considered for the design of siRNA delivery agents for difficult-to-transfect cells and for the translation of siRNA therapies into clinics.

In Chapter 2, with the aim of designing effective non-viral carriers for difficult-to-transfect and suspension growing leukemic cells, the use of lipid-substituted polyethylenimine (PEI) was investigated in RS4;11 and SUP-B15 cells *in vitro* and also in ALL primary cells. In this study, we investigated the use of different lipids for polymer modification, degree of lipid substitutions and polymer molecular weights, and variations in the complex formation to identify suitable characteristics for efficient transfection of RS4;11 and SUP-B15 cells. The siRNA carrier consisting of linoleic acid (LA) and lauric acid (Lau) substitutions on PEI (2PEI-LA6 and 1.2PEI-

Lau8), although they induced some cytotoxicity after transfection, they showed effective siRNA delivery and silencing in ALL cells, which decreased the gene target (STAT5) mRNA and increased cell death after treatment. This study demonstrated the proof-of-principle for the potential of lipid-substituted polymers for a functional therapeutic outcome in ALL cell lines.

With the purpose of evaluating whether lipid-modified polymers may be successfully translated into their use in clinics, we evaluated the siRNA delivery and transfection effect (silencing and biological outcome) of these polymers in ALL primary cells. Those polymers that afforded higher siRNA uptake in ALL cell lines, were selected for further evaluation. STAT5 siRNA delivery and transfection were explored in different ALL patient samples by MTT, cell count, colony assays and qPCR. These studies revealed that the STAT5 gene level and the cell survival *in vitro* can be significantly decreased; and that these effects, are comparable to those found with the ALL cell lines. Although these are preliminary studies with ALL patient cells, these results show further translation potential of these lipid-modified polymers into clinics. Due to constrains with cell numbers, in these studies mononuclear differentiated cells rather than immature and primitive cell portion were used for allowing enough cell numbers for polymer screenings.

As promising results were achieved in ALL cell lines and also primary cells by siRNA-mediated STAT5 silencing, the therapeutic effects of combination of siRNA therapy and currently-used chemotherapeutics for ALL including dexamethasone, doxorubicine, vincristine, and also TKIs such as nilotinib and dasatinib were investigated in ALL cell lines presented in chapter 3. First, IC_{50} of each drug was identified by MTT assay and then three concentrations of each chemotherapeutic agent along with one STAT5 siRNA concentration (60 nM) and one polymer:siRNA ratio of (6:1) were selected to treat ALL cells and evaluate the synergistic effect

of this combination therapy. The outcomes revealed that no synergistic effect was observed when cells treated with the combination of STAT5 siRNA and chemotherapeutics compared with individual siRNA or drug groups. As desired results were not obtained by MTT assay in ALL cell lines, we did not continue this study.

Lastly, in Chapter 4, we presented the overall conclusions of this thesis work and summarized the identified main characteristics needed from the polymeric siRNA carriers to enact an effective gene-mediated biological effect in ALL cells. We also mentioned the challenges that we overcame with our work and the impact these results had in the field, as well as the areas that would require further improvement for the development of siRNA therapeutics in ALL.

Chapter 1

Advances in biology of ALL and therapeutic implications[1]

[1] A version of this chapter was published in: Mahsa Mohseni, Hasan Uludag, Joseph M Brandwein*. Advances in biology of acute lymphoblastic leukemia (ALL) and therapeutic implications. American Journal of Blood Research. 2018 Dec; 8(4), 29–56. (*Corresponding author)

1.1 Introduction

Leukemic cancers arise from genetic abnormalities occurring in normal hematopoietic stem or progenitor cells, resulting in impaired regulation of proliferation, apoptosis, differentiation, and survival of malignant cells. In 2020, an estimated 6900 new cases (4100 males and 2800 females) are expected to be diagnosed with leukemia in Canada and an estimated 3000 Canadians are expected to die of leukemia (1800 males and 1200 females) [1]. The US National Cancer Institute calculated an overall 5-year relative survival (between 2003 and 2009) rate of 56.0% for various leukemias combined [2]. The front-line therapy in leukemia is chemo (drug) therapy [3]; current therapeutic strategies comprise broad-spectrum drugs to inhibit the proliferation of rapidly dividing cells and molecular therapies using small-molecule inhibitors targeting specific signal transduction pathways [4]. Leukemic cells generally respond well to chemotherapy at the beginning of the treatment, but the drug effectivity is lost over a period of 6-12 months. It is well perceived now that development of drug resistance to current chemotherapeutics is inevitable, but recent evidence also showed that even the most advanced molecularly targeted drugs lose their efficacy because of the development of resistance in a relatively short time. The inherent cell plasticity along with different mechanisms of resistance cause cancer cells to naturally alter by mounting an effective resistance against the therapies. Moreover, the high rate of relapse in leukemia patients has been associated with the existence of a rare population of leukemia stem cells (LSC) resistant to conventional drug therapies [5].

By increasing the knowledge and understanding of molecular changes occurring in malignant transformations, treatments that target tumor-specific alterations are expected to result in more successful treatments. For this purpose, the RNA interference (RNAi) mechanism can be utilized

to silence the aberrant genes that are involved in the initiation of this disease and therefore, develop a highly specific leukemia therapy [6,7]. There are different tools in RNAi for gene inhibition, including a plasmid encoding for short hairpin RNA (shRNA; a single strand RNA with a hairpin loop structure), small interfering RNA (siRNA; a double strand RNA), and antisense oligonucleotides (a single DNA or RNA strand). Antisense oligonucleotides perform gene silencing either by promoting mRNA degradation through hybridizing with the target mRNA and forming a heteroduplex that activates RNase H, or by physically translation inhibition [8,9]. Transcription of shRNA is carried out within the cellular nucleus, followed by transferring into the cytoplasm, and then processing by the cellular-machinery into a siRNA-like molecule, which is finally incorporated by cytoplasm molecules for gene inhibition [10,11]. shRNA molecules can be expressed continuously by the host cells therefore; their effect can persist indefinitely [10]. However, siRNA molecules are exogenously delivered to the cells and then loaded onto the RNAi machinery for silencing activity, therefore, the processing steps of shRNA and the transcription are no longer needed. Hence, siRNA silencing effect is transient while they are safe as they cannot be integrated into the host cell's DNA [12–15]. This chapter will focus mainly on siRNA as one of the RNAi mechanisms of gene silencing. In the siRNA mediated silencing of target genes, the siRNA duplexes assemble into a pre-RISC (RNA-induced silencing complex) in the cytosol that consists of specific proteins, including argonaute proteins (AGO1, 3 or 4) [16,17], which is subsequently guided to target desired mRNA based on complementary base pairing [16]. Then, endonucleoyltic cleavage and/or translational inhibition of the mRNA silences the target protein. siRNA can be essentially applied as a pharmaceutical 'drug' that can inhibit effectively any single gene expression within the cells, differing from antibodies or tyrosine kinase inhibitors that only

target and bind to surface antigens and tyrosine kinases, so that the range of possible molecular targets is widely expanded. [16,17].

However, for a successful siRNA therapy, developing effective delivery systems is an absolute necessity since the siRNA molecules are highly sensitive to serum nucleases and their large (~13 kDa) and anionic nature (due to its phosphodiesterase backbone) prevents the siRNA from traversing cellular membranes. Viral vectors for siRNA delivery were initially utilized, but the unfavorable side-effects of viral delivery limited their therapeutic use in a clinical setting. Alternatively, cationic biomolecules as carriers are able to bind and neutralize the anionic charges of siRNA molecules and package them into nano-scale complexes, leading to the safe delivery of nucleic acids without any risk of interactions with genomic materials of the host cells which makes them more likely for clinical implementation [18]. siRNA carriers proper for cellular delivery include: (i) liposomes that are formulations of multiple lipids and siRNA for the formation of solid lipid nanoparticles (NPs); (ii) polycationic polymers that condense siRNA to form NP complexes; and, (iii) carriers consisted of several domains, including cationic, lipophilic, hydrophilic and targeting (e.g. antibody-derivatized) moieties.

In addition, for an effective cancer targeted therapy, we need to have a tumor-specific target. Among different molecular targets, transcription factors including Signal Transducer and Activator of Transcription (STAT) protein family are one of the best targets especially for hematological malignancies as multiple signalling pathways converge on a limited groups of transcription factors leading to activation of expression of oncogenes. Hence, targeting a single transcription factor may inhibit the effect of multiple upstream signaling pathways [19]. One of the best transcription factors to target in ALL, is STAT5 which is a nuclear transcription factor and it conveys signals from cytokines and growth factor receptors to the nucleus. It has been

observed that STAT5 is constitutively activated in hematologic malignancies especially in high-risk B-ALL patients resulting in aberrant cell survival and proliferation of leukemic cells [20,21].

In this chapter, I will focus on a type of leukemia, namely acute lymphoblastic leukemia (ALL); which is the most common childhood cancer. Then, advances in the understanding of ALL biology and recent studies evaluating molecular and immune-based targeted therapies will be summarized. I will next focus on potential novel therapeutic strategies based on our understanding of disease biology and I will summarize the attempts reported in the literature to deliver siRNA molecules using non-viral carriers in leukemia. Most of the work of siRNA treatment in leukemia has been done in *in vitro* cell models using cell lines, with fewer studies in primary cells and animal models. The literature review in this chapter will be focused on advances in biology of ALL and therapeutic implications. In conjunction, potential molecular targets in ALL especially STAT family transcription factors and the technology of effective siRNA delivery to inhibit this protein target will be explored.

1.2 Acute lymphoblastic leukemia (ALL) and current therapeutic approaches

Acute lymphoblastic leukemia (ALL) is a malignancy of hematopoietic stem cells that originates from B- and T-lineage lymphoid precursors and is driven by a spectrum of genetic aberrations including mutations, chromosome translocations and aneuploidy in genes involved in the development of lymphoid cells and regulation of cell cycle progression [22]. ALL is the most common childhood cancer (Fig. 1.1) accounting for 74% of all leukemia diagnosed in people under age 20. ALL has the most incidence rate in children younger than five, but adults have the most death rate (about four out of five), and in adults, the risk of ALL increases after age 50. In 2020, an estimated 1,520 deaths from ALL will occur (860 men and boys and 660 women and girls).

Though the number of new cases of ALL increased by 1% yearly between 2007 and 2016, the rate of death declined by 1% annually between 2008 and 2017 [2,23]. The 5-year survival rates for children diagnosed with ALL has greatly raised over time and is now about 90% overall [14,24,25], and in adolescents and young adults is 75-85%. The outcomes in older adults are inferior, with overall survival rates of 35-55% in middle age adults and under 30% in those over age 60 [26–29]. Specifically, B-ALL that develops from the B-cell precursors accounts for 75% of adult ALL cases, with the remainder of cases consisting of malignant T-cell precursors [30]. It has been observed that two molecular subtypes of B-ALL, BCR-ABL$^+$ and BCR-ABL-like subgroups, are associated with high relapse rates and inferior survival in both childhood and adult B-ALL [31]. Most of the clinical indications of ALL reveal the accumulation of malignant, poorly differentiated lymphoid cells (blast cells) within the bone marrow (BM) or peripheral blood (PB), and the diagnosis is established by the presence of 20% or more lymphoblasts.

The standard front-line therapeutic approach for treatment of pediatric and adult ALL patients consists of multiagent chemotherapy regimens, followed by hematopoietic stem cell transplantation (HSCT) in high-risk groups [26,30,32–34]. Chemotherapy consists of induction, consolidation and long-term maintenance, with central nervous system (CNS) prophylaxis given at intervals throughout therapy. The goal of induction therapy is to achieve complete remission (CR). The backbone of induction therapy typically includes vincristine, corticosteroids and an anthracycline [26,30]. To remove the remaining leukemic cells, consolidation therapy is applied after induction therapy using different combinations of cytotoxic agents. High-dose methotrexate plays a very important role in preventing relapses involving the CNS. At the end of consolidation, re-induction or delayed intensification is given that is a similar combination with induction therapy (steroids, vincristine, asparaginase, and anthracycline). After consolidation therapy, subsequent

maintenance therapy is given and typically persists for 1–2 years. Daily 6-mercaptopurine and weekly methotrexate are the standard combinations, and some maintenance therapies are intensified with vincristine and steroid [35]. Although there have been major advances in the treatment of ALL that has resulted in improved overall survival (OS), significant drawbacks of conventional therapies persist, including severe toxicities and the development of chemoresistance leading to relapse. Relapse is the leading cause of treatment failure occurred in 11 to 36% of those with high-risk B-ALL [36]. Accordingly, to address these limitations, development of novel therapeutic strategies targeting tumor-specific changes is of utmost importance to improve the outcomes of treatment modalities [14,37–39]. This requires a thorough understanding of the biology of this heterogeneous group of diseases. Both B-ALL and T-ALL subtypes harbor distinct groups of chromosomal rearrangements and sequence mutations affecting lymphoid development, tumor suppression, cytokine receptors, and kinase and other signaling pathways [40] (Table 1.1). Differential gene expression patterns in various ALL subtypes have been identified by a number of techniques, including polymerase chain reaction (PCR), genome-wide sequencing, single nucleotide polymorphism (SNP) array analysis and microarrays. These have provided valuable insights into the biology and pathogenesis of ALL, permitting differentiation into prognostic subgroups, and have highlighted potential therapeutic strategies [41] (Table 1.1).

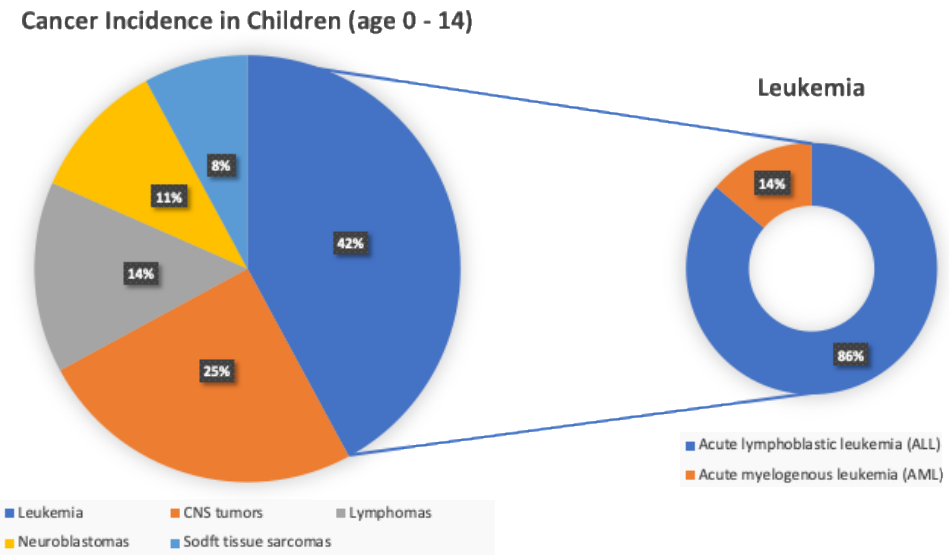

Figure 1.1. Percent of childhood cancer cases (2006-2013). Adopted from "Canadian Cancer Society-Childhood leukemia statistics" and "Childhood cancer incidence and mortality in Canada" by Lawrence Ellison and Teresa Janz (released in September 2015) [243].

1.3 Genetic subsets of B-cell lymphoblastic leukemia/lymphoma (B-ALL)

1.3.1 BCR-ABL1$^+$ ALL

The t(9;22)(q34;q11) translocation, associated with the Philadelphia (Ph) chromosome, is the most common cytogenetic abnormality in adult ALL. The frequency increases with age, occurring in 2-5% of pediatric ALL cases, and 20% of young adults and 30-40% of older adult patients [42,43]. The Ph chromosome encodes the BCR-ABL1 fusion oncogenic protein with constitutively active tyrosine kinase activity. The major breakpoint, which creates a 210-kDa protein, is detected in 24–50% of adult Ph+ ALL [20, 21], but is rare in childhood Ph+ ALL [46]. The minor breakpoint, which encodes a 190-kDa protein, is more prevalent and can be identified in 50–77% of adult Ph+ ALL [42,45] and more than 90% of pediatric cases [47].

Upregulation of *BCR–ABL1* fusion gene leads to activation of multiple signaling pathways such as MAPK, Ras, NF-kB, c-Myc, PI-3 kinase, and JAK-STAT [48]. It also promotes

proliferation of lymphoblasts by the alteration of pro- and anti-apoptotic proteins [37]. One of the main genetic alterations in *BCR-ABL1* positive patients is the mutations and deletions in *IKZF1* gene, encoding for the transcription factor Ikaros which is associated with the unfavorable outcomes and poor prognosis in both Ph^+ and Ph^- ALL [17, 25, 26]. One study on 83 Ph^+ patients demonstrated that 10% lacked *IKZF1* due to chromosome 7 monosomy. Moreover, 63% of patients had a 7p12 deletion of *IKZF1* with different patterns. The most frequent deletions were the loss of exons 4 to 7, detected in 37% of patients, and the loss of exons 2 to 7, detected in 20%. This type of abnormality led to shorter disease-free survival (DFS) compared to patients with *IKZF1* wild type (10 vs. 32 months, P = 0.02) [51]. In addition, the time of cumulative incidence of relapse (CIR) was significantly shorter in patients with *IKZF1* deletions versus patients without this aberration (10.1 vs. 56.1 months, respectively; P= 0.001) [51].

BCR-ABL positive ALL has been associated with an adverse prognosis and is virtually incurable with chemotherapy alone. The advent of *BCR-ABL1*-directed tyrosine kinase inhibitors (TKIs) has significantly improved the response rates and overall survival rates, particularly when used in combination with chemotherapy, although relapse remains a problem [52,53].

1.3.2 BCR-ABL1–like (Ph-like) B-ALL

This high-risk subtype of ALL was first detected by Mullighan and colleagues from the Children's Oncology Group (COG) and St. Jude Children's Research Hospital, (SJCRH) and den Boer and colleagues from the Netherlands in 2009. This subtype is characterized by a gene expression pattern similar to that of the *BCR-ABL1* positive ALL cases [41,54,55], but without *BCR-ABL1* expression. This so-called Ph-like ALL is more prevalent in adolescents and young adults with B-ALL, comprising about 15% of pediatric B-ALL patients age 12-18 and 20-25% of young adult B-ALL cases [39,56–59]. It has been associated with an adverse response to induction

chemotherapy, a higher frequency of persistent minimal residual disease (MRD) and poor survival [49,56,60]. It is the most frequently occurring pediatric and young adult ALL subtype associated with an unfavorable prognosis, with a 5-year disease free survival of about 60% [41,56].

Different types of genomic alterations have been identified in Ph-like ALL, which are involved in the activation of kinase and cytokine receptor signaling. In addition, more than 80% of Ph-like ALL cases have deletions and/or mutations in genes involved in B-cell development including *IKZF1* (the most frequent aberration), paired box 5 *(PAX5), EBF1*, transcription factor 3 (*TCF3*) and *VPREB1* which encodes the immunoglobulin iota chain [30,61].

Translocations of *CRLF2* such as *P2Ry8–CRLF2* fusion (detectable by RT-PCR) or *IGH–CRLF2* rearrangements (detectable by FISH), or translocations resulting in truncation and activation of the erythropoietin receptor (*EPOR*) involving four partner genes (*IGH, IGK, LAIR1* and *THADA*), are the common genomic characteristics of Ph–like ALL [62–66]. It has been observed that *EPOR* rearrangements, overexpression of *CRLF2* (detectable by flow cytometry), translocations and point mutations involved in activating JAK proteins, rare deletions of *SH2B3* (encodes the *JAK2*-negative regulator *LNK*) and activation mutations of *IL7R* result in the constitutive activation of JAK–STAT signaling, which explain the resemblance of kinase activity profiles to those of Ph^+ ALL [49]. B-ALL children with Down syndrome (30-50% of cases) are more likely to have CRLF2 translocations along with point mutations in *JAK* genes (*JAK1* (V617F), *JAK2* (R683G), and *JAK3*) [39,62–64,66–70]. Upregulation of the thymocyte stromal lymphopoietin receptor (TSLPR) encoded by *CRLF2* gene can be detected by flow cytometry in leukemic cells. This receptor, which is induced by the cytokine *TSLP*, is involved in the activation of numerous signaling pathways, including PI3K/AKT/mTOR and JAK/STAT, that are associated with aberrant proliferation and survival of ALL blasts. Therapeutic approaches targeting

PI3K/mTOR, JAK-STAT, and BCL2 signaling pathways have been effective in preclinical models. With the development of *JAK* inhibitors, *JAK* mutations can be considered as potential targets for treatment of this subgroup of ALL patients [55,71–74].

Another Ph-like-associated genetic aberration involves ABL-class fusion genes, including translocations of *ABL1* (with partners other than *BCR*), *ABL2, PDGFRB* and *CSF1R* (encoding the macrophage colony-stimulating factor receptor) which have been observed in cases with translocations in tyrosine kinases genes. These types of abnormalities have been detected in about 3-5% of childhood ALL patients and 2-3% of adult ALL patients [55,56,74]. Preclinical studies suggest that TKIs, including imatinib and dasatinib, may represent effective treatment options for the Ph-like ALL patients with ABL-class fusions [52,55,56,74]. In addition, cases with *ETV6-JAK2* and *BCR-JAK2*–rearrangements are considered as Ph-like variants. It has also been suggested that TKI therapy could be very effective for patients with *EBF1-PDGFRB* translocations [39,49,52,55,56,74,75].

Other rearrangements involving kinase genes such as *ETV6-NTRK3* fusion, *FGFR1, TYK2, IL2RB, BLNK, DGKH, LYN, PTK2B, FLT3* and *RAS* subfamily genes are identified in Ph-like ALL cases [39,56,57,76–79]. Patients with tyrosine receptor kinase (TRK) fusions are sensitive to TRK inhibitors [80] and ponatinib, which is a kinase inhibitor of *FGFR1* can be considered for cases with *FGFR1* fusion [55].

1.3.3 MLL rearrangements

The t(4;11)(q21;q23) translocation, resulting in the *MLL-AF4* fusion gene, is the most frequently occurring aberration in infants with ALL. This abnormality is also detected in 3–7% of adult ALL cases and confers a poor prognosis [19, 57]. Other *MLL* gene rearrangements can be seen, including *MLL-AF10* in t(10;11), *MLL-AF9* in t(9;11) and *MLL-ENL* in t(11;19). The *MLL*

gene, also called *KMT2A*, codes for a histone methyltransferase that regulates gene transcription. Gene expression profiling has identified distinct *MLL*-associated gene signatures in ALL [82].

1.3.4 Hypodiploid B-ALL

This group, characterized by having less than 44 chromosomes, composes 2-3% of ALL patients, and is associated with poor outcomes [83]. It has different subtypes with distinct genetic alterations: Low hypodiploid ALL, with 32 to 39 chromosomes, is associated with several abnormalities, including *TP53* mutations (in 91% of patients), *RB1* (41% of cases) and *IKZF2* alterations (53% of cases). Another subset is the near-haploid ALL with 24 to 31 chromosomes, associated with aberrations such as *IKZF3* alterations (13% of patients) and mutations involved in Ras signaling pathway activation (71% of cases). In pediatric hypodiploid ALL cases, aberrations detected by next-generation sequencing (NGS) include RAS signaling (*NRAS, KRAS, FLT3* and *NF1*), receptor tyrosine kinases (70% of near haploid cases), *IKZF2* and *TP53* mutation of low hypodiploid cases) [50,84].

As Ras and PI3K signaling pathways are activated in both near haploid and low-hypodiploid subtypes, these pathways can be considered as potential targets in the treatment of hypodiploid ALL [30,84,85].

1.3.5 B-ALL with intrachromosomal amplification of chromosome 21 (iAMP21)

The amplification of a portion of chromosome 21, which can be recognized by FISH using the *RUNX1* gene probe, is the main characteristic of this subtype of ALL. The result of metaphase FISH reveals ≥5 or ≥3 extra copies of genes on a single anomalous chromosome 21. This subtype rarely occurs in adults, but accounts for about 2% of childhood ALL with a higher incidence rate in older children, and patients typically have low WBC counts and a poor prognosis. Patients with

this abnormality have been categorized as a standard-risk group; however, studies have revealed a shorter event-free survival (EFS) and overall survival upon treatment based on the standard-risk protocols [11, 62, 63]. More intensified chemotherapy regimens appear to improve the poor prognosis of this entity [64].

1.3.6 B-ALL with *DUX4* and *ERG* deregulation

This recently identified subgroup of B-ALL accounts for about 7% patients. It is characterized by deregulation of the double homeobox 4 gene (*DUX4*) and the *ETS* transcription factor gene (*ERG*). In this subtype, the expression of a truncated isoform of *DUX4* has been identified in the B-cell lineage as a result of *DUX4* rearrangement, while *DUX4* cannot be expressed in normal B cells. This isoform has the ability to bind to the *ERG* intron 6, leading to the deregulation of *ERG* and expression of a noncanonical first exon and transcript, *ERGalt*. This results in the inhibition of the transcriptional activity of wild-type *ERG* and plays an important role in the early initiation of leukemogenesis [40,88–91]. In general, *DUX4/ERG* deregulated ALL is associated with a good response; however, the presence of other genetic aberrations, including *IKZF1* deletions along with *DUX4/ERG* deregulation, is associated with an unfavorable outcome [40,92,93].

1.3.7 Other molecular aberrations in B-ALL

Hyperdiploidy occurs in about 25% of childhood ALL and has a favorable prognosis. These cases have been associated with an approximately ten-fold over-expression of the *SH3BP5* gene, which encodes *SH3*-binding protein 5 located on chromosome 3p24. This protein may be involved in transferring signals from Bruton's tyrosine kinase (BTK) receptor, suggesting this receptor or its downstream signaling pathways may constitute potential therapeutic targets [41,94]. Disruptions in *CREBBP* gene have also been identified in relapsed hyperdiploid cases [95].

The t(12;21)(p13;q22) translocation, resulting in the *TEL–AML1* (*ETV6–RUNX1*) fusion gene, accounts for 20-25% of childhood B-ALL. The erythropoietin receptor, which is found in myeloid lineage progenitor cells, can be expressed ~7-fold higher in *ETV6–RUNX1*-positive cases. The upregulation of this gene implies that either *ETV6–RUNX1*-positive cases can express myeloid associated markers, or this gene might have non-erythropoietin related functions [41,96–98]. Translocation (1;19)(q23;p13.3), occurring in adult B-ALL subtype, generates a chimeric *E2A-PBX1* gene, is seen in ~5% of cases, and has been associated with poor outcome in some studies [43,99].

About 80% of ALL cases have epigenetic alterations, such as promoter hypermethylation of tumor-suppressor genes, leading to inactivation of tumor-suppressor genes or hypomethylation of oncogenes. Evaluation of epigenetic changes by the use of methylation-specific PCR in ALL is of the utmost importance because different steps involved in this process can be potential targets for many current chemotherapeutic agents including DNA methyltransferase inhibitors [26,100–105].

1.4 Genetic alterations in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL)

T-ALL, comprising 10-15% of ALL cases, has been associated with a number of genetic lesions [85,106]. The most common T-cell receptor (*TCR*) breakpoint is at 14q11.2, which are the alpha and delta T-cell receptor loci [*TRA* and *TRD*]. In adult T-ALL, *HOX11* gene overexpression resulting from t(10;14)(q24;q11.2) is the most frequent TCR rearrangement [43,107]. However, in childhood T-ALL, t(1;14)(p32;q11.2) leading to *SCL* (also called *TAL1* or *TCL5*) overexpression

(identified in about 3% of cases) and the *SIL-SCL* deletion (chromosome 1 deletion juxtaposing *SCL* and *SIL* genes, which is recognized in 6–26% of cases) are the most common aberrations [107]. Moreover, upregulation of *HOX11L2*, resulting from a cryptic t(5;14)(q35;q32) translocation, is detected in 20–30% of pediatric T-ALL [108,109]. The *TCR* gene can also be rearranged to other fusion partners such as *TAL2, LYL1, OLIG2, LMO1, LMO2, NKX2-1, NKX2-2, NKX2-5, HOXA* genes, *MYC*, and *MYB* [40,110]. In addition, in-frame fusion genes encoding oncogenic proteins such as *PICALM-MLLT10*, *MLL* gene rearrangements, *SET-NUP214* fusion, *EML1-ABL1, ETV6-ABL1* and *NUP214-ABL1* fusion formed on episomes, can be caused by chromosomal rearrangements [50].

Common sequence mutations in T-ALL detected by re-sequencing and NGS consist of those in translocation-associated Notch homolog 1 (*NOTCH1*) (detectable in >60% of cases), *FBW7* (detectable in >20% of cases), *PTPN2*, and *MYB*, genes involved in the RAS/PI3K/AKT (*NRAS, KRAS*, and *PTEN*) and JAK-STAT (*JAK1, JAK3, IL7R*, and *STAT5B*) pathways, in transcription regulators (*BCL11B, LEF1, WT1*, and *ZEB2*), in epigenetic regulators (*SUZ12, PHF6, EZH2, TET2, H3F3A*, and *KDM6A*) and in genes associated with the maturation of mRNA and activity of ribosomes (*CNOT3, RPL5*, and *RPL10*) [111,112]. Cases with mutations in *NOTCH1,* a gene expressing a transmembrane receptor involved in T-cell development, and *FBW7* genes are considered as low-risk patients, while patients who lack these mutations or have mutations involving *RAS/PTEN* are defined as high-risk cases [113–115]. Moreover, *JAK1* mutations are associated with poor prognosis in T-ALL [116,117].

Early T-precursor (ETP) ALL has recently been identified as a distinct subtype. This heterogeneous subtype shows limited early T-cell differentiation phenotype and has some myeloid and hematopoietic stem cell associated genetic and immunophenotypic features. This subset

overexpresses myeloid transcription factors such as *CEBPA, CEBPB, CEBPD* and a group of micro-RNAs, including miR-221, miR-222 and miR-223 [50,118–122].

Studies of ETP ALL blasts have revealed the presence of mutations in several cellular pathways including Ras, kinase and cytokine receptor signaling genes (*NRAS, KRAS, IL7R, JAK1, JAK3, NF1, PTPN11*, and *SH2B3*), myeloid-associated genes (*FLT3, DNMT3A, IDH1, IDH2* and *ETV6*), genes involved in lymphoid and hematopoietic development (*RUNX1, IKZF1, GATA3*, and *EP300*) and epigenetic regulators with loss-of-function mutations (*EZH2, SUZ12, EED*, and *SETD2*). In this subtype, common gene mutations in typical T-ALL such as mutations in *NOTCH1* (detectable in >60% of T-ALL cases) or *CDKN1/2* are rarely observed [121]. In general, this subgroup has an unfavorable response to standard therapy; however, current risk adapted therapy may improve the therapeutic outcome [123,124]. As JAK-STAT and PRC2 pathways are active in ETP-ALL, *JAK* inhibitors and chromatin-modifying agents may be potentially beneficial as therapeutic options [125].

Table 1.1: Genetic aberrations and targeting agents associated with different ALL subtypes.

ALL subtype	Genetic Aberrations	Targeting Agents
BCR-ABL$^+$ ALL	t(9,22) (q34,q11) translocation resulting in BCR-ABL1 fusion protein	Tyrosine kinase inhibitors
	MAPK, Ras, NF-Kβ, C-Myc-PI3K-JAK-STAT pathways, Src family kinases	JAK inhibitors,

		Proteasome inhibitors
	Mutations and deletions of *IKZF1* gene	-
Ph-like B-ALL	Mutations and deletions of *IKZF1, PAX5, EBF1, TCF3, VPREB1* genes	-
	Overexpression of *CRLF2,* Translocations of *CRLF2* such as P2RY8-CRLF2 and IGH-CRLF2 rearrangements	JAK inhibitors
	EPOR rearrangements, *EPOR* translocations involving 4 partner genes (*IGH, IGK, LAIR1, THADA*)	JAK inhibitors
	Point mutations of *JAK* genes (*JAK1* (V617F), *JAK2* (R683G), *JAK3*)	JAK inhibitors
	Rare deletions of *SH2B3*	-
	Mutations of *IL7R*	-
	Activation of numerous signaling pathways including PI3K/AKT/mTOR, JAK-STAT, BCL2	JAK inhibitors, mTOR inhibitors

	ABL class fusion genes including translocations of *ABL1, ABL2, PDGFRB, CSF1R*	Tyrosine kinase inhibitors
	Rearrangements including *ETV6-JAK2, BCR-JAK2, EBF1-PDGFRB, ETV6-NTRK3*	JAK inhibitors, Tyrosine kinase inhibitors
	Translocations involving *FGFR1, TYK2, IL2RB, BLNK, DGKH, LYN, PTK2B, FLT3*, and *RAS* subfamily genes	-
Low hypodiploid ALL	*TP53* mutations	-
	RB1 alterations	-
	IKZF2 alterations	-
Near haploid ALL	*IKZF3* alterations	-
	Mutations in Ras signalling pathways and PI3K	mTOR inhibitors
B-ALL with intrachromosomal amplification of chromosome 21 (iAMP21)	Amplification of a portion of chromosome 21	-

B-ALL with DUX4 and ERG deregulation	Deregulation of DUX4 and ERG	-
B-ALL cases with hyperdiploidy	Overexpression of *SH3BP5* gene	-
	Disruptions in *CREBBP* gene	-
Other abnormalities in B-ALL subtypes	MLL rearrangements specially *MLL-AF4* fusion gene	BCL-2 inhibitors
	TEL-AML1 fusion gene	-
	E2A-PBX1 fusion gene	-
	Epigenetic alterations	HDAC inhibitors, DNMT inhibitors
T-ALL	TCR rearrangements with fusion partners including *TAL2, LYL1, OLIG2, LMO1, LMO2, NKX2-1, NKX2-2, NKX2-5, HOXA*, *MYC*, and *MYB*	-
	Overexpression of *HOX11, HOX11L2,* and *SCL* genes	-
	Deletion of *SIL-SCL* gene	-
	In-frame infusion genes such as *PICALM-MLLT10*, *MLL* gene rearrangements, *SET-NUP214* fusion,	JAK inhibitors, BCL-2 inhibitors

	EML1-ABL1, ETV6-ABL1 and *NUP214-ABL1*	
	Sequence mutations in genes such as *NOTCH1*, *FBW7*, *PTPN2*, *MYB*, *NRAS, KRAS*, *PTEN*, *JAK1, JAK3, IL7R*, *STAT5B*, *BCL11B, LEF1, WT1*, *ZEB2*, *SUZ12, PHF6, EZH2, TET2, H3F3A*, *KDM6A CNOT3, RPL5*, *RPL10*	JAK inhibitors
ETP ALL	Overexpression of *CEBPA, CEBPB, CEBPD,* miR-221, miR-222 and miR-223	JAK inhibitors and chromatin modifying agents
	Mutations in genes including *NRAS, KRAS, IL7R, JAK1, JAK3, NF1, PTPN11*, and *SH2B3*, *FLT3, DNMT3A, IDH1, IDH2, ETV6*, *RUNX1, IKZF1, GATA3*, *EP300, EZH2, SUZ12, EED*, *SETD2*	JAK inhibitors and chromatin modifying agents

1.5 Targeted therapeutic approaches in ALL

1.5.1 Small molecule inhibitors

1.5.1.1 BCR-ABL1-directed TKIs

This class of agents has revolutionized the treatment of BCR-ABL$^+$ B-ALL. When used as single agents combined with corticosteroids, imatinib or dasatinib can produce complete responses in virtually 100% of cases, but usually leads to the rapid emergence of resistant clones, most commonly due to point mutations within the *BCR-ABL* kinase domain, resulting in relapse [52,53]. Other mechanisms of resistance include increased drug efflux and recruitment of alternative active cell signaling pathways and kinases including *Src*-family kinases leading to cell proliferation and inhibition of apoptosis [126,127]. These agents have also resulted in significant improvement in treatment outcomes when used in combination with conventional chemotherapy [5, 11, 13]. Imatinib mesylate combined with chemotherapy dramatically increased the 3-year EFS rate from 35% to 80% in pediatric Ph$^+$ ALL compared with chemotherapy alone [128]. The COG AALL0031 study, using the combination of imatinib and intensive chemotherapy, reported equivalent or better 5-year DFS (70%) compared to those who received allogenic HSCT from related or unrelated donors (65% and 59%, respectively) [129]. Children with refractory Ph$^+$ B-ALL demonstrated remissions following treatment with imatinib and dasatinib in combination with chemotherapy [130,131]. Dasatinib and nilotinib, which are more potent and have activity in some imatinib-resistant clones, have produced remissions in some cases that have relapsed on imatinib therapy [26,132,133].

