Potential of siRNA Therapy in Chronic Myeloid Leukemia

Juliana Valencia-Serna, Breanne Landry, Xiaoyan Jiang and Hasan Uludag

Abstract Leukemic cancers arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, apoptosis and survival of the malignant cells. A range of molecular alterations is beginning to be elucidated in specific types of leukemias, providing potential targets for molecular modulation as the basis of a therapy. With the advent of RNA interference (RNAi) and, in particular, the short interfering RNA (siRNA) as its pharmacological mediator, it is becoming possible to specifically modulate desired leukemic targets at will. This chapter will summarize the current attempts to utilize siRNAs in leukemic therapy using chronic myeloid leukemia (CML) as a prototypical disease model. We first provide a brief background on the CML disease with particular emphasis on molecular mediators critical in this disease and the current drug therapy. The limitations of current drugs and potential of RNAi are presented. We then provide a summary of delivery efforts employed to deliver siRNA to CML cells, with emphasis on non-viral delivery approach due to its better safety profile for utility in a clinical setting. Important factors involved in intracellular delivery of siRNA are highlighted, emphasizing features critical for non-viral delivery. We conclude with a perspective on the future of siRNA therapy for the CML disease.

J. Valencia-Serna · H. Uludag (✉)
Department of Biomedical Engineering, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB T6G 2V2, Canada
e-mail: huludag@ualberta.ca

B. Landry · H. Uludag
Department of Chemical and Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, AB T6G 2G6, Canada

X. Jiang
Terry Fox Laboratories, British Columbia Cancer Agency, Department of Medical Genetics, University of British Columbia, Vancouver, BC V5Z 1L3, Canada

H. Uludag
Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB T6G 2G6, Canada

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Abbreviations

RNAi	RNA interference
dsRNA	Double stranded RNA
CML	Chronic myeloid leukemia
AML	Acute myeloid leukemia
LSC	Leukemic stem cells
RISC	RNA-induced silencing complex
siRNA	Short interfering RNA
mRNA	Messenger RNA
shRNS	Short hairpin RNA
TKI	Tyrosine-kinase inhibitors
SDF-1	Stromal cell-derived factor-1
CXCR4	C-X-C chemokine receptor type 4
IC50	Concentration required for 50 % loss of cell viability
PRAME	Preferentially expressed antigen of melanoma
STAT	Signal transducer and activator of transcription
PPP2R5C	Protein phosphatase 2, Regulatory subunit B′, gamma
PEI	Polyethylenimine
PLL	Poly-l-lysine
MW	Molecular weight
kDa	Kilo Dalton
PEI2	2 kDa PEI
PEI2LA	Linoleic acid substituted 2 kDa PEI
PEI1.2PA	Palmitic acid substituted 1.2 kDa PEI
GFP	Green fluorescent protein
MSC	Mesenchymal stem cells
ECM	Extracellular matrix
CPP	Cell penetrating peptides
PTD-DRBD	Peptide transduction domain and double-stranded RNA-binding domain

1 Introduction

Leukemic cancers arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, apoptosis and survival of malignant cells. The US National Cancer Institute calculated an overall 5-year relative survival (between 2003 and 2009) rate of 56.0 %

for various leukemias combined (Fast Stats 2013). The front line therapy in leukemia is chemo (drug) therapy (Estey 2013; Stefanachi et al. 2012); current therapeutic approaches include broad-spectrum drugs against fast-proliferating cells and small-molecule inhibitors targeting specific signal transduction pathways, so called molecular therapies (Ferrara 2012). Leukemic cells generally respond well to drug therapy at the onset of the treatment, but the drugs lose their effectiveness over a period of 6–12 months. It is well recognized now that the resistance to conventional (broad-spectrum) therapeutic agents is inevitable, but recent evidence also indicated that even the most advanced molecularly-targeted drugs lose their efficacy as a result of resistance development in a relatively short time. The inherent plasticity of the cells combined with diverse resistance mechanisms make malignant cells naturally adapt by mounting an effective resistance against the drugs. The high relapse rate in leukemia patients has been additionally attributed to existence of a rare population of leukemia stem cells (LSC) resistant to current drug therapies (Ishikawa et al. 2007; Mikkola et al. 2010).

With better understanding of molecular changes in malignant transformations, treatments that target tumor-specific changes are expected to lead to more effective therapies as the normal cells transform into malignant cells. Towards this end, a highly specific leukemia therapy can be developed by exploiting the RNA interference (RNAi) mechanism to silence the aberrant protein(s) responsible for the disease (Iorns et al. 2007; Rossbach 2010). There are two main approaches for RNAi, using either a plasmid encoding for short hairpin RNA (shRNA) or delivering small interfering RNA (siRNA) where the shRNA transcription and processing steps can be omitted (Guo 2010). The use of siRNA is a more practical approach bypassing the need to express the shRNA at sufficient quantities in hard-to-transfect primary cells. The siRNA essentially acts as a pharmaceutical 'drug' in this respect. In cytosol, the siRNA duplexes assemble into a pre-RISC (RNA-induced silencing complex) containing specific proteins, including argonaute proteins (AGO1, 3 or 4) (Yoda et al. 2010; Wang et al. 2009), which is subsequently guided to target desired mRNA based on complementary base pairing (Yoda et al. 2010). Endonucleoyltic cleavage and/or translational repression of the mRNA (Yoda et al. 2010; Wang et al. 2009) then silences the protein target. Delivery systems, however, are an absolute necessity for effective use of siRNA since the molecules are highly sensitive to serum nucleases and their large ($\sim$13 kDa) and anionic nature (due to its phosphodiesterase backbone) prevents the siRNA to traverse cellular membranes. Viral means to deliver siRNA has been emphasized initially, but the undesirable side-effects of viral delivery in a clinical setting makes this approach highly risky for therapeutic use. Alternatively, cationic biomolecules capable of binding and neutralizing the anionic charges of siRNA and packaging the siRNA into nano-sized complexes can serve as effective siRNA carriers (Abbasi et al. 2013). Formulations of cationic biomolecules, such as lipids, small amines or polymers, with siRNA, typically results in nano-sized particles that are suitable for systemic administration and cellular uptake.