A number of studies have evaluated the use of imatinib, dasatinib and nilotinib, in combination with chemotherapy in adults with Ph$^+$ B-ALL [134–136], which was recently

reviewed [137]. Although results are superior to previous studies with chemotherapy alone, relapse due to the emergence of resistant clones remains a problem. The use of dasatinib has been associated with a high frequency of relapse with resistant T315I mutations [136]. More recently, the addition of ponatinib, which is active against resistant T315I clones, to chemotherapy has produced encouraging 3-year EFS in adults with Ph^+ ALL [138,139], and a number of further studies with this agent are either in progress or are being planned.

In Ph-like ALL patients with ABL-class fusions, adding imatinib or dasatinib to combination chemotherapy regimens has resulted in the induction of remissions and clearance of minimal residual disease (MRD) in isolated cases [140–143]. Furthermore, dasatinib was effective in a pediatric Ph-like ALL case with persistent post-transplant MRD [144]. A Phase II clinical trial by MDACC (NCT02420717) is evaluating the use of either ruxolitinib or dasatinib, based on molecular profiling, as initial monotherapy, followed by the addition of Hyper-CVAD chemotherapy [55,145]. A COG AALL1131 trial (NCT02883049) is currently evaluating the efficacy of dasatinib in Ph-like ALL patients with ABL-class mutations.

1.5.1.2 PI3K/mTOR inhibitors

Several small molecule inhibitors have been developed to efficiently target different aberrantly activated signaling pathways [30,37,38]. One of the constitutive activated signaling pathways in B- and T-ALL is the PI3K/Akt/mTOR pathway which can promote drug resistance, cell proliferation and metabolism. Mammalian target of rapamycin (*mTOR*) which is a downstream target of Akt, functions as a serine/threonine kinase and comprises a core component of two protein complexes: mTORC1 and mTORC2 [14,30]. mTOR kinase activity can be inhibited by small molecules such as everolimus, temsirolimus, and sirolimus [14,30]. Studies reported that everolimus induced apoptosis in B-ALL cell lines and reduced enzyme phosphorylation in Akt

and mTOR signaling [146–148]. In addition, a Phase I/II trial in relapsed childhood ALL treated with everolimus in combination with hyper-CVAD (fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone) reported a CR rate of 25% [149]. Temsirolimus is being evaluated in combination with intensive re-induction therapy through a phase I COG study ADVL1114 in relapsed ALL cases (NCT01403415), and also in combination with etoposide, cyclophosphamide, and dexamethasone for children with refractory ALL (NCT01614197). The efficacy of sirolimus plus corticosteroids is under investigation in a Phase I trial in relapsed ALL (NCT00874562).

Dactolisib (BEZ235) is the first dual PI3K/mTOR inhibitor whose efficacy alone and in combination was investigated in clinical trials for hematological malignancies [150–153]. A preclinical study evaluated the effect of BEZ235 on the resistance mechanisms to glucocorticoids which is mediated by constitutive activation of the PI3K/mTOR signaling pathway in T-ALL. The findings demonstrated that BEZ235 enhanced the cytotoxic activity of dexamethasone in various T-ALL cell lines and xenograft models through inhibition of *AKT1* leading to upregulation of pro-apoptotic protein BIM and downregulation of anti-apoptotic protein MCL-1 in an AKT-inactivation independent manner. This study suggested that BEZ235 could be a potential therapeutic option, capable of increasing dexamethasone-induced apoptosis and reversing glucocorticoid resistance in children with T-ALL [154].

1.5.1.3 BTK inhibitors

BTK, which is a member of the BCR signaling pathway and is involved in the B cell development, can be irreversibly inhibited by ibrutinib. It was reported that ibrutinib could significantly reduce cell proliferation in mouse xenograft models and BCR-positive human ALL

cell lines [155–158]. A Phase II trial is assessing ibrutinib efficacy in combination with blinatumomab for adults with relapsed B-ALL (NCT02997761).

1.5.1.4 JAK/STAT inhibitors

In Ph-like ALL with JAK/STAT pathway alterations, including *CRLF2* rearrangements, JAK mutations, *JAK2* fusions and *EPOR* rearrangements, using ruxolitinib as a selective *JAK* inhibitor, *USP9X* inhibitors, and givinostat as a histone deacetylase inhibitor can be considered as potential therapeutic strategies [55,72,159–162]. Currently, two clinical trials, COG AALL1521 Phase II trial (NCT02723994) and a subset of SJCRH Total XVII, are evaluating the efficacy of adding ruxolitinib to multi-agent chemotherapy in Ph-like patients with JAK pathway lesions. Moreover, ruxolitinib in combination with chemotherapy is also being investigated by the MD Anderson Cancer Center (MDACC) trial (NCT02420717) in adult and adolescent Ph-like patients with JAK pathway lesions. In this trial, patients receive 3 weeks of ruxolitinib monotherapy followed by multi-agent chemotherapy for patients with an incomplete response. A combination of ruxolitinib, dasatinib and dexamethasone is being assessed in a Phase I trial (NCT02494882) in older patients with newly diagnosed Ph^+ ALL.

1.5.1.5 MEK inhibitors

MAPK/ERK pathway deregulation has been observed in hematologic malignancies as well. 40% of children with relapsed ALL and 6% of Ph-like ALL patients contain mutations in *KRAS* and *NRAS* genes and MAPK signaling pathway [14,37,55,56,163]. Selumetinib, which is an inhibitor of mitogen-activated protein kinase (*MEK*), has been effective in *RAS* mutated ALL cell line models [14,163]. Moreover, the cytotoxic effect of concomitant inhibition of MEK and PI3K/AKT pathways was revealed in T-ALL cells [164]. Pimasertib and trametinib are other inhibitors of *MEK1/2*; however, their effects have not been clinically evaluated in ALL [37]. A

preclinical study investigated the efficacy of trametinib alone and in combination with BCL-2 inhibitors, ABT-199 and ABT-263, in different B-ALL cell lines and primary B-ALL patient cells. The results demonstrated that trametinib alone could modestly affect the cell viability; however, a combination of *MEK* and *BCL-2* inhibitors showed a synergistic effect and significantly suppressed proliferation and induced apoptosis in B-ALL cells through a MEK/ERK signaling-dependent mechanism mediated by the pro-apoptotic factor *BIM* [27].

1.5.1.6 *FLT3* inhibitors

While mutations of FMS-like tyrosine kinase 3 (*FLT3*) are uncommon in ALL, *FLT3* is highly expressed and often mutated in ALL with MLL rearrangement and in childhood hyperdiploid ALL [165,166]. Midostaurin, quizartinib, and lestaurtinib are kinase inhibitors with anti-*FLT3* activity [37]. The Therapeutic Advances in Childhood Leukemia & Lymphoma (TACL) study (NCT01411267) has evaluated quizartinib in relapsed pediatric ALL. Lestaurtinib is also being explored in infants and young children by a laboratory biomarker study (NCT01150669) and a Phase III trial (NCT00557193), respectively.

1.5.1.7 *PARP* inhibitors

Veliparib is an inhibitor of poly (ADP-ribose) polymerase (*PARP*), a protein involved in DNA repair, genomic stability, and programmed cell death. It was observed that veliparib could inhibit cell proliferation by apoptosis induction in human T-ALL cell line models [37,167]. Multiple clinical trials are now evaluating veliparib in combination with other inhibitors and chemotherapeutic agents in various ALL subgroups including a Phase I, multi-center trial (NCT01139970) investigating veliparib and temozolomide, another Phase I study (NCT00588991) assessing veliparib and topotecan with or without carboplatin and a Phase I/II trial (NCT01326702) evaluating veliparib, bendamustine, and rituximab combination.

1.5.1.8 Apoptosis inhibitors

YM155 is a small molecule inhibitor of survivin [168], which is a member of the Inhibitor of Apoptosis Protein (IAP) family involved in the cancer development. It was originally suggested to act as a transcriptional suppressor of survivin, but recent evidence is suggesting a multitude of activities in its mechanism of action [169]. Since survivin overexpression was observed in relapsed childhood ALL,[170] the efficacy of YM155 in combination with dasatinib was evaluated in various subtypes of primary ALL samples and ALL cell lines including Ph^+ ALL. The results revealed significant sensitivity of ALL cells to YM155 treatment. In addition to its action on downregulation of survivin expression, its activation of the DNA damage pathway is leading to apoptosis induction and chemo-sensitivity in ALL cells [171].

The B cell lymphoma-2 (*BCL-2*) proteins are involved in the cell death regulation and can prevent apoptosis through binding to anti-apoptotic proteins. *BCL-2* inhibition by selective inhibitors can induce apoptosis in malignant cells. Navitoclax (ABT-263) is believed to inhibit the binding of BCL-2 protein to apoptotic effectors Bax and Bak proteins [172]. It has been evaluated through preclinical xenograft models of B-ALL, T-ALL and *MLL*-mutated ALL and the results were promising [37,173]. However, navitoclax could not be evaluated in childhood ALL due to dose-limiting thrombocytopenia. Venetoclax (ABT-199) is also a second generation of *BCL-2* inhibitor which showed promising activity in xenograft models of ALL [173]. One study reported that high level of *BCL-2* expression induced by *MLL/AF4* fusion protein was significantly decreased by venetoclax in *MLL*-rearranged ALL cells and also showed a synergistic effect of venetoclax and standard-induction-type chemotherapeutic agents on both *MLL*-rearranged cell lines and xenograft models [174]. A Phase I study has opened evaluating venetoclax and navitoclax in combination with chemotherapy in children and adults with relapsed B-ALL (NCT03181126).

Moreover, *BCL-2* can be upregulated through the tyrosine kinase 2 (*TYK2*) signaling and its downstream effector phospho-*STAT1*. It has been observed that inhibition of heat shock protein 90 (*HSP90*) by its specific small molecule inhibitor NVP-AUY922 (AUY922) resulted in the blockage of *TYK2* signaling and downregulation of phospho-*STAT1* and *BCL-2* in T-ALL cells. In addition, pro-apoptotic proteins BIM and BAD were upregulated by AUY922 which in combination with *BCL-2* downregulation led to induction of apoptosis in T-ALL cells [175].

1.5.1.9 Proteasome inhibitors

Another aberration in ALL is the deregulation of nuclear factor kappa-B (*NF-KB*), a transcription factor involved in the expression of oncogenes leading to protecting cells from apoptosis. It was observed that *NF-KB* is constitutively activated in different malignancies; inhibition of the activity of *NF-KB* by proteasome inhibitors including bortezomib, carfilzomib, and ixazomib can induce apoptosis in tumor cells or increase the sensitivity of cells to anti-tumor agents [14,37,176].

Bortezomib, which inhibits the 26S proteasome reversibly, has been effective in combination with other chemotherapeutics such as dexamethasone, asparaginase, vincristine, doxorubicin, and cytarabine in pre-clinical studies of ALL [177–180]. Different clinical trials have investigated bortezomib in combination with re-induction chemotherapy in ALL patients. A Phase I/II trial of the TACL recruited children with relapsed ALL, with a response rate of 73% [179]; a Phase II AALL07P1 COG study in relapsed B-ALL (61 cases) and T-ALL (17 cases) patients showed CR rates of 69% and 65%, respectively [181]. A Phase II study (NCT01769209) in adult ALL patients, another Phase II trial (NCT02535806) in childhood ALL patients, and a randomized Phase III COG trial (AALL1231) recruiting newly diagnosed young T-ALL patients or stage II-IV T-ALL cases receiving chemotherapy with or without bortezomib (NCT02112916), are investigating the

effectiveness of bortezomib. The efficacy of bortezomib in combination with other inhibitors including HDAC inhibitors which target epigenetic-related abnormalities has been evaluated; however, the results are yet to be published.

Carfilzomib, a more potent proteasome inhibitor with higher specificity [37], was investigated by a Phase I trial (NCT01137747) for AML and ALL cases in 2014 but the outcomes were not published. Moreover, a Phase I study (NCT02293109) is evaluating the optimal tolerable dose of carfilzomib in combination with hyper-CVAD, and another study (NCT02228772) is exploring the tolerability and safety of carfilzomib combined with re-induction chemotherapy in patients with refractory ALL. Ixazomib, another proteasome inhibitor, is under investigation in combination with chemotherapy by a Phase I trial (NCT02228772) for ALL patients.

1.5.1.10 Inhibitors of epigenetic modifications

Epigenetic abnormalities have been also considered a significant source of transformations in ALL. Deacetylation of lysine residue on histones mediated by histone deacetylase (HDACs) is an epigenetic abnormality leading to silencing of the transcription of tumor suppressor genes [176]. Therefore, inhibition of HDACs can stop cell proliferation and induce programmed cell death. Belinostat, vorinostat, and panobinostat are HDAC inhibitors, [37] which have been investigated through several clinical trials in ALL patients. Relapsed ALL patients were treated in a Phase II study (NCT01483690) with a combination of vorinostat, decitabine and chemotherapy; however, high toxicity resulted in the termination of the study. A Phase I trial (NCT00348985) investigated the effect of the combination of belinostat and bortezomib on adult patients with refractory T-ALL, but the outcomes were not published. The effectiveness of panobinostat in combination with bortezomib, liposomal vincristine and salvage therapy is being evaluated in a Phase II study (NCT02518750) in children and young adults with refractory T-ALL.

DNA methylation of cytosine-phosphate diesterguanine (CpG) islands by DNA methyltransferases (DNMTs) is another epigenetic silencing mechanism resulting in suppression of tumor suppressor genes. Cytosine analogs, including azacitidine and decitabine, are hypomethylating agents which inhibit DNMTs through incorporation into DNA and RNA and induction of apoptosis in abnormal hematopoietic cells. Azacitidine and decitabine with and without HDAC inhibitors demonstrated promising results in relapsed ALL cell line models [182]. Furthermore, patients with refractory AML and ALL were treated in a Phase I TACL trial with azacitidine in combination with chemotherapy and was well-tolerated. However, the high toxicity associated with the combination of decitabine, vorinostat and chemotherapy in children with relapsed ALL caused termination of the pilot TACL study (NCT01483690).

1.5.2 Antibody-based immunotherapy

Recently, several mAbs have been developed to target specific markers mostly expressed on B-cell lymphoblasts including CD20, CD19, CD22 and CD52. In addition, cytotoxic T-cell responses can be activated by new immunotherapeutic approaches [14,30,37].

1.5.2.1 Anti-CD20 mAbs

CD20, which is expressed on the surface of 30-50% of B cell lymphoblasts, can be targeted by rituximab, ofatumumab, and obinutuzumab [37,183]. Rituximab is a chimeric anti-CD20 mAb which was approved in 1997 for non-Hodgkin lymphoma [184]. Binding of rituximab to CD20 removes B cells from circulation through complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), and apoptosis induction. It has been observed that rituximab improved the efficacy of induction and consolidation chemotherapy and increased the CR and OS in $CD20^+$ ALL patients [37,183]. In the GRAALL-2005 study, rituximab was combined with

chemotherapy and improved 2-year EFS and 2-year OS (65% vs. 52%; P = 0.038 and 74% vs. 63%; P = 0.018, respectively) in patients with Ph^-, $CD20^+$ B-ALL [185,186].

Ofatumumab, which was approved in 2009 for CLL [187], is a humanized type I mAb, and obinutuzumab (GA101) is a glycoengineered humanized type II anti-CD20 mAb [164, 165]. Ofatumumab targets the small-loop epitope on CD20 and it has higher complement-dependent cytotoxicity compared to rituximab, while obinutuzumab has lower complement- and antibody-dependent cytotoxicity and little direct cytotoxicity [188,189]. Ofatumumab is being investigated in a Phase II clinical trial (NCT02419469) in combination with the augmented BFM in B-ALL patients. In another Phase II trial, ofatumumab in combination with hyper-CVAD increased the 3-year continuous CR rate to 78% and the 3-year OS to 68% in newly diagnosed ALL patients [190]. In addition, preclinical studies revealed that the cell death was induced in rituximab-sensitive or rituximab-resistant precursor B-ALL xenografted mice by obinutuzumab [191].

1.5.2.2 Anti-CD19 mAbs

CD 19, which is highly expressed on the surface of >90% of B-ALL blasts, is the target for blinatumomab and denintuzumab mafodotin [37]. Blinatumomab is a bispecific T cell engager (BiTE) antibody, which binds to CD3 on T cells and CD19 on B lymphoblasts, resulting in the release of inflammatory cytokines, proliferation of T cells and $CD19^+$ cell lysis. Initial studies in relapsed/refractory pediatric B-ALL patients demonstrated promising results, with 40-60% of patients achieving CR [192]. In 2016, blinatumomab was approved by the US FDA for the treatment of adult patients with relapsed Ph^- ALL based on Phase II data [193]. It has also been shown to be capable of eradicating MRD in ALL [194], and this may improve outcomes post-HSCT [195]. The Phase III TOWER study assessed the efficacy of blinatumomab compared to the standard chemotherapy in adults with relapsed/refractory B-ALL [196]. This trial demonstrated a

superior CR rate and blinatumomab improved OS and CR rates compared to standard therapy (7.7 vs. 4 months and 39 vs. 19%, respectively). Treatment was more effective in patients with lower tumor burdens. Other trials (NCT02143414, NCT02003222 and NCT02807883) are evaluating blinatumomab in different settings, including frontline and maintenance therapy. Side effects associated with blinatumomab, including cytokine release syndrome and neurologic toxicities, necessitate close observation during the early stages of infusion [37].

Denintuzumab mafodotin (SGN-CD19A) which is an antibody drug conjugate (ADC) links a humanized anti-CD19 antibody to the monomethyl auristatin F (MMAF), a drug that induces apoptosis by inhibiting the microtubule assembly and triggering G2/M arrest upon binding to CD19 and internalization [197]. Denintuzumab mafodotin was applied for the treatment of relapsed B-ALL patients in a Phase I trial, and 22% and 35% of patients demonstrated CR or PR following the weekly treatment and once in 3 weeks of treatment, respectively [198].

ADCT-402 is also an ADC which links a humanized anti-CD19 antibody to a cytotoxic dimer, pyrrolobenzodiazepine, leading to inhibition of both DNA replication and proliferation of $CD19^+$ blasts. ADCT-402 demonstrated significant cytotoxicity in $CD19^+$ cell lines and improved the survival of xenograft models [199]. The efficacy of ADCT-402 is currently under investigation for relapsed B-ALL cases in a Phase I clinical study (NCT02669264).

DT2219 is a bispecific recombinant mAb targeting both CD19 and CD22 and contains the catalytic and translocation domains of diphtheria toxin (DT390) and two sFv subunits recognizing CD19 and CD22. In vitro studies demonstrated favorable results in B cell leukemia models [200,201]. In a Phase I dose escalation study, only one patient out of 25 adolescents and adults with relapsed $CD22^+$, $CD19^+$ B-ALL achieved a partial response [202]. Efficacy of DT2219 is now being evaluated in a Phase II trial in adults and children older than 12 years (NCT02370160).

1.5.2.3 Anti-CD22 mAbs

CD22 is another specific B cell lineage antigen expressed by 90% of B-ALL lymphoblasts and is a potential target for inotuzumab ozogamicin, moxetumomab pasudotox, coltuximab ravtansine, and epratuzumab [37,183,203]. Inotuzumab ozogamicin, which is an ADC, includes a humanized anti-CD22 IgG4 mAb and calicheamicin, a cytotoxic agent resulting in DNA breakage following the linkage and internalization of CD22 and anti-CD22 [180, 181]. In the Phase III INO-VATE trial (NCT01564784), relapsed/refractory $CD22^+$ ALL patients treated with inotuzumab ozogamicin experienced higher CR rates and superior OS compared to a control group treated with standard chemotherapy (80.7 vs. 29.4%; $P < 0.001$, 7.7 vs. 6.7 months; $P = 0.04$) [206]. This agent has also been approved by the FDA and other regulatory bodies. Other ongoing clinical trials are also evaluating inotuzumab ozogamicin in combination with chemotherapy in relapsed ALL patients (NCT01925131), in combination with frontline chemotherapy regimens, and in combination with bendamustine, fludarabine +/- rituximab as conditioning therapy with an allogeneic HSCT (NCT01664910).

Moxetumomab pasudotox is a recombinant antibody comprising the variable fragment of an anti-CD22 antibody and part of a Pseudomonas exotoxin which can induce apoptosis upon internalization [207]. This agent is being studied in a Phase I/II study in refractory ALL patients (NCT01891981). Coltuximab ravtansine (SAR3419) is also an ADC which includes an anti-CD22 antibody and maytansinoid (DM4), a cytotoxic agent that induces cell cycle arrest through the inhibition of microtubule assembly and tubulin polymerization following internalization. However, a phase II trial in relapsed ALL did not yield favorable results [183].

Epratuzumab is a humanized anti-CD22 mAb that is internalized upon binding to the third extracellular domain of CD22. It can induce CD22 phosphorylation, inhibition of proliferation, B-

cell activation and cytotoxicity. The results of a Phase II COG trial showed that combining epratuzumab with standard re-induction chemotherapy could not improve the rates of achieving a second CR in relapsed B-ALL [13, 185]. Epratuzumab is being evaluated in combination with cytarabine and clofarabine in another Phase II trial (NCT00945815) in refractory ALL. Adult with relapsed ALL have shown an overall response rate of 40-52% following treatment with epratuzumab and chemotherapy [209].

1.5.2.4 Anti-CD52 mAb

Alemtuzumab is a recombinant mAb against CD52, expressed in 36–66% of leukemic blasts, which causes ADCC-mediated lysis of $CD52^+$ cells [37,183,210]. However, there are limitations and side effects associated with its use in ALL patients, including lymphopenia resulting in severe and prolonged immunosuppression, and it did not show promising results in several trials [183]. Alemtuzumab is currently being applied in a clinical trial for refractory chronic or acute adult T-cell leukemia in combination with recombinant human IL-15 (NCT02689453).

1.5.2.5 Anti-PD-1 mAb

Nivolumab, which is a humanized mAb against programmed cell death protein-1 (PD-1), can induce immunosurveillance of malignant cells [37,211]. The efficacy of this mAb in combination with dasatinib is being assessed in a Phase I study for relapsed Ph^+ ALL patients (NCT02819804). Furthermore, the poor-risk relapsed $CD19^+$ B-ALL patients treated with the combination of blinatumomab and nivolumab with or without ipilimumab, are being evaluated by a Phase I study (NCT02879695).

1.5.3 Cellular immunotherapy

A novel targeted therapeutic approach for B-ALL utilizes chimeric antigen receptor-modified T-cells (CAR T-cells) which are specific for B-cell antigens. In this approach, an

antibody is expressed by the patients' own genetically engineered cytotoxic T cells recognizing B-cell antigens. In the CAR structure, an extracellular antigen-recognition domain from a mAb fragment (scFv) is linked to the intracellular signaling domains of the T-cell receptor complex by CARs. This results in the activation of T-cells in a major histocompatibility complex–independent manner yielding a potent cytotoxic response [14,212]. In the second and third generations of CAR T-cells, one (second) or two (third) costimulatory domains including CD28 or CD27, CD137 (4-1BB), ICOS, and CD134 (OX40) are incorporated to increase the persistence of engineered T cells and achieve higher cytokine production and replicative capacity [213–216]. Preclinical data indicated that CD19-targeted CAR T-cells display enhanced cytotoxicity in vitro and in murine xenograft models [217].

CAR T-cell therapy has yielded remarkable activity in patients with relapsed and refractory B-ALL, with CR rates in the 70-90% range [218–220]. In a Phase I study in 2014, 21 ALL patients (children and young adults) were treated with CD19 CAR T-cells (maximum tolerated dose: 1×10^6 CD19-CAR T-cells/kg), 70% achieving CR [220]. In another report in 2014, 90% (27 out of 30) of childhood and adult patients with relapsed ALL achieved CR with CTL019 cells, and these cells were detectable at least for 6 months in 68% of cases [221]. In Sept. 2017, the FDA approved the first CAR T-cell therapy (Tisagenlecleucel) for relapsed/refractory B-ALL in patient under age 25, and many other CAR T trials in B-ALL are in progress. CAR T-cell therapy is associated with several acute adverse effects including hypotension related to the inflammatory cytokine level in serum, fevers and cytokine release syndrome (CRS), which can develop into macrophage activating syndrome [221,222]. These effects can be mitigated by tocilizumab, an ani-IL6 monoclonal antibody [223,224].

The outcome of CAR T therapy is affected by the durability of CAR T-cells in patients. This limitation can be addressed by reinfusion of 'fresh' CAR T-cells [225,226]. Emergence of CD19 negative B cells is another reason for CAR-T therapy failure. A study by Lacey *et al.* identified a relapsed CD19 negative leukemia originated from a CTL019 treated clone [227]. These CD19 negative B-cells escaped from CAR T therapy by downregulation of surface CD19 target antigen in a cell autonomous manner. Developing CAR T-cells to target additional antigens on the surface of B-cells is a promising approach to prevent the relapse. CAR T-cells targeting CD22 have been explored to treat CD19 negative B-cells in some studies, and the results revealed that CD22 is a promising target in relapsed B-ALL patients pre-treated with CD19 CAR T-cells [228–230]. Innovative strategies to improve the efficacy of CAR T-cell therapy include using combined CD19 and CD22 CAR T-cells, vaccination to increase the CAR T-cells persistence, use of allogeneic T-cells or even cord blood T-cells, induction of apoptosis in CAR T-cells, and sleeping beauty transposon system [231,232].

1.5.4 RNAi-mediated and related therapy

The development of drug resistance leading to relapse and toxicity due to off-target effects are serious limitations associated with conventional therapeutic strategies [14,30,33,233,234]. To address these limitations and develop a more specific approach, RNA interference (RNAi) based therapy is being explored with promising results in pre-clinical settings [235,236]. The RNAi targets the complementary mRNA for degradation or inhibition of translation and, therefore, can selectively silence the expression of the aberrant proteins involved in uncontrolled cell proliferation. As RNAi functions at a molecular level to downregulate its target mRNA with a high degree of specificity, its activity is minimally affected by point mutations which results in drug resistance [234,237]. RNAi-mediated therapy can be carried out by delivery of short hairpin RNA

(shRNA) encoded by an expression vector (viruses or plasmid DNA for non-viral means), antisense oligonucleotides (ASO; typically 16-20 b.p. single-stranded DNA polynucleotides) or double stranded small interfering RNA (siRNA; typically 19-27 polynucleotides) [234,238].

ASOs (typically inhibit translation) and siRNAs (typically promote mRNA degradation) act through different intracellular pathways and therefore, demonstrate different gene silencing potentials. In the process of shRNA-mediated gene silencing, shRNA transcription is performed within the cellular nucleus, followed by transporting into the cytoplasm, and then processing by the cellular-machinery into a siRNA-like molecule, which is finally incorporated by cytoplasm molecules for gene inhibition [12] [13]. The persistent expression of shRNA molecules by the host cells has made their effect remain indefinitely [12]. However, siRNA molecules are exogenously introduced to the cells and then loaded onto the RNAi machinery for silencing activity, which in turn, shRNA transcription and subsequent processing steps are no longer required. Therefore, siRNA silencing effect is transient while it is safe as the RNAi agents cannot be integrated into the host cell's DNA [14]-[16]. In my thesis work, we focus mainly on siRNA as one of the RNAi mechanisms of gene silencing.

RNAi pathway is induced by a specific enzyme belonging to RNAse III family called DICER. Upon delivery of a long double stranded molecule into a cell; DICER chops down it into small RNA pieces. Small RNA molecules are classified into two types: microRNA (miRNA) and siRNA. siRNA molecules bind and target the mRNAs with complementary gene sequences and change their activity. Each siRNA gets unwounded to two singly stranded molecules named passenger strand and guide strand. The passenger strand is degraded by cytosolic enzymes while the guide strand binds the RNA induced silencing complex (RISC). This complex promotes gene silencing by aligning its complementary sequence (the portion offered by guide strand) with post

transcriptional mRNA and inducing cleavage by Argonaute protein. Exogenously administered siRNA directly interacts with RISC and acting together, the siRNA-RISC complex damages the target mRNA [239]. miRNAs are doubly stranded endogenous non-coding RNA molecules (typically 19 to 25 nucleotides with partial base pairing) that are involved in regulating the expression of genes as well as other mRNAs [236,238]. miRNAs are able to control the expression of multiple proteins through inhibiting mRNA translation instead of promoting its cleavage as in case of siRNA which differentiates them from siRNAs [240]. However, recently counter evidence has also been found which recognizes mRNA degradation to be the contributing mechanism behind miRNA-induced gene silencing [241]. In mammalian cells, the effect of RNAi lasts only for an average of 66 hours as a result of its dilution during cell divisions [242], and therefore, repeated administration is necessary to achieve a persistent effect [243].

RNAi has reached two clinical trials for leukemia. In the first case, (NCT00257647), siRNA against a fusion gene was delivered by a viral vector, simian virus 40 (SV40), to CML patients but there is no published data from this study. The second trial was a nonviral delivery of siRNA using a liposomal formulation explored in one CML patient.

A number of potential targets in B-ALL have been identified for downregulation by RNAi (Table 1.2). These targets can be categorized based on their role in the development of leukemia and include those mediating proliferation, apoptosis, chemo-sensitivity, mediating B-cell differentiation and regulating the mobility of cancer cells [234,244]. One of the potential targets in ALL is CD22 without exon 12 (CD22ΔE12) which normal B cells lack. Therefore, siRNA mediated silencing of this specific target will not affect the normal B cell function. One study showed that CD22ΔE12 siRNAs delivered by liposome and cationic peptide carriers reduced clonogenicity of B-ALL cells and added to the cytotoxic effects of chemotherapy agents *in vitro*.

Furthermore, infusion of this construct inhibited the growth of B-ALL in a mouse xenograft model [216, 217] (Table 1.2).

High expression of certain isoforms of histone deacetylases (HDAC) is also associated with poor outcomes in ALL. One study reported that the chemo-sensitivity of T-cells increased in an ALL model treated by HDAC siRNA [245]. Another study investigated silencing of MAX dimerization protein 3 (MXD3) which is a basic-helix-loop-helix-leucine-zipper transcription factor involved in cellular proliferation [140,246,247]. It has been demonstrated that MXD3 functions as an anti-apoptotic protein, and therefore, downregulation of MXD3 may be beneficial for B-ALL [248,249].

Ab-mediated delivery of polynucleotides, which can specifically target the leukemic cells, is also promising, as it reduces their non-specific delivery into non-leukemic cells. To knockdown *MXD3*, a nanoparticle (NP) formulation of super paramagnetic iron oxide (SPIO) with *MXD3* ASO conjugated to anti-CD22 Ab (αCD22 Ab) was developed to target B-ALL cells. The results revealed significant *in vitro* and downregulation of *MXD3* mRNA, with induction of apoptosis and sensitization to chemotherapeutics; it also demonstrated anti-leukemic activity in B-ALL xenograft mouse models [250] (Table 1.2).

Hsp32, which functions as a survival factor in cancer cells, is another target for RNAi-mediated therapy. The role of Hsp32 was explored in ALL patient and cell line models by Cerny-Reiterer *et al* [251]. ATL1102 is a second-generation antisense oligonucleotide against human α4 integrin (CD49d/ITGA4) RNA. CD49d is involved in signal transduction, adhesion and proliferation of cells [252,253] and its silencing by ATL1102 in chemo-resistant human Kasumi-2 B-ALL cells decreased the expression of CD49d protein; however, these results could not be confirmed in mouse xenograft models [254] (Table 1.2).

A recent preclinical study evaluated the therapeutic effect of lipid NP encapsulated *TCF3-PBX1* siRNA *in vitro* on the *TCF3-PBX1*-expressing 697 cells and *in vivo* on a patient-derived xenograft (PDX) model from a *TCF3-PBX1*-positive B-ALL patient [230, 231]. *TCF3-PBX1* was significantly downregulated by the specific siRNA at both mRNA and protein levels. Moreover, an efficient uptake of the NP/siRNA formulation was observed in difficult-to-transfect patient CML and ALL cells, which confirmed the potency of the siRNA delivery system. *In vivo* studies demonstrated that siRNA-mediated silencing of the *TCF3-PBX1* fusion oncogene improved survival in *TCF3-PBX1* dependent B-ALL PDX mice compared to the control group [255] (median OS 45 days vs. 32 days, P = 0.0026) (Table 1.2).

RNAi prodrugs were recently derived from modified short interfering Ribonucleic Neutrals (siRNNs), which can enter the cells without a delivery system. Upon internalization, cytoplasmic enzymes cleave the siRNNs into regular siRNAs which are capable of inhibiting their target mRNAs [257]. The therapeutic efficacy of RNAi prodrugs against polo-like kinase 1 (*Plk1*), which has a key role in mitosis regulation and its upregulation is associated with unfavorable outcomes [258–261], was evaluated by a preclinical study in pediatric T-ALL cell line models and patient samples. *PlK1* siRNNs significantly inhibited *PlK1* at both mRNA and protein levels and induced apoptosis and G2/M arrest in T-ALL patient cells, with less toxic effect on normal cells [260].

There is also a new category of non-coding RNAs known as long non-coding RNAs (lncRNAs). lncRNAs are not RNAi but they are capable of regulating different cellular processes leading to tumorigenesis. Recent studies have reported that some specific lncRNAs are deregulated in pediatric B-ALL and they can be utilized as potential therapeutic targets [238,262–265]. *RP11-137H2.4* is a lncRNA which plays an important regulatory role in apoptosis, proliferation, and

migration of leukemic blasts. It has been observed that silencing the expression of *RP11-137H2.4*, through its specific siRNA, induced apoptosis and inhibited migration in NALM6 B-ALL cells. In addition, transducing Reh prednisolone-resistant B-ALL cells with a shRNA targeting *RP11-137H2.4*, sensitized cells to glucocorticoids by modulating the expression of MAPK cascade genes [262] (Table 1.2).

Table 1.2: A list of molecular targets, delivery systems and cell models applied to investigate nucleic acid-based therapy in ALL

Molecular Targets	**Delivery agent**	**Cell models**
CD22ΔE12	Liposome (DOTAP/DOPE) and Cationic peptides (PVBLG)	B-ALL xenograft models
HDAC	Electroporation	Human T-ALL cell line (CCRF-CEM)
MXD3	Super paramagnetic iron oxide nanoparticles	Jurkat: T-ALL, Reh: B-ALL cells
CD49d	Nucleofection/Incubation	Chemo-resistant human Kasumi-2 B-ALL cells/Mouse model of B-ALL
HSP 32	Lipofection	ALL patient cells

TCF3-PBX1	Lipid nanoparticles	*TCF3-PBX1*-expressing 697 cells/ patient-derived xenograft (PDX) model from a *TCF3-PBX1*-positive B-ALL patient
RP11-137H2.4	Nucleofection	NALM6 B-ALL cells, Reh prednisolone-resistant B-ALL cells
PlK1	Self-delivery of siRNNs	T-ALL cell line models and patient samples
E2A-PBX1	Nucleofection	697 pre-B leukemia cell line
BCL11B	Nucleofection	Molt-4 human T-cell leukemia cells, 3 T-ALL patient cells
HIF-1α and Notch1	Lipofectamine 2000 reagent	Human T-ALL cell lines: Jurkat and Sup-T1 cells
MCL-1	Cationic lipids	Jurkat T-ALL cells
PlK1	Self-delivery of siRNNs	Pediatric B-ALL patient cells
PYK2	Nucleofection	Jurkat T-ALL cells

HOXB7	Turbofect (cationic polymer)	CCRF-CEM (T-ALL) and NALM6 B-ALL, and ALL primary cells
FOXP3	Electroporation	KOPT-K1 and Jurkat T-ALL cells

1.6 STAT5 as a potential therapeutic target for siRNA therapy in ALL

One of the most promising therapeutic targets in lymphoid leukemias is transcription factor genes that are involved in the development of blood cells [19]. As multiple oncogenic signalling pathways are connected by a few groups of nuclear transcription factors associated with the activation of oncogenes' expression resulting in the development of the malignancy, the impacts of several upstream signalling pathways might be inhibited by targeting a single transcription factor [19]. Reports on the genetic abnormalities in leukemia revealed that persistent activation or overexpression of functionally normal transcription factors plays an important role in the pathobiology of lymphoid leukemia and induces aberrant proliferation and impaired differentiation of leukemic cells [19,266]. It is noteworthy to mention that as a potential molecular target for cancer therapy, a transcription factor should meet certain (desirable) requirements. First, it must be constitutively active in most of the cells of various kinds of tumours. The activity of transcription factor should affect the expression of genes involved in inducing all malignant features including proliferation, survival, migration and invasion, and immune evasion. Moreover, it should be feasible to target the transcription factor and inhibit its activity by the current strategies for targeted therapy and the tumor cells' activity should be influenced more by the function of the

transcription factor rather than the normal cells to prevent undesirable side effects [19]. By considering all above-mentioned criteria, STAT (signal transducer and activator of transcription) proteins, especially STAT5, are one of the most promising molecular targets for the treatment of hematologic malignancies [19]. This family of proteins and their mechanisms of action were originally discovered in the context of interferon (IFN)-mediated gene regulation in the early 1990s and includes seven members: STAT1 to STAT4, STAT6, and the closely related STAT5A and STAT5B proteins [19,267]. These proteins are involved in both conveying cytoplasmic signals from cytokines and growth factors' receptors with tyrosine kinase activity to nucleus and acting as nuclear transcription factors to activate the expression of genes promoting malignant progression [19,267].

The structure of STAT family members consists of several domains (Fig. 1.2). The amino-terminal domain (N-terminal) facilitates the tetramer formation by providing the interaction of two STAT dimers; this interaction leads to a stable attachment of the tetramer to binding sites in DNA [19,266]. The coiled-coil domain promotes the interactions with other transcription factors and regulatory proteins. The STAT proteins are able to bind to STAT-binding sites in gene promoters with the common core sequence of TT (N4–6) AA though their DNA-binding domain [19,266]. STAT proteins in the form of dimers are only able to bind to DNA to activate the gene expression and dimer formation is mediated by the reciprocal interactions between the SRC-homology 2 (SH2) domain of one STAT monomer and the phosphotyrosine (pY) residue of another STAT molecule [19,266]. The transcriptional activation of target genes is induced and modulated by the transactivation domain of STAT proteins through its interactions with other proteins/transcription cofactors/activators including histone acetyltransferases, the bromodomains and extra-terminal domain (BET) family of bromodomain-containing proteins and centrosomal P4.1-associated

protein (CPAP), as well as cell-specific transcription factors, such as the glucocorticoid receptor (GR) and CCAAT/enhancer binding protein (C/EBP) in adipocytes [19,266]. This transcriptional activity can increase in some STATs by the phosphorylation of a serine residue located in the carboxy-terminal domain (C-terminal domain). STAT5A and STAT5B are closely related isoforms of STAT5 encoded by separate genes [19,266].

STAT5 regulates expression of target genes that induce cell proliferation including cyclin D and serine/threonine kinase Pim-1, as well as those that promote apoptosis such as Janus kinase-binding protein (JAB) [268].

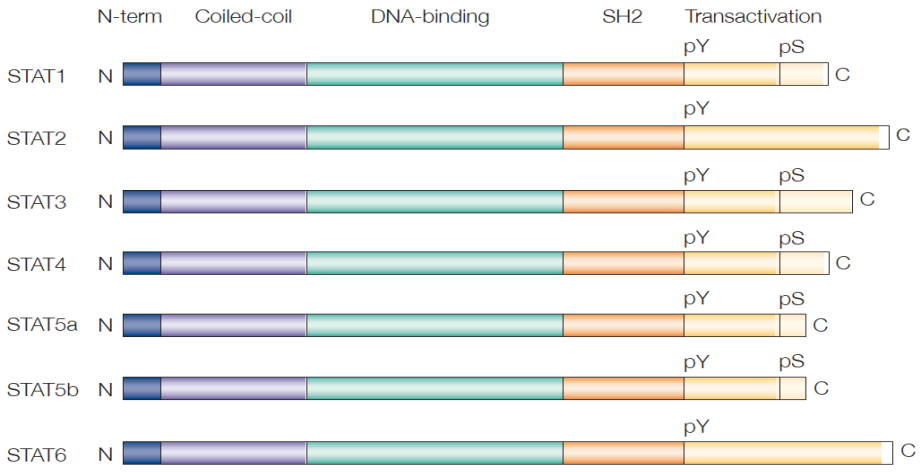

Figure 1.2. The domain structure of the STAT proteins [19].

In the process of STAT5 signalling pathway, binding of growth factors or cytokines to their receptors results in the activation of intrinsic receptor-tyrosine-kinase or of receptor-associated kinases such as janus kinases (JAK) or SRC tyrosine kinases [266,269]. The cytoplasmic tails are then phosphorylated by these tyrosine kinases to recruit and activate the STAT5 monomers through tyrosine phosphorylation [266,269]. Oncoproteins SRC and BCR-ABL that are non-receptor tyrosine kinases can also phosphorylate STAT5 molecules individually without receptor

involvement [266,269]. The phosphorylated STAT5 proteins are fully activated when they either homo- or heterodimerize and then translocate to the nucleus where the dimers can bind to the interferon gamma activated sites (GAS) of the gene promoters regulated by STAT5 proteins (Fig. 1.3) [266,269]. Multiple proteins can also negatively regulate the STAT5 signalling pathway including the suppressors of cytokine signalling (SOCS), phosphatases like SHP-2, and protein inhibitors of activated STAT (PIAS) [269]. SOCS is able to bind JAKs and receptors to prevent phosphorylation and activation of STAT5. Phosphatases such as SHP-2 induce dephosphorylation of activated JAKs and STAT5 molecules, and PIAS can inhibit the binding of activated STAT5 dimers to GAS sites and disrupt its dimerization (Fig. 1.3) [269]. As tyrosine kinases are among the most common overactivated oncoproteins in cancer cells due to different genetic or epigenetic abnormalities, they result in the persistent activation of STAT5 causing alterations in the expression level of genes regulating the major cellular activities [266,269].

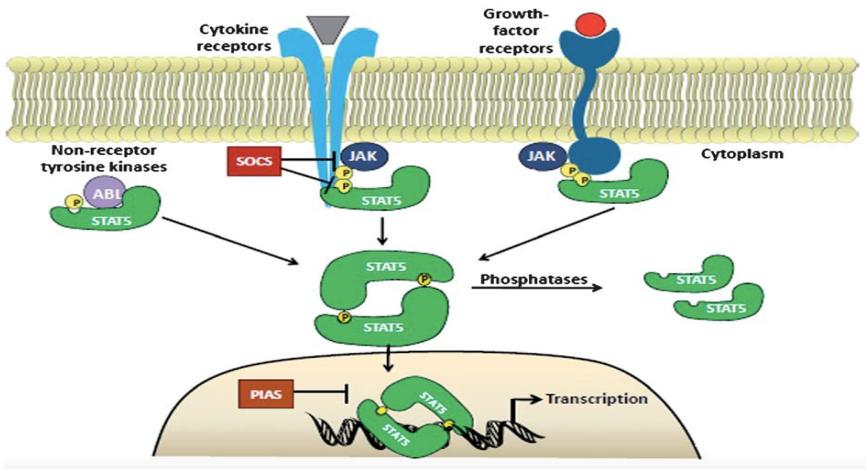

Figure 1.3. Signalling Pathway of STAT5 [270].

1.6.1 Structural differences between STAT5A and STAT5B isoforms

STAT5, initially known as the mammary gland factor (MGF), was originally discovered in lactating mammary cells as a transcription factor for the β-casein gene [271]. It was recognized as a member of the STAT protein family by the cloning of MGF. The STAT5 protein contains 794 amino acids and could be activated by phosphorylation on its Tyr694 residue mediated by JAK2 in the prolactin (PRL) signalling [272]. Expanding the understanding of molecular genetics revealed that two separate genes located on human chromosome 17 encode for STAT5 resulting in the expression of two different isoforms, STAT5A and STAT5B. Both isoforms are 96% identical in their amino acid sequences and they have the same structural domains (Fig. 1.4) [273,274]. The original STAT5 discovered in PRL signalling was considered as STAT5A with 794 amino acids; however, STAT5B protein has 786 amino acids, and is activated by the phosphorylation on its Tyr699 while Tyr694 is phosphorylated in STAT5A [274]. The main difference is in their C-terminal domain and STAT5A has 20 unique amino acids in its C-terminal domain while STAT5B has eight unique amino acids in the same region [274]. Tyr694 phosphorylation and Threonine 92 glycosylation play a significant role in STAT5A activity. The mutation of Serin 710 to Phenylalanine (in the C-terminal domain) in STAT5A enhances its transcriptional activity [273,274]. STAT5A/B indicates distinction cell-specific regulation. Expression of STAT5A is mostly restricted to the mammary tissue (breast) and its inactivation inhibits terminal differentiation of the mammary gland whereas, STAT5B expression mainly occurs in liver and muscle tissues and its deletion leads to loss of sexual dimorphism in response to growth hormone signalling and reduced body growth [274]. It has been observed that both STAT5A/B are not able to affect the lymphocyte function. STAT5A/B double knockout mouse models demonstrated early post-natal lethality, infertility in female mice, deficiencies in body

growth rate, hematopoiesis and breast development [273]. As these two isoforms have roughly the same structure, they have the same effect on regulating genes involved in some cellular activities including cell proliferation and apoptosis [274]. However, they also indicate distinctive functions because they have some structural differences on their C-terminal domains, for instance, STAT5A mediates the expression of genes involved with neural development and STAT5B regulates the expression of genes associated with T-cell development [274].

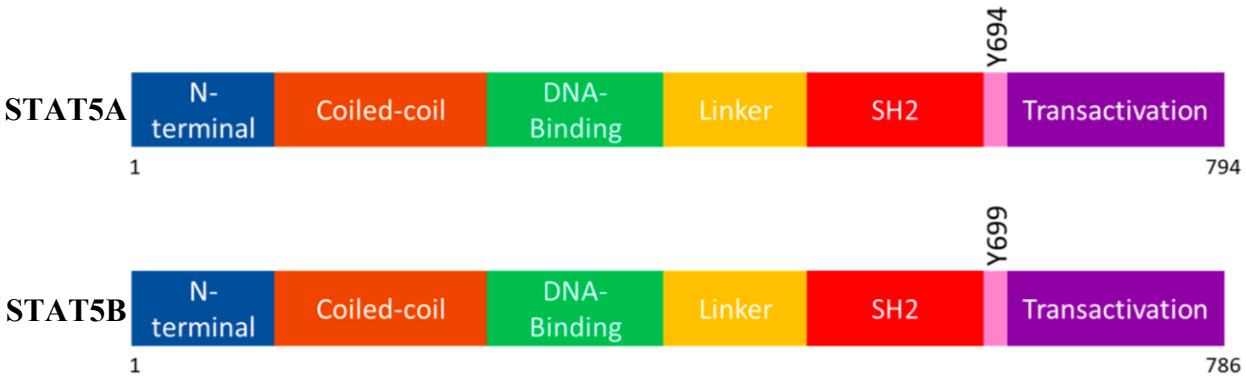

Figure 1.4. Domain structure of two STAT5 isoforms, STAT5A and STAT5B [269].

1.6.2 Role of STAT5 in physiology and B-cell development

During the pregnancy, development of normal mammary glands and lactation is essentially dependent on STAT5 signalling pathway [275]. STAT5A is involved in inducing the mammary epithelial cell survival, proliferation and differentiation of alveolar cells during lactation and milk production [275]. STAT5B promotes the antigen re-stimulation T cell death (RICD) of effector memory T cells, which assists to sustain T cell homeostasis [276]. In addition, STAT5 is associated with modulating haematopoiesis through IL-3 signalling which results in the proliferation, differentiation and apoptosis of haematopoietic cells [277]. STAT5-mediated cell proliferation is promoted by Pim-1 expression independently of IL-3 signalling while, cell differentiation and

apoptosis happen through STAT5-induced p21 and JAB expression in the presence of IL-3 [277]. Moreover, development of functional and mature B lymphocytes from differentiation of lymphoid primed multipotent progenitors (LMPPs) to common lymphoid progenitors (CLPs) and subsequent lineage commitment depends on a network of transcription factors including E2A and early B cell factor (EBF1) that modulate B cell specification and lineage and Pax5 that regulates B-lineage commitment [273]. The differentiation and developmental stages of lymphocytes are partly controlled by cytokine-activated signaling pathways including interleukin 7 (IL-7), as well as by alterations in the expression of genes regulated via STAT5 [273]. In the IL-7R signaling, STAT5 activation and phosphorylation through binding IL-7 to the γ_c chain of its receptor and subsequent JAK1/JAK3 phosphorylation, leads to activating the expression of prosurvival *Mcl-1* gene to promote cell survival and also activating the B-cell regulatory genes *Ebf1 and Pax5* [273]. Activated STAT5 also directs the rearrangement of immunoglobulin gene by blocking Igk recombination in pro-B cells [273]. Consequently, it collaborates with the pre-B cell receptor (pre-BCR) to induce pre-B cell expansion from pro-B cells by activating the expression of cyclin D3 [273]. Pre-BCR signaling can prevent degradation of cyclin D3 by PI3K/AKT pathway activation [273]. Therefore, IL-7/STAT5 signaling is activated only in some stages of early B-cell development and in other stages, prosurvival protein Bcl-xL controls the survival of small pre-B cells to eventually generate the mature B-cells [273].

1.6.3 Role of STAT5 in developing different cancers

The critical role of STAT5 in the development of different cancers has been comprehended recently. Dysregulated STAT5 signalling pathway, mainly because of its persistent activation, promote tumor survival, growth, metastasis and drug resistance [278]. It has been observed that

STAT5 is associated with the pathology of several cancers including breast, colorectal, lung, prostate, and liver cancers, as well as hematological malignancies, that are among the top 10 cancers with the highest death rates [278].

In breast cancer, overactive STAT5 has been identified in all three types of breast cancers, including estrogen receptor (ER)-positive, HER2-positive, and triple-negative breast cancer (TNBC) [279]. Recent evidence showed that STAT5 has dual roles as the tumor suppressor and oncogene in breast cancer which depends on the type of cancer and various circumstances. When STAT3 is activated in breast cancer as an oncogene, STAT5 acts as a tumor suppressor causing reduction in cell proliferation and breaking the drug resistance [279]. For example, STAT5 expression and activity improved the outcomes of hormone therapy and increased the overall survival of patients with ER-positive breast cancer [280]. Moreover, migration and invasion of cancer cells can be inhibited by STAT5 expression through the downregulation of matrix metalloproteases (MMP) 2 and 9, and upregulation of E-cadherin expression on the cell surface [281]. STAT5 can also inhibit the expression of the activator protein 1 (AP-1), that induces cell survival, proliferation, angiogenesis and invasion [282]. However, as an oncogene, JAK/STAT5 signalling can promote the expression of heat shock protein 90 alpha (HSP90α) and cyclin D1, inhibit apoptosis and increase tumor formation and survival in breast cancer [283,284]. STAT5 activation by binding of the upregulated transforming growth factor (TGF) to epidermal growth factor receptor (EGFR), could develop hyperplasia and tumors in STAT5-expressing mouse models compared to control groups [285]. In addition, FYN that is a Src family kinase (SFK), activates STAT5 which leads to the metastasis of TNBC cells [286].

In colorectal cancer, it has been observed that STAT5 overexpression and constitutive activation result in poor prognosis and reduced survival [287–289]. STAT5 activation by IL-23

downregulates SOCS3, p16, p21, p27, and E-cadherin but induces the expression of cyclin D1, Bcl-2, MMP-2, vascular endothelial growth factor (VEGF) and survivin leading to cell proliferation, survival, invasion, metastasis and inhibition of apoptosis in colorectal cancer [287,288,290–292]. Moreover, STAT5 silencing can restore the sensitivity of cancer cells to the commonly used chemotherapeutics cisplatin and 5-fluorouracil [293].

In lung cancer, STAT5 overexpression was detected in different subgroups of non-small cell lung cancer (NSCLC) including squamous cell carcinoma, adenocarcinoma and large cell carcinoma [294]. It was observed that upregulated STAT5 could induce the nuclear expression of Bcl-xL and it correlated with distant metastases. Bcl-xL as an anti-apoptotic protein, could promote survival and proliferation of lung cancer cells through inhibition of apoptosis [294]. Moreover, cyclooxygenase-2 (COX-2) overexpression is mediated by EGF signalling pathway that activates STAT5 and facilitates disease progression through inflammation [295,296]. In NSCLC, IL-6 signalling, JAK1, JAK2, c-Src, and PIAS3 downregulation are able to activate STAT5 [295,297]. Suppressing STAT5 resulted in reduced cell proliferation, and enhanced G1 phase cell cycle arrest and apoptosis in NSCLC that reveals the potential therapeutic role of STAT5 silencing in NSCLC [297].

In prostate cancer, patients with persistent activity of STAT5 face with early disease development, and poor survival as well as higher relapse rates [298]. STAT5 is activated and phosphorylated through either JAK2 or erythropoietin receptor (EpoR) in prostate cancer which increases the expression of oncogenes involved in inducing proliferation and metastasis and inhibiting apoptosis [299,300]. Moreover, STAT5 acts in concert with the androgen receptor (AR) and protects it from proteasomal degradation that leads to synergistic effect on overexpression of their target genes and also disease progression [301]. It has been shown that STAT5 inhibition in

prostate cancer could induce apoptosis and decrease cell proliferation and tumor growth that highlights the importance of STAT5 as a promising target in prostate cancer [299,302].

In hepatocellular carcinoma (HCC), STAT5 performs both as a tumor suppressor when STAT3 signalling is active and as an oncogene to promote tumor growth, invasion, proliferation and chemoresistance in other circumstances which is similar to its role in breast cancer [303]. When acting as a tumor suppressor, STAT5 regulates the levels of both STAT3 and STAT5-mediated signalling pathways to prevent tumor growth and also increases the expression of pro-apoptotic proteins including p53 upregulated modulator of apoptosis (PUMA) and the Bcl-2-interacting mediator of cell death (BIM), as well as NADPH oxidase 4 (NOX4) enzyme which produces the reactive oxygen species (ROS) to induce apoptosis [303,304]. As an oncogene, activation of STAT5 by mammalian target of rapamycin (mTOR) can initiate the HCC through overexpression of sterol regulatory element binding protein-1 (SREBP1) involved in lipid synthesis in the liver [305]. Additionally, STAT5 overexpression mediated by the cholesterol transporter, GRAM domain-containing 1A (GRAMD1A) leads to high expression of cyclin D1, Bcl-2, c-Myc and c-Jun, as well as downregulation of caspase 3 and poly (ADP-ribose) polymerase (PARP) to promote cell survival, proliferation and drug resistance [306].

STAT5 also plays a critical role in the development of different hematological malignancies including AML, CML, B-ALL and T-ALL [269]. In CML and BCR-ABL positive ALL, STAT5 is persistently activated either directly by BCR-ABL oncoprotein or indirectly by JAK2 that results in overexpression of growth-promoting Pim-1 kinase protein and antiapoptotic proteins MCL-1 and Bcl-2 while downregulates the expression of DNA repair proteins, ataxia telangiecstasia mutated (ATM) and tumour protein p53-binding protein 1 (TP53BP1), as well as proapoptotic protein Bim, promoting cell proliferation and leukemogenesis [31,307–309]. In addition,

upregulation of Bcl-xL and cyclin D1/D2 by activated STAT5 was observed in CML. However, STAT5 silencing resulted in the downregulation of Bcl-2 and MCL-1, as well as upregulation of Bim expression [31]. In AML, STAT5 is activated by Flt-3 receptor tyrosine kinase leading to upregulation of DNMT3A, Bcl-2, Bcl-xL and cyclin D1, as well as DNA hypermethylation of the tumor suppression gene, PTEN, and activation of PI3K/Akt signaling pathway to promote cell survival through inhibition of apoptosis [310,311].

Moreover, STAT5 mutations, especially STAT5B mutations, are particularly identified to promote its constitutive activation in different leukemias rather than in solid tumors [312]. The outcomes of one study revealed that somatic mutations in STAT5B including the mutation of SH2 domain N642H, repressed interferon-α/γ (IFNα/γ) signalling in BCR-ABL positive leukemia that induced tumor formation and growth [313,314]. The gain-of-function mutations of STAT5B was also observed in T-ALL and natural killer cell (NKC) T-ALL while no STAT5A/B mutation was identified in B-ALL. Hence, STAT5 constitutive activation and mutations in leukemia leads to aberrant cell proliferation, survival, chemoresistance and apoptosis inhibition [307,315].

1.7 Therapeutic strategies to target STAT5 in cancer

As STAT5 plays an important role in pathogenesis and progression of multiple cancers with high rate of incidence and mortality, targeting STAT5 to inhibit its activity can be considered as a potential therapeutic approach for cancers that are dependent on the constitutive activation of STAT5 signalling pathway. STAT5 activity can be abrogated by inhibitors that interact with STAT5 and target any of the steps that is required for its activation including inhibitors that (I) block tyrosine phosphorylation of STAT5, (II) target SH2 domain to prevent formation of STAT5

dimers, and (III) inhibit the transcriptional activity of STAT5 by binding to transcriptional regulator bromodomain containing 2 (BRD2) and counteracting STAT5-BRD2 interaction. In addition, the upstream signalling molecules that are involved in STAT5 activation including JAK, Flt3 and BCR-ABL can be targeted to affect and inhibit STAT5 signalling [269].

Post-transcriptional gene silencing mechanisms such as employing antisense ODN, shRNAs and siRNAs to silence/suppress STAT5 by degrading its mRNA can also be considered as a specific and safe strategy to inhibit STAT5 expression [234].

Regarding inhibitors of tyrosine phosphorylation, one study explored the effect of pimozide that is an antipsychotic drug to inhibit STAT5 in CML cell lines KU812 and KU562. The results indicated that direct interaction of pimozide with STAT5 led to prevention of tyrosine phosphorylation in STAT5 and subsequent downregulation target genes including Bcl-xL, Pim-1, MCL-1 and cyclin D1 that decreased cell viability and induced apoptosis in CML cell lines [316]. In addition, combination of pimozide and imatinib as a TKI showed a synergistic effect and improved the CML treatment [316]. Similarly, pimozide was able to inhibit STAT5 in Flt3 mutated AML cells resulted in blocking mTOR activity, decreasing the expression of MCL-1 and breaking the Flt-3-mediated resistance of cells to PI3K/Akt inhibitors. Synergistic effect of applying pimozide along with a TKI, PKC412 or sunitinib was also observed in AML cells [317]. *In vivo* study in an AML mouse model demonstrated that treatment with pimozide caused a reduction in subcutaneous tumor size as well as improved survival of mice compared to control groups with negligible effect on body weight highlighting the safety of pimozide administration *in vivo* [317,318]. However, further investigations are required to determine the mechanism of action of pimozide in the process of STAT5 inhibition.

Therapeutic effect of the other class of STAT5 inhibitors which targets SH2 domain of STAT5 to inhibit its full activation was investigated in CML and AML [319,320]. The benefit of these compounds is that they can specifically supress STAT5 as the SH2 domain structure is considerably different from that of other STAT proteins. BP-1-108, 13a and AC-4-130 are salicylic acid-derived materials that block STAT5 activity by decreasing STAT5 phosphorylation and dimerization [319–321]. The inhibitory effect of BP-1-108 was explored in MV-4-11 AML and K562 CML cell lines and the outcomes showed the induction of apoptosis in leukemic cells by downregulation of STAT5 target genes including cyclin D1, cyclin D2, MCL-1 and Myc, while having negligible toxicity on normal bone marrow cells even at high concentrations [319]. The compounds 13a and AC-4-130 also promoted the induction of apoptosis, cell cycle arrest in the G0/G1 phase and cell growth inhibition in MV-4-11 cells by decreasing the expression of STAT5 target genes cyclin D2, Bcl-2 and Myc [320,321]. The *in vivo* studies in an AML xenograft mouse model indicated that AC-4-130 was able to inhibit tumor growth and AML progression without affecting normal blood cells [320]. Concurrent administration of TKIs including dasatinib and ruxolitinib as well as AC-4-130 demonstrated a synergistic effect on decreasing viability of MV4-11 and MOLM-13 cells to below 50% in 24 h at lower concentration of all compounds (1 μM) [320,322]. In addition, Nicotinoyl hydrazine is a small molecule inhibitor which is capable of reducing STAT5 phosphorylation and subsequently its DNA biding ability [323]. This inhibitor promoted significant decrease of cell proliferation at high concentrations (200-400 μM) in breast cancer cell line T47D [322]. Further studies are required to evaluate the safety and therapeutic potential of STAT5 SH2 domain inhibitors in other types of cancers *in vitro* and *in vivo.*

The transcriptional activation of STAT5 target genes is mediated by the interaction of STAT5 with other transcriptional cofactors such as BRD2, a transcriptional regulator of the BET

family of proteins [324]. Some molecules can prevent this interaction and thus inhibit the transcriptional activity of STAT5. JQ1 is a member of this group of inhibitors that could downregulate the expression of STAT5 target genes Bcl-xL, PIM and CIS, in ALL cell lines and primary cells resulted in reduced cell viability especially when combining with TKIs [324–326]. Moreover, a CML study revealed that a 21-mer decoy oligodeoxynucleotides (dODN) was capable of inhibiting STAT5 transcriptional activity by occupying to DBD of STAT5 to block its binding to the promoter of its target genes. The results showed that the decreased expression of both STAT5A and STAT5B genes led to cell growth inhibition, cell cycle arrest and apoptosis induction [327]. Similarly, the effect of inhibitors reducing STAT5 transcriptional activity should be explored *in vitro* and *in vivo* in other cancers to determine their efficacy and safety.

To implement RNAi-mediated gene silencing, STAT5 gene expression can be specifically supressed by using siRNAs, shRNAs and antisense ODNs to prevent the activation of subsequent STAT5-induced signalling pathways and oncogene expression. As these inhibitory mechanisms perform at the post-transcriptional stage and interact with mRNA molecules, they are quite specific methods to be considered as potential therapeutic options especially for hematological malignancies [234]. The results of a CML study showed that inhibition of both STAT5A and STAT5B using their specific siRNAs could significantly downregulate STAT5 expression at both mRNA and protein levels and also induce apoptosis in K562 cells while this inhibitory effect was not observed in ODN treated cells, therefore, siRNA-mediated STAT5 silencing was more effective compared to ODN treatment [328]. Moreover, a preclinical study evaluated the effect of STAT5 inhibition by its specific shRNA in BCR-ABL$^+$ and BCR-ABL-like B-ALL cell lines and primary cells derived from newly diagnosed as well as relapsed/TKI-resistant BCR-ABL-like ALL patients and also in mouse models. The results revealed that STAT5 silencing suppressed cell

growth, induced apoptosis, and inhibited leukemogenesis *in vitro* and *in vivo* by downregulating the expression of STAT5 target genes Pim-1, Mcl-1 and Bcl-2 [31]. In an AML study, the findings demonstrated that the activation and phosphorylation of STAT5 through Flt3-ITD could activate the PI3K/Akt pathway to upregulate Mcl-1 expression at the protein level that resulted in impaired survival of leukemic stem cells (LSCs) in AML. Based on these observations, siRNA-mediated STAT5 silencing could reduce the expression of Mcl-1 at both mRNA and protein levels and thus, promoted apoptosis induction and cell growth inhibition of Flt3-ITD positive MV4-11 cells and AML primary cells [329]. Additionally, a study on lung cancer indicated that STAT5 activation by epidermal growth factor (EGF) can stimulate the expression of COX2 in human lung adenocarcinoma A549 cells that leads to tumor development and disease progression. The results of this study showed that STAT5 inhibition by its specific siRNA significantly downregulated EGF-mediated COX-2 expression, and STAT5 phosphorylation in A549 cells which can be considered as a beneficial therapeutic approach for the treatment of lung cancer [330]. In conclusion, the therapeutic potential of STAT5 silencing by nucleic acid-based strategies is required to be further evaluated in clinical trials for the treatment of cancers that are dependent on the STAT5 persistent activation including leukemias.

1.8 Delivery systems for siRNA therapy

A successful siRNA-mediated gene silencing requires an efficient delivery system to help siRNA molecules to reach the target mRNA in the cytoplasm; since polynucleotides are highly unstable in serum due to presence of nucleases, and their anionic nature prevents them from traversing cellular membranes on their own. In the process of siRNA delivery, carriers need to interact with the siRNAs and form siRNA nanoparticles to protect the siRNAs from serum

nucleases, promote their interaction with cell membrane and internalization, facilitate the siRNA release from endosomes into the cytoplasmic environment to eventually allow the siRNA incorporation into the RISC protein complex and target mRNA with the complementary sequence [331].

In the process of transfection, exogenous polynucleotides are introduced into target cells by a carrier. An effective transfection can only be observed if the polynucleotide escapes intracellular degradation and carries out its function typically in the cytoplasm. An ideal method of transfection delivers the polynucleotide to all target cells while causing minimal or negligible toxicity [332]. Although, different strategies have been developed for transfection, there is no universally effective material and/or method that can be applied for all cells. Therefore, different transfection approaches are required to use based on the type of target cells and also polynucleotide needed to be delivered to the cells. Transfection methods can be categorized into physical and chemical (biomaterial-mediated) methods.

In the physical methods of transfection, delivery of "naked" polynucleotides is promoted through making pores in the cell membrane with physical forces including electric or magnetic field, ultrasound waves and high pressure. Different methods in physical transfection of target cells consist of (I) direct injection of polynucleotide into the cell cytoplasm or nucleus using specialized tools including a glass microneedle or micropipette, (II) polynucleotide delivery utilizing high pressure by gene guns, jet injection, or hydrodynamic injection, (III) electroporation-mediated gene transfer, (IV) magnetic field-mediated gene transfer (magnetofection), (V) ultrasound-mediated gene delivery (sonoporation), and (VI) polynucleotide delivery by using laser beams (optoporation or laserfection) [333]. It is typical for electroporation to be used for leukemia studies

where siRNAs (and other nucleic acids) are delivered into the cells – this approach allows study of the outcomes with siRNA delivery but cannot be readily translated into a clinical setting.

In general, physical approaches do not introduce additional reagents, which sometimes introduces cytotoxicity to the system for interactions with the cell membrane for successful delivery. In addition, using physical methods, polynucleotide delivery may bypass the endosomal processing resulting in evading lysosomal degradation. These approaches are not affected by cell type and can be applied to all cells as they use mechanical forces to temporarily permeabilize cells. However, the drawbacks are the limited capacity for scale-up and the extreme loss of membrane integrity leading to cytotoxicity. Some techniques including microinjection, gene gun, and jet injection are restricted to single cells, but other methods can be applied for a larger number of cells. Moreover, these techniques need specialized equipment and skilled operators making them costly that should be considered. Currently, they are more applicable for the administration of polynucleotide-based vaccines to superficial tissues, evaluating the biology and effect of gene depletion by RNAi in attachment-independent cells and generating cell lines to produce recombinant proteins. There are some limitations associated with clinical application of physical methods including toxicity and invasive procedures required to access non-superficial tissues [238]. Some therapeutic approaches including clinical immunotherapy of hematological malignancies, as well as a few other diseases, utilize physical methods for *ex vivo* manipulation of patient cells followed by re-introduction of the modified cells into patients. In particular, electroporation has been widely used for *ex vivo* mRNA or siRNA transfection of suspension-growing natural killer (NK) [334,335] and T cells in animal studies [336–338].

Viral vectors have been effectively used to deliver polynucleotides, but they indicate significant safety risk because of integration into their host's genome or promote lethal immune

responses and inflammation [331,339]. This study focused on the of non-viral delivery of siRNA in ALL cells using biomaterials that interact with siRNA molecules to form nanoparticles.

In chemical (biomaterial-mediated) methods, carriers are rationally designed to overcome extracellular and intracellular hurdles. These carriers either electrostatically condense polynucleotides to a suitable size for internalization or encapsulate and protect them from endonucleases by masking their negative charge. Internalization of nanoparticles is conducted through different endocytosis mechanisms or cell surface interaction with anionic glycosaminoglycans (GAGs) or lipid rafts for larger lipophilic particles. The biomaterials should subsequently have features that promote endosomal escape to release their payload intracellularly and initiate the post-transcriptional gene silencing [238].

1.8.1 Nonviral biomaterials for siRNA delivery to leukemic cells

1.8.1.1 Cationic lipid-based carriers

Among different carrier systems, cationic lipids are the most frequently used vehicles for intracellular delivery of anionic siRNA molecules as they offer safer delivery of siRNA unlike viral vectors; and they can be chemically modified for specificity for different purposes such as cancer applications. There are some commercially available cationic lipids for gene delivery including N-[1-(2,3-dioleoyloxy)propel]-N,N,N-trimethylammonium (DOTMA, Lipofectin); 2,3-dioleyloxy-N-[2- spermine carboxamide] ethyl-N,N-dimethyl-1- propanammonium trifluoroacetate (DOSPA); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); N-[1-(2,3-dimyristyloxy)propyl]-N,N- dimethyl-N-(2-hydroxyethyl) ammonium bromide (DMRIE); 3β-[N-(N,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol); dioctadecyl amidoglyceryl

spermine (DOGS, Transfectam); and dimethyldioctadecylammonium bromide (DODAB) [238] (Fig. 1.5).

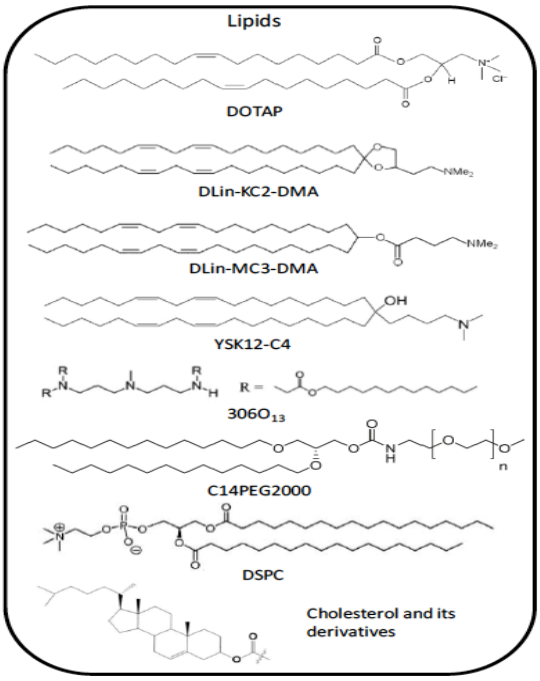

Figure 1.5. Chemical structures of different cationic lipids used for polynucleotide delivery to suspension cells [238].

Cationic lipids are able to interact with siRNA molecules to form either liposomes or lipid nanoparticles (LNP), which are also known as stable nucleic acid lipid particles (SNALP). Liposomes are defined as the nanosized vesicular structures consisting of one or more continuous lipid bilayers separating the inner aqueous core from the external aqueous medium. Cationic lipids may or may not be accompanied by co-lipids in liposomes. However, LNPs have a solid electron dense lipid core with aqueous pockets [340]. In addition, to increase the efficacy of the delivery system, neutral helper lipids, cholesterol, and a polyethylene glycol (PEG)ylated lipid are applied in the structure of LNPs along with the cationic lipid. The neutral helper lipids have fusogenic trait that assists towards particle structure, uniformity, stability, and endosomal escape [341].

Cholesterol contributes to form rigid and stable particles and also promotes cellular internalization [342]. PEG lipids protect particles from external agents and help to increase circulation half-life and biodistribution [343]. Structure of SNALPs consists of siRNA molecules surrounded by a lipid bilayer containing the cationic lipid (1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane, DMA) and the neutral lipid (such as cholesterol) coated with PEG lipids. The results of one study demonstrated that use of alkylated DMA/DMA as cationic lipids increased the LNPs transfection efficacy compared to DMA only in AML (MOLM13, THP1) and CML (K562) cell lines, yielding silencing more than 90%. The silencing in more challenging to transfect (KG1) cells was ~20%. These alkylated DMA-containing LNPs showed low toxicity and high stability [344].