In this chapter, we will summarize the attempts to deliver siRNA molecules using non-viral carriers in leukemia. We will focus on a particular type of

leukemic cancers, namely chronic myeloid leukemia (CML), since it is one of the major classes of leukemia and it is well understood at molecular level. The siRNA therapy has not reached clinical setting in CML treatment, so that we will review the literature on preclinical studies exploring the potential of siRNA therapy in this disease. In conjunction, we will explore the technology of siRNA delivery, investigating the critical issues pertinent to effective siRNA delivery.

2 Chronic Myeloid Leukemia and Current Drug Therapies

Myeloid leukemias (46 % of all leukemias) affect the myeloid cells of the bone marrow, which normally go on to form the blood cells. Thirteen percent of those cases account for the CML. Approximately 350,000 people worldwide are diagnosed with leukemia annually, with ~250,000 death resulting from leukemia each year. Most leukemia occur in the elderly and peaks between the ages of 75 and 79 (Elert 2013). CML is a myeloproliferative disease initialized at the hematopoietic stem cells that is thought to arise due to translocation of chromosomes 9 and 22, which results in a fusion between *ABL* and *BCR* genes, or in the so-called Philadelphia (Ph) Chromosome (Sloma et al. 2010; Kumar et al. 2009; Bocchia et al. 2005). Once the normally regulated tyrosine kinase of the ABL protein is permanently activated by the juxtaposition of the BCR sequence, it leads initially to a chronic phase characterized by myeloid cell expansion, while allowing differentiation of expanded cells in the peripheral blood. As the disease progresses, either by increased *BCR-ABL* expression or activation of other pathways, patients enter a more aggressive disease phase (blast crisis), which is characterized by a progressive loss of the capacity of hematopoietic cells to differentiate and increased expansion and accumulation of immature blast cells in the bone marrow and spread to the bloodstream (Ito 2013; Melo and Barnes 2007). The Philadelphia chromosome is not specific for CML, since it can also be found in ~5 % of children with acute lymphoblastic leukemia (ALL), the most common childhood cancer (DeWeerdt 2013).

Current therapies for CML are based on the use of tyrosine-kinase inhibitors (TKIs) and allogeneic hematopoietic stem-cell transplantation. Stem-cell transplantation therapy is an option when the treatment with TKIs fails; however, this therapy has substantial risk of mortality due to chronic graft-versus-host disease (Goldman and Melo 2003; Baccarani et al. 2009). TKIs, such as Imatinib, have revolutionized CML therapy. Imatinib binds to the ABL kinase domain with the formation of a bond that impedes ATP binding, subsequently blocking or preventing the interaction of the ABL kinase with substrates and therefore from activating its oncogenic pathways (Zhang and Li 2013; Deininger et al. 2000). Targeted therapy with Imatinib has transformed the survival potential for patients with chronic phase of CML; it has significant impact on patients with accelerated phase but a minimal impact for those patients at the blastic phase stage (Giles 2006). However, CML patients, especially those in advance-stage disease, can

develop TKI resistance leading to relapse (Baccarani et al. 2009). This acquired drug resistance could be due to the amplification of *BCR-ABL* gene, and overexpression of *BCR-ABL* mRNA and protein (Weisberg et al. 2007). However, resistance most often results from point mutations in the kinase domain of BCR-ABL protein that affect drug binding to the protein, thereby reducing the ability of Imatinib to block the tyrosine kinase activity. More than 50 distinct *BCR-ABL* mutations have been reported to-date and the current repertoire of TKIs can cover all known mutations leading to resistance; however, no single drug can prevent all forms of resistance (Zhang and Li 2013), necessitating the use of TKI cocktails to overcome any possible resistance.

Next-generation drugs such as Dasatinib and Nilotinib, are more potent TKIs and produce more rapid declines in CML disease burden than the Imatinib, which translates into more durable cytogenetic (absence of metaphase Ph^+ cells) and hematological remissions (achievement of normal white blood and platelet cell counts and, no signal of CML symptoms) (Weisberg et al. 2007; Sawyers 2013). Nilotinib is ~30 fold more potent than Imatinib as an ABL inhibitor. Dasatinib is a potent inhibitor of ABL kinase Src-family kinases, which are know to be involved in multiple intracellular signal transduction pathways including oncogenesis and disease progression (Weisberg et al. 2007). Ponatinib is a newer drug that has the unique property of inhibiting both the native (un-mutated) and mutated BCR-ABL proteins, specially those including the T315I mutation, which confers resistance to all other CML drugs (including Nilotinib and Dasatinib) and seems to translate in worst overall survival compared with other mutations found in Imatinib-treated patients (Weisberg et al. 2007; Sawyers 2013; Cortes et al. 2012). A strategy of combing two or three ABL inhibitors with non-overlapping *BCR-ABL* mutations resistance profiles, such the example of Ponatinib exemplified above, could prevent the emergence of drug resistance (Sawyers 2013). However, it is expected that treatment with these new ABL inhibitors could also lead to new point mutations that overcome the resistance of these new drugs (Weisberg et al. 2007), given the plasticity of leukemic stem cells.