In the structure of cationic lipids, a linker connects a cationic head group to a hydrophobic lipid anchor group. The cationic head group facilitates cellular uptake and endosomal escape through electrostatic interactions with anionic cell membranes as well as encapsulating anionic polynucleotides [345]. The electrostatic interaction between cationic head groups and polynucleotides should be sufficiently strong to protect its payload during delivery, but also weak enough to release the polynucleotides inside the target cell. The hydrophobic lipid anchor contains aliphatic hydrocarbon chains or multicyclic moieties including cholesterol. The length and configuration of the chains play an important role in the transfection efficacy. In addition, siRNA complexation is adversely impacted when there are more than three double bonds per chain, with linoleyl lipid being optimal [346]. The nature and structure of the linker have also a strong effect on stability, biodegradability, and transfection efficacy [347].

In the process of cationic lipid complexation with siRNA, cationic lipids provide a positive surface charge that can bind to siRNA complexes, contributing to particle interaction with anionic cell membranes [343]. This surface charge of the nanoparticles is required to be high enough to

promote cell association, but not excessive to avoid any immune system responses and excess toxicity on target cells [348]. Coating cationic lipid/nucleic acid lipoplexes with hydrophilic PEG molecules aids in avoiding immune system stimulation by masking excessive positive surface charge, but this also reduced uptake to some extent.

One study reported that PEG insertion after siRNA complexation with cationic lipid DOTAP resulted in higher siRNA encapsulation and subsequently higher transfection efficacy in suspension growing primary effusion lymphoma BCBL-1 cells [349]. Moreover, coating DODAB: monoolein (MO)-siRNA lipoplexes with PEG-Ceramide (PEG-Cer) has been investigated to increase lipoplex stability. The outcomes revealed that the Pegylated LNPs released less siRNA in human serum, protected the siRNA from displacement by serum proteins and prevented lipoplexes aggregation in serum compared to non-pegylated LNPs [350].

1.8.1.2 Cell penetrating peptides (CPPs)

Cell penetrating peptides (CPPs) are short sequences of amino acids (typically 5 – 30 amino acids long), and different peptides that are able to deliver polynucleotides inherently through cell membranes [351] (Fig. 1.6). Cationic peptides with basic amino acids including arginine and lysine, and amphipathic peptides having both hydrophobic and hydrophilic amino acids are two main groups of CPPs. The CPPs bind to nucleic acids via electrostatic charges and form complexes as in lipid carriers. In the process of internalization of CPPs, several factors such as size, amount and density of charge, hydrogen bonds and secondary structure of CPPs can influence their interaction with cell membrane [352,353]. The uptake of CPP/polynucleotide particles can be carried out by endocytosis, direct transportation through cell membrane and receptor-induced delivery to target cells depending on the concentration of cationic CPPs [352,354,355]. Typically,

endocytosis happens at low concentrations and direct translocation occurs at high concentrations of cationic CPPs including oligoarginine (R9), penetratin, lactoferrin, and HIV-1 protein Tat-derived peptides [356,357]. The electrostatic interaction of cationic CPPs with the anionic cell membrane promotes cellular uptake of CPPs [358]. Moreover, interaction of hydrophobic amino acids such as tryptophan (Trp) with the membrane bilayer increases the peptide delivery by inducing membrane destabilization [359]. As delivery systems, CPPs can be also directly conjugated to nucleic acids instead of electrostatically interact with phosphate backbone of polynucleotides to form NPs. However, the conjugates and NPs are observed to be unstable in serum when facing opportunistic competitors. Therefore, to increase the stability of NPs and protect them against extracellular components, CPPs can be modified with fatty acids or cholesterol [360]. It has been observed that a group of amphipathic CPPs called PepFect peptides were able to successfully deliver not only ASO and siRNA molecules but also larger plasmids [361,362]. Moreover, PepFect 6 peptide containing multiple trifluoromethylquinoline (TFMQ) residues in its structure revealed efficient siRNA delivery compared to commercial transfection agents to numerous leukemic suspension cells through increase in endosomal release [363,364]. One study applied HIV Tat-derived CPPs fused with a cationic membrane lytic peptide (LK15) to generate a fused peptide (Tat-LK15 peptide) for siRNA delivery that resulted in significant p210 BCR-ABL gene silencing (~70%) in CML K562 cells. In addition, this fused peptide increased the transfection efficiency by two folds in comparison with Tat peptide alone in several cell lines [339]. Outcomes of another study indicated a marked reduction of the mean GFP fluorescence (90%) and a significant mRNA silencing (90%) in GFP positive Jurkat T-cells using GFP siRNA delivered with a carrier comprised of a TAT peptide transduction domain (PTD) and double-stranded RNA-binding domain (PTD-DRBD). However, Lipofectin led to a decrease in mean

fluorescence by 40-50% and reduction in mRNA levels by ~50%. No cell toxicity was observed with this delivery system in primary human umbilical cord vein endothelial cells (HUVEC) [365]. In a recent study, two CPPs derived from dengue virus capsid, pepM and pepR, were evaluated for siRNA delivery in BV173 leukemia cells. The results showed successful delivery of siRNA as well as anti-cancer activity observed with both pepM and pepR by changing signalling pathways involved in cell cycle and proliferation [366].

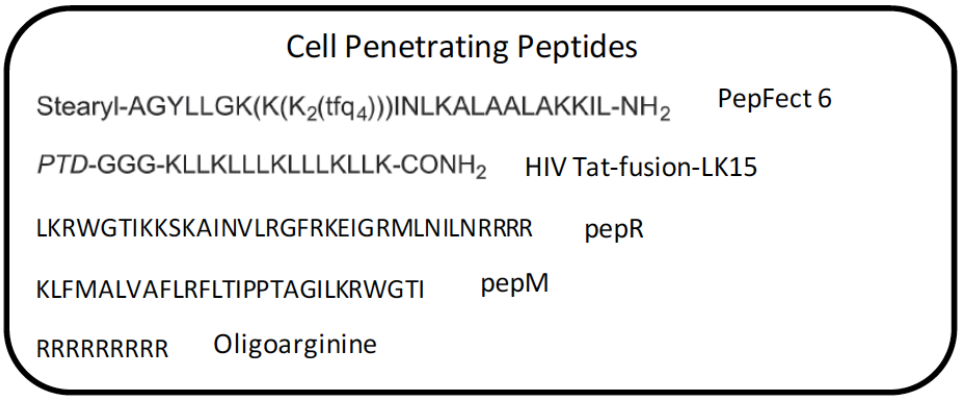

Figure 1.6. Chemical structures of different CPPs applied for polynucleotide delivery to suspension cells [238].

1.8.1.3 Polymer-based carriers

Polymers are a various group of natural or synthetic materials, either biodegradable or non-biodegradable, with a broad range of size, molecular weight (MW), structures and chemistries [238]. Among other non-viral delivery systems for nucleic acids, cationic polymers have been recognized to be effective carriers as they can be readily engineered and chemically modified for different applications [367]. Cationic polyamines, polyethylenimines (PEIs) are one of the most promising polycationic carriers for the transfection of suspension cells. They are able to condense polynucleotides into stable, spherical NPs through electrostatic interaction with the anionic phosphate groups of the nucleic acids. The resulting surface positive charge on polyplexes allows

particle endocytosis by interacting with the anionic cell membranes and facilitates cellular uptake of NPs [367]. The high transfection efficiency of PEI is associated with its high buffering capacity necessary for endosomal escape triggered by the so called “proton-sponge” effect. In the acidic pH of endosome, amine groups of PEIs get protonated, which subsequently induces an influx of protons, chloride ions and water into endosome. The high osmotic pressure within the endocytic vesicles promotes swelling and eventually endosomal rupture which results in the polyplex dissociation and release of the nucleic acids into the cytoplasm [368]. Moreover, MW of the PEI plays a critical role in the transfection efficiency and cytotoxicity. The cytotoxicity and transfection efficiency are known to inversely depend on the PEI MW. High MW (~25 kDa) PEI shows higher transfection efficacy; however, leads to membrane damage, excessive endosome rupture and cell toxicity. Therefore, using low MW PEI might be more suitable for gene delivery due to the lower degree of interactions with plasma membranes and thereby lower toxicity [369]. To improve the effectivity of the low MW PEIs, hydrophobic modification of these polymers has been attempted by employing the amine groups of PEIs for substitutions [370]. For this purpose, Uludag’s Lab developed lipid-substituted low MW PEIs (0.6 – 2 kDa) using aliphatic lipids with different chain lengths and degree of unsaturation for effective delivery of siRNA molecules (Fig. 1.7). Though these lipopolymers revealed slightly more toxicity than unmodified low MW PEIs, they were able to effectively encapsulate and deliver siRNA molecules to anchorage-independent leukemic cells [371]. These modified polymers are known to be generally effective, but their gene delivery efficiency can vary among leukemic cell lines which necessitates the optimization of substituent lipids based on the type of leukemic cells.

The efficacy of these polymeric-based siRNA carries has been evaluated in our research group in different leukemic cells including cutaneous T-cell lymphoma (CTCL), CML and AML

cell models. The CTCL study reported that siRNA-mediated silencing of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) or cyclin-depended kinase 18 (CDK18) genes by employing 2PEI-LA and 2PEI-caprylic acid (CA) as siRNA delivery systems induced cell death in Hut78 cells [372]. In the AML study, the same lipopolymers were capable of downregulation of the C-X-C chemokine receptor type 4 (CXCR4) gene as well as cell proliferation inhibition in THP-1 cells [373] and AML primary cells [374]. In another study on AML models, hyaluronic acid receptor CD44 was successfully silenced by 2PEI-LA/siRNA polyplexes leading to increased apoptosis and decreased cell adhesion in $CD34^+$ cell lines KG-1 and KG-1a and $CD34^+$ AML patient cells [375]. In CML studies, significant BCR-ABL gene downregulation was achieved by different siRNA/polymer complexes including palmitic acid (PA), α-linolenic acid (αLA) and cholesterol (Chol)-substituted PEIs (PEI-PA, PEI-αLA and PEI-Chol, respectively) which decreased cell viability, induced apoptosis and drug sensitivity in imatinib (IM)-resistant K562 cells [370,376,377] and PEI-LA, PEI-αLA and PEI-Chol polymer/siRNA groups promoted the reduction of BCR-ABL mRNA expression and colony formation inhibition in CML primary cells [378]. Furthermore, the tumor growth was arrested in a K562-derived xenograft mouse model using BCR-ABL siRNA/PEI-αLA lipopolymer [379]. These outcomes revealed the therapeutic potential of the lipid-modified PEIs as the siRNA carries to silence leading targets in various leukemic cell line models, patient-derived cells as well as xenograft mouse models for the first time.

Biodegradable charged polyester-based vectors (BCPV) are also a group of cationic polylactides explored for polymeric-mediated siRNA delivery in K562 cells [380]. Results indicated low cell toxicity (10% cell death) and high siRNA biding capacity (13-fold higher) with BCVP/siRNA complexes compared to Lipofectamine® 2000. Moreover, a significant reduction in

BCR-ABL mRNA levels was observed (~76%) which accompanied by marked cell growth inhibition (50%) compared to 17% with Lipofectamine®, and an apoptotic induction of 12.4% (vs. 5% with Lipofectamine® 2000). These outcomes of this study suggest a potent delivery agent for effective siRNA delivery to promote siRNA-mediated BCR-ABL silencing, decrease cell proliferation and induce apoptosis in K562 cells [380].

Another polymeric delivery agent for the transfection of suspension cells is the naturally derived chitosan which is generated by partial deacetylation of chitin as a linear polysaccharide. The constituent units of this polymer consist of D-glucosamine (deacetylated) and N-acetyl-D-glucosamine (acetylated) linked by randomly distributed β-(1,4) glycosidic bonds [381]. The MW of chitosan and degree of deacetylation play an important role in the transfection efficiency which control the size and the positive charge of the polyplexes [382,383]. High MW chitosan encapsulates nucleic acids into more stable complexes through both electrostatic interactions and chain entanglement. Though, polynucleotide release inside the cells might be affected by this tight complexation [382]. Therefore, to effectively deliver and protect the nucleic acids, these parameters should be considered and optimized. High transfection efficiency was also observed with high degree of deacetylation (>80%) resulting in high charge density and subsequently increased interaction with polynucleotides [384]. Chitosan mediated delivery of siRNA to leukemic cells was reported by several studies [385,386], and one study could achieve significant protein downregulation by Western Blot (~90%) in K562 cells using this delivery system [387].

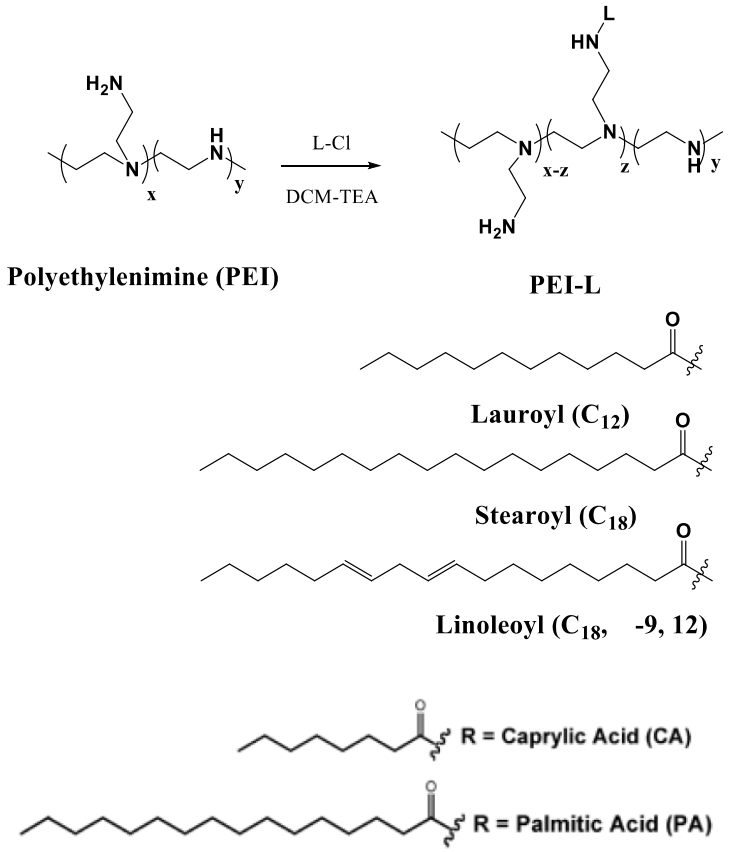

Figure 1.7. Schematic illustration for synthesis of lipid-substituted PEIs applied for polynucleotide delivery to suspension cells and chemical structures of different aliphatic lipids [238,388].

1.8.1.4 Cell-specific nucleic acid delivery

To improve the effectiveness of the delivery system, decrease the non-specific transfection of non-leukemic cells and thereby reduce the cytotoxicity, antibody or ligands that are specific for target receptors (antigens) on the surface of leukemic cells have been conjugated with NPs which permit the direct delivery of polynucleotides to a specific type of cells [389,390]. The success of this approach depends on the identification of highly expressed markers on the surface of leukemic cells [250]. In the process of uptake, these complexes enter the target cells through either receptor-mediated endocytosis or receptor-mediated direct penetration when for instance CPPs serve as

delivery agents [389]. Some barriers associated with nanoparticle binding and internalization include returning of the cell membrane to its original form and cell membrane-induced hydrophobic exclusion of polar surfaces [389]. These limitations required to be considered when designing the delivery systems.

One of the highly expressed receptors in cancer cells including AML and CML cells, is the transferrin receptor (TrfR), also called CD71, which is a cell membrane-associated glycoprotein capable of inducing endocytosis upon binding to its ligand, transferrin (Trf). In one study, this receptor was utilized for targeted delivery of BCR-ABL siRNA to K562 and LAMA-84 cells through the TrfR-targeted SNALPs consisting of Chol/DSPC/DODAP/PEG-Cer as the delivery agent [391]. The results revealed the effective siRNA uptake through TrfR-mediated endocytosis accompanied by a significant decrease in cell viability, BCR-ABL mRNA and protein levels in a dose-dependent manner (~60%) with TrfR-targeted SNALPs in both K562 and LAMA-84 cells compared to non-targeted SNALPs as the control groups [391]. In an AML study, transferrin (Trf) conjugated cationic lipid NPs explored for the delivery of LOR-1284 siRNA targeting the R2 subunit of ribonucleotide reductase (RRM2), into MV4-11cells. Outcomes indicated high siRNA uptake (91.5%) along with cell growth inhibition and marked reduction of R2 mRNA (80%) and protein levels in MV4-11 cells compared to control groups. In addition, *in vivo* studies were performed by intravenous (i.v.) administration of Trf-NPs in a murine xenograft model. The results showed tumor growth inhibition and prolonged circulation time of siRNA by 3 folds when encapsulated by Trf–NPs. The mRNA and protein levels of R2 dramatically reduced (86%) in the Trf–NP–LOR1284 treatment group compared with the control group [392].

In another receptor-mediated targeted siRNA therapy for AML and multiple myeloma (MM), a specific delivery system was developed using type-A CpG- oligodeoxyribonucleotide

(ODN) to encapsulate siRNA molecules against signal transducer and activator of transcription 3 (STAT3) and promote siRNA internalization through Toll-like receptor 9 (TLR9) expressed by several hematologic malignancies. The findings revealed that CpG(A)-siRNA complexes promoted TLR9-dependent STAT3 gene downregulation in 3 MM and 7 AML cell lines. Moreover, this formulation was able to induce apoptosis and inhibit tumor growth in AML mouse models and also to generate strong immune responses by increasing the ratio of $CD8^+$ T cells resulted in eliminating AML *in vivo*.

STAT3 is another transcription factor that is persistently activated in leukemic and tumor-associated immune cells leading to aberrant proliferation and survival of leukemic cells. It was observed that STAT3 is a negative regulator of TLR9 which is involved in enhancing immune responses by increasing the antigen-presenting performances or inducing apoptosis of leukemic cells. Therefore, siRNA-mediated STAT3 silencing along with TLR9 triggering through its agonists, CpG oligonucleotides, can generate potent TLR9-induced immune reactions against AML and break the tumor resistance *in vivo* [393,394].

Regarding the antibody-mediated polynucleotide delivery systems, uptake efficiency is associated with the carrier employed as carriers are directly involved in the interactions with cell membrane and particle internalization.

In one leukemia study, the efficacy of the immunopolyplexes developed by applying biotinylated antibodies (anti-CD3 and anti-CD19) linked through a streptavidin bridge to PEI (25 kDa) polyplexes was evaluated in Jurkat T cells ($CD3^+/CD19^-$), Granta B-cell line ($CD3^-/CD19^+$) and J.RT3/T3.5 cells (a $CD3^-/CD19^-$ T-cell line). The results showed the selective delivery of immunopolyplexes to cells compared to the control naked polyplexes: anti-CD3 immunopolyplexes only transfected Jurkat cells and anti-CD19 immunopolyplexes only

transfected Granta cells. While the transfection efficiency was low (~11% in Jurkat and ~2% in Granta cells) which highlights the importance of carrier and cell type. Moreover, anti-CD3 immunopolyplexes was identified in only Jurkat cells (80% of $CD3^+$ cells) when a combination of Jurkat and J.RT3/T3.5 cells employed. These immunopolyplexes were also able to reduce cell viability of Jurkat (50%) and J.RT3/T3.5 cells (10%) that underscores the association of cytotoxicity with targeting activity of immunopolyplexes as they were less toxic for untargeted J.RT3/T3.5 cells than for targeted Jurkat cells [395].

Another targeted delivery system was generated by conjugating an anti-JL1 minibody (leukemia cell-specific minibody) to a CPP oligo-9-Arg peptide (9R) for the selective siRNA delivery to JL1-positive T leukemic cells (CEM and Jurkat cells). High siRNA uptake was observed with these immunonanoplexes in JL1-over-expressing CEM cells (96%) compared to JL1-negative control groups. In addition, this formulation indicated efficient targeted siRNA delivery in mouse models. In this study, silencing and cell growth inhibition were not investigated [396].

An AML study evaluated the efficacy of an antibody targeted cyclodextrin-based siRNA delivery vector (CD.DSPE-PEG-Fab) in KG1 AML cells. In this formulation, CD123 which is also known as IL-3 receptor α-chain (IL-3Rα) and is expressed on the surface of human AML cells, is targeted by Fab to induce siRNA-mediated inhibition of bromodomain-containing protein 4 (BRD4) as an AML therapeutic target involved in affecting transcription through binding to acetylated histones.

The outcomes demonstrated significant downregulation of BRD4 mRNA (40%) and protein (~50%) compared to control siRNA groups in KG1 and also AML primary cells accompanied by inducting apoptosis and myeloid differentiation. In addition, combination of this

formulation and cytarabine resulted in a synergistic effect on leukemic cells in which ~80% cell death was observed with the combination treatment while the drug or targeted formulation alone led to 20% and ~40% cell death, respectively [397].

Consequently, the findings of the above-mentioned studies indicated that exploiting targeted antibody-mediated vectors to deliver nucleic acids resulted in the improvement of selectivity and efficacy of the delivery systems developed for leukemia therapy. Selecting a proper carrier for these targeted delivery systems is of utmost importance as it can increase the transfection efficacy and decrease cytotoxicity by using less siRNA concentrations.

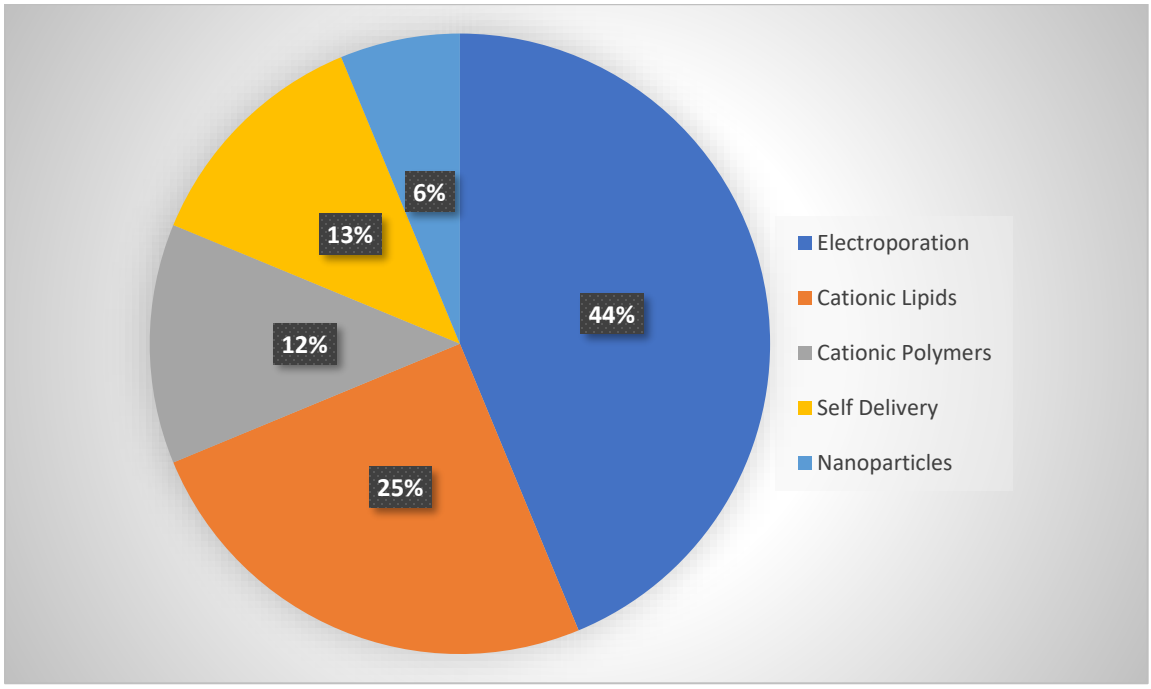

Figure 1.8. A summary of different delivery systems applied to deliver RNAi reagents. The percentages show the relative use of a specific kind of delivery system in the studies mentioned in Tables 1.2.

1.9 Outlook of siRNA therapy in ALL

New functional and potent carriers promoting effective delivery of gene-based materials (i.e., siRNA) in a controlled and non-toxic way are inspiring researchers to find physiological solutions for ALL treatment. A better understanding of the mechanisms involved in the uptake and intracellular trafficking of siRNA nanoparticles in the challenging suspension growing leukemic cells will further assist in this effort. The impact of carrier features including molecular size, degree of substitution (or modification) and optimal balance of the lipophilic-cationic moieties should be better appreciated not only on siRNA delivery efficiency, but also on toxicity, intracellular trafficking and cell specificity. This in conjunction with the recognition of novel siRNA targets that can be applied along with classical siRNA targets in ALL to inhibit genes involved in the activation of different survival pathways in ALL should prove beneficial. The combinational delivery, where several targets are simultaneously suppressed, is likely going to improve therapeutic outcomes in ALL. Regardless of the target, however, non-viral siRNA delivery is more likely to be the clinically applicable, given the relatively safe nature of such a delivery approach. The siRNA therapy could be applied in combination with the chemotherapeutics currently used to improve their efficacy or re-sensitize the cells to current drugs.

However, the siRNA therapy could also serve as an individual therapy if LSC could be specifically targeted. The delivery systems used for ALL cells could also be applied to other types of leukemias, but this will need a different set of biomaterials effective in a particular type of leukemia. There is only little information on the molecular details for effective delivery systems in different leukemias so that this should be a fruitful avenue of exploration in the future.

Since the suspension-growing cells are more difficult to transfect than the attachment-dependent cells, added pressure exists for non-viral delivery to be functional for leukemic diseases.

The siRNA nanoparticles need to be effective at a 20-50 nM range in culture for a practical translation to preclinical animal models. It is typical for reported delivery system to be used at concentrations beyond this range, including in our lab group work [370]. Intensive effort to lower efficacious doses will be beneficial in this regard. In addition to efficacy, target the cells of interest is of utmost importance so that critical genes in normal cells are not affected. By considering the cationic nature of these nanoparticles, they could theoretically attach to a group of cells *in vivo*. However, 'biochemical' targeting could overcome this limitation to some extent: only highly expressed genes in ALL cells or other supporting mediators, could be the target of RNAi, so that nanoparticles entering 'normal' cells might not result in inhibiting important targets. On the other hand, to increase the specificity of siRNA delivery, carriers could be conjugated with ALL-specific ligands, such as antibodies, to deliver the siRNA to only certain populations of cells. For example, carriers could be coupled with an anti-CD19 antibody to target at least most of the ALLL cells. However, these antibodies need to be chosen with care, so the delivery system is not too limited to certain cell populations. These antibody ligands need to be also exclusively or substantially over-expressed in the target cells to minimize nanoparticle binding to normal cells. A modular design could be envisioned where a delivery system optimized for general cellular uptake is further functionalized with leukemic specific cell surface binding molecules.

Finally, little information exists on siRNA delivery to primary cells, either healthy or malignant cells from ALL patients. It is necessary not only to investigate the efficacy in human cells, but also to evaluate the off-target effects of the siRNA delivered and cytotoxic effect of the carriers. While cell lines are preferred (due to practical reasons) in the design and optimization of carriers, characteristics including endocytosis rate and intracellular trafficking pathways are

expected to be significantly different in leukemia primary samples. Misleading directions could be avoided by using ALL primary cells early on in the development of process.

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Chapter 2

Therapeutic Delivery of siRNA with Polymeric Carriers to Down regulate STAT5A Expression in High-Risk B-cell Acute Lymphoblastic Leukemia (B-ALL)[1]

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2.1 INTRODUCTION

Acute lymphoblastic leukemia (ALL), the most common type of childhood cancer [1], is characterized by overproduction and accumulation of malignant lymphoid progenitor cells within the bone marrow. Current multiagent chemotherapy regimens have improved the treatment outcomes in people under age 20, with 5-year survival rate of 89%. However, outcomes in adults remain inferior, largely owing to the development of chemoresistance leading to relapse [2]. In older adults, severe toxicities limit the ability to administer intensive chemotherapy. In B-ALL, which accounts for 80% of ALL, two molecular subtypes, BCR-ABL$^+$ and BCR-ABL-like subgroups, are associated with high relapse rates and inferior survival in both childhood and adult B-ALL [3,4]. These two subtypes account for over 60% of adult ALL in some series [3,5,6]. Therefore, developing novel treatment modalities is of utmost importance to improve treatment outcomes in these high-risk patients [7,8].

Transcription factors are one of the most promising molecular targets for siRNA-based cancer therapy, as multiple oncogenic signalling pathways converge on a limited group of transcription factors which promote cell growth and survival [9]. One such key group of transcription factors is the Signal Transducer and Activator of Transcription (STAT) family proteins. These proteins act as nuclear transcription factors that activate the expression of a diverse set of genes, including some that are involved in cancer cell development, growth and survival [9,10]. Certain STATs, such as STAT3 and STAT5, are often constitutively active when one or more upstream tyrosine kinases become overactive due to different genetic alterations leading to a variety of solid tumors and blood malignancies [10]. In the normal process of early B-cell development in bone marrow, STAT5 is required to induce cell survival and B-cell expansion [11–13]. Studies in BCR-ABL-like B-ALL cells have demonstrated constitutive activation of a variety

of key signal transduction pathways, including JAK, EPOR, ABL and PDGFR. Activation of these pathways induces overexpression of STAT5, leading to uncontrolled proliferation and/or survival of leukemic cells [14]. Similarly, STAT5 is activated indirectly by BCR-ABL1 in Philadelphia $(Ph)^+$ B-ALL cells leading to its overexpression [15–17]. In BCR-ABL^+ chronic myeloid leukemia (CML), high levels of STAT5 mRNA correlate with tyrosine kinase inhibitor (TKI) resistance, regardless of the presence of tyrosine kinase domain (TKD) BCR-ABL1 mutations [18,19]. A preclinical study in BCR-ABL^+ and BCR-ABL-like B-ALL cell lines and primary cells derived from newly diagnosed and relapsed/TKI-resistant BCR-ABL-like ALL patients, found that STAT5 silencing suppressed cell growth, induced apoptosis, and inhibited leukemogenesis [3]. These studies suggest that STAT5 signaling is a potentially attractive therapeutic target in high-risk B-ALL [3,9,20,21]; however, only a few number of targeted small molecule inhibitors of STAT5 are currently available.

RNA interference (RNAi) has emerged as an alternative approach to inhibit signaling pathways. RNAi is a process by which double-stranded small interfering RNA (siRNA) can induce sequence-specific, post-transcriptional gene silencing [22]. To control the expression of genes involved in these malfunctioning processes, synthetic siRNA can be delivered into diseased cells to interact with the target mRNA of aberrant genes for degradation or inhibition of translation, thereby silencing their expression [22]. However, siRNA therapy requires an efficient delivery system since naked siRNA molecules are susceptible to degradation by endogenous nucleases in serum, and they are not able to pass through cellular membranes due to their anionic nature. Among different delivery systems, cationic polymers are safer carriers than the viral vectors for intracellular delivery of anionic siRNA molecules as they do not have the capacity to integrate into host genome. Moreover, cationic polymers can be chemically engineered and functionalized

according to the needs of the application [22–24]. Lipid substituted low molecular weight (MW) polyethylenimine (PEI) is a promising cationic polymer to undertake siRNA delivery into leukemic cells as it effectively condenses siRNA molecules into nanoscale particles due to its high charge density, facilitates cell internalization through electrostatic interaction with plasma membranes, and displays high buffering capacity needed for endosomal escape [25–27]. The lower MW PEI does not display the disadvantages of the high MW (>25 kDa) PEIs such as high cytotoxicity and limited biodegradability. However, low MW PEIs need to be modified for effective siRNA delivery to provide the required stability for the self-assembly process during complexation with polynucleotides and increase the interactions with the plasma membrane to facilitate the cellular uptake. Therefore, we have utilized lipid substitution on the amine groups of low MW PEIs to improve the efficacy of siRNA delivery into the cells [28,29].

In this study, the impact of STAT5A inhibition on B-ALL cell lines and ALL patient-derived cells were assessed by screening a library of lipid-substituted polymeric nanoparticles and determined the most effective polymeric carriers for STAT5A siRNA delivery. We focused on the silencing activity of siRNA delivery in selected cell models as the physicochemical properties of the lipopolymers were earlier reported [30].

2.2 Materials and Methods

2.2.1 Materials

The low MW PEIs (0.6, 1.2, and 2.0 kDa), anhydrous dimethyl sulfoxide (DMSO), formaldehyde, chloroform, 2-Mercaptoethanol, doxorubicin hydrochloride (product number: 44583), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Iscove's Modified Dulbecco's Medium (IMDM), Roswell Park Memorial Institute

Medium (RPMI) 1640 medium with L-glutamine, fetal bovine serum (FBS), GlutaMAX, Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), penicillin, streptomycin, and UltraPure DNase/RNase-free dH2O were obtained from ThermoFisher Scientific (Ottawa, Canada). Interleukin 3 (IL3), IL6, IL7, FMS-like tyrosine kinase 3 ligand (Flt3-L), and stem cell factor (SCF) were supplied by PeproTech (Rocky Hill, NJ, USA). BIT 9500 serum substitute was purchased from StemCell Technology (Vancouver, BC, Canada). Negative control scrambled siRNA (Cat. No. DS NC1), the 6-carboxyfluorescein (FAM) labeled scrambled siRNA, STAT5A specific dicer-substrate siRNA (5′-CCCGAUUUCUGAGUCACUAAAGCGCAA-3′ and 3′-GGGCUAAAGACUCAGUGAUUUCGCG5′-5′), and a custom-synthesized BCR-ABL specific dicer-siRNA (5′-GCAGAGUUCAAAAGCCCU-3′ and 3′-GUCUCAAGUUUUCGGGAA-5′) were obtained from Integrated DNA Technologies (IDT) (Coralville, IA, USA). SensiFAST cDNA Synthesis Kit was from FroggaBio Inc. (Toronto, ON, Canada). Luna® Universal One-Step qPCR Kit was ordered from New England Biolabs ® (NEB), Inc. (Ipswich, MA, USA). Human methylcellulose enriched media (Cat. No. HSC005) and human methylcellulose base media (Cat. No. HSC002) were supplied by R&D systems, Inc. (Oakville, ON, Canada). Trizol used for total RNA extraction and Lipofectamine™ RNAiMAX Reagent were from Invitrogen (Carlsbad, CA).

2.2.2 Cell models and cultures

Acute lymphocytic RS4;11 and SUP-B15 leukemia cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). SUP-B15 Cells were cultured in IMDM supplemented with 20% FBS, 0.05 mM 2-Mercaptoethanol, 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. RS4;11 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. All cell

lines were maintained at 37 °C and 5% CO_2. To sub-culture the cells after reaching 80% confluency, cells were centrifuged at 900 rpm for 5 min and passaged at a 20% concentration of the original count.

Ten frozen ALL patient cells were obtained from the biobank at the University of Alberta Hospital (Edmonton, AB, Canada) with the approval of the institutional Health Research Ethics Board. Patient samples with specific genetic abnormalities were selected according to the World Health Organization guidelines for categorizing the ALL subtypes. The clinical characteristics of patients are summarized in Table 2.1. To culture the mononuclear (MN) cells obtained from ALL frozen samples, the cryovial was quickly thawed in a water bath at 37 °C (without dissolving ice completely). Thawed cells were then carefully added dropwise to 4 mL of the recovery medium containing DNase I solution (0.5 ml), PBS (2 ml) and FBS (1.5 ml) (for every 1 mL of cell suspension) followed by incubating for 2–4 min at room temperature (RT) to dissolve any clumps completely. Cell suspension was then transferred to 1.5 mL tubes to spin down at 100 rcf for 10 min at 4 °C. Supernatants were carefully removed and cells were resuspended in the remaining fluid. Pellets were combined and cell viability was assessed by trypan blue staining in a hemocytometer before cell culture. The MN cells were maintained in IMDM serum free media, supplemented with 20% BIT 9500 serum substitute, GlutaMAX (2 mM), IL3 (10 ng/mL), IL6 (10 ng/mL), IL7 (10 ng/mL), Flt3- ligand (20 ng/mL), SCF (30 ng/mL) and 10−4M 2-Mercaptoethanol (0.1 mM) and incubated at 37 °C and 5% CO_2 for at least 4 h to allow sufficient cell recovery prior to siRNA delivery studies.