3 Insensitivity of CML Stem Cells to TKIs

Although Imatinib inhibits the production of ~99 % of differentiated leukemic cells, it fails to deplete the LSCs (Weisberg et al. 2007). Studies have shown that despite the complete depletion of *BCR-ABL* transcript levels in these LSCs with TKIs, the cells remain viable. These data indicate that even in the presence of Imatinib, especially in accelerated and advanced states of the disease, CML has progressed and evolved so these LSCs no longer require BCR-ABL activity to maintain their viability (Kumar et al. 2009; Zhang and Li 2013; Corbin et al. 2011; Muvarak et al. 2012), and anti-apoptotic and pro-survival signals are provided by

alternate pathways. Thus, the drivers of cell proliferation and survival, probably influenced by BCR-ABL in the early stages of CML, now operate autonomously and could lead to CML relapse (Savona and Talpaz 2008). Thus, it is clear that not only BCR-ABL inhibition is needed for the eradication of progenitor CML cells, but BCR-ABL-independent survival mechanisms of LSCs also needs to be targeted for a complete CML eradication.

One characteristic common to all LSCs is that they require the particular microenvironment of bone marrow, the stroma, to thrive. The stroma bathes the leukemic cells in growth factors, chemical signals and cell-surface ligands that keep the cells in a dormant phase resistant to drug therapy. Part of what keeps these cells entrenched in the bone marrow is a chemical signal sent by the stroma, called stromal cell-derived factor (SDF-1). This signal binds to a protein located at the surface of the stem cells, called C-X-C chemokine receptor type 4 (CXCR4) (Willyard 2013). In the case of CML, BCR-ABL protein seems to be involved in the inhibition of SDF-1-induced migration and signaling which allows an abnormal release of immature myeloid cells from the bone marrow into the circulation (Jin et al. 2008). On the other hand, it has been shown that under Imatinib, CXCR4 expression in $CD34^+$ can be reversibly up-regulated, hence allowing these cells to home to bone marrow, helping them to become quiescent and to become insensitive to TKIs (Jin et al. 2008; Copland 2009). Down-regulation of CXCR4 expression along with TKIs therapy could enhance the eradication of LSCs in CML.

Researchers are also developing drugs that target a key property of stem cells, namely their self-renewal potential. One signaling pathway that seems to play an important role in self-renewal of CML LSCs hinges on two proteins: Wnt and beta-catenin (Copland 2009; Zhao et al. 2007). In 2012, Armstrong et al. reported that a small molecule that inhibits beta-catenin, given in combination with Imatinib, reduces CML survival and eliminates leukemia stem cells in a CML mouse model (Willyard 2013; Heidel et al. 2012). Finally, *AHI-1* is a newly discovered oncogene that is highly expressed in primitive hematopoietic CML stem and progenitor cells, and whose overexpression has been shown to promote abnormal differentiation and proliferative activity of myeloid cells in CML. Zhou et al. showed that *AHI-1* overexpressing $BCR\text{-}ABL^+$ cells (CML cells transduced with an *AHI-1* construct) showed greater resistance to growth inhibition effects of Imatinib in comparison to control cells. Suppression of *AHI-1* by transduction of an AHI-1 silencing construct (AHI-1/sh4) resulted in increased sensitivity to Imatinib. AHI-1 was also found to significantly increase or reduce protein expression and phosphorylation of BCR-ABL, JAK2 and STAT5 once *AHI-1* is overexpressed or suppressed, respectively. Suppression of *AHI-1* in primary $CD34^+$ CML cells was also shown to increase Imatinib sensitivity especially in Imatinib-resistant and blast crisis patients who express relatively higher levels of AHI-1 (Zhou et al. 2008).

4 Down-Regulation of Protein Targets by RNAi in CML

RNA interference (RNAi) is a process by which double-stranded small interfering RNA (siRNA) induces sequence-specific, post-transcriptional gene silencing (De Paula et al. 2007). Endogenous RNAi is triggered by the transcription of long pieces of double-stranded RNA (dsRNA), which are cleaved into the smaller (21–23 nucleotides long) fragments by the enzyme Dicer. In practice, siRNA is synthetically produced and then directly introduced into the cell, thus circumventing Dicer mechanics (Whitehead et al. 2009). Once siRNA is present in the cytoplasm of the cell, it is incorporated into the protein complex RISC (RNA-induced silencing complex). Thereafter, Argonaute, a protein contained within RISC, cleaves the sense strand of the siRNA, thereby releasing it from RISC. The now activated RISC, which contains the antisense strand of the siRNA, selectively seeks out and cleaves mRNA that is complementary to the antisense strand (De Paula et al. 2007; Whitehead et al. 2009). The activated RISC complex is not affected by this reaction and can move on to destroy additional mRNA targets, which further propagates the silencing of gene expression. In mammalian cells, RNAi persists effectively only for an average of 66 h due to its dilution during cell divisions (De Paula et al. 2007), and so repeated administration is necessary to achieve a persistent effect if needed (Whitehead et al. 2009).

The shortcomings of current leukemia treatments call for development of new strategies to deliver more efficacious drugs into CML cells. Owing to increasing knowledge of CML at a molecular level, RNAi is a promising approach for leukemia treatment. To control the expression of *BCR-ABL* and other genes involved in these cellular malfunctioning processes, synthetic small interfering RNA (siRNA) can be delivered into diseased cells to interact with the target mRNA of aberrant genes and silence their protein expression. However, a delivery carrier that helps these siRNA moieties to reach the mRNA in the cytoplasm is needed. In order to achieve this purpose, carriers need to interact with the siRNA moieties to form siRNA nanoparticles that protect the siRNA from serum nucleases and facilitate their cell membrane interaction, internalization via endocytosis and escape from endosomes, in order to ultimately be released in the cytoplasm (Prokop 2011; Dominska and Dykxhoorn 2010; Mintzer and Simanek 2009).