Table 2.1: Clinical characteristic of ALL patient samples

Patient samples	Phenotype	Molecular Abnormality	Cytogenetics	(%) blasts	Prior treatment
P1	**B-ALL**	**BCR-ABL1 Positive (b2a2, P210)**		76	chemoRx for breast Cancer
P2	**B-ALL**	**BCR-ABL1 Negative**	45,XX,add(1)(q21),der(1)del(1)(p13p32)t(1;9)(q42;q34),del(3)(q11.2q13),der(6)t(6;21)(q21;q)dup(21)(q),der(8)t(1;8)(q21;p21),-9,del(12)(p13),der(16)t(1;16)(p13.3),-21,der(22)t(6;22)(p11.1),+mar[cp23]/46,XX[3].ish ,add(1)(q21)(wcp1-),der(1)del(1)(p13p32)(wcp1+)t(1;9)(q42;q34)(wcp1-,ABL1+),der(6)t(6;21)(q21;q)qdp(21)(q)(wcp6+,wcp6-,RUNX1 amp),der(8)t(1;8)(q21;q21)(wcp1+),del(12)(p13p13)(ETV6-),der(16)t(1;16)(p13.3)(wcp	95	none

			1+),der(22)t(6;22)(p11.1)(wcp6+,BCR+)		
P3	**B-ALL**	**BCR-ABL1 Negative, ABL rearranged. Likely BCR-ABL-like**	46,XY,t(2;9)(q11.2;q34)[12]/46,idem,add(5)(q35),del(11)(p11.2)[4]/46, XY[7].ish t(2;9)(ABL1+;ABL1+)[7]	93	none
P4	**B-ALL**	**MLL-AF4 t(4;11), MLL rearranged**	47,XX,+X,t(4;11)(q21;q23)	81	none
P5	**B-ALL**	**BCR-ABL1 Positive (e1a2, P190)**		100	none
P6	**B-ALL**	**BCR-ABL1 Positive (b3a2, P210)**		85	none
P7	**B-ALL**	**BCR-ABL1 Negative**	46,XX,t(1;16)	76	none
P8	**B-ALL**	**BCR-ABL1 Positive (b3a2, P210)**		68	none
P9	**B-ALL**	**BCR-ABL1 Positive (e1a2, P190)**		95	none
P10	**B-ALL**	**BCR-ABL1 Negative**	cytogenetics failed	85	none

2.2.3 Polymer synthesis and polymeric nanoparticle preparation for cell delivery

Lipid-modified PEIs were synthesized according to the previously published protocols [30]. Lauric acid (Lau), linoleic acid (LA) and stearic acid (St) were used as specific lipids to modify the amines of low MW PEIs and the lipid substitution levels in final products were analyzed by ^{1}H-NMR (Fig. 1). Lipofectamine™ RNAiMAX Reagent was used as the lipid- based commercial reagent in all the experiments. The lipid-modified polymers and desired siRNAs were dissolved in nuclease-free water at 1 and 0.14 μg/μL, respectively. siRNA-lipid-modified polymer complexes (polymeric nanoparticles) were prepared by adding siRNA solutions to serum-free medium to get the final siRNA concentration of 60 nM in cell suspension. The polymers were then added to the siRNA solutions to give the desired polymer:siRNA weight ratios (3:1, 6:1 and 9:1, designated as R3, R6 and R9, respectively) bringing the final volume to 330 μL, followed by mixing briefly and incubating for 30 min at room temperature to allow for siRNA and polymer interaction with each other and forming complexes. Lipofectamine™ RNAiMAX Reagent-siRNA complexes were prepared at 2.5:1 lipid-to-siRNA (weight/weight) ratio (as suggested by the manufacturer) with similar siRNA concentration and incubated for 30 min at room temperature. The siRNA-polymer complexes (100 μL/well) were then added in triplicate to Falcon™ Polystyrene 48-well Microplates (Thermo Scientific, Lafayette, CO, USA) and then the cells suspended in 300 μL of culture media were added on top of the complexes. Non-treatment (NT) control groups were exposed to serum-free medium alone (no complexes), while the negative control groups were exposed to control (scrambled) siRNA/lipid-polymer nanoparticles. Cells were collected 3 and 6 days after polymeric nanoparticle treatment and analyzed for STAT5A silencing efficiency and viability.

2.2.4 siRNA uptake by flow cytometry

To determine the most effective polymeric carriers for siRNA delivery into hard to transfect leukemic cells, a library of lipid-modified PEIs was screened with FAM-labeled siRNA/polymer complexes at three polymer:siRNA ratios of 3:1, 6:1, and 9:1 and a final siRNA concentration of 30 nM in 48-well plates for 24 h. RNAiMAX was used as the reference carrier at 2.5:1 RNAiMAX:siRNA ratio. Non-labeled scrambled siRNA was used as a negative control. After 24 hours of incubation time, cells were collected in microcentrifuge tubes and centrifuged (1800 rpm for 8 min), washed twice with HBSS (pH 7.4) and re-suspended in formalin at a final concentration of 3.7% in HBSS. The uptake efficiency of complexes was quantified by a BD Accuri^{TM} C6 Plus flow cytometer using the FL1 channel (10,000 events per sample) and calibrating the instrument so that autofluorescence of the negative control (untreated cells) gave ~1% of the total cell population as the background. The mean fluorescence of the recovered cell population and the percentage of cells showing FAM-fluorescence were measured to evaluate the siRNA delivery in cells.

2.2.5 Reverse transcription quantitative polymerase chain reaction (qPCR) for assessing the silencing activity of siRNAs

We used qPCR analysis for investigation of siRNA silencing since protein level detection of silencing (by western blots) was going to require 3-4 fold increased cell mass, which was not available for most primary patient samples. To explore the silencing effect of desired siRNAs, the leukemic cells were transfected in 6-well plates with complexes prepared with STAT5A, BCR-ABL and control siRNAs at a 60 nM siRNA concentration and the effective polymers at 3:1 and 6:1 polymer:siRNA weight ratios for 3 and 6 days. After the incubation time, cells were collected

in microcentrifuge tubes, spun down, and total RNA was extracted using TRIzol reagent following manufacturer's instructions. The quantity and integrity of total extracted RNA was then assessed by optical density measurement (A260/A280 ratio) using spectrophotometer (GE Nanovue). One microgram of total RNA was converted into cDNA using SensiFAST cDNA Synthesis Kit according to manufacturer's recommendations. Finally, using a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA), real-time PCR analysis with 2× SYBR green master mix and ROX (MAF Center, University of Alberta) was performed to follow the fluorescence intensity. Specific primers applied to detect the expression levels of human beta-actin (housekeeping endogenous gene) (forward: 5′-GCGAGAAGATGACCCAGAT-3′; reverse: 5′-CCAGTGGTACGGCCAGA-3′), STAT5A (forward: 5′-CCGACGGGACCTTCTTGTTG-3′; reverse: 5′-TGCGTTCCGGGGAGTCAAAC-3′), and BCR-ABL (forward: 5′-CATTCCGCTGACCATCAATAA G-3′; reverse: 5′-GATGCTACTGGCCGCTGAAG-3′) were designed by the NCBI Primer-BLAST and supplied by IDT. A 10 µL volume containing 5 µL of 2× master mix SYBR Green, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer and 3 µL of cDNA template (5 ng/µL) for each sample (three independent biological replicates) were transferred to a Fast Optical 96-well plate. The cDNA template was omitted from qPCR reaction as a negative control in qPCR. The amplification cycle consists of heating the reaction mixtures for 5 min at 95 °C before going through 40 cycles of a denaturation step (15 s at 95 °C) and an annealing/elongation step (1 min at 65 °C). The level of target gene expression was determined by $2^{-\Delta\Delta CT}$ method using the non-treatment groups as the calibrator. Target gene CT values were normalized against beta-actin CT values and the results were reported as the relative quantity of transcripts.

2.2.6 MTT assay to evaluate inhibition of cell growth following STAT5A silencing

The efficacy of STAT5A silencing on cell growth inhibition was investigated using MTT assay. In 48-well plates, 45,000 RS4;11 cells, 50,000 SUP-B15 cells and 2×10^5 ALL primary cells suspended in 300 μL of culture medium, were transfected with the indicated siRNA-polymer complexes for 3 and 6 days. At desired time points, MTT solution (5 mg/mL) was added to the wells to give a final concentration of 1 mg/mL and the cells were incubated for 2 h further at 37 °C, after which the cells were collected by centrifugation and the medium was removed. The formed formazan crystals were then dissolved with 100 μL of DMSO and the absorbance of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments, VT, USA) at 570 nm. The cell viability percentage was calculated as follows: 100% × (absorbance of polymeric nanoparticle treated cells/absorbance of untreated cells).

2.2.7 Trypan blue exclusion assay for cell viability

To evaluate cell growth inhibition, cells were transfected for 3 and 6 days with complexes. After incubation, trypan blue exclusion assay was performed by mixing 20 μL of cell suspension with 20 μL of trypan blue dye and injecting into a hemocytometer. The numbers of viable cells that exclude the dye were counted and cell concentration (the number of viable cells per ml) was then calculated.

2.2.8 Colony-forming cell (CFC) assay

To assess the inhibition of cell proliferation induced by siRNA/polymer complexes, leukemic cells were transfected with complexes at a polymer:siRNA ratio of 6:1 and final siRNA concentration of 60 nM. 24 hours after the transfection, trypan blue exclusion staining was used to

count the viable cells in a hemocytometer. 400 RS4;11 and SUP-B15 cells, or 15,000 ALL primary cells were then mixed in 0.5 mL of methylcellulose media and seeded in the center wells of the 24-well plates. For the CFC assay of ALL primary cells, human methylcellulose-enriched media supplemented with final recombinant human EPO (3 IU/mL), recombinant human IL-6 (20 ng/mL), recombinant human IL-3 (20 ng/mL), recombinant human GM-CSF (20 ng/mL), recombinant human G- CSF (20 ng/mL), recombinant human SCF (50 ng/mL), 2-Mercaptoethanol (5 x 10^{-5} M), L-Glutamine (2mM), 2% Bovine Serum Albumin (BSA), 25% FBS, and 1.4% IMDM was applied while for ALL cell lines, human methylcellulose-based media containing 1.4% IMDM, 25% FBS, 2% BSA, 2-Mercaptoethanol (5 x 10^{-5} M) and L-Glutamine (2mM) was used. The total number of colonies were observed and counted with optic microscopy after 14 days of incubation at 37 °C in a fully humidified incubator with 5% CO_2.

2.2.9 Combinational siRNA therapy

Combinational siRNA delivery was performed in ALL BCR-ABL positive primary cells using STAT5A and BCR-ABL siRNAs at total siRNA concentration of 60 nM (30 nM each) with a 6:1 polymer:siRNA weight ratio. Individual STAT5A or BCR-ABL siRNA at total 30 and 60 nM concentrations with 6:1 polymer:siRNA weight ratio was delivered by 1.2PEI-Lau8 polymer in ALL patient cells as well. The efficacy of combinational siRNA therapy to inhibit cell growth and induce gene silencing was investigated after 72 hours by MTT assay, cell counting, and qPCR as described above, respectively. Scrambled siRNA was used as the negative control in the experiments.

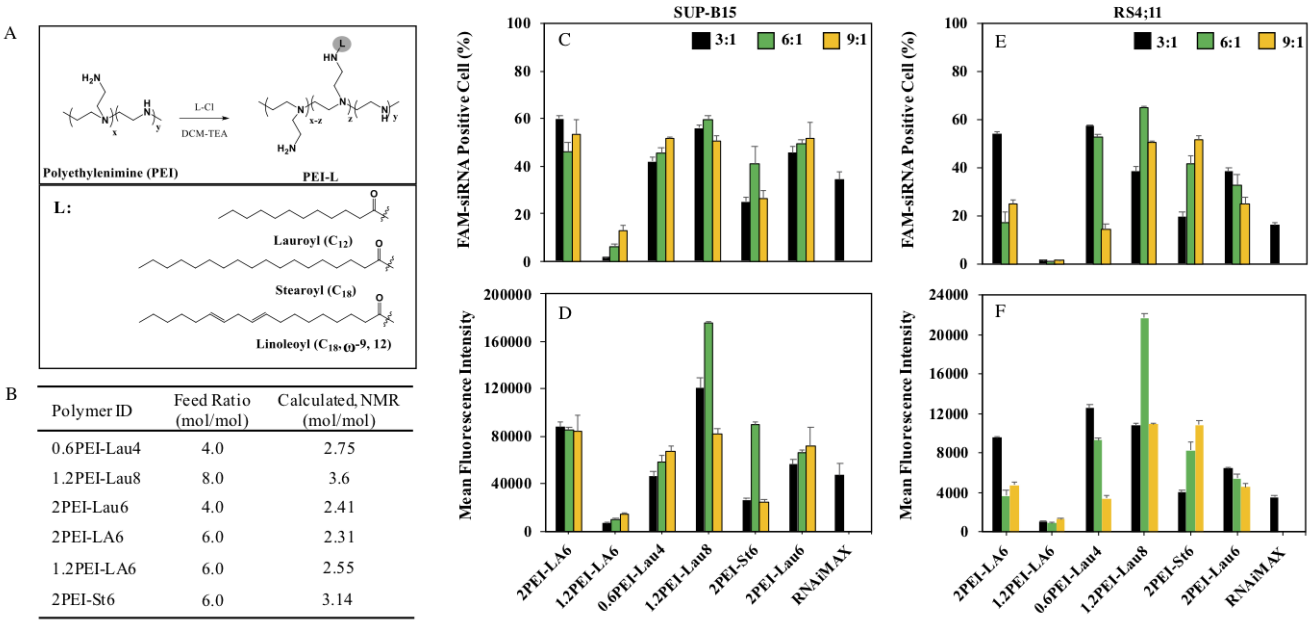
2.2.10 Statistical analysis

All results were reported as mean ± standard deviation (SD). Statistical analysis was performed by unpaired Student's t test, where significantly different groups were determined by an asterisk (*) in figures. A value of $p \leq 0.05$ was considered statistically significant and it was defined by comparing specific siRNA/polymer-treated groups to that of control siRNA/polymer-treated groups.

2.3 RESULTS

2.3.1 siRNA uptake in RS4;11 and SUP-B15 cells

We evaluated and compared the potential of a library of lipid-substituted low MW PEI derivatives to transfect ALL cells. The scheme for synthesis of lipid-substituted PEIs and lipid substitution levels as a function of feed ratio are displayed in Fig. 2.1A and B, respectively. The level of substitution ranged between 2 and 4 lipids/PEI and the MW of PEI backbone did not affect the level of substation among the tested carriers. In SUP-B15 cells, four polymer groups exhibited high FAM-siRNA uptake at most ratios (Fig. 2.1C). A similar observation was made in RS4;11 cells (Fig. 2.1E), among which 1.2PEI-Lau8 was the highest, with an overall range between 40-50% FAM positive cells. Considering the mean fluorescence intensity in both the cell lines, 1.2PEI-Lau8 at ratio 6:1 gave the highest uptake which was significantly ($p \leq 0.005$) different when compared to other ratios (3:1 and 9:1) (Fig. 2.1D and F). The commercial reagent RNAiMAX gave lower FAM-siRNA positive cells and mean fluorescence intensity compared to the polymeric delivery systems.



B

Polymer ID	Feed Ratio (mol/mol)	Calculated, NMR (mol/mol)
0.6PEI-Lau4	4.0	2.75
1.2PEI-Lau8	8.0	3.6
2PEI-Lau6	4.0	2.41
2PEI-LA6	6.0	2.31
1.2PEI-LA6	6.0	2.55
2PEI-St6	6.0	3.14

Figure 2.1: Schematic illustration of the synthesis of lipid-substituted PEIs (A). The obtained lipid substitutions as a function of lipid:polymer feed ratio during synthesis (B). (C-E) The uptake of FAM-labelled siRNA at 30 nM with polymer:siRNA ratios of 3:1, 6:1 and 9:1 after 24 hours of treatment. The percentage of cells that has taken up the siRNA is shown as FAM-positive SUP-B15 cells and RS4;11 cells (C and E, respectively). The mean fluorescence intensity in SUP-B15 and RS4;11 cells are also shown (D and F, respectively). Lipofectamine RNAiMAX was used as a reference delivery reagent at polymer:siRNA ratio of 2.5:1. FAM-siRNA positive cells and mean fluorescence were determined by flowcytometry.

2.3.2 STAT5A gene knockdown

STAT5A silencing was evaluated at the mRNA level using qPCR. In SUP-B15 cells, two of the polymer groups that showed high siRNA uptake, 1.2PEI-Lau8 and 2PEI-LA6, were chosen to evaluate gene silencing. The 1.2PEI-Lau8 polymer was able to silence 70% and 36% of STAT5A gene expression at ratio 6:1 on both day 3 and day 6, respectively, whereas 2PEI-LA6 at the same conditions, silenced 92%, at ratio 6:1 on day 3 compared to the CTRL siRNA groups (Fig. 2.2A). In RS4;11 cells, 1.2PEI-Lau8 was effective in lowering the STAT5A gene expression (32%) only

at ratio 6:1/day 3 (Fig. 2.2B). RNAiMAX exhibited 71% silencing, only on day 3 in SUP-B15 cells in comparison with the CTRL siRNA groups (Fig. 2.2A).

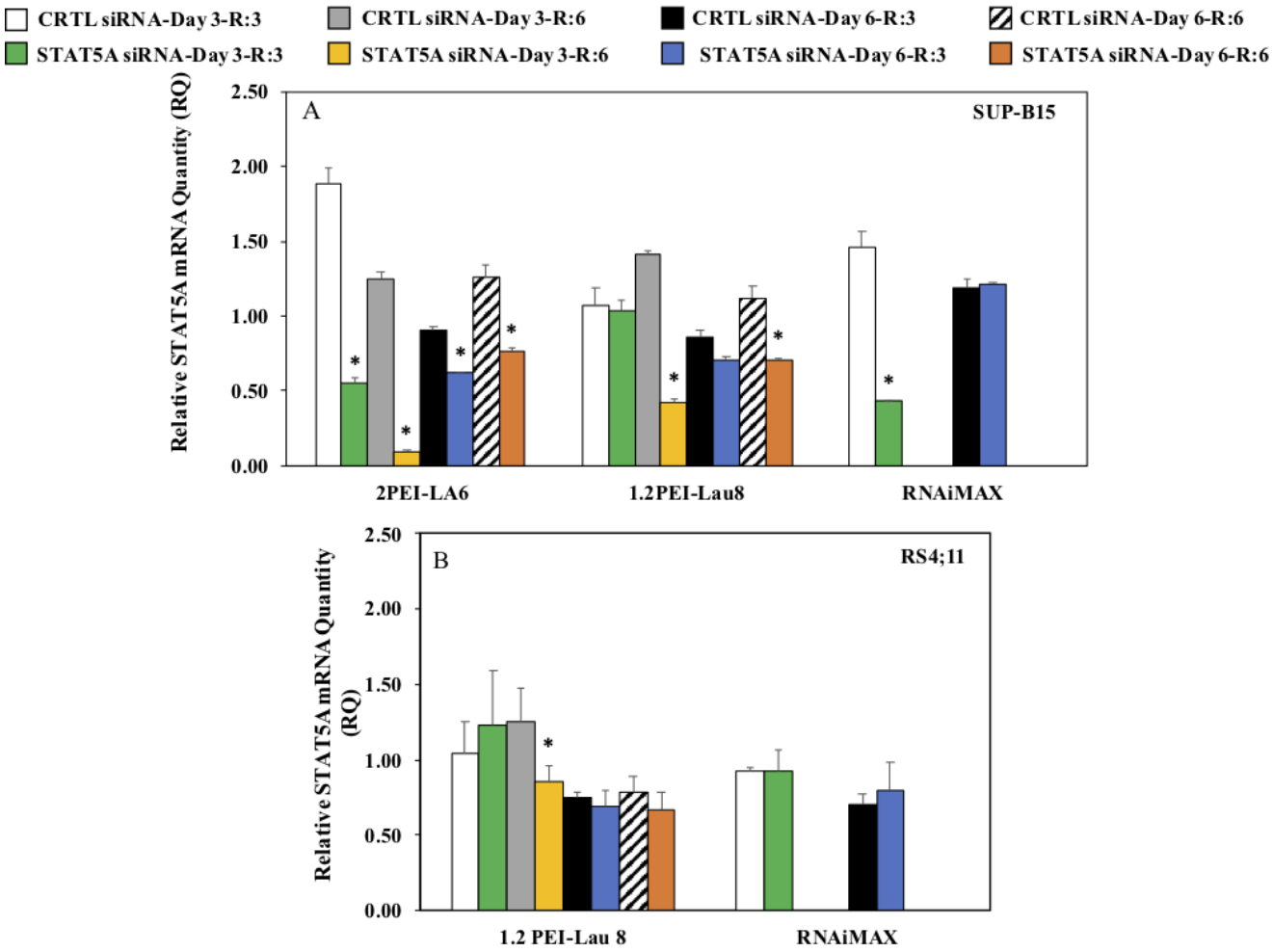


Figure 2.2: STAT5A mRNA levels after siRNA treatment. The relative STAT5A mRNA levels (relative to β-actin as an internal control) were quantified through qPCR. SUP-B15 (A) and RS4;11 (B) cells were treated with 60 nM of STAT5A/CTRL siRNAs for 3 and 6 days and RQ of mRNA are plotted relative to non-treatment group. Lipofectamine RNAiMAX was used as a reference delivery reagent. *$p \leq 0.05$ vs. Control siRNA.

2.3.3 Cell growth inhibition by STAT5A silencing

In the cell viability assessment, the 1.2PEI-Lau8/STAT5A complexes were able to inhibit the growth by 39% in SUP-B15 cells on day 3 and 24-27% in RS4;11 cells on day 3 and day 6 compared to polymer/CTRL siRNA groups ($p \leq 0.05$; Fig. 2.3A and B). On the other hand, 2PEI-LA6/STAT5A complexes could only exhibit minimal decrease in the cell viability in both SUP-B15 cells (20%; Fig. 2.3A) and RS4;11 cells (15-18%; Fig. 2.3B) irrespective of the time points.

Using trypan blue exclusion assay in both cell lines, 2PEI-LA6/STAT5A siRNA complexes were able to exhibit a 1.5-fold significant decrease in the live cell count compared with CTRL siRNA complexes on both time points ($p \leq 0.05$; Fig. 2.4A, D). The leading 1.2PEI-Lau8/STAT5A siRNA complexes showed a stronger effect on both cell lines with 1.7 and 2.7-fold decrease in live SUP-B15 cells and 2 and 2.7-fold decrease in RS4;11 cells on day 3 and day 6, respectively ($p \leq 0.05$; Fig. 2.4B, E).

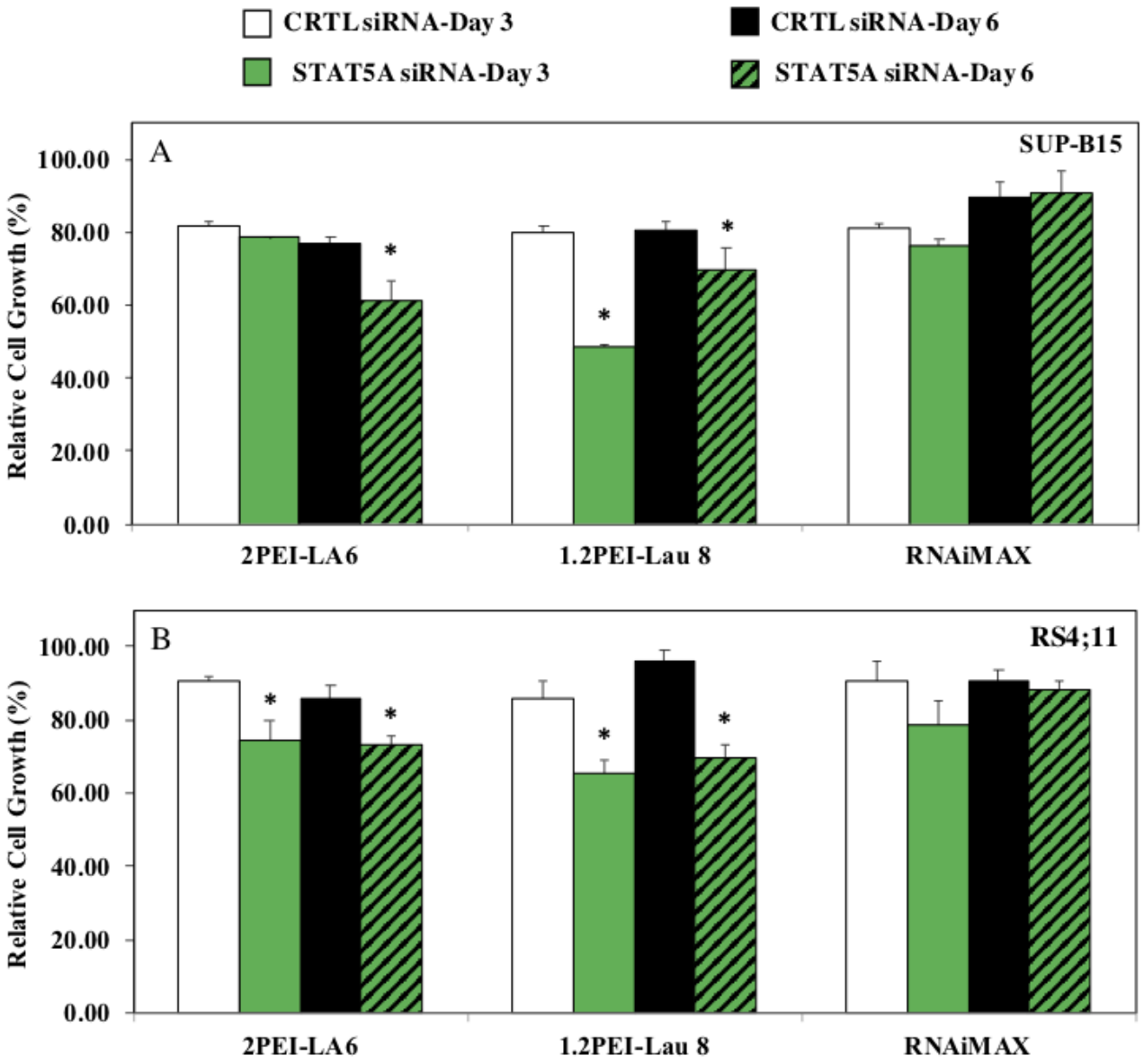


Figure 2.3: Effect of siRNA/polymer complexes on proliferation of SUP-B15 (A) and RS4;11 (B) cells. Cells were treated with polymer/siRNA ratio of 6:1 and 60 nM of Control/STAT5A siRNA complexes for 3 and 6 days and cell growth inhibition was assessed by the MTT Assay and expressed relative to non-treated cells (taken as 100%). The data are the mean ± SD. *$p \leq 0.01$ compared with the complexes with Control siRNA.

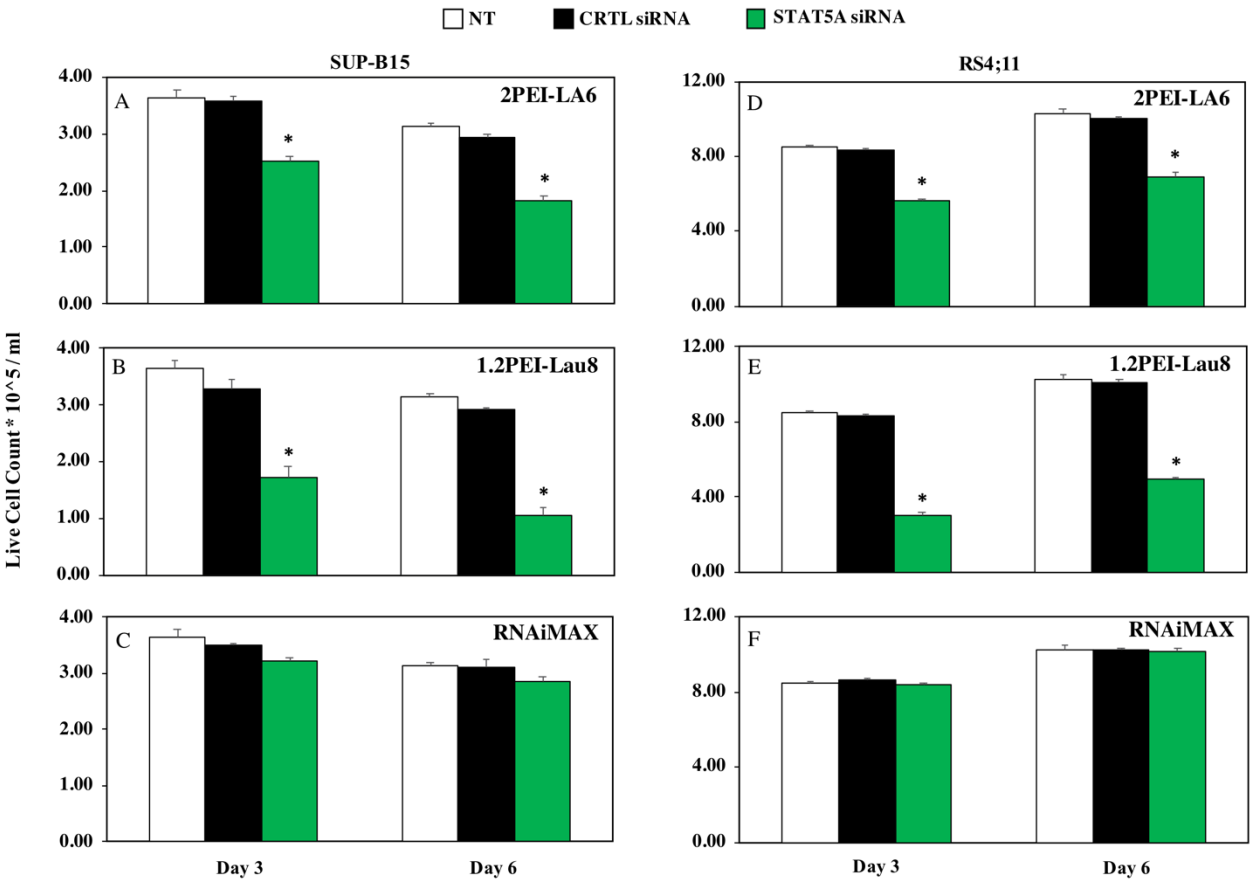


Figure 2.4: The growth inhibition of cells by complexes. Cells were transfected with CTRL/STAT5A siRNA complexes and live cells were counted by trypan blue exclusion assay after 3 and 6 days of treatment. Y-axis indicates the live cell count (x 10^5) per ml. Live cell counts in SUP-B15 and RS4;11 cells using 2PEI-LA6 (A and D), 1.2PEI-Lau 8 (B and E) polymers along with RNAiMAX (C and F) are indicated at two time points and values are shown as means ± SD; *p ≤ 0.05 versus control siRNA.

2.3.4 Inhibition of cell growth by CFC assay following STAT5A knockdown

In CFC assay, 2PEI-LA6 polymer was able to show 2.7-fold and 2-fold significant decrease in colony counts in SUP-B15 and RS4;11 cells, respectively, compared to the control siRNA group ($p \leq 0.05$; Fig. 2.5A, B). Moreover, a 3.9-fold and 5.4-fold marked reduction in the colony formation was induced by 1.2PEI-Lau8 in SUP-B15 and RS4;11 cells, respectively ($p \leq 0.05$; Fig. 2.5A, B).

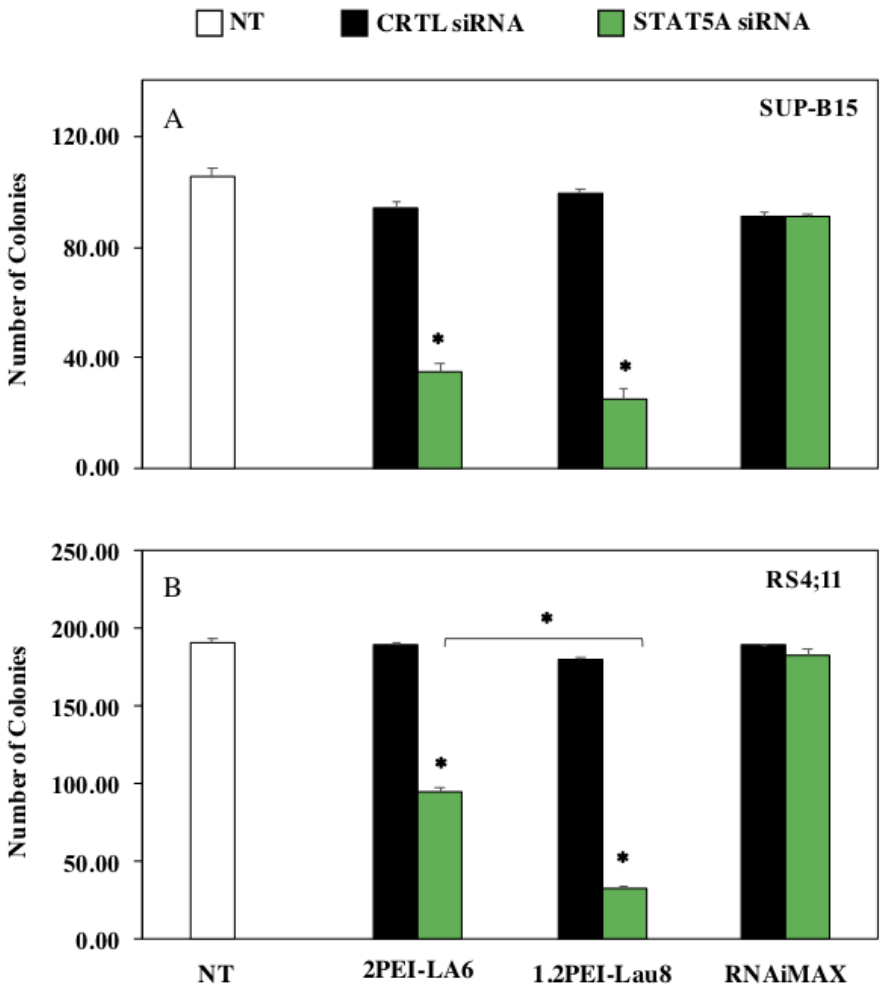
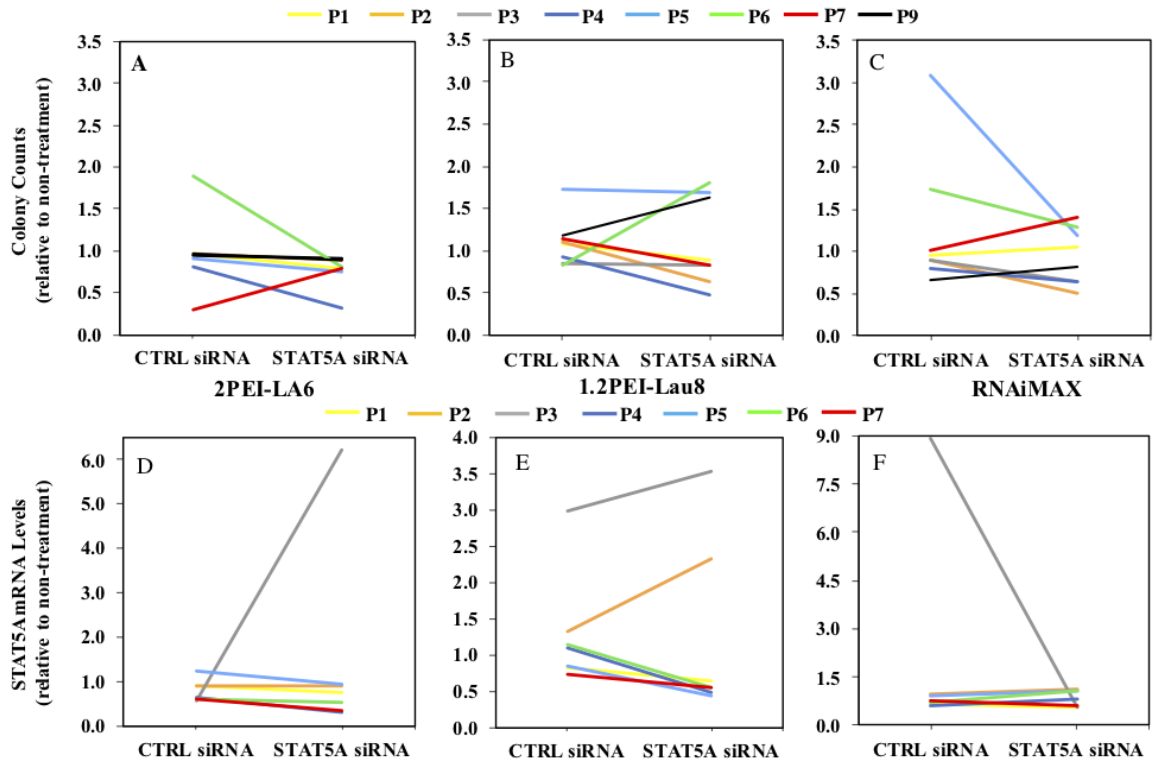


Figure 2.5: Effects of transfection with CTRL/STAT5A siRNA/polymer complexes on colony formation in leukemic cell lines. Colony counts were performed two weeks after treatment at concentration of 60 nM and at polymer:siRNA ratio of 6, (RNAiMAX:siRNA ratio: 2.5:1). (A) Total number of colonies formed by SUP-B15 cells (A) and RS4;11 cells (B). *: $p \leq 0.05$.