Several potential targets have been pursued for siRNA therapy of CML cells (Table 1). Silencing specific targets has been used as a tool to elucidate their functional role in CML and the biological outcome upon depleting the selected target. The main aim of these studies was identification of novel targets to decrease proliferation rates and induce programmed cell death that can be used in combination with conventional drugs to improve drug sensitivity. In one of the first studies to explore siRNA therapy, Wohlbold and co-workers targeted *BCR-ABL* expression in *BCR-ABL*-transduced cells. This siRNA treatment resulted in a significant reduction of BCR-ABL protein, which led to a reduced regulatory effect of its substrates, reducing the expression of antiapoptotic Bcl-X_L protein and increasing the expression of cell cycle inhibitor p27. BCR-ABL silencing led to a

Table 1 siRNA targets shown to be beneficial in CML. siRNA studies which reported significant anti-survival effects in CML cells were selected from a PubMed 'CML siRNA' keyword search

References	Target	Rationale	siRNA carrier (concentration)	Outcome
Wilda et al. (2002)	BCR-ABL	Compare efficiency of cell killing by Imatinib to that of silencing of *BCR-ABL* with siRNA	Oligofectamine (unknown)	Reduction of mRNA and protein were found with apoptosis levels 2.5x higher than controls. Apoptosis rate of BCR-ABL siRNA treated cells was at the same level as cells treated with Imatinib or ~5 times more than control cells
Wohlbold et al. (2003)	BCR-ABL	Inhibit *BCR-ABL* expression and evaluate sensitization to imatinib	Electroporation (200–800 nM)	Decreased cell viability and sensitization of imatinib-resistant K562 cells to imatinib
Rangatia and Bonnet (2006)	BCR-ABL	Study anti-leukemic properties of BCR-ABL by RNAi	Electroporation (1 μg per 5×10^5 cells)	60 % reduction of *BCR-ABL* mRNA expression. Slight increase of apoptosis. 2-fold increase of DNA fragmentation. Caspase-7 and -9 activated. Cells unable to actively divide for at least 2 weeks after silencing
Arthanari et al. (2010)	BCR-ABL	To assess efficacy of Tat-LK15 peptide in delivering siRNA to target *BCR-ABL*	Tat-LK15 peptide: fusion of HIV-Tat-derived peptide to cationic peptide LK15 (1 to 30 μg siRNA/mL—24–729 nM calculated)	Expression of p210 BCR-ABL was reduced for all concentrations. Cytotoxicity due to siRNA nanoparticles ranging from 0 % (10 μg) to 30 % (30 μg). No silencing detected after 48 h
Wang et al. (2008)	Cyclin A2	Deliver cyclin A2 siRNA with SWNTs and evaluation of cyclin A_2 role upon doxorubicin treatment.	Functionalized single wall carbon nanotubes (f-SWNTs) (25 nM in culture)	A positive correlation between ability of doxorubicin to induce apoptosis and up-regulation of cyclin A_2
Gioia et al. (2011)	Syk and Axl	Identify downstream effectors of Lyn involved in resistance to nilotinib	Nucleofection (unknown)	Silencing Lyn's downstream effectors Syk and Axl restored capacity of nilotinib to inhibit cell proliferation

(continued)

Table 1 (continued)

References	Target	Rationale	siRNA carrier (concentration)	Outcome
Tanaka et al. (2011)	PRAME	Investigate function of PRAME in CML progression by RNAi in K562 cells	Nucleofection (1.5 μM siRNA/1.5 × 10^6 cells)	70 % knockdown of *PRAME* mRNA. Significant inhibition of cell proliferation and decrease of clonogenic growth. 60 % of apoptotic cells in comparison with 15 % of controls
Kosova et al. (2010)	STAT5A	Effects of STAT5A siRNA knockdown on cell growth and apoptosis induction	HiPerFect (unknown)	~75 % suppression of *STAT5A* mRNA. Resistant K562 cells became ~4 times more sensitive to Imatinib. An increase in caspase-3 activation was seen
Zhang et al. (2011)	GCS or MDR1	Relation of GCS and MDR1 to regulation P-gp gene expression and function activity in drug retention	Lipofectamine 2000™ (unknown)	Silencing of GCS can affect MDR1 expression and inhibit P-gp efflux. Silencing of GCS or MDR1 sensitized drug-resistant cells to chemotherapy and increased drug retention
Koldehoff et al. (2013)	BCR-ABL and GFI1B	Anti-leukemic additive effect of co-silencing of BCR-ABL and GFI1B	DOTAP, liposomal transfection (175 pM for GFI1B and 54 pM for BCR-ABL)	Additive effect in the inhibition of cell growth and in the increase of apoptosis in comparison with transfection of either siRNA alone
Shen et al. (2013)	PPP2R5C	Effect of PPP2R5C down-regulation in imatinib-sensitive and –resistance CML cells	Nucleofection (3 μg)	Inhibition of the proliferation of CML cells. Rendered imatinib-resistant cells more sensitive to TKIs

significant reduction of cell viability in a dose-dependent manner. A significant drop in the IC_{50} values of Imatinib (3.4-fold drop) was observed in K562 cells transfected BCR-ABL siRNA but not in untreated K562 cells (Wohlbold et al. 2003). Effective *BCR-ABL* silencing was also obtained by Rangatia et al, where ~50 % decrease at the mRNA level was found after 72 h of siRNA delivery. This silencing resulted in a two-fold increase of sub-G1 cell population as well as an increase of DNA fragmentation and mitochondrial-induced apoptosis. Although only a transient mRNA reduction was seen with siRNA treatment, a long-term effect was observed in proliferation of targeted cells: cells were unable to actively divide for at least 2 weeks in comparison with untreated cells. The reason for this was the cell cycle arrest in G1 phase, which is observed by decrease in cyclin D1 and increase in p21 and p27 cell cycle inhibitors (Rangatia and Bonnet 2006).

The related cell cycle mediator cyclin A_2 was targeted by RNAi in CML K562 cells. Silencing cyclin A_2 in doxorubicin-treated K562 cells led to a significant decrease in growth inhibition, apoptosis induction and increased erythroid differentiation. This suppression also caused a small fraction of K562 cells to differentiate along megakaryocytic and monocyte-macrophage pathways upon doxorubicin treatment. A positive correlation between the ability of doxorubicin to induce apoptosis in K562 cells and upregulation of cyclin A_2 was seen; the higher the cyclin A_2 expression, the higher the sensitivity to doxorubicin was. These results indicated a pro-apoptotic role of cyclin A_2 and its ability to regulate cell differentiation in CML (Wang et al. 2008).

Upregulated expression of Lyn has been suggested as an additional mechanism of cell resistance to nilotinib (Mahon et al. 2008). Gioia et al. investigated the role of Lyn kinase signalling as a mediator of resistance to nilotinib. Tyrosine kinase Lyn was overexpressed ~8 times more in nilotinib-resistant K562 cells in comparison with TKI-sensitive K562 cells. The proteins spleen tyrosine kinase Syk, UFO receptor Axl, and an adaptor protein CDCP-1 were found to have increased tyrosine phosphorylation in Lyn-overexpressing cells. Co-immunoprecipitation studies showed that Lyn interacted with Syk and Axl proteins in both cell lines. An increase in Syk phosphorylation was detected in nilotinib-resistant cells (with no significant difference of expression). Inhibition of Syk either by addition of Syk inhibitor R406, Syk shRNA or Syk siRNA increased (or restored) the sensitivity to nilotinib. Silencing of Axl and CDCP-1 by siRNA increased the sensitivity to nilonitib partially, suggesting that Axl and CDCP-1 may be mediators of Syk/Lyn signaling pathways. An overexpression of Lyn, CDCP-1 and Axl was also detected in nilotinib-resistant $CD34^+$ patient cells. The role played by Syk and Axl in the nilotinib resistance identifies these genes as potential targets as a combinatorial therapy for CML (Gioia et al. 2011).

Tanaka et al. investigated the function of preferentially expressed antigen of melanoma (PRAME) in leukemia. PRAME acts as a repressor of retinoic acid receptor (RAR) signalling and thus the functional repression of PRAME was investigated in K562 cell line in the absence of retinoic acid. Three days after siRNA delivery, ~70 % knockdown of *PRAME* mRNA and a complete inhibition of protein expression was achieved, which resulted in a significant inhibition of

proliferation and clonogenic growth. This PRAME knockdown also lead to a significant increase of cells in G_0/G_1 phase and a related decrease in cells in S phase in comparison with the control group, which suggests a relationship between PRAME and cell cycle arrest in the G_0/G_1 phase. This cell cycle arrest was followed by a gradual increase in apoptotic cells and caspase-3 activation. Overexpression of PRAME was also found to prevent the cells from erythroid differentiation (Tanaka et al. 2011).

Kosova et al. studied the effect of STAT (signal transducer and activator of transcription) knockdown in apoptosis and proliferation in sensitive and Imatinib-resistant K562 cells. STAT5 is involved in the development of myeloproliferative diseases while STAT3 is implicated in malignant transformation; both STAT5 and STAT3 are constitutively expressed in haematological malignancies (Bromberg 2002; Turkson 2004). Quantification of mRNA levels revealed a significant increase in *STAT5B,* and *STAT5A* (>50 %), but not *STAT3* level (4 %) in Imatinib-resistant cells as compared to Imatinib-sensitive cells. Transient knockdown of STAT5A by siRNA led to sensitization of Imatinib-resistant and Imatinib-sensitive cells by 4.5 and 1.2 times to Imatinib treatment (Kosova et al. 2010). When Imatinib-resistant cells were treated with 5 μM Imatinib, cell viability was decreased by ∼20 %, while the same concentration of Imatinib with STAT5A-siRNA-treated cells decreased cell viability by ∼60 % (Kosova et al. 2010).

The sphingolipid ceramide plays an important role in apoptotic signalling in response to anticancer drugs. Intracellular levels of pro-apoptotic ceramide were shown to increase when cells respond to drugs, contributing to their anti-cancer efficacy. However, multidrug-resistant cells accumulate ceramide due to an enhanced activity of glugosyceramide synthase (GCS), which converts the available ceramide in glucosylceramide (GlcCer). This conversion impedes ceramide from being involved in activation of apoptosis (Baran et al. 2011; Zhang et al. 2011). Although controversial, down-regulation of GCS has been shown to down-regulate expression of P-glycoprotein (P-gp) (Zhang et al. 2011), an efflux pump that decreases intracellular levels of drugs. With the aim of decreasing the multidrug resistance in doxorubicin-resistance K562 cells, Zhang and co-workers targeted GCS or P-gp by siRNA. Upon silencing GCS or P-gp with specific siRNAs, the transporter activity was significantly decreased, suggesting a linkage between GCS and P-gp expression, and providing potential therapeutic targets in CML therapy (Zhang et al. 2011).

Growth factor independence-1B (GFI-1B) is a transcription factor that controls the development and differentiation of erythroid cells and megakaryocytes at the erythro-megakaryocytic progenitor stage (Randrianarison-Huetz et al. 2010). *GPI1B* mRNA expression was found to be overexpressed in leukemic cells (Elmaagacli et al. 2007). Koldehoff et al. investigated whether antileukemic effect of *BCR-ABL* silencing can be further increased by co-silencing of *GFI1B* (Koldehoff et al. 2013). A significant drop in cell viability was evident with the combination of GFI1B and BCR-ABL siRNAs, as well as *BCR-ABL* mRNA levels after co-silencing. An additive induction of apoptosis after co-silencing was observed. Similar results of the inhibition of mRNA levels of *BCR-ABL* and

GFI1B were found in advanced CML patient cells. The co-silencing led to a significant reduction of *MDR1* (P-gp) and *c-Myc* mRNA levels, suggesting *BCR-ABL* and *GFI1B* to be connected to other critical mediators involved in cancer transformation (Koldehoff et al. 2013).