2.3.5 Functional outcome of STAT5A silencing in ALL primary cells

The inhibition in cell growth and proliferation observed in cell lines was further investigated in ALL patient cells by the CFC assay (Fig. 2.6A, B and C) and qPCR (gene silencing) (Fig. 2.6D, E and F). Some of the frozen patient samples (P8 and P10) had limited number of viable cells due to the poor cell recovery in the thawing process; therefore, some assays could not be performed (denoted as n/a [not available] in Fig. 2.6G and Fig. 2.7E). In 2PEI-LA6/STAT5A complex

treatment groups, two samples, P4 and P6, showed a significant decrease in the colony counts in comparison with their control siRNA groups (P4: 61.7% and P6: 56.6%, $p \leq 0.05$; Fig. 2.6A and G). With 1.2PEI-Lau8/STAT5A siRNA complexes, the colony counts decreased significantly by 42.3%, 48.39 and 28% in P2, P4 and P7, respectively ($p \leq 0.05$; Fig. 2.6B and G).



G	Colony Counts			qPCR			
Time point	14 Days			3 Days			
siRNA type and	STAT5A	STAT5A	STAT5A	STAT5A	STAT5A	STAT5A	Patient subtype/
concentration	60 nM	60 nM	60 nM	60 nM	60 nM	60 nM	Genetic Abnormality
Polymer Group	2PEI-LA6	1.2PEI-Lau8	RNAiMAX	2PEI-LA6	1.2PEI-Lau8	RNAiMAX	
P1	ns	ns	ns	+	ns	+	BCR-ABL1 Positive (b2a2, P210)
P2	ns	+	++	ns	ns	ns	BCR-ABL1 Negative
P3	ns	ns	+++	ns	ns	ns	BCR-ABL1 Negative, ABL mutated
P4	+++	+++	ns	+++	+++	ns	MLL-AF4 t(4;11), MLL rearranged
P5	ns	ns	+++	+	+	ns	BCR-ABL1 Positive (e1a2, P190)
P6	+++	ns	+++	ns	+++	ns	BCR-ABL1 Positive (b3a2, P210)
P7	ns	+++	ns	+++	+	+	t(1;19)
P9	ns	ns	ns	n/a	n/a	n/a	BCR-ABL1 Positive (e1a2, P190)

ns: not significant, n/a: not available, +: $p<0.05$ (one-side t-test), ++: $p<0.01$ (one-side t-test), +++: $p<0.001$ (one-side t-test)

Figure 2.6. Effects of treatment with STAT5A siRNA/polymer complexes on colony formation (A, B and C) and STAT5A mRNA expression (D, E and F) in ALL primary cells. Colony counts were performed two weeks after transfection at a siRNA concentration of 60 nM and polymer:siRNA ratio of 6 and RNAiMAX:siRNA ratio of 2.5 for one day. STAT5A mRNA levels

were assessed by qPCR after 3 days of transfection on frozen MN patient samples. (G) Summary of statistical analysis for the results from qPCR and CFC assays.

Based on the qPCR assay, 2PEI-LA6 complex treatment significantly decreased STAT5A mRNA levels in four patient samples (P1, P4, P5 and P7) by 18%, 51.6%, 24.2% and 44.8%, respectively, compared to their control siRNA groups ($p \leq 0.05$; Fig. 2.6D and G). In the 1.2PEI-Lau8 treatment groups, there was a marked reduction in STAT5A mRNA levels in P4, P5, P6 and P7 by 57.3%, 47.6%, 51.3% and 23.6%, respectively ($p \leq 0.05$; Fig. 2.6E and G). STAT5A silencing with RNAiMAX was evident in P1 (17.6%) and P7 (22.8%) in comparison with control siRNA groups ($p \leq 0.05$; Fig. 2.6F and G).

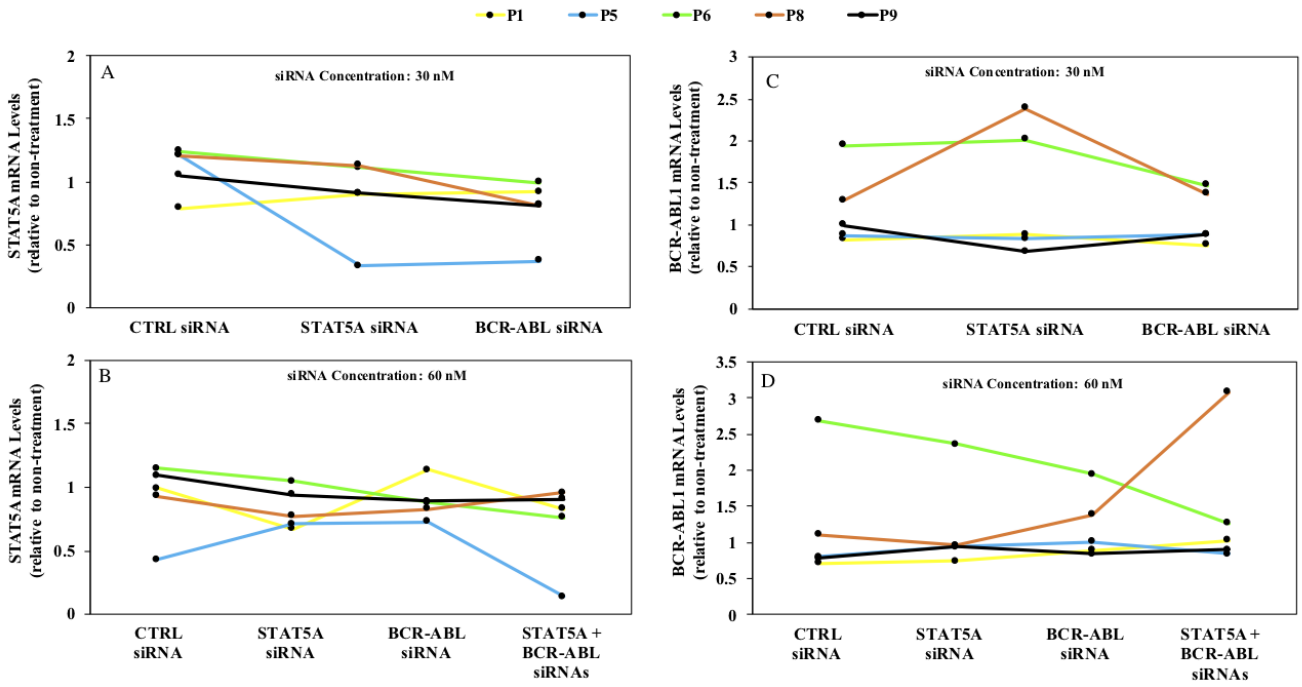
2.3.5.1 Combinational silencing of BCR-ABL and STAT5A in BCR-ABL positive ALL patient cells

Among the 10 patient samples available in this study, 5 samples were BCR-ABL positive and, therefore, the extent of both STAT5A and BCR-ABL gene silencing and cell growth inhibition was investigated by qPCR, MTT and trypan blue exclusion assays. The STAT5A mRNA levels significantly decreased with 30 nM of STAT5A siRNA (72.7%) as well as with 30 nM of BCR-ABL siRNA (69.4%) only in the P5 sample, in comparison with the control siRNA group (Fig. 2.7A and E; $p \leq 0.05$). The combination of STAT5A (30 nM) and BCR-ABL (30 nM) siRNAs resulted in the downregulation of STAT5A gene in samples P1 (16.2%) and P5 (67.4%) compared to their control siRNA groups (Fig. 2.7B and E; $p \leq 0.05$).

Using the MTT assay, STAT5A siRNA at both 30 nM and 60 nM showed a significant cell growth inhibition in 3 out of 5 patient samples compared to their respective control groups; P1, P5

and P6 (Fig. 2.8A, B, C; $p \leq 0.05$). By trypan blue exclusion assay, the live cell number was significantly reduced compared to their respective controls in 2 of these samples, P5 and P6, at both 30 nM and 60 nM (Fig. 2.8G, H; $p \leq 0.05$). After transfected with BCR-ABL siRNA, cell growth was markedly inhibited in only one patient sample at 30 nM and 2 patient samples (P1 and P6) at 60 nM (Fig. 2.8A, B, C; $p \leq 0.05$).

By trypan blue exclusion, transfection with BCR-ABL siRNA led to marked decrease in live cell counts in only one patient sample (P6) at 30 nM and 60 nM siRNA (Fig. 2.8H). The combination of STAT5A and BCR-ABL siRNAs resulted in a significant reduction in cell growth in comparison with their control siRNA groups in 3 samples (P1: 32.6%, P5: 31.6%, P6: 45.2%) (Fig. 2.8A, B, C; $p \leq 0.05$). This correlated with a significant drop in the live cell number by MTT assay in the same 3 patients: P1: 46.4%, P5: 49.3% and P6: 35.6%, (Fig. 2.8F, G and H).

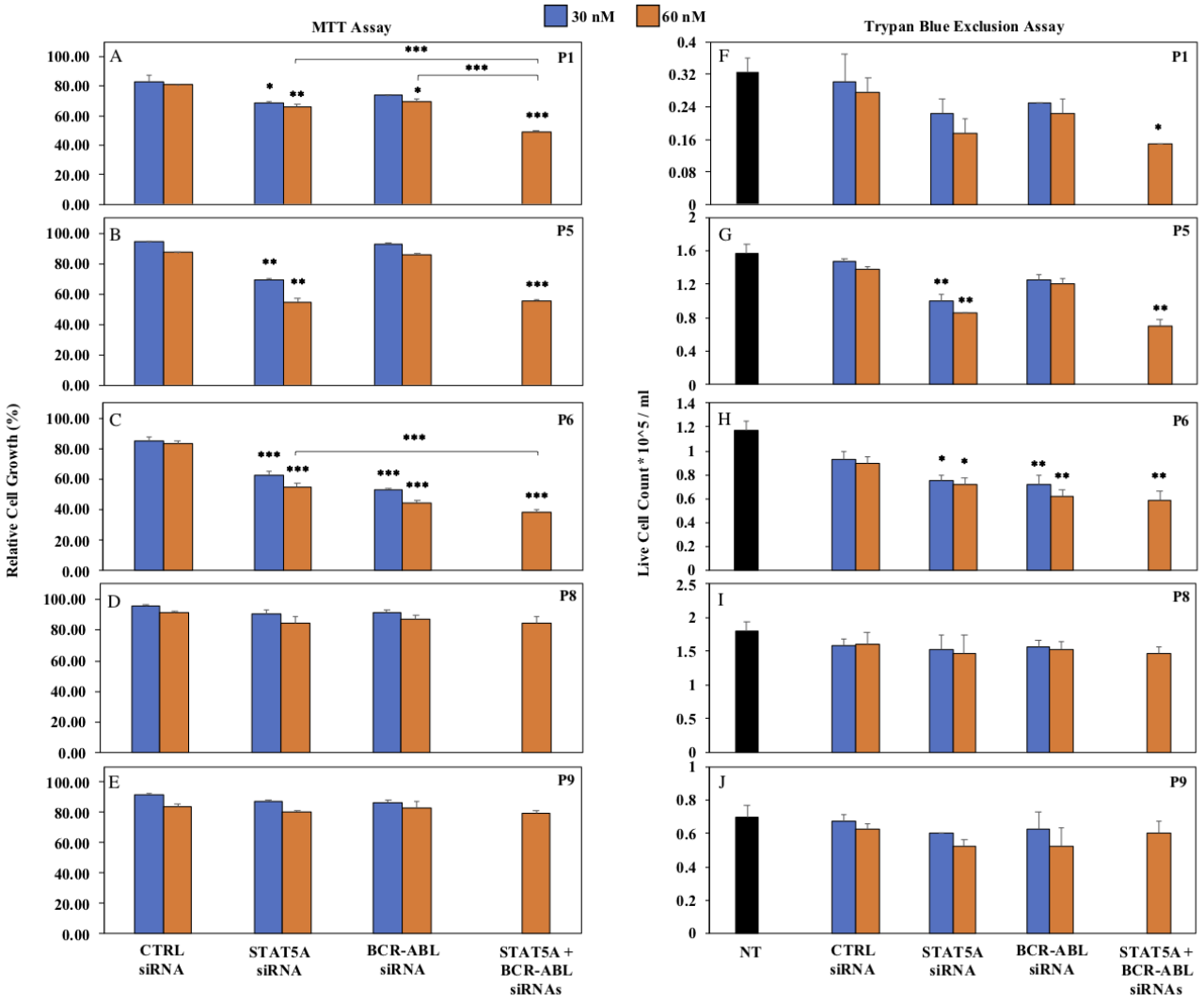


E

qPCR with STAT5A and BCR-ABL siRNA combination											
Time point	3 Days										
siRNA type and concentration	STAT5A 30 nM		BCR-ABL 30 nM		STAT5A 60 nM		BCR-ABL 60 nM		STAT5A + 30 nM	BCR-ABL + 30 nM	Patient subtype/ Genetic Abnormality
Polymer Group					1.2PEI-Lau8						
mRNA	STAT5A	BCR-ABL	STAT5A	BCR-ABL	STAT5A	BCR-ABL	STAT5A	BCR-ABL	STAT5A	BCR-ABL	
P1	ns	ns	ns	ns	++	ns	ns	ns	+++	ns	BCR-ABL1 Positive (b2a2, P210)
P5	++	ns	++	ns	ns	ns	ns	ns	+	ns	BCR-ABL1 Positive (e1a2, P190)
P6	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	BCR-ABL1 Positive (b3a2, P210)
P8	ns	ns	ns	ns	n/a	ns	ns	ns	ns	ns	BCR-ABL1 Positive (b3a2, P210)
P9	ns	ns	ns	ns	n/a	ns	+	ns	ns	ns	BCR-ABL1 Positive (e1a2, P190)

ns: not significant, n/a: not available, +: $p<0.05$ (one-side t-test), ++: $p<0.01$ (one-side t-test), +++: $p<0.001$ (one-side t-test)

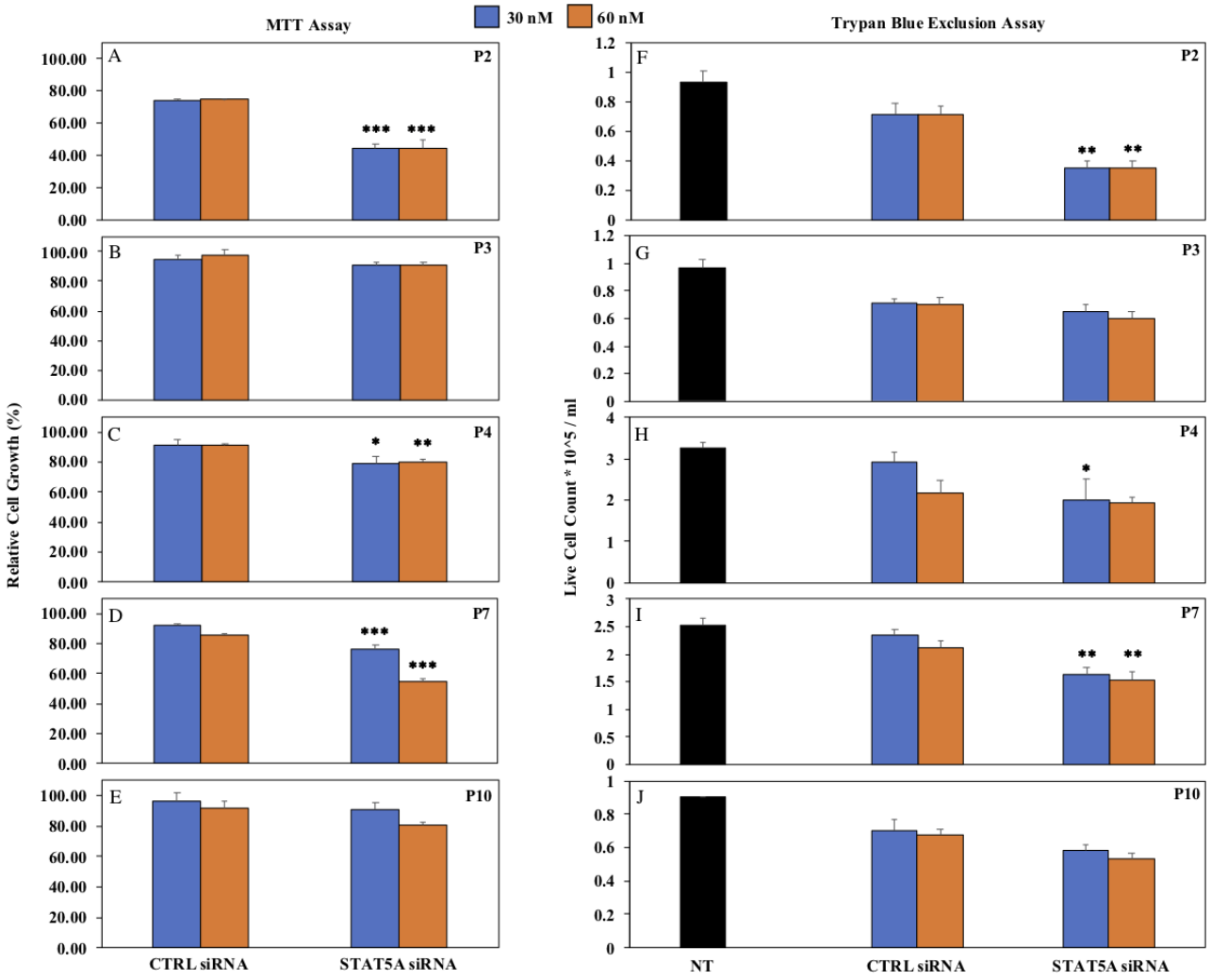
Figure 2.7. Effects of treatment with STAT5A and BCR-ABL siRNA/polymer complexes on STAT5A (A and B) and BCR-ABL (C and D) mRNA expression in ALL primary cells. mRNA levels were assessed 3 days after transfection at siRNA concentrations of 30 and 60 nM and 1.2PEI-Lau8 polymer:siRNA ratio of 6 by qPCR. (E) Summary of statistical analysis for the results from qPCR results of combination treatment.



K	MTT					Cell Counts					
Time point	3 Days					3 Days					
siRNA type and concentration	STAT5A 30 nM	STAT5A 60 nM	BCR-ABL 30 nM	BCR-ABL 60 nM	STAT5A + BCR-ABL 60 nM	STAT5A 30 nM	STAT5A 60 nM	BCR-ABL 30 nM	BCR-ABL 60 nM	STAT5A + BCR-ABL 60 nM	Patient subtype/ Genetic Abnormality
Polymer Group	1.2PEI-Lau8					1.2PEI-Lau8					
P1	+	++	ns	+	+++	ns	ns	ns	ns	+	BCR-ABL1 Positive (b2a2, P210)
P5	++	++	ns	ns	+++	++	++	ns	ns	++	BCR-ABL1 Positive (e1a2, P190)
P6	+++	+++	+++	+++	+++	+	+	++	++	++	BCR-ABL1 Positive (b3a2, P210)
P8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	BCR-ABL1 Positive (b3a2, P210)
P9	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	BCR-ABL1 Positive (e1a2, P190)

Figure 2.8: Effect of siRNA/polymer complexes on proliferation of BCR-ABL positive patient cells explored by MTT assay (A-E) and trypan blue exclusion assay (F-J). Cells were treated with 1.2PEI-Lau8/siRNA ratio of 6:1 and 30 nM and 60 nM of Control, STAT5A and BCR-ABL siRNAs for 3 days. Cell growth inhibition was assessed by MTT Assay and expressed relative to non-treated cells (taken as 100%). In addition, live cells were counted by trypan blue exclusion assay. Y-axis indicates the live cell count (x 10^5) per ml. Values are shown as means ± SD; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ versus control siRNA. (K). Summary of significant differences in MTT and cell counts in BCR-ABL positive patient samples.

In BCR-ABL negative ALL primary cells, STAT5A siRNA decreased the cell viability by MTT assay in 3 out of 5 patient samples compared to their respective control groups (P2, P4 and P7) at both 30 nM and 60 nM. (Fig. 2.9A, C and D). By trypan blue assay, the live cell number significantly reduced with STAT5A siRNA 30 nM in the same 3 patient samples (Fig. 2.9F, H and I), and in samples P2 and P7 at 60 nM (Fig. 2.9F and I).



K	MTT			Cell Counts		
Time point	3 Days			3 Days		
siRNA type and	STAT5A	STAT5A		STAT5A	STAT5A	Patient subtype/
concentration	30 nM	60 nM		30 nM	60 nM	Genetic Abnormality
Polymer Group	1.2PEI-Lau8			1.2PEI-Lau8		
P2	+ +	+++		+ +	+ +	BCR-ABL1 Negative
P3	ns	ns		ns	ns	BCR-ABL1 Negative, ABL mutated
P4	+	+ +		+	ns	MLL-AF4 t(4;11), MLL rearranged
P7	+++	+++		+ +	+ +	t(1;19)
P10	ns	ns		ns	ns	Cytogenetics failed

Figure 2.9: The growth inhibition of BCR-ABL negative patient cells evaluated by MTT assay (A-E) and trypan blue exclusion assay (F-J). Cells were transfected with 1.2PEI-Lau8 polymer/CTRL and STAT5A siRNA complexes at ratio of 6:1 and 30 and 60 nM siRNA concentrations for 3 days. In MTT assay, cell growth inhibition was expressed relative to non-treated cells (taken as 100%). Furthermore, live cells were counted by trypan blue exclusion assay. Y-axis shows the live cell count (x 10^5) per ml. The data are the mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared with the complexes with Control siRNA. (K). Summary of significant differences in MTT and cell counts in ALL primary cells.

2.4 DISCUSSION

RNAi has become a promising therapeutic approach for cancer and its success can be observed at various stages of clinical trials in different malignancies [31–33]. In ALL, various targets have been successfully silenced by RNAi, such as Polo-like kinase 1 (Plk1), CD22ΔE12, CSF1R, JAK1 and FLT3 [34–36], all of which highlights the versatility of using RNAi for ALL therapy. In this study, we have shown the feasibility of siRNA-mediated STAT5A silencing in ALL cell lines SUP-B15 and RS4;11 as well as ALL patient cells from different donors, using 2 lipid modified PEIs. We have demonstrated a strong reduction in mRNA levels which led to a sharp decrease in live cell counts and colony formation in ALL cell lines. The 1.2PEI-Lau8 polymer emerged as the leading candidate from this screen. The same polymer, when previously applied in breast cancer studies, only exhibited moderate performance for siRNA delivery [37], indicating the need to tailor

delivery systems for specific cell phenotypes. The other effective polymer 2PEI-LA6 was successful in other cancer types such as AML, CML and breast cancer [25,37–39], indicating a more universal applicability of this delivery system. The smaller LA derivative of PEI (1.2PEI-LA6) did not demonstrate effective siRNA delivery in both cell lines, presumably due to smaller size and relatively less efficient binding to siRNA; however, in previous reports, the same polymer was able to effectively deliver siRNA to MDA-MB-231 and MCF7 breast cancer cells. This emphasizes that different polymer formulations might be required for different cell types and the functionality of the same polymer could vary among various cell lines owing to the different cell properties [37,40]. Other studies showed that the efficiency of these polymers was related to their physiochemical characteristics including the optimal degree of lipid substitution, type of the lipid substituent, MW, charge and size of the siRNA nanoparticles [28,30,41], which were not repeated in this study. The reason(s) for low efficacy observed with some delivery agents was not explored here, but could be attributed to low binding or poor complexation ability with siRNA leading to reduced entry into the cells, weak interactions of complexes with cell membrane, unsuccessful endosomal escape leading to lysosomal degradation or tight binding resulting in the lack of siRNA dissociation inside the cytoplasm [42,43].

The siRNA uptake for SUP-B15 was ~10-fold higher to that observed in RS4;11 cells (e.g., 1.2PEI-Lau8 at ratio 6:1 gave uptake of ~200,000 au in SUP-B15 vs. ~24,000 in RS4;11 cells; Fig. 1B and D). This difference was reflected in the STAT5A mRNA reduction levels, where both polymers showed strong silencing in SUP-B15, whereas the effect was attenuated in RS4;11 (Fig. 2). This supports previous reports on the correlation between uptake and silencing efficacy [26,39,40]. STAT5A silencing efficiency of 2PEI-LA6 was considerably higher than that of

1.2PEI-Lau8 at ratio 6:1 in SUBP-B15 even though the latter polymer showed significantly higher uptake. This could be attributed to a better siRNA release in the cytoplasm with 2PEI-LA6 than 1.2PEI-Lau8. 2PEI-LA6 did not exhibit any difference in uptake with SUP-B15 at different ratios; however, the silencing was much stronger at ratio 6:1, which could point to a better siRNA release in the cytoplasm at this particular ratio. 2PEI with LA substitution has been previously shown a tendency to dissociate more rapidly due to its large linoleoyl in studies with cutaneous T cell lymphoma and AML cells [38,44]. This emphasizes the importance of releasing free siRNA in the cytoplasm for incorporation into RISC to induce the cleavage and degradation of target mRNA. Although the details of trafficking was not investigated here, a recent study by our group indicated the effective internalization of siRNA/PEI-LA complexes by confocal microscopy in K562 cells and CML primary cells [39]. 1.2PEI-Lau8 showed successful silencing at ratio 6:1 that was expected from its highest siRNA uptake at this ratio. The same polymer did not reveal any reduction of STAT5A mRNA levels at ratio 3:1, even though, its uptake was considerably higher, which could be due to low siRNA loading ability at this ratio. In RS4;11, 1.2PEI-Lau8 showed significant reduction of STAT5A mRNA only on day 3 at ratio 6:1 which was consistent with the uptake results. It is noteworthy that the polymeric delivery systems were able to sustain STAT5A mRNA silencing up to day 6, whereas RNAiMAX could not exhibit this efficacy. Moreover, the safety profile of our polymeric delivery systems was previously reported using normal human skin fibroblasts and the effect of treatments was negligible in these cells [45].

Subsequent studies in both cell lines demonstrated that these two PEI's complexed to STAT5A siRNA were able to effectively inhibit cell growth, as measured by MTT assay, and to reduce cell viability by trypan blue exclusion Figs. 3 and 4). This highlights the important role of

STAT5A in the survival of these ALL cells. The reduction of live cells with 1.2PEI-Lau8 was more evident at both time points (with stronger effect on day 6) compared to 2PEI-LA6. The live cell count appears to slightly decrease on day 6 for SUP-B15, whereas in RS4;11 cells, the day 6 counts were higher irrespective of the carrier used. This observation could be due to the differences in the doubling time of 60 vs. 35 hours for SUP-B15 vs. RS4;11 cells, respectively [46].

A recent study also demonstrated high siRNA uptake, marked reduction of STAT5A mRNA levels as well as increase in cell death using lipid-modified PEIs in breast cancer cell lines, which is consistent with our findings here and supports the efficacy of our delivery system [47]. The different siRNA silencing efficiencies between the ALL cell lines could be explained by differences in endosomal processing pathway or endocytic activities and also different expression levels of STAT5A in the target cells, as reported previously [3,48,49]. Both 2PEI-LA6 and 1.2PEI-Lau8 displayed a significantly stronger colony inhibition than the cell viability assays; the results were most profound with 1.2PEI-Lau8, especially in RS4;11 cells ($p \leq 0.05$). These outcomes highlight the high efficacy of the polymer/siRNA complexes as well as the key role of STAT5A in cell proliferation. In addition, our results corroborate other studies on STAT5 as a therapeutic target in ALL [3,50].

With respect to patient samples, we observed significant variability in the response to the siRNA treatments. Five of 8 samples showed significant STAT5 silencing with at least one of the delivery systems (Fig. 5). Similarly, colony formation was significantly reduced in 6 of 8 samples with at least one polymeric construct (Fig. 5). With our leading polymeric delivery system, 1.2PEI-Lau8, 4 of 8 samples showed significant reduction of STAT5A levels based on qPCR assessment

and 3 of 8 showed a reduction in colony formation. This emphasizes the importance of individualizing such treatment approaches. The expression levels of target genes might have played an important role; low STAT5 expression, strong upregulation or the presence of redundant signaling pathways could have hindered the silencing effect, thereby inhibiting the ability to affect the cell growth and proliferation [51]. We also could not exclude variability in cellular uptake between different patient samples.

In BCR-ABL positive samples, silencing using STAT5A siRNA (60 nM) was successful in one case (P1), accompanied by a reduction in cell growth by MTT assay; however, there were no changes in colony and cell counts. Notably, though BCR-ABL silencing was not evident by itself, the combination of siRNA to both BCR-ABL + STAT5A resulted a statistically significant reduction in cell growth in three samples, consistent with the observation that STAT5 is a downstream target of BCR-ABL for phosphorylation [52]. However, in other samples no downregulation was observed at the concentrations used. As we did not measure cellular uptake in patient samples due to limitations in cell numbers, the possibility that uptake may have been suboptimal in those cases could not be excluded.

The P4 sample, with an MLL rearrangement, consistently gave significant reduction in STAT5A mRNA and colony counts with 2PEI-LA6 and 1.2PEI-Lau8. Furthermore, cell growth was inhibited with STAT5A siRNA in MTT and cell counts (at 30 nM siRNA). Some studies have found that, in MLL-rearranged ALL, STAT5 is persistently activated by FLT3-ITD which in turn induces the expression of its downstream target [53,54]. Due to presumably sustained activation of STAT5 in this MLL-rearranged ALL, STAT5A silencing was more beneficial and promoted

proliferation inhibition, possibly by downregulating PIM-2 [54,55]. It was also shown that a potent STAT5 SH2 domain inhibitor, AC-4–130, was beneficial against FLT3-ITD-mediated activation of STAT5 for supressing proliferation and colony formation in FLT3-ITD$^+$ AML primary cells *in vitro* and *in vivo* [55]. The P7 sample, which had translocation t(1;19), gave a strong reduction in STAT5A mRNA levels with all polymer/siRNA complexes, with a decrease in colony counts using 1.2PEI-Lau8, accompanied by suppression of cell growth. The role of STAT5-mediated signaling in ALL patients with t(1;19) has not been investigated and our findings suggest that STAT5A may be important in this subset of ALL patients.

Other reasons for the heterogeneity of responses may include variations in qPCR assay conditions [56,57] and variable time to observe responses; a more frequent analysis of mRNA levels might better link changes in target mRNA levels to growth assessments. While differences between the MTT and cell count results observed in some patients (e.g., P1, P2 and P4) could be due to alterations of cell metabolism (i.e., up- or down-regulation of enzymatic activity), distinctly different from membrane integrity events [58], these differences are more likely due to underlying differences in STAT5 related signaling events. Since the patient cells exhibited different cytogenetics and likely possess different genetic and signalling profiles, it is not surprising that STAT5A siRNA treatments did not lead to uniform responses in all patient samples. Such response heterogeneity also exists with current drugs for ALL patients. These findings highlight the importance of developing individual approaches for ALL treatment [59–61], whether it is conventional drug or siRNA based.

In conclusion, we have demonstrated, for the first time, the successful delivery of STAT5A siRNA via polymeric carriers into ALL cell lines, which was accompanied by marked inhibition of STAT5A expression and reductions in cell viability and proliferation. This outcome was also reflected in some patient-derived ALL primary cells, where STAT5A knockdown decreased the total number of colonies and inhibited cell growth. These data support the importance of STAT5A as a potential therapeutic target in ALL as well as the potential role of this polymer-based delivery system. It was clear that the extent of lipid modification in polymers and complex formulation details (in particular polymer:siRNA ratio) are important and requires attention for the final efficacy of gene silencing. Additional primary ALL cells should be evaluated to more thoroughly investigate the response heterogeneity, and to correlate the responses with cell uptake, baseline protein expression and genetic profiles. Effects on normal bone marrow and peripheral mononuclear cells should also be evaluated to get a better sense of undesired effects of STAT5 silencing. Furthermore, strategies using chemotherapeutic agents and tyrosine kinase inhibitors combined with STAT5A siRNA should be explored, both in vitro and in animal models, which could potentially improve the efficacy of existing ALL therapies and help circumvent drug resistance.

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Chapter 3

The effect of combination of siRNA therapy and chemotherapeutic agents on B-ALL cells

3.1 Introduction

The established cytotoxic chemotherapy applied to treat pediatric leukemia leads to high cure rates in pediatric ALL patients but is suboptimal in the treatment of adult ALL. The cure rate of ALL is now over 80% in children but only 30-40% in adults and elderly patients with the appropriate therapeutic options [1]. The challenges for the treatment of adult ALL may be attributed to adults harboring higher-risk features at diagnosis, enhanced comorbidities, and the development of chemoresistance after relapse [2]. ALL treatment for adults is mostly established after designing the multiagent chemotherapy regimen as the first line treatment for pediatric ALL. Almost all chemotherapeutics applied as the standard of care for pediatric ALL were developed prior to the 1980s. This multiagent chemotherapy regimen includes induction, consolidation, and maintenance therapy and CNS prophylaxis. The reinduction chemotherapy consists of vincristine combined with an anthracycline, and glucocorticoids (GCs) such as prednisone or dexamethasone [3].

Among the different subtypes of B-ALL, Philadelphia chromosome-positive ALL (Ph^+ ALL), associated with high expression of the BCR-ABL oncoprotein, is one of the most prevalent and aggressive forms of B-ALL, and is one of the most unfavorable prognostic factors [4]. Ph chromosome is the most common cytogenetic abnormality in adult B-ALL, accounting for 20–30% of cases, and results from the reciprocal translocation t(9:22) (q34; q11.2) in leukemic cells. The inhibition of activity of BCR-ABL fusion oncoprotein using small molecular TKIs, including imatinib (first generation), nilotinib (NL), and dasatinib (DA) (second generation), has significantly improved the treatment of ALL patients [5]. Imatinib mesylate (formally STI571; Glivec and Gleevec) has been incorporated into treatment protocols of Ph^+ ALL patients since the early 2000s and it acts as an selective inhibitor of ABL and ABL fusion kinase (e.g., BCR-ABL), c-kit, and PDGF receptors-a/-b [6,7]. TKIs are aminopyrimidine-based ATP-competitive

inhibitors that target the kinase domain of the ABL1 moiety and supress the autophosphorylation of BCR-ABL fusion oncoprotein leading to growth inhibition of Ph^+ cells and apoptosis induction by blocking the cell surface receptor [7]. However, it was observed that over the course of therapy, the response to a single-agent TKI has been transient in Ph^+ ALL patients, due to acquired resistance mediated by ABL1 kinase domain mutations, reactivation of BCR-ABL, pharmacogenomic/pharmacokinetics changes, and the activation of alternative cellular pathways [5,7,8]. Consequently, in clinical setting, combination of TKIs and other targeted therapeutic approaches including siRNA therapy may break the resistance of leukemic cells to TKIs and improve the efficacy of treatment by targeting complementary approaches responsible for oncogenic transformations.

Vincristine (VCR), a member of the vinca alkaloids, is considered as a microtubule-targeting agent (MTA) and is an important component of ALL treatment protocols. VCR exerts its anticancer effects primarily by inducing microtubule depolymerization that results in mitotic arrest and eventually cell death, providing a rationale for its use as an antitumor agent. Cells treated with VCR die either in mitosis or after mitosis without proper chromosome separation or cytokinesis, known as mitotic slippage. Following slippage, cells may die in interphase, or arrest and survive, or continue cycling [9] (Fig. 3.1A).