Protein Phosphatase 2, Regulatory Subunit B', Gamma (PPP2R5C) levels were over-expressed in peripheral blood mononuclear cells from chronic phase CML patients, and *PPP2R5C* expression was significantly decreased in patients undergoing remission (Zheng et al. 2011). *PPP2R5C* plays an important role in cell proliferation, differentiation, and transformation based on its induction of the dephosphorylation of p53 at various residues, which negatively modulates its apoptotic activities, and thus promoting cell survival (Zheng et al. 2011). It was possible to reduce *PPP2R5C* mRNA and protein levels in K562 and resistant-K562 cells with specific siRNA treatment. *PPP2R5C* mRNA levels in CML primary cells was also reduced with specific siRNA treatment, leading to reduced proliferation rate in both K562 and CML primary cells. An increase in apoptosis rate in K562 cells was also evident. These results indicate that down-regulation of *PPP2R5C* could significantly inhibit the proliferation of CML cells and more importantly, could render imatinib-resistant cells more sensitive to TKIs (Shen et al. 2013).

Taken together, it is evident that several promising protein targets are available for siRNA-mediated silencing. Effective functional responses have been obtained, in the form of reduced proliferation, apoptosis induction as well as sensitization to CML drugs, after targeting individual or combination of the appropriate targets. Whether this approach could be applied clinically remains to be seen and it is generally assumed that effective delivery of siRNA is the limiting step.

5 Biomaterials in siRNA Delivery

Biomaterials such as cationic lipids and polyamines have been used to condense nucleic acids for delivery into cells. However, existing transfection and delivery methods are more suitable for attachment-dependent cells (e.g., breast cancer cells) rather than attachment-independent CML cells. Physical treatments such as electroporation on the other hand, although helpful to investigate the effect of gene depletion by RNAi, especially on difficult-to-transfect cells such as primary or suspension-growing cells (Gioia et al. 2011; Tanaka et al. 2011; Kosova et al. 2010), cannot be translated in vivo because of the significant toxicity they induce to cells after transfection, and because they have only been designed for an in vitro setting (Rangatia and Bonnet 2006; Merkerova et al. 2007). Electroporation (and related 'nucleofection') is the most common method to deliver siRNA for experimental purposes. Viral vector have been effectively used but these present significant safety risk because they can integrate to the host's genome or cause lethal immune responses and inflammation (Mintzer and Simanek 2009; Arthanari et al. 2010).

Cationic polyamines are desirable for siRNA delivery because they are capable of condensing anionic siRNAs into spherical, stable nano-particles (De Paula et al.

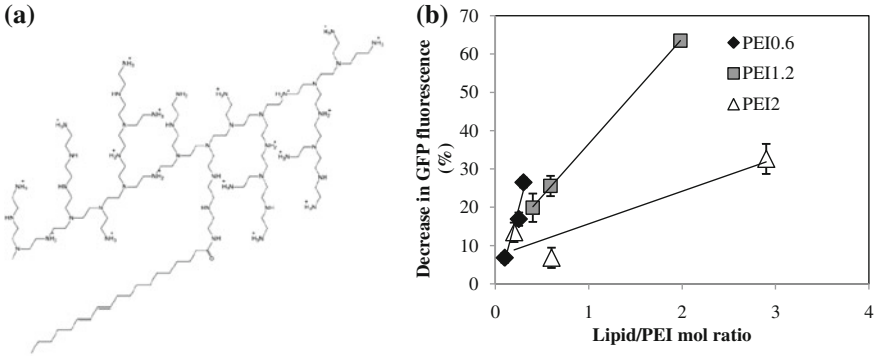

Fig. 1 **a** LA-substituted 2 kDa PEI. **b** GFP silencing in GFP-K562 cells with PA-substituted polymers (72 nM siRNA concentration). Decrease in mean GFP fluorescence was evaluated by flow cytometry 3 days after transfection. PEI1.2PA (lipid substitution of 1.98 PEI/PA) stands out showing a 63 % decrease in the mean GFP fluorescence and a milder effect on the cell counts decreasing it by 40 %. A strong correlation between the extent of lipid substitution and the GFP silencing was found with all the PEIs used. GFP silencing was also found to be dependent on the molecular weight (MW) of PEI used: the lower the MW of the PEI, the greater the increase in GFP silencing at increasing lipid substitutions

2007). Polyethylenimines (PEIs) with various molecular weights (MWs) and modifications have been used for transfection of nucleic acids in different cell lines and live animals (De Paula et al. 2007; Aliabadi et al. 2011, 2012). The high transfection efficiency of PEI is attributed to its "proton-sponge" effect, by which PEI once in the endosome attracts ions that lead to swelling and bursting of the endosome (Whitehead et al. 2009), which results in the release of the nucleic acids into the cytoplasm. This high transfection efficiency is mostly seen with high MW ($\sim$25 kDa) PEI where cellular delivery of nucleic acid cargo is efficient (unlike low MW PEIs). However, excessive endosome rupture leads to cell toxicity, thus limiting the dose of siRNA that can be delivered (Aliabadi et al. 2011; Wang et al. 2010). Moreover, an inverse relationship between cytotoxicity and transfection is observed in PEI, such that low MW (2–5 kDa) PEIs are considered to have better safety profiles due to non-toxicity, but are ineffective for nucleic acid delivery (Neamnark et al. 2009). By using the amine groups of the PEI that allow conjugation with other ligands, the Uludag group investigated the effect of lipid substitutions on 2 kDa PEI (PEI2) in an attempt to increase the polymer interaction with the cell membrane and nucleic acids delivery (Fig. 1a). It was found that the relatively nontoxic but ineffective PEI2 polymer carrier can be transformed into an effective delivery agent by grafting a lipid molecule onto the polymer (Aliabadi et al. 2011; Neamnark et al. 2009). Although generally effective, the gene delivery efficiency of these modified polymers can vary among cell lines (Aliabadi et al. 2011; Abbasi et al. 2008; Farrell et al. 2007; Alshamsan et al. 2009). Our recent studies on AML cells indicate that LA substitution (and to a lesser extent caprylic acid substitution in recent studies) sustained most silencing among the lipid-

substituted 2 kDa PEIs (PEI2LA) for down-regulation of Green Fluorescent Protein (GFP) reporter gene and endogenous CXCR4 gene (Landry et al. 2012). Similarly, PEI2LA polymer also showed the highest silencing performance with GAPDH and P-glycoprotein expression when targeted in the MDA-MB-435 breast cancer cells among the different lipid-substituted PEI2s utilized (Aliabadi et al. 2011). However, the polymers that were effective in CML cells were different; we found a particular polymer (1.2 kDa PEI) substituted with a relatively high amount of palmitic acid (PEI1.2PA; 2.0 PA per PEI1.2) to be effective. The ability of PEI1.2PA to deliver siRNA intracellularly was high (Fig. 1b), explaining its relative efficiency. The oncogene *BCR-ABL* was also effectively silenced with this polymer, resulting in the expected apoptosis induction in the targeted cells (Valencia Serna et al. 2013). It is presently not known if this is a unique combination, or other molecular weight PEIs and/or lipids can substitute for its efficiency. The liposomal agent Lipofectamine™ 2000, however, seems to be equally effective in the K562 cell model of CML.

To better explore the range of delivery systems suitable for CML, we can also inspect the siRNA delivery attempts in a related leukemia, namely acute myeloid leukemia (AML). These studies are summarized in Table 2, where the promising siRNA targets involved in cell survival (66) were categorized based on the physiological role of the target chosen (e.g., proteins involved in cell cycle regulation, bone marrow microenvironment interactions, drug sensitization, regulating transcription, phosphorylation as well as common AML mutations and related proteins). One can again see a range of non-viral carriers that have been used for delivering different siRNAs, but electroporation, as an experimental approach, has again dominated the delivery attempts (Fig. 2). Both polymeric and liposomal reagents were successfully employed for siRNA delivery in this leukemia and one can see the relatively limited studies involving non-viral carriers. The major focus in AML studies remained on elucidating suitable targets, which is expected to be analogues in the case of CML as well. As expected, there was a general increase in these attempts in recent years (Fig. 3a) but the effective dose of the siRNA delivery formulations (whether formulated with a carrier or delivered naked typically with electroporation) did not significantly change over time (Fig. 3b). One would have liked to see an improved effective dose (i.e., lower effective dose) with recently developed delivery systems, but this did not appear to be the case. This issue is discussed in more detail below.

6 Mechanism of Uptake and Intracellular Processing of siRNA Nanoparticles in Leukemic Cells

Cell membrane is the first interface that siRNA nanoparticles need to interact with for internalization. The lipid bilayer acts as an impermeable membrane to entry of unwanted materials from the external environment (including siRNA nanoparticles) and as selectively permeable, by the control of protein channels and pores, to the entry of nutrients and exit of metabolites (De Paula et al. 2007; Whitehead

Table 2 siRNA targets shown to be beneficial in AML models

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Cell cycle				
Yang et al. (2013)	SGOL1 (cell cycle—mitosis)	SGOL1 is a centromeric protein overexpressed in leukemia's including AML	Electroporation (unknown)	Decreased proliferation; mitotic arrest, intrinsic apoptosis
Tibes et al. (2012)	WEE1, CHEK1, PKMYT, ATR (cell-cycle check-points and DNA-damage repair proteins)	siRNA kinase/cytarabine screen to determine chemosensitizing targets to use with cytarabine	A cationic lipid-based transfection reagent (unknown)	Increased cytarabine efficacy
Bone marrow (BM) microenvironment interaction				
Fernandez-Vidal et al. (2006)	CDC25A (cell cycle)	Effects on cell adhesion and proliferation	Electroporation (8 pmol per 6×10^6 cells)	Decreased adhesion-dependent proliferation
Landry et al. (2013)	CXCR4 (BM micro-environment interaction)	CXCR4 mediated adhesion of AML cells	CA-PEI 2 kDa (25–50 nM)	Decreased proliferation
De Toni et al. (2006)	GSK3beta, p65 subunit NF-κB	Resistance due to adhesion molecules/integrin and morphogen Wnt soluble factors in AML	Electroporation (200 nM)	Restored chemosensitivity (daunorubicin)
Hu et al. (2011)	IGFBP7 (tumor suppressor)	To investigate the role of the known solid tumor suppressor (IGFBP7), in childhood AML	Lipofectamine 2000 (unknown)	Decreased adhesion, migration, invasion, proliferation. Role in BM microenvironment interaction was apparent
Despeaux et al. (2012)	FAK (stem cell pathway)	Over expression; FAK pathway deregulated in cancers (replaces Wnt3a-controlled canonical pathway)	Electroporation (200 nM)	Decreased survival