Anthracyclines are fundamental components of cancer therapy with well-established effectiveness against several hematopoietic malignancies and solid tumors. Doxorubicin (DOX) is one of the most frequently used anthracycline chemotherapeutic agents since the 1960s. DOX has been applied for the treatment of various cancers including breast, ovary, bladder, thyroid and small cell lung cancers. It has also been widely used in the treatment of different hematologic malignancies such as ALL and lymphomas in adult and pediatric patients [10,11]. DOX has a

multidirectional mechanism of action. The main mechanism of action of DOX includes the drug's ability to intercalate between DNA base pairs, leading to DNA double strand breaks and inhibition of both DNA and RNA synthesis. Doxorubicin inhibits the enzyme topoisomerase II, resulting in DNA damage and apoptosis induction in tumor cells. DOX can also induce free radical-mediated oxidative damage to DNA via combining with iron and cause further inhibition of DNA synthesis. It also produces oxygen free radicals through oxidation-reduction reactions promoting the accumulation of proteins that are oxidatively altered [10,11] (Fig. 3.1B).

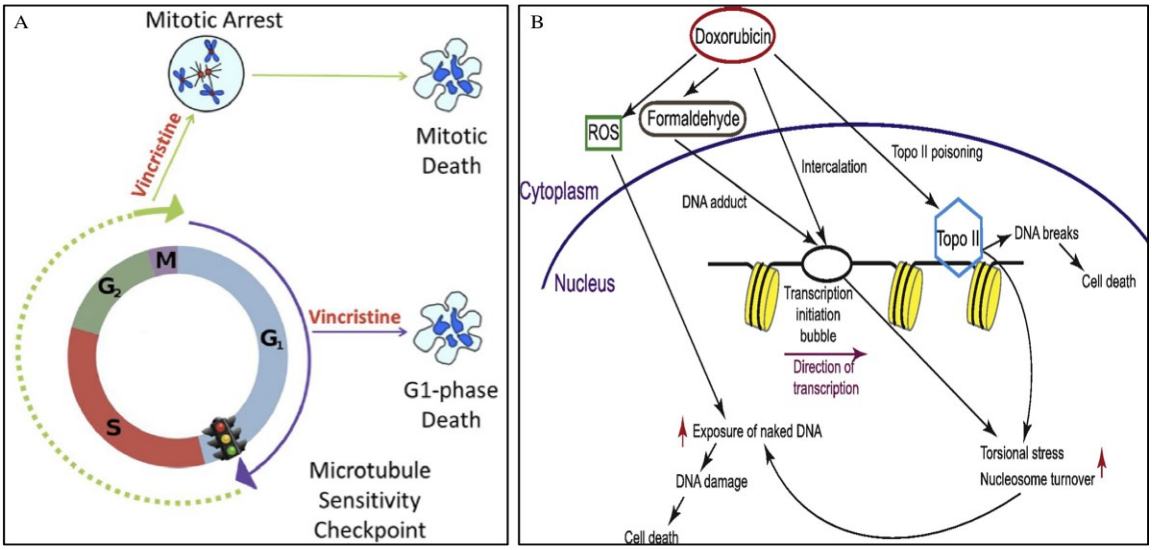

Figure 3.1. (A) Mechanism of action of VCR in ALL cells. Cell death happens directly when cells are in G1 phase, whereas cells that have passed a putative "microtubule sensitivity checkpoint" in late G1/early S phase continue to cycle and die after mitotic arrest [403]. (B) Mechanism of action of DOX in tumor cells. DOX traps topoisomerase II at breakage sites resulting in DNA double strand breaks. The exposure of naked DNA to DNA damaging agents including reactive oxygen species (ROS) causing DNA damage and cell death. [406].

Glucocorticoids (GCs) such as dexamethasone (DEX) or prednisone play a pivotal role in the treatment of hematologic malignancies for more than 50 years as they are capable of apoptosis

induction in leukemic cells [13–15]. Although prednisone administration was more common in the past, DEX is now more frequently administered because of decrease in central nervous system and bone marrow relapses. DEX is the most frequently used chemotherapeutic agent that promotes remission in ALL patients. In addition, during induction therapy, DEX is considered as a key component in the treatment protocols of pediatric ALL with the specific antileukemic activity due to its ability to eradicate leukemic blasts [13–15]. Response to DEX during induction phase is essential to assess further treatment options and is commonly used to predict patient outcomes. The activity of GCs on their target cells is initiated by binding to the cytosolic glucocorticoid receptor (GR) NR3C1, which is a member of the nuclear receptor family of ligand-dependent transcription factors. Upon a conformational change, NR3C1 then dissociates from the large protein complex that prevents its activation in the cytoplasm. The activated receptor translocates to the nucleus, where it directly interacts with specific palindromic DNA sequences known as glucocorticoid response elements (GREs), mostly in a homodimerized form. This leads to the transactivation or suppression of many target genes including NR3C1 itself, BCL2, KLF13, GILZ, and PER1. The monomeric form of activated NR3C1 can also bind to other transcription factors including activating protein-1 (AP-1) or nuclear factor-κB (NF-κB) and inhibit their activity. Mechanistically, DEX is known to upregulate the expression of pro-apoptotic proteins including Bim and several inhibitors of various signaling pathways such as the NF-κB and the RAS-MAPK pathways, thereby promoting caspase and endonuclease activation, and apoptosis in leukemic cells [13,15,16] (Fig. 3.2).

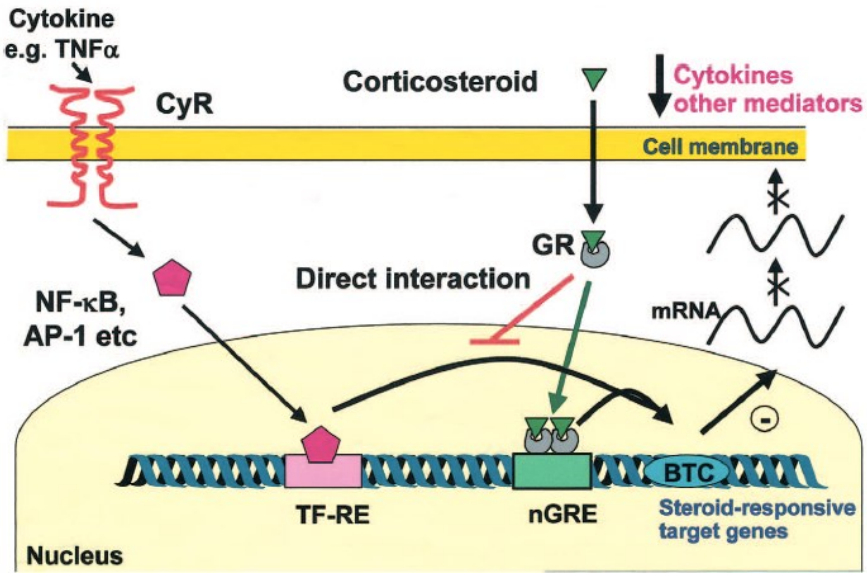

Figure 3.2. Mechanism of action of GCs in tumor cells [411].

Despite increased overall survival and remarkable improvements in the primary therapy for pediatric leukemia during the past 30 years, ~20% of children and up to 65% of adults relapse following initial therapy and the outcomes after relapse are poor [2,3]. Conventional therapeutic strategies for ALL are not optimally effective and fail to achieve clinical remission. Relapse and resistance to chemotherapy are the leading causes of treatment failure and death in ALL. Patients with relapsed or refractory ALL normally have a median overall survival of 2 to 6 months and a 3-to-5-year survival of less than 10% [18]. Intensive chemotherapy along with hematopoietic stem cell transplantation (HSCT) are the treatment options for relapsed or refractory ALL patients who experience an early bone marrow relapse or suboptimal response to reinduction chemotherapy [3]. Additional intensification of current therapeutic regimens is not desired as it results in the high toxicity, and thereby severe short-term and long-term adverse effects, including lethal infections, neurobehavioral side effects, osteonecrosis, and growth defects [15]. In addition, depending on the

therapeutic dose, most chemotherapeutic agents have potentially serious side effects. In the case of VCR, neurotoxicity is a prominent adverse effect that has a direct impact on the life quality of patients during and after treatment and may limit the dose of the drug or causes treatment discontinuation in some cases that can also influence survival. Common indications of neurotoxicity are sensory and motor dysfunctions including muscle weakness, muscle pain, paresthesia, or hyperesthesia [19,20]. The VCR toxicity on the autonomic nervous system observed especially after inadvertent overdoses of VCR can also lead to developing symptoms such as constipation, abnormal fluctuations in blood pressure and bladder dysfunction. Moreover, VCR has further peripheral neurological adverse effects including sensory loss, hearing changes, tingling and numbness [19,20].

Side effects are also associated with DOX administration, which include fatigue, alopecia, nausea and vomiting. Bone marrow suppression and an enhanced risk of developing secondary malignancy may happen. The use of DOX is also dose limited by observing significant cardiotoxicity, with up to 60% of patients exposed to high doses of DOX developing subclinical cardiac abnormalities which limits the long-term use of the drug. There are three types of DOX-mediated cardiotoxicity: (i) an acute form that initiates within days of the drug's administration and happens in nearly 11% of patients who receive the drug (ii) an early-onset chronic cardiotoxicity that develops within 1 year after DOX administration, and (iii) a late-onset CT that appears several years after usage and it is known as the most serious and potentially lethal side effect induced by DOX administration [10,11,21]. The incidence of chronic cardiotoxicity is ~1.7% and congestive heart failure could also be observed [10,11,21]. The mechanism of developing DOX-mediated cardiotoxicity is different from the antitumor activity of the drug. It is

mainly promoted by increased oxidative stress, inhibition the expression of cardiac-specific genes, and induction of cardiac myocyte apoptosis by DOX [10,11,21].

Patients also suffer from dose-related adverse effects of the DEX that could be short- and long-term. The most common side effect in ALL patients is the presence of insomnia after DEX administration [22]. DEX is known as disruptive to child behavior and mood including sleep patterns [22]. Some other prevalent side effects include fractures, osteonecrosis, acne, weight gain, indigestion, increased appetite, anorexia, hyperglycemia, hypertension and depression. Moreover, the development of resistance to DEX is a common feature of relapsed ALL that is much more frequent in adult ALL [13,14,22].

By considering these limitations associated with the conventional chemotherapy, especially adult patients, the development of novel and effective therapeutic modalities is warranted to overcome drug resistance, decrease risk of relapse and therefore, improve the treatment outcomes and overall survival of ALL patients [1,13]. Approaches to target individual signaling pathways may not be enough to inhibit the proliferation and metastasis of cancer cells owing to cellular plasticity to restore the activities of affected pathways or to employ alternative pathways for fundamental cellular activities [23]. For this purpose, a new approach utilizing combination therapy, which involves the co-delivery of various kinds of therapeutic agents is emerging [24]. Combination therapy offers several important theoretical advantages including targeting different phases of the cell cycle that leads to increased number of cells exposed to cytotoxic effects, decreasing the possibility of chemoresistance development, allowing lower doses of components to be employed and thereby, reducing the possible toxicities associated with clinical doses of individual drugs [25]. The overall aim of the combination therapy is to generate a better efficacy (ideally a synergistic effect) with no additional side effect by delivering multiple types of

therapeutic agents such as conventional drugs, antibodies, nucleic acids and molecular inhibitors. Targeted therapeutic strategies including nonviral gene therapy with specific siRNAs, as articulated in Chapter 2, can be an alternative and/or supportive therapy for ALL as synthetic siRNAs can sensitize resistant cells against various chemotherapeutics by overcoming the compensatory strategies of cancer cells through different signaling pathways [7]. With regard to the benefits provided by siRNA-mediated gene silencing in cancer cells, incorporation of siRNA therapy in the chemotherapy protocols may increase the efficacy of commonly used drugs for ALL.

As our previous studies in Chapter 2 indicated that significant STAT5A silencing mediated by polymeric delivery of STAT5A siRNA could inhibit cell growth and colony formation in RS4;11 and BCR-ABL$^+$ SUPB-15 cells, we decided to further investigate the effects of combination of STAT5A siRNA and the commonly used chemotherapeutic agents (VCR, DOX and DEX) and TKIs (NL and DA) on the leukemic cells and explore if any increased cell growth inhibition could be observed with dual treatment approach.

3.2 Materials and Methods

3.2.1 Materials

Doxorubicin hydrochloride (Cat No 44583), DEX-water soluble (Cat No D2915), vincristine sulfate salt (Cat No V8879), anhydrous dimethyl sulfoxide (DMSO), 2-mercaptoethanol, and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Roswell Park Memorial Institute Medium (RPMI) 1640 medium with L-glutamine, Iscove's Modified Dulbecco's Medium (IMDM), Hank's Balanced Salt Solution (HBSS), penicillin/streptomycin, and UltraPure DNase/RNase-free dH_2O were purchased from ThermoFisher (Ottawa, Canada). Negative control scrambled siRNA (Cat. No. DS NC1) and

STAT5A specific siRNA (5′-CCCGAUUUCUGAGUCACUAAAGCGCAA-3′ and 3′-GGGCUAAAGACUCAGUGAUUUCGCG5′-5′) were designed and synthesized by IDT (Coralville, IA). The polymers used in this study was described before [26].

3.2.2 Cell culture

Two B-ALL cell lines with the high-risk feature were selected for this study. RS4;11 cells have t(4;11) leading to MLL-AF4 fusion gene and BCR-ABL$^+$ SUP-B15 cells have t(9;22) and these genetic abnormalities cause poor prognosis in ALL patients. These cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). RS4;11 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin, and 100 µg/mL streptomycin. SUP-B15 cells were cultured in IMDM supplemented with 20% FBS, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained at 37 °C and 95/5% air/CO_2. Cells were subcultured after reaching 80% confluency by centrifugation at 900 rpm for 5 min and diluting the cells at 1:10.

3.2.3 Preparing the polymeric nanoparticle for STAT5A siRNA delivery to leukemic cells

Lipid-modified low MW PEIs were generated by following the previously reported protocols [26]. The amine groups of low MW PEIs (0.6 and 1.2 kDa) were modified by employing lauric acid (Lau) as a specific lipid and the levels of lipid substitution in final products were confirmed by ^{1}H-NMR. For designations, the polymers were labeled with the MW of the polymer and feed ratio (i.e., lipid to amine ratio) during synthesis; e.g., 0.6 PEI-Lau4 indicates polymer backbone of 0.6 kDa and Lau:polymer amine feed ratio of 4. As a reference/commercial transfection reagent,

RNAiMAX from Invitrogen (ThermoFisher) was used as the commonly recommended transfection reagent for siRNA delivery.

To obtain the desired stock concentrations of 0.14 μg/μL for the siRNAs and 1 μg/μL for the lipopolymers, these reagents were dissolved in ultra-pure dH_2O. In the process of preparing the siRNA/polymer complexes, siRNA solutions were first added to serum-free medium to get the final siRNA concentration of 60 nM in cell suspension. The polymers were then added to the siRNA solutions to give the polymer:siRNA weight ratio of 6:1, followed by briefly mixing and incubating for 30 min at room temperature to allow for the formation of siRNA and polymer complexes. Lipofectamine™ RNAiMAX Reagent was used as the lipid-based commercial reagent (positive control) in all the experiments and RNAiMAX/siRNA complexes were prepared at 2.5:1 lipid-to-siRNA (weight/weight) ratio (as suggested by the manufacturer) with the same siRNA concentration. The siRNA/polymer complexes (100 μL/well) were then added in triplicate to Falcon™ Polystyrene 48-well Microplates (Thermo Scientific, Lafayette, CO, USA) and then the 300 μL of cells were added on top of the polyplexes. No treatment (NT) control groups were treated with serum-free medium alone (no complexes), while the negative control groups were transfected with control (scrambled)-siRNA/lipid-polymer nanoparticles. Cells were collected after 3 days of transfection with polymeric nanoparticles and analyzed for growth inhibition by STAT5A silencing.

3.2.4 Evaluating the effect of STAT5A siRNA/polymer pre-treatment on the cytotoxicity of chemotherapeutics in leukemic cells

The stock solutions of VCR, DOX, DEX, nilotinib (NL), and dasatinib (DA) were prepared in ultra-pure DNase/RNase-free dH_2O at 1 mg/mL concentration and diluted in serum-free

medium to obtain the desired concentrations (0-250 nM). The cytotoxicity of drugs against RS4;11 and SUP-B15 cells was evaluated by the MTT assay. The RS4;11 cells (45,000/well) and SUP-B15 cells (50,000/well) were seeded in 48-well plates (300 µL/well) and incubated for 24 h. The drugs were then added to the wells in triplicates and treated cells were incubated at 37 °C with 5% CO2 for 48 h, followed by MTT assay. After the incubation time, MTT solution (5 mg/ml) was added to the wells to give a final concentration of 1 mg/mL and the cells were incubated for 2 h further at 37 °C, after which the cells were collected in microcentrifuge tubes, spun down at 1,400 rpm for 5 min, and the medium was removed. The formed formazan crystals were then dissolved with 100 µL of DMSO and the absorbance of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments, VT, USA) at 570 nm. The cell viability percentage was calculated as follows: 100% × (absorbance of polymeric nanoparticle treated cells/absorbance of nontreated cells).

These experiments were then followed by exploring the effect of combination of STAT5A siRNA and these anticancer drugs on leukemic cells. After seeding the cells in 48-well plates as above for 24 h, cells were transfected with STAT5A/scrambled siRNA-polymer complexes at a siRNA concentration of 60 nM and a polymer:siRNA ratio of 6:1 (wt/wt) for 24 h followed by treating with the desired drug concentrations (DEX= 1, 10 and 30 nM, DOX= 20, 30, and 50 nM and VCR= 5, 8 and 12 nM). Cells were incubated for another 48 h and were processed for the MTT assay. In each analysis, the relative cell growth was reported with regard to the nontreated cells (taken as 100%).

3.2.5 Statistical analysis

All results were indicated as mean ± standard deviation (SD). Statistical comparisons were made using unpaired Student's t test, where a value of $P \leq 0.05$ was considered as statistically significant and it was calculated by comparing specific siRNA-treated groups to that of control siRNA-treated groups.

3.3 Results

3.3.1 Cytotoxic effects of VCR, DOX, DEX and TKIs on ALL cells

The cytotoxicity of chemotherapeutic agents and the TKIs was first explored by MTT assay to determine the IC_{50} and optimal doses of each drug in RS4;11 and SUP-B15 cells. In BCR-ABL negative RS4;11 cell, treatment with increasing concentrations (0.1-1000 nM) of DOX, DEX and VCR for 48 h resulted in decrease in cell viability in a dose-dependent manner (Fig. 3.3). Cells were more sensitive to VCR and cell viability reduced from 89.8% at 5 nM to 50% at 10 nM (Fig. 3.3), while the cytotoxic effects of DOX and DEX were observed at higher concentration (200 nM) in RS4;11 cells (Fig. 3.3). Furthermore, the dose–response curves appeared to reach a plateau at higher concentrations of all drugs, which is generally seen with cytostatic agents. In RS4;11 cells, the IC_{50} values of DOX, DEX and VCR were 130, 150 and 10 nM, respectively.

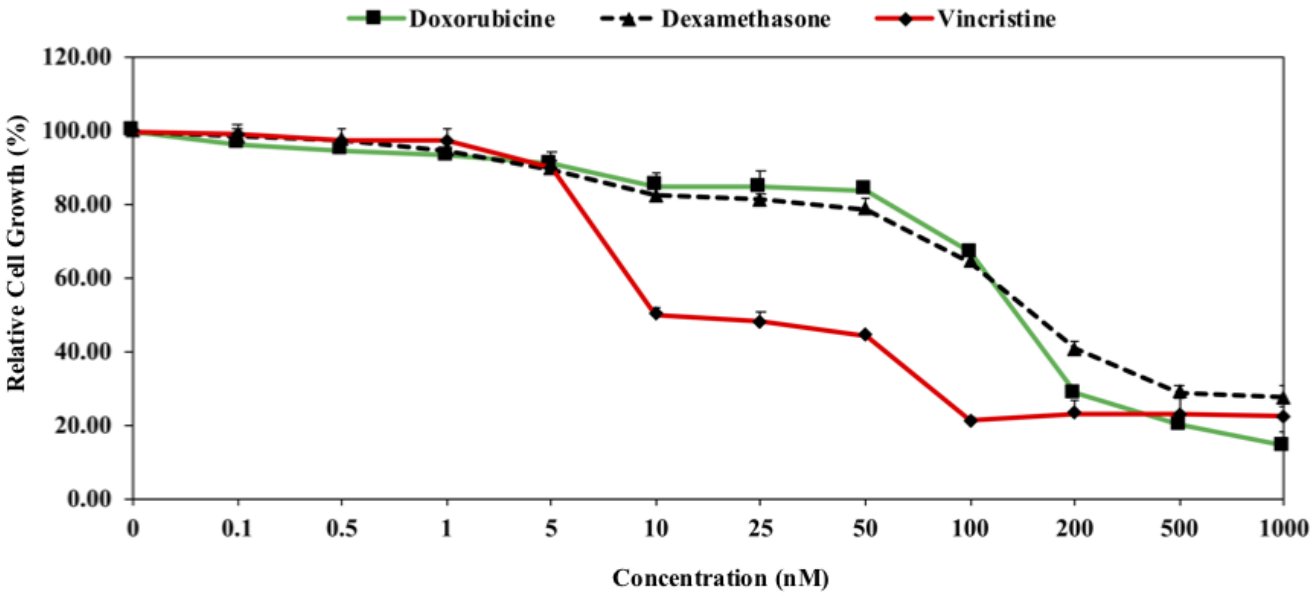

Figure 3.3. Growth inhibition of RS4;11 cells after treatment with different concentrations (0-1000 nM) of DOX, DEX and VCR. Cells were treated with increasing doses of drugs and incubated for 48 h before proceeding for the MTT assay. Cell growth was expressed relative to NT cells (taken as 100%).

The BCR-ABL positive SUP-B15 cells were treated with different concentrations of VCR (0.1-200 nM), DOX (0.1-200 nM) (data not shown) and DEX (0.5-200 nM) (Fig. 3.4) as well as two potent members of second generation TKIs, NL (0.1-250 nM) and DA (0.1-250 nM) for 48 h (Fig. 3.5). The results revealed that all drugs and TKIs led to the decrease in the viability of cells in a dose-dependent manner. VCR caused a marked drop in cell viability from 60.4% at 5 nM to 25.9% at 10 nM (data not shown). The same observation was achieved when cells treated with DOX and the cell viability decreased from 83.6% (25 nM) to 48.2% (50 nM; data not shown). Therefore, to determine the accurate IC_{50} values of these two anticancer agents, cells were incubated with a narrower range of concentrations of VCR (5-50 nM) and DOX (20-75 nM) for 48 h (Fig. 3.4). Similarly, the dose–response curves reached a plateau at higher concentration of

all drugs. In this cell line, the IC_{50} doses of VCR, DOX and DEX were 12, 50 and 30 nM, respectively. The SUP-B15 cells were more sensitive to DEX and DOX compared to RS4;11 cells and IC_{50} values were observed at lower doses.

Treatment with NL and DA induced gradual increase in cell death with increase in drug concentration. Both NL and DA showed the same IC_{50} dose of 200 nM in this cell line.

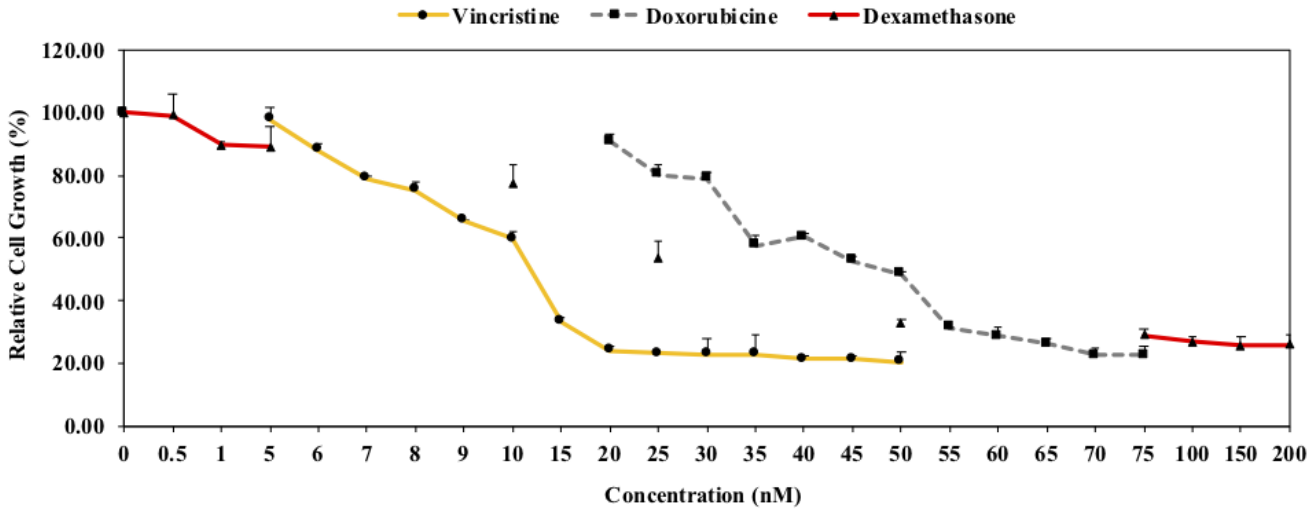

Figure 3.4. The growth inhibition of SUP-B15 cells by treatment with different ranges of concentrations of VCR (0-50 nM), DOX (0-75 nM) and DEX (0-200 nM). Cells were treated with the drugs for two days and the MTT assay was conducted to evaluate the growth inhibition. Cell growth was expressed relative NT cells.

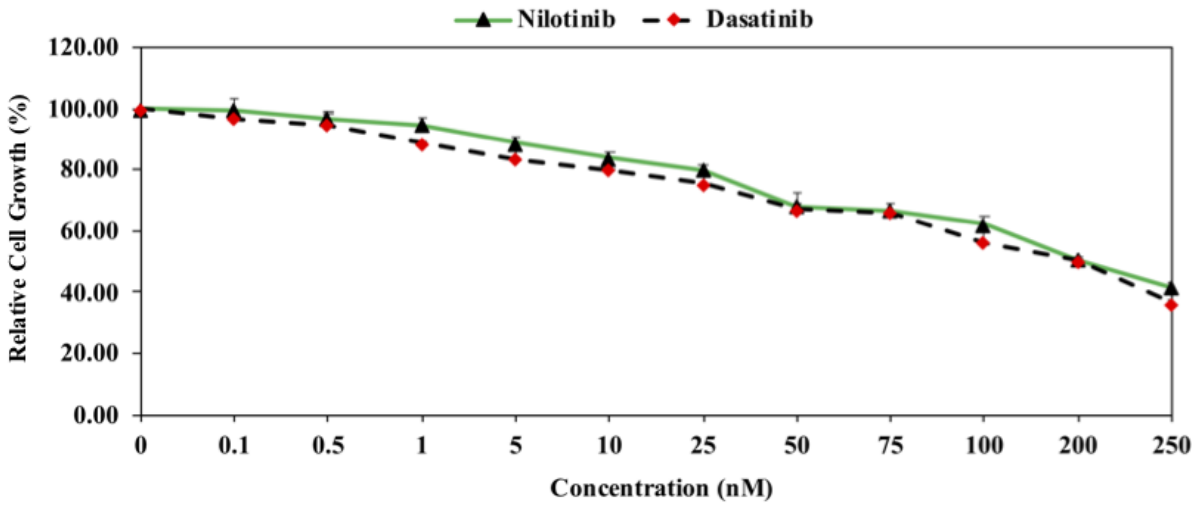

Figure 3.5. The growth inhibition of SUP-B15 cells treated with TKIs. Cells were incubated with the indicated concentrations of NL and DA for 48 h and growth inhibition was assessed by the MTT assay and expressed relative to NT cells.

3.3.2 Effect of STAT5A silencing and drug combinations on ALL cell lines

Since our previous results showed that siRNA-mediated STAT5A silencing using effective lipid modified-PEIs as delivery systems led to marked growth inhibition and reduced cell count and colony formation in both RS4;11 and SUP-B15 cells, we further explored the effect of STAT5A inhibition on the cytotoxicity of VCR, DOX, DEX and TKIs in the ALL cells. In the combinational formulation, cells were treated with three doses of each drug (IC_{50} and two lower concentrations with negligible cytotoxic effect) along with 60 nM of siRNA concentration and cell growth inhibition was assessed by the MTT assay. Three delivery systems were used for siRNA delivery, namely 0.6 PEI-Lau4, 1.2 PEI-Lau8 and RNAiMAX.

In RS4;11 cells, 0.6 PEI-Lau4 /STAT5A siRNA complexes could significantly inhibit the cell growth by 18-20% compared to polymer/CTRL siRNA groups ($p \leq 0.05$; Fig. 3.6 A, B and C) and combination of 0.6 PEI-Lau4 /STAT5A siRNA complexes and different concentrations of

DOX (25, 75, and 130 nM) caused 17% decrease in the cell viability in comparison with polymer/CTRL siRNA groups ($p \leq 0.05$; Fig. 3.6 A). Similarly, the same polymer along with three doses of DEX (25, 75, and 150 nM) could significantly suppress cell growth by 18-19% compared to control groups ($p \leq 0.05$; Fig. 3.6 B). Combination of three concentrations of VCR (1, 5, and 10 nM) and 0.6 PEI-Lau4 /STAT5A siRNA complexes reduced the cell viability between 17%-26% compared to control groups ($p \leq 0.05$; Fig. 3.6 C).

The growth of RS4;11 cells was significantly inhibited by 1.2 PEI-Lau8/STAT5A siRNA polyplexes between 21-24% in comparison with polymer/CTRL siRNA groups ($p \leq 0.05$; Fig. 3.6 A, B and C). Combination of DOX and the same polyplexes inhibited cell growth by 20-22% compared to control groups ($p \leq 0.05$; Fig. 3.6 A). In addition, the cell viability significantly reduced by 20-21% when cells treated with DEX and 1.2 PEI-Lau8 /STAT5A siRNA complexes ($p \leq 0.05$; Fig. 3.6 B). The same polymer/siRNA group along with VCR resulted in decrease in cell viability by 19.4%-21.5% compared to polymer/CTRL siRNA groups ($p \leq 0.05$; Fig. 3.6 C).

On the other hand, at all compositions, RNAiMAX could only exhibit minimal decrease in the viability of RS4;11 cells compared to control groups (Fig. 3.6 A, B and C). Therefore, we observed that combinational formulation of 10 nM of VCR and 0.6 PEI-Lau4 /STAT5A siRNA was the most effective formulation in inhibiting the growth of RS4;11 cells; however, all other compositions were less effective in affecting the cell viability than polymer/STAT5A siRNA complexes.

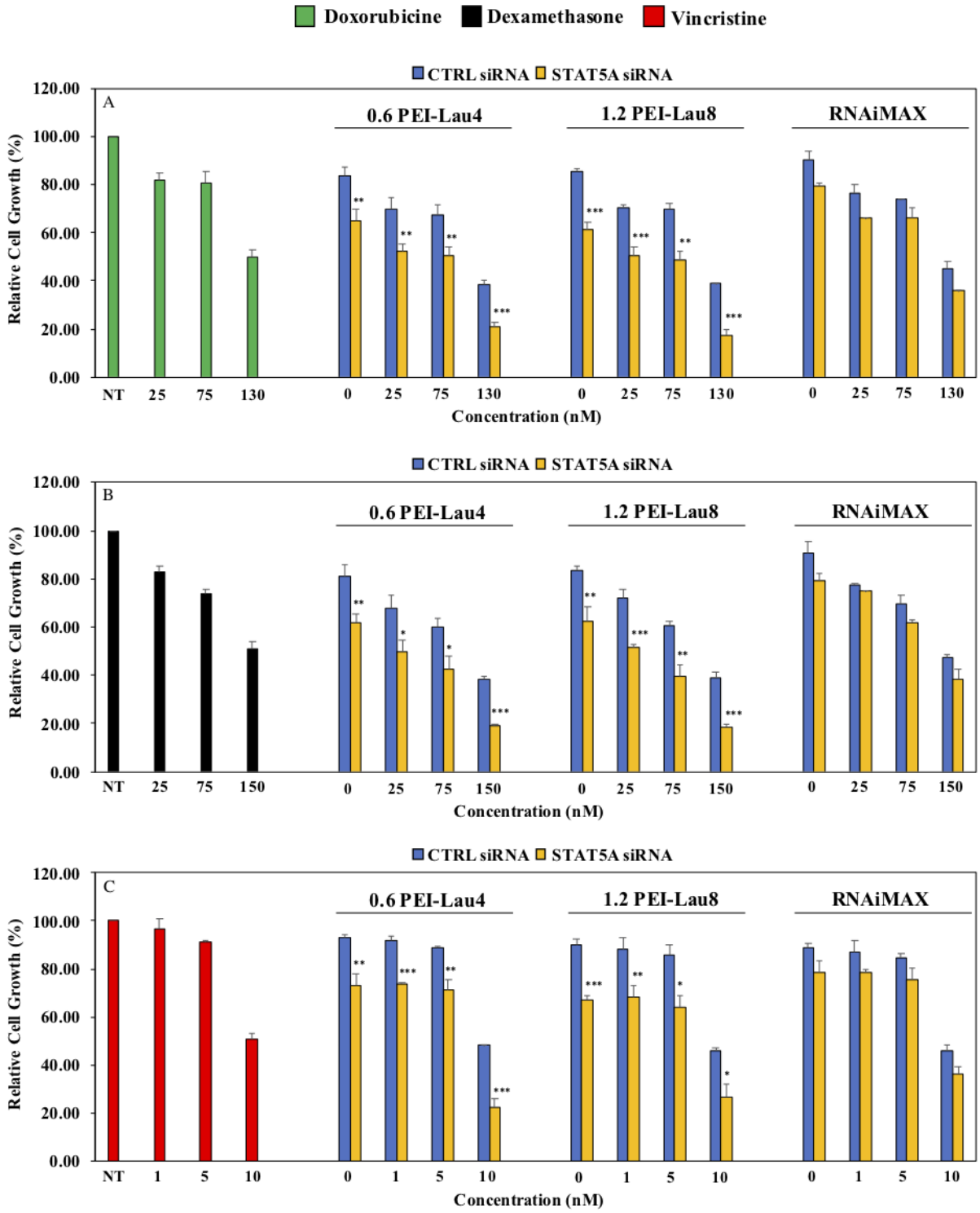

Figure 3.6. (A-C): The effect of STAT5A silencing on the growth of RS4;11 cells in combination with three different concentrations of DOX, DEX and VCR treatment. Cells were transfected with the indicated siRNA/polymer complexes (1.2 PEI-Lau8, 0.6 PEI-Lau4/siRNA ratio of 6:1 and RNAiMAX/ siRNA ratio of 2.5:1) and 60 nM of Control/STAT5A siRNA for 24 h and then treated

with drugs and incubated for another 48 h before proceeding for the MTT assay. Cell growth was expressed relative to NT cells (taken as 100%). The data are the mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared with the complexes with Control siRNA.

In SUP-B15 cells, 0.6 PEI-Lau4/ STAT5A siRNA complexes gave a significant growth inhibition of 16-19% compared to the CTRL siRNA groups ($p\leq0.05$; Fig. 3.7 A-C and Fig. 3.8 A and B). Similarly, combining the siRNA complexes with the same polymer and three doses of DEX (1, 10 and 30 nM), DOX (20, 30 and 50 nM), VCR (5, 8 and 12 nM), and TKIs, NL (1, 25 and 200 nM) and DA (1, 50 and 200 nM), reduced the cell viability by 16-19%, 16-19%, 15-18%, 16-19%, and 15-17%, respectively ($p\leq0.05$; Fig. 3.7 A-C and Fig. 3.8 A and B). Cell growth was also inhibited significantly through 1.2PEI-Lau8/STAT5A siRNA polyplexes (30-33%) compared to control groups ($p\leq0.05$; Fig. 3.7 A-C and Fig. 3.8 A and B). However, applying this polymer along with DEX, DOX, VCR, NL and DA could not exhibit an additional effect on decreasing the cell viability (21-33%) in comparison with the control groups ($p\leq0.05$; Fig. 3.7 A-C and Fig. 3.8 A and B). The performance of RNAiMAX in suppressing cell growth was marginal at all formulations in this cell line (Fig. 3.7 A-C and Fig. 3.8 A and B). Therefore, the effect of combining STAT5 siRNA and anticancer drugs on increasing the cytotoxicity of drugs and inhibiting the cell growth was not observant in SUP-B15 cells.