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Recher et al. (2004)	FAK (cell motility and survival)	FAK involvement in AML	Electroporation (200 nM)	Decreased migration, increased chemosensitivity to daunorubicin, decreased FAK did not improve resistance due to fibronectin adhesion
Sansonetti et al. (2012)	MCL-1 (anti-apoptosis)	Survival effects of adhesion interactions with BMSCs. (Induced CD44 expression upregulated MCL-1)	Lipofectamine RNAiMax (50 nM)	Increased apoptosis
Kim et al. (2013)	SDF1 (BM micro-environment interaction)	Role of SDF-1 in survival and proliferation in AML	HiPerFect (5–25 nM)	Decreased proliferation
Drug sensitizing targets				
Konopleva et al. (2004)	BCL2 (anti-apoptosis)	Determining CDDO (novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid) mechanisms in AML	Electroporation (100–500 nM)	Decreased cell proliferation and increased apoptosis with co-treatment of CDDO (but not without) in CDDO resistant cells
Rao et al. (2011)	BCL2 (anti-apoptosis)	Involvement in curcumin action in daunorubicin insensitive CD34+ AML	Lipofectamine 2000 (50 nM)	Increased chemosensitivity of daunorubicin in CD34+ AML
Cluzeau et al. (2012)	BCL2L10 (anti-apoptosis)	BCL210 over-expression in azacitidine resistant cells	Electroporation (50 nM)	Sensitized cells to azacitidine
McLornan et al. (2013)	C-FLIP$_L$ (anti-apoptosis)	Higher expression of C-FLIP (drug resistance role) correlated with decreased patient survival	Electroporation (1.5 μg/1–2.5 × 10^6 cells)	Increased apoptosis, sensitization to rTRAIL induced apoptosis

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Wang et al. (2010)	COT1	COT1 increases effect of silibinin/1,25-dihydroxyvitamin D3 combinations	Electroporation (5,000 nM)	Increased G1 arrest and differentiation caused by Silibinin/1, 25-dihydroxyvitamin D3 combinations
Kasper et al. (2012)	MCL-1 (anti-apoptosis)	MCL-1 is over expressed in FLT3-ITD cell lines	Electroporation (unknown)	Increased chemosensitivity in FLT3-ITD+ AML
Wang et al. (2013)	MCL-1 (anti-apoptosis)	Involvement in arsenic trioxide effect in AML	Unknown	Increased arsenic trioxide-induced mitochondrial apoptosis (chemosensitivity)
Nishioka et al. (2009)	MEK-1	Study of 5-AzadC (DNA methyltransferase inhibitor) and AZD6244 (MEK inhibitor) in AML	Electroporation (unknown)	Decreased viability with 5-AzadC co-treatment but not without
Altman et al. (2010)	Mnk1, Mnk2	Involvement in cytarabine mechanism of action	Unknown	Decreased leukemic colony formation with cytarabine treatment but not without
Nishioka et al. (2010)	4E-BP1 (MEK/ERK pathway) and MCL-1 (anti-apoptotic)	AZD6244 causes apoptosis and suppresses 4E-BP1 and MCL-1 in HL-60 cells but not in EOL-1 and MOLM13 cells	Electroporation (300 nM)	Decreased MCL-1 expression and increased apoptosis with AZD6244 (4E-BP1). Increased apoptosis with/out AZD6244 (MCL-1)
Transcription factor				

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Pan et al. (2012)	Gli1 (transcription factor—activator/hedgehog pathway)	Effects of aberrant expression and inhibition of Gli	Jet-PEI (100 nM)	Decreased proliferation and decreased survival
Elmaagacli et al. (2007)	GFI1B	Role of GF1IB in erythropoietic and megakaryocytic malignancies	TransMessenger (0.1–2.4 μg/24-well plate well)	Decreased proliferation in AML, CML and normal CD34+ cells and increased apoptosis in AML and CML cells
Rushworth and MacEwan (2008)	HO-1, Nrf2, c-FLIP	Involvement in NF-κB and TNF-induced apoptosis in AML	Electroporation (30 nM)	Susceptible to TNF-induced cell death (HO-1, Nrf2), Susceptible to TNF but not with NF-κB inhibitor BAY 11–7082 (c-FLIP)
Rushworth et al. (2010)	NF-κB and HO-1	Inhibition of highly expressed NF-κB did not cause apoptosis due to HO-1	Electroporation (30 nM)	Increased apoptosis after targeting both HO-1 and NF-κB in AML cells but not in CD34+ non-malignant cells
Carvalho et al. (2007)	NF-κB subunit p65, IKK1, IKK2, NEMO	Understanding the role of NF-κB activation in AML	Electroporation (unknown)	Increased apoptosis
Braun et al. (2006)	NF-κB subunit p65	NF-κB is continuously activated in P39 MDS/AML cells	Electroporation (unknown)	Increased apoptosis
Zhang et al. (2013)	STAT3	Development of targeted STAT3 (role in cancers) siRNA delivery in TLR9+ hematopoietic cells	TLR9 antagonist CpG(A)-siRNA (100–500 nM in vitro, 100 μg (5 mg/kg) every 24 h IT for in vivo)	In vivo, decreased tumor growth. The delivery system is immunostimulatory and can contribute to overall anti-cancer effects

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Gao et al. (2011)	WT1 (transcription factor and tumor suppressor)	Involvement in miR-15a and miR-16-1 tumor suppressors	HiPerFect (50 nM)	Decreased proliferation
Elmaagacli et al. (2005)	WT1 (transcription factor and tumor suppressor)	WT1 is overexpressed in leukemia	TransMessenger (0.8 μg, 1×10^5 cells/well)	Decreased proliferation, increased apoptosis in AML and CML (but not in normal CD34+ cells). Increased anti-survival effects when WT1 and BCR-ABL were targeted in K562 cells
Tyrosine kinase related				
Park et al. (2013)	Axl (receptor tyrosine kinase, various pathways)	Determine role of Axl in FLT3 signaling in AML	Electroporation (unknown)	Inhibited cell growth, arrested cell-cycle, induced apoptosis and differentiation in FLT3-ITD+ AML
Gu et al. (2007)	CSFIR	Identification of tyrosine-phosphorylated proteins in AML M7 (AMKL)	Electroporation (unknown)	Decreased proliferation and increased apoptosis in AML M7 MKPL1 cells but not in CML K562 cells. (C-KIT siRNA did not decrease