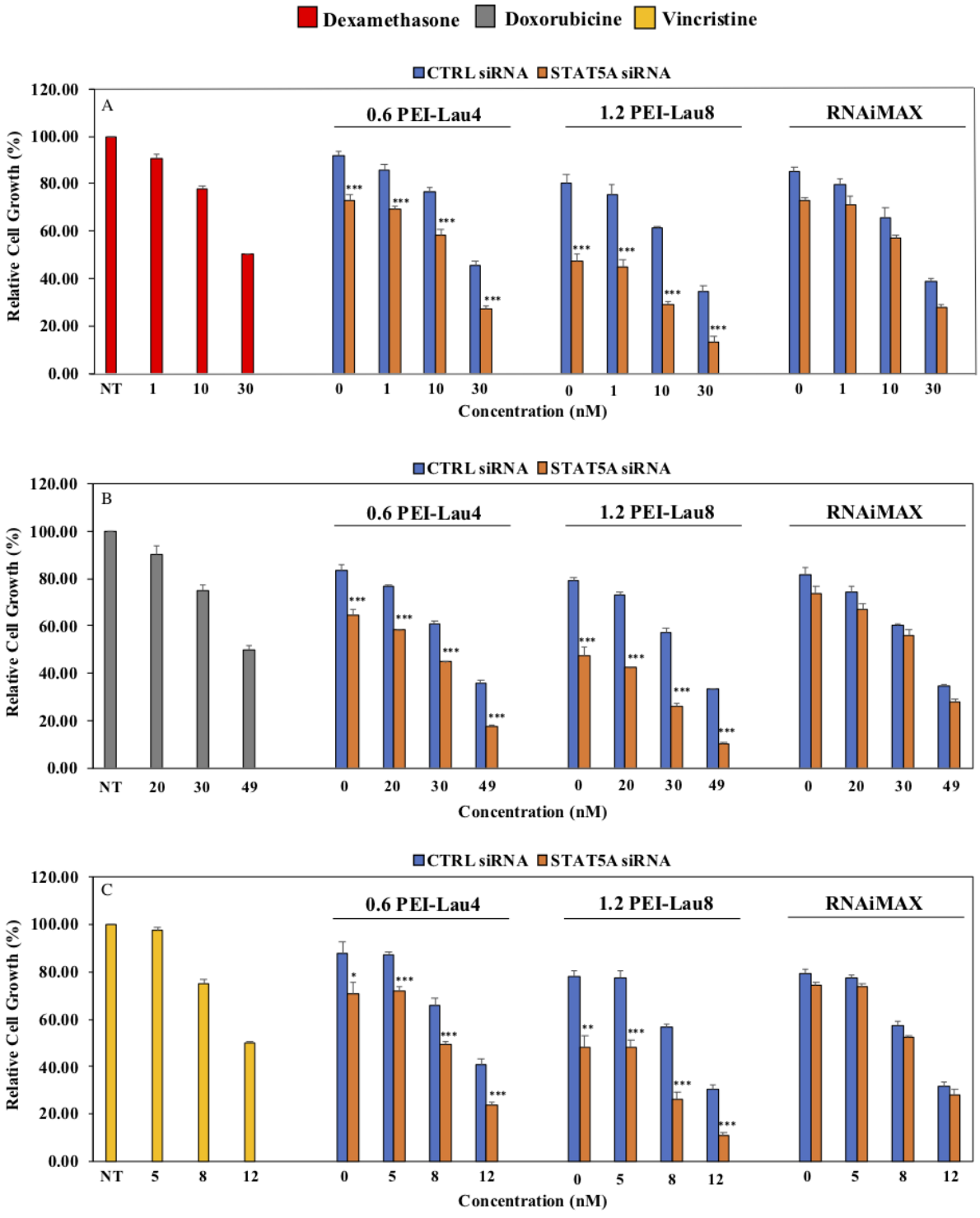
Dexamethasone
Doxorubicine
Vincristine
CTRL siRNA
STAT5A siRNA
A
B
C
0.6 PEI-Lau4
1.2 PEI-Lau8
RNAiMAX
Relative Cell Growth (%)
Concentration (nM)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
NT
1
10
30
20
49
5
8
12
0

Figure 3.7. The effect of cotreatment with siRNA/polymer complexes and drugs on the growth of SUP-B15 cells. Cells were first transfected with polymer/siRNA complexes (ratio 6) and 60 nM of Control/STAT5A siRNA for one day and then treated with three different doses of DEX (A), DOX (B), and VCR (C) for two more days. In the MTT assay, cell growth inhibition was expressed relative to NT cells (taken as 100%). The data are the mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared with the complexes with Control siRNA.

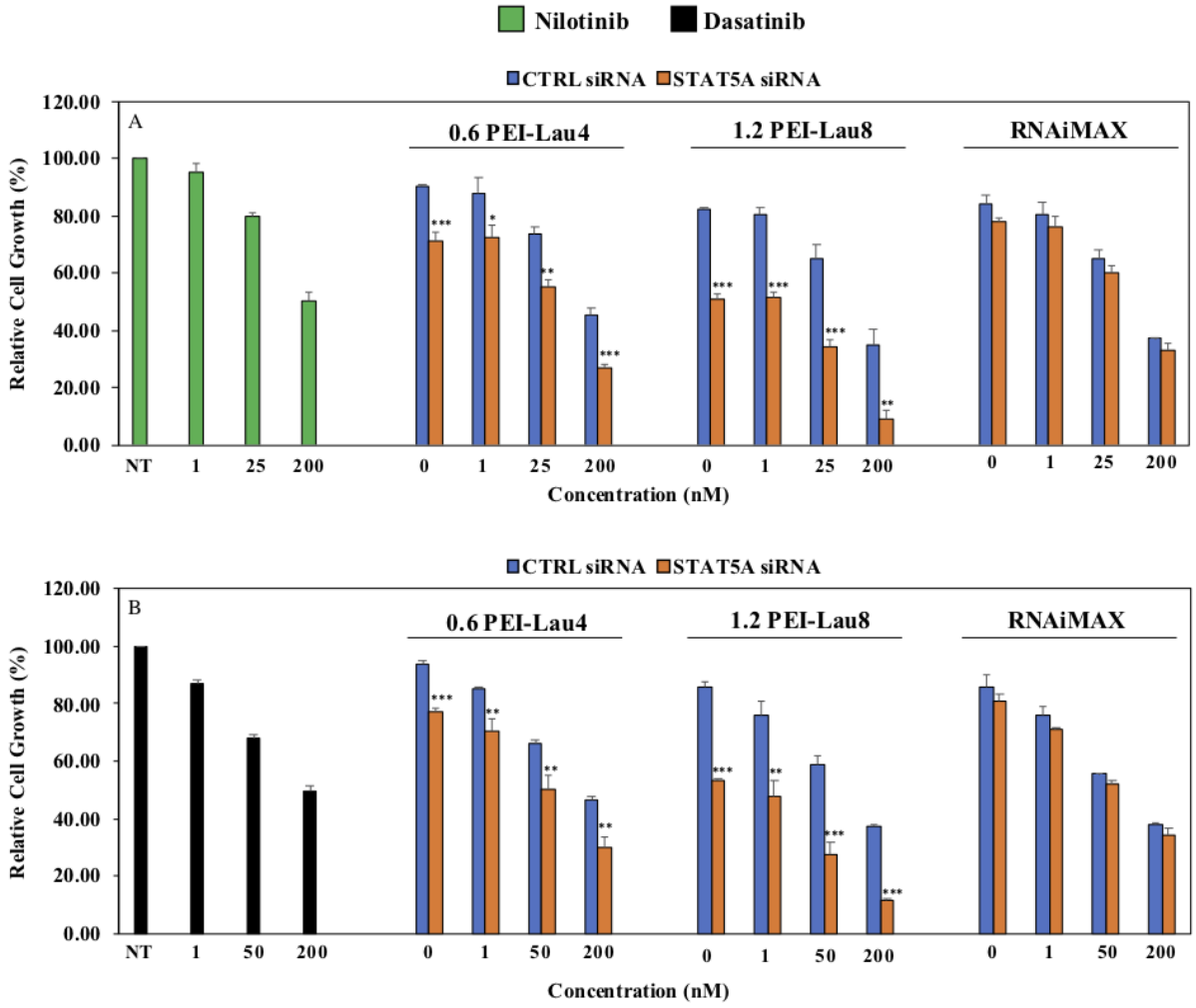

Figure 3.8. The effect of combination of TKIs and STAT5A silencing on the growth of SUP-B15 cells. Cells were treated with polymer/siRNA ratio of 6:1 and 60 nM of Control/STAT5A siRNA for 24 h and then incubated with three concentrations of NL (A) and DA (B) for another 48 h before proceeding for the MTT assay. Cell growth inhibition was expressed relative to NT cells (taken as 100%). The data are the mean ± SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared with the complexes with Control siRNA.

3.4 Discussion and Conclusions

The efficacy of existing ALL therapies is limited by rapid development of resistance to currently available anticancer agents that is responsible for poor prognosis, high relapse rate, and high mortality rate in ALL patients especially adults with B-cell ALL [2,27]. STAT5 is a nuclear transcription factor that is closely related to B cell proliferation and differentiation [28]. Constitutive activation of STAT5A, by JAK/STAT signalling pathway or BCR-ABL oncoprotein, promotes high expression of genes linked to aberrant proliferation and survival of leukemic cells, thereby resulting in the development of hematologic malignancies including B-ALL [29,30]. Novel therapeutic modalities using specific siRNAs to selectively target and silence STAT5 can be a promising approach to prevent uncontrolled cell proliferation and survival [31]. To have an effective siRNA therapy, utilizing non-viral delivery systems such as cationic polymers is of utmost importance as they can condense anionic siRNA molecules into nanoparticles and protect them against the extracellular and intracellular barriers of gene delivery [31]. Our previous study indicated that downregulation of STAT5A by lipid-substituted low MW PEI/siRNA complexes could significantly inhibit cell growth and reduce the live cell count and colony formation in RS4;11 and SUP-B15 B-ALL cell lines and also ALL primary cells. Our results were consistent with an ALL study showing that successful STAT5 silencing could inhibit cell proliferation and induce apoptosis in B-ALL cell lines, patient-derived cells and ALL mouse models [32]. Moreover, we reasoned that combining standard cytotoxic chemotherapy or TKIs with siRNA therapy could improve the outcomes of first-line treatment of ALL patients. In most cases, the efficiency of chemotherapy in inhibiting cell growth is dependent on various factors including drug activity in the target cell, administered dosage of drug and genetic profile of the malignancy [25]. Deploying combinational therapy makes it plausible to target complementary signaling

pathways, enhance the silencing of a specific molecular target, inhibit the activity of several components in a single pathway and abolish multiple mechanisms that are involved in tumor growth and metastasis [33]. Therefore, we further explored the effect of combination of siRNA therapy on the cytotoxicity of commonly used anticancer drugs in ALL therapy.

The B-ALL RS4;11 and SUP-B15 cells were first treated with a wide range of concentrations of each chemotherapeutic agent for 48 h to evaluate the cytotoxicity of drugs and determine the IC_{50} values. The results revealed that all chemotherapeutic agents along with TKIs were able to decrease the cell viability as a single agent in a dose-dependent manner in both cell lines. SUP-B15 cells were more sensitive to DOX and DEX and IC_{50} values of these two drugs were obtained at lower doses compared to RS4;11 cells. The VCR showed similar cytotoxic effect in both cell lines and IC_{50} doses were comparable. In BCR-ABL positive SUP-B15 cells, two potent TKIs, NL and DA could gradually reduce the cell growth by increasing the concentration and a same IC_{50} value (200 nM) was determined for both NL and DA. Other studies also demonstrated the cytotoxicity of DEX, VCR and DOX in a concentration-dependent manner in both cell lines; however, they identified the IC_{50} of these drugs at lower doses, which might be due to different experimental conditions, treatment protocols, source of drugs, and experimental assays used [9,34,35]. High IC_{50} value of NL and DA in SUP-B15 cells was previously attributed to resistance in SUP-B15 cells to second generation TKIs NL and DA; the cells did not carry any mutations in the kinase domain of BCR-ABL and displayed constitutive activation of PI3K/AKT1/mTOR pathway through a BCR-ABL-independent mechanism [36].

In our combinational studies, difficult-to-transfect B-ALL leukemic cell lines were first transfected with polymer/siRNA complexes. We previously demonstrated the effectiveness of lipid-modified PEIs for delivery of selected siRNAs in *in vitro* models. In this study, two effective

lipopolymers, 0.6 PEI-Lau4 and 1.2 PEI-Lau8 were used for STAT5A siRNA delivery based on our previous uptake assay results. The results indicated that siRNA-mediated STAT5A silencing could only show its effect on inducing cell death compared to the scrambled (CTRL) siRNA/polymer groups. The extent of decrease in cell viability in all groups treated with the combination of STAT5A siRNA and drugs was comparable to that of STAT5A siRNA/polymer control groups which exhibited the successful and significant STAT5A silencing by effective siRNA delivery to cells. However, no stronger cytotoxic effect was observed by combining STAT5A siRNA and anticancer agents at different doses, and cytotoxicity of drugs was not increased by siRNA therapy. 1.2 PEI-Lau8 polymer/siRNA groups were more effective in cell growth inhibition than 0.6 PEI-Lau4 polymer/siRNA groups. This observation was consistent with our uptake assay findings that showed higher siRNA uptake with 1.2PEI-Lau8 polymer than with 0.6PEI-Lau4 polymer. Applying 0.6PEI-Lau4/STAT5A siRNA along with 10 nM of VCR could decrease the cell viability more than other combination formulations in RS4;11 cells; however, this effect was not synergistic. In SUP-B15 cells, 1.2 PEI-Lau8 polymer was able to promote higher STAT5A silencing compared to RS4;11 cells that confirmed the uptake assay results in which this polymer showed significantly higher siRNA uptake in these cells.

Several studies have explored the effect of combinational formulations including simultaneous cell treatment with different drugs, small molecule inhibitors/monoclonal antibodies plus anticancer agents and siRNAs along with dugs/TKIs in different types of leukemias. The combination of DEX and the proteasome inhibitor bortezomib was synergistic in RS4;11 cells only at high concentrations and was additive or antagonistic at lower concentrations [1]. It has been shown that DEX-induced cell death is mediated through the activation of autophagy, which lies upstream of mitochondrial dysfunction and subsequent cell death. DEX treatment can cause the

dephosphorylation of Akt that is not only a well-known inhibitor of apoptotic cell death, but it is also a major negative regulator of autophagy. Downregulation of the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-XL at the mRNA level and also upregulation of BIM, a pro-apoptotic protein, is mediated by DEX that contributes to the induction of autophagy in human ALL cells [37]. One leukemia study evaluated the effect of siRNA-mediated B cell chronic lymphocytic leukemia/lymphoma 11 A (BCL11A) silencing plus VCR in SUDHL6 cells derived from germinal center B cell-like diffuse large B cell lymphoma (DLBCL), the most common type of non-Hodgkin lymphoma (NHL) [38]. BCL11A is a transcription factor that is closely related to B cell proliferation and differentiation and its overexpression promotes proliferation and survival of B cells resulting in the development of B cell lymphoma and B cell leukemia. The inhibition of BCL11A expression by siRNA was capable of inducing apoptosis and decreasing proliferation of SUDHL6 cells by downregulation of BCL-2 and MDM2 and upregulation of BIM [38]. Moreover, combination of BCL11A siRNA and VCR significantly increased apoptosis and inhibited the growth of SUDHL6 cells in comparison with VCR or BCL11A siRNA treatment alone and negative control siRNA plus VCR treatment; thus, significantly enhanced the therapeutic efficacy of VCR in SUDHL6 cells ($P < 0.05$) [38]. Similarly, another study indicated that silencing the antiapoptotic protein Bfl-1 by siRNA in a DLBCL cell line induced apoptosis and enhanced the apoptosis induction promoted by drugs including DOX, VCR, cisplatin and fludarabine [39]. In addition, silencing of *BCR-ABL* gene by siRNA in combination with TKI treatment displayed synergism to generate a strong antileukemic activity in TKI-resistant K562 cells [7]. Although our results were contrary to those of studies in different leukemias; the reason behind this contradiction was unclear. Our assumption is that the discrepancy might be due to (i) insufficient magnitude of target protein downregulation, (ii) presence of alternate non-STAT5 intracellular signalling

pathways, or (iii) other anti-apoptotic effects in RS4;11 and SUP-B15 cells that prevents the siRNA-mediated STAT5A silencing from enhancing the cytotoxic effects of the drugs.

We could not also observe any significant cell growth inhibition with STAT5A siRNA delivery by RNAiMAX mediated delivery (compared with our polymeric delivery systems) when applied alone or in combination with anticancer agents in both RS4;11 and SUP-B15 cells. These outcomes supported our previous findings in which this common commercial transfection reagent could not show high siRNA uptake and following STAT5A silencing in both cell lines; thereby significant cell growth inhibition, reduced live cell count and total colony counts could not be achieved. These observations indicate a lower cellular delivery of STAT5A siRNA by RNAiMAX, so that intracellular release of siRNA will not be efficient and sufficient enough with this reagent to silence STAT5A. Similar to our results, a CML study showed that RNAiMAX-mediated delivery of BCR-ABL siRNA could not silence BCR-ABL expression at mRNA level and promote cell growth inhibition in K562 cells [7].

In conclusion, our results demonstrated that siRNA-mediated STAT5A silencing by utilizing our polymeric delivery systems could significantly inhibit the growth of B-ALL cell lines; however, combination of siRNA therapy and chemotherapeutic agents/TKIs could not exhibit further cytotoxic effects compared to control groups in our *in vitro* models. Our data suggest that STAT5A inhibition can be individually deployed as a novel and potential targeted therapeutic approach to potentially improve the outcomes of current therapies in ALL patients. However, further investigation of a wide range of ALL cell types and *in vivo* studies confirming the efficacy of this treatment paradigm are necessary. It also remains to be seen whether other chemotherapeutics, protein kinase inhibitors and antibodies can be explored as a synergistic pair

with STAT5A siRNAs to develop a replacement for conventional therapies against drug-resistant ALL phenotypes.

3.5 Acknowledgments

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Chapter 4

Overall conclusions, discussion and future directions

4.1 Overall conclusions and discussion

RNAi approach provides a potential and promising alternative for the treatment of ALL when conventional therapeutic modalities fail as a result of development of drug resistance and subsequent emergence of the malignancy. This dissertation explored the potential of siRNA therapy by applying non-viral delivery systems in ALL cells to promote therapeutic effects and create alternative/supportive therapeutic strategies that overcome the current limitations of ALL treatment. In **Chapter 1**, we provided a comprehensive overview of recent studies exploring the current status of developing novel therapeutic approaches for ALL based on the latest progressions in the understanding of ALL biology including the identification of putative therapeutic targets. In the other sections of this chapter, we focused on the STAT5 nuclear transcription factor as a potential therapeutic target for siRNA therapy as the constitutive activation of STAT5 results in the upregulation of oncogenes involved in uncontrolled proliferation and survival of cancer cells [1,2]. Inhibiting of this important gene was applied here as a proof of principle for siRNA silencing and the delivery method. We further described various types of delivery systems for siRNA therapy in **Chapter 1**. Currently-in-use non-viral carriers comprise liposomes, lipoplexes, peptides and cationic polymers. These carriers have been formulated for the siRNA delivery to the cytoplasm and allow for siRNA activity by the RNAi mechanism to overcome various extracellular and intracellular delivery obstacles from siRNA encapsulation and protection, internalization, endosomal escape and eventually functional release of the siRNA [3,4]. For this purpose, we applied tailored lipid-modified PEIs as non-viral siRNA delivery agents to target and silence STAT5A in B-ALL cell lines and primary cells and revealed the importance of formulation details while preparing the siRNA/polymer complexes (**Chapter 2**). Based on the uptake results of a library of lipid-substituted low MW PEI derivatives, two polymer groups, 2PEI-LA6 and 1.2PEI-

Lau8 showed high uptake of FAM-siRNA in both RS4,11 and SUP-B15 cells compared to other lipopolymers and commercial reagent, RNAiMAX **(Chapter 2)**. Therefore, these two polymers were considered as promising candidates for further experiments in RS4,11 and SUP-B15 cells as well as ALLL primary samples. siRNA-mediated silencing of STAT5A using the effective polymer groups indicated significant decrease in STAT5A mRNA levels in SUP-B15 (36-92%) and RS4,11 (32%) compared to control siRNA groups. FAM-siRNA uptake was higher with 1.2PEI-Lau8 polymer in SUP-B15 cells compared to that observed in RS4;11 cells and this difference was reflected in the levels of STAT5A mRNA decrease where both polymer groups revealed higher levels of silencing in SUP-B15 cells. This observation was consistent with previous studies on the correlation between the siRNA uptake and silencing efficiency [5–7].

Moreover, strong STAT5A silencing at mRNA level resulted in marked cell growth inhibition (MTT assay) and reduced live cell counts (trypan blue assay) and total colony numbers (CFC assay) in ALL cell lines highlighting the important role of STAT5A in the survival of these ALL cells. 1.2PEI-Lau8 was more effective in decreasing live cell counts and total colony counts than 2PEI-LA6 in both cell lines which was expected based on the higher siRNA uptake observed with this polymer. Several studies reported that 1.2PEI-Lau8 polymer indicated moderate siRNA delivery to breast cancer cells while 2PEI-LA6 was able to successfully deliver siRNA molecules to AML, CML and breast cancer cells and silence the target genes which shows that different cell types require different polymer formulations for effective siRNA delivery [6,8,9]. Moreover, physiochemical properties of lipopolymers including the degree of lipid substitution, type of the lipid substituent, MW, charge and size of the siRNA nanoparticles have a direct impact on the efficacy of cell transfection by siRNA/polymer complexes [10,11]. Different siRNA silencing efficacies observed in the ALL cell lines with two polymer groups could be attributed to different

endosomal processing pathways or endocytic activities and also different expression levels of STAT5A in the target cells [1,12,13].

Regarding the studies with patient-derived ALL cells, significant variability was observed in the response to transfection with siRNA/polymer complexes. Five of eight samples demonstrated significant decreases in STAT5 mRNA levels, and six of eight samples showed reduced ability in colony formation with at least one of the polymeric delivery systems (**Chapter 2**). In BCR-ABL positive samples, combination of STAT5A and BCR-ABL siRNAs caused a significant cell growth inhibition in three of five samples. Since the ALL patient cells revealed different cytogenetics and likely possess different genetic and signalling profiles, it is not surprising that STAT5A siRNA treatments did not cause uniform results in all patient samples. Such response heterogeneity has been also observed in response to commonly used drugs for ALL patients. These outcomes emphasize the importance of establishing individual approaches for ALL treatment. It would be beneficial to evaluate greater number of ALL patient-derived cells in order to more comprehensively explore the response heterogeneity and relate the outcomes to cellular uptake and genetic abnormalities.

Based on the promising results achieved in **Chapter 2,** we decided to further evaluate the impact of combination of STAT5A siRNA and frequently used anticancer agents for the treatment of ALL such as dexamethasone, doxorubicine and vincristine as well as TKIs including dasatinib and nilotinib on ALL cell lines to investigate if we could observe more reduction in cell viability and increase in cell sensitivity to drugs with this combination protocol (**Chapter 3**). The results indicated that all chemotherapeutics along with TKIs were able to inhibit the growth of RS4;11 and BCR-ABL$^+$ SUP-B15 cells as a single agent in a dose-dependent manner when cells treated with different concentrations of drugs for 48h. SUP-B15 cells exhibited more sensitivity to

dexamethasone and doxorubicine (lower IC_{50} doses) than RS4;11 cells while vincristine had the similar cytotoxic effects in both cell lines. Moreover, SUP-B15 cells showed resistance to the second generation TKIs, nilotinib and dasatinib, as these agents gradually decreased the cell viability with increase in the concentration and IC_{50} values were observed at higher doses (200 nM) for both TKIs. This observation supports the outcome of one study regarding the resistance of SUP-B15 cells to nilotinib and dasatinib through a BCR-ABL-independent mechanism [14].

The siRNA and drug combination results revealed that the simultaneous treatment of cells with STAT5A siRNA/polymer complexes and 3 different concentrations of drugs could not reduce the cell viability more than the individual siRNA/polymer groups (**Chapter 3**). The only combination formulation with which the cell viability reduced more than other combination treatments was observed in RS4;11 cells treated with 0.6PEI-Lau4/STAT5A siRNA along with 10 nM of vincristine; however, this effect was not synergistic but simply additive (**Chapter 3**). Therefore, STAT5A silencing was effective in inhibiting cell growth while combination of siRNA and drugs could not improve this effect. Some studies have reported successful synergistic effects of various combination formulations such as drugs, monoclonal antibodies, small molecule inhibitors as well as siRNA therapy. It was shown that in RS4;11 cells, applying dexamethasone and proteasome inhibitor bortezomib (i.e., no siRNA involvement) induced synergistic effects at high concentrations of drug [15], moreover, siRNA-mediated silencing of BCR-ABL along with TKI treatment promoted a synergistic response in TKI-resistant K562 cells [16]. This difference observed between our results and outcomes of other leukemia studies could be attributed to the fact that we targeted different mechanisms in the cells as STAT5 siRNA and the selected drugs had different mechanisms of action in promoting cell death, while targeting the same mechanism with two agents could result in a synergistic response. Furthermore, the enhanced activity of other

oncogenic signalling pathways in target cells could promote cell survival and thereby nullify the effects of combination of STAT5A siRNA and anticancer agents. Therefore, our findings suggest that individual siRNA therapy can be considered as an alternative therapeutic modality for ALL to improve the treatment outcomes in ALL patients. However, it would be beneficial to investigate the therapeutic effects of other combination formulations including monoclonal antibodies, other protein kinase inhibitors and anticancer agents as well as STAT5 siRNA to develop an effective targeted therapy for ALL. Targeting two different STAT5-related mechanisms (e.g., inhibiting STAT5 activity with small molecule drugs along with silencing STAT5 gene with siRNA) can reveal the possible synergistic effect(s) of combination of siRNA therapy and other drugs. So far, a handful of small molecule inhibitors of STAT5 activity including IST5-002, 13a, and BP-1-108, have been developed that disrupt SH2 domain–mediated docking of STAT5 to the receptor-kinase complex and subsequent phosphorylation and dimerization with no off-target kinase activity [17–19]. The efficacy of these small molecule inhibitors has been evaluated in different leukemic cell lines including CML, AML and ALL cells, patient-derived samples and animal models and they were able to selectively inhibit the transcriptional activity of STAT5, suppress nuclear translocation of STAT5, binding to DNA and the expression of STAT5 target genes including cyclin D1, cyclin D2, C-MYC, and MCL-1 resulting in the induction of apoptosis and inhibition of cell proliferation and colony formation [1,17–19]. They also indicated negligible cytotoxic effects in normal bone marrow cells, so that they can be considered as potential candidates for combination with STAT5 siRNA therapy to explore the likelihood of promoting any synergistic antileukemic activity.

4.2 Future directions

4.2.1 *In vivo* leukemic models

Besides the *in vitro* studies that we concentrated here, bio-distribution and toxicity of siRNA carries as well as the validity of this siRNA therapy need to be evaluated *in vivo* to better understand pharmacokinetics and efficacy of siRNA/polymer complexes. While subcutaneous tumor models have been established as first models to explore siRNA delivery, protein silencing and subsequent effects *in vivo*, researchers are realizing the importance of employing more clinically relevant animal models for better assessment of siRNA therapy. The bone marrow niche(s) plays a complex role in leukemogenesis and should be included into the leukemic models for *in vivo* studies. Typical leukemic engraftment (primarily in the peripheral blood, bone marrow and the spleen) can be conducted with human ALL, CML and AML patient cells and some ALL, AML and CML cell lines (such as SUP-B15, Z181, HL-60, KG-1, K562) in NOD/SCID (NS) and/or NOD/SCID/IL-2Rγnull (NSG) mice with and without pre-irradiation through intravenous (and sometime intraperitoneal) injection of cells [1,20,21]. NOD/SCID mice lack mature T cells, B cells, and natural killer (NK) cells while adding the IL-2Rγ deficiency further impairs development of NK cells and has extra adverse impacts on innate immunity. The reported ranges for engraftment of human leukemic MNCs could be over 70%. Better engraftment is usually associated with poor prognosis. However, engraftment of cell lines is very variable which depends on the mouse model applied and the type of leukemic cell line [1,20,21]. By developing *in vivo* xenograft models, it would be plausible to inject our polymer/siRNA nanoparticles subcutaneously, intraperitoneally or intravenously and assess the response of established leukemic populations within the mouse peripheral blood and bone marrow environments and evaluate the toxicity to normal tissues as well. Other potential *in vivo* models can be developed using leukemic

stem cells, MLL-AF9 fusion gene initiating AML and the intracellular domain of NOTCH-1 leading to T-ALL [22,23], which would also allow for further evaluation specifically in stem cell type cells *in vivo* and the role of these cells in initiation and progression of leukemia.

4.2.2 Better assessment of siRNA delivery systems

Pharmaceutical and biomedical researchers have been attempting to develop different types of functional carriers that can assemble siRNA molecules into nano scale complexes. However, good comparisons have to be made among the available delivery systems to truly evaluate their potential for therapy. To identify carriers with the highest efficacy, it is necessary to comprehend their relative behavior in well-controlled experimental systems. The latter could be determined based on the desirable siRNA concentration for effective inhibition of the target gene or amount of carrier to be deployed for therapeutic purposes. Some of the questions in this field regarding the efficacy of potential carrier agents can be replied by utilizing dose-response studies clearly indicating the IC_{50} of the synthesized systems. This is required not only in *in vitro* studies but also in preclinical studies (similar to any pharmacological agent to be developed for clinical testing). Moreover, it would be beneficial to explore polymers modified by different lipid molecules/additives and degree of lipid substitutions to improve physicochemical characteristics and optimize effective siRNA delivery systems for different clinical samples as non-specific effects of siRNA could be minimized with specific formulation of siRNA/polymer complexes. This could be also advantageous to develop siRNA carriers that could potentially distinguish between normal and cancer cells to reduce the side-effects of non-specific impact of carriers and/or siRNA exposure. Additionally, to achieve complete downregulation of target genes, studies will be needed to focus on issues associated with the fate of intracellularly delivered siRNA/polymer

complexes including the dissociation of polyplexes inside the cytoplasm, long term fate of dissociated carriers and the fraction of delivered siRNA molecules that remain functional and are used up in silencing.

We have mainly performed *in vitro* studies with 2D cell culture and identified the outcome of siRNA delivery. siRNA delivery in 3D cell culture may indicate the distribution of siRNA/polymer complexes better as 3D cell culture can mimic the tumor microenvironment [24]. Aljitawi *et al.* evaluated the cytotoxic and apoptotic effects of chemotherapeutic drugs, doxorubicin and cytarabine on HL-60, Kasumi-1 and MV411 cell lines, co-cultured with human bone marrow mesenchymal stem cells (hu-BM-MSCs) in an experimentally designed 3D cell culture model. In this 3D microenvironment, a synthetic scaffold, polyglycolic acid/poly-l-lactic acid (PGA/PLLA) 90/10 copolymer, was applied to co-culture AML cells with expanded hu-BM-MSCs [25]. The results of this study revealed that leukemic cells cultured in 3D were more resistant to drug-induced apoptosis compared to cells cultured in 2D or in suspension and this differential responses to chemotherapy in 3D might be due to the expression of N-cadherin in the co-culture system [25]. Therefore, this 3D cell culture model is more predictive of *in vivo* responses to chemotherapy, as it considers the capability of leukemic cells to interact with the bone marrow microenvironment as well as their ability to form niches, while the responses reported by current 2D cell culture models have led to unsatisfactory clinical outcomes. By considering the advantageous of 3D models, leukemic cell culture in 3D spheroids should be developed and the efficacy of siRNA delivery and its distribution should be investigated to achieve more physiologically relevant information.

4.2.3 Efficacy of siRNA therapy

Increasing the dose of siRNA in transfection methods would naturally enhance the silencing and pharmacological impact on the target cells, while other factors including cost, cytotoxicity, and off-targets effects are necessary to be considered in the development of siRNA-based therapies. Therefore, other approaches should be taken into account other than using high concentrations of siRNA in treatments. The effective range of siRNA concentration for clinical translation would be 10-50 nM [26]. One promising approach to enhance the efficacy and specificity of siRNA therapy is to link polynucleotides to antibodies against the highly expressed markers on the surface of leukemic cells (B-lymphoblasts) including anti-CD19/CD22 as it can potentially target B-ALL cells and reduce the non-specific delivery of polynucleotides into non-leukemic cells. So far, several antibody-drug conjugates (ADC) including denintuzumab mafodotin (linking a humanized anti-CD19 antibody to the monomethyl auristatin F) and inotuzumab ozogamicin (binding a humanized anti-CD22 IgG4 mAb to calicheamicin) have been successfully developed and they showed favorable responses in clinical trials for B-ALL patients [27, 28]. Therefore, by utilizing this approach to design a siRNA delivery system which includes an anti-CD19/CD22 and cationic polymers, the efficacy of siRNA therapy can be potentially improved.

One of the most significant challenges that is required to be controlled in any siRNA therapy is the off-target effect. Sequence-specific is one of the types of off-target effect which can happen as a result of partial sequence complementarity of the siRNA guide strand with sequence motifs from 3’ untranslated regions of mRNA present in the cell. False positive results can be potentially observed due to this partial binding, which is similar to the activity of microRNAs, as it leads to non-specific silencing of transcript [29]. The inflammatory response is another type of off-target

effect, and it initiates when monocytes identify siRNA molecules or even siRNA delivery systems such as cationic lipids as pathogens through their toll-like receptors (TLR) situated in the endosomes (TLR7/8). This receptor activation leads to an undesired production of cytokines [29]. The factors that can influence the potency of off-target effects and immune response have been determined and the effect of these parameters can be moderated by chemical modifications, and control of designing the specific siRNA sequences without affecting the siRNA silencing efficacy and specificity [30].

Another challenge could be as a result of siRNA-mediated targeting of normal cells/tissues in which STAT5 is expressed resulting in unwanted silencing of STAT5 and possibly disruption of normal activity of targeted tissues. However, this effect might be negligible as the expression of STAT5 is highly regulated in other normal cells (i.e., lower levels of STAT5 expression in normal cells compared to cancer cells), and by controlling the dose of siRNA, we can address this issue. The effects of STAT5 downregulation on normal bone marrow should be investigated *in vitro,* as well.

Moreover, to have a better understanding of the heterogeneity of responses commonly observed in patient samples, it would be beneficial to (i) perform comprehensive genetic profiling of patient ALL cells to identify the exact genetic abnormalities of B-ALL subtypes including BCR-ABL-like profiles, (ii) measure the STAT5 expression in cells, (iii) evaluate the differences in siRNA uptake and (iv) try to correlate all these with effects on mRNA expression and cell viability/growth.

4.2.4 Silencing other molecular targets beyond STAT5

Although STAT5 plays a significant role in the development and progression of ALL, not all leukemic cells are dependent on STAT5 activity to thrive. Approaches are required to target other survival mechanisms in ALL cells. Several potential new targets have been already determined (**Chapter 1**). Inhibiting these potential targets by siRNA along with STAT5 silencing could be advantageous for the elimination of leukemic cells. Whether the same polymeric carriers could be applied to deliver siRNA and silence other molecular targets is an open issue remains to be investigated. The outcomes of this thesis work suggest considerable opportunities in this regard that could be beneficial to target and inhibit other oncogenes and signalling pathways in leukemic cells for therapeutic purposes. Moreover, to improve treatment outcomes, this siRNA therapy can be deployed as a potential treatment modality (either alone or in combination with chemotherapy/other therapeutic strategies) for other types of leukemias including CML and AML as well as other kinds of cancers in which STAT5 is a disease driver.

4.3 References

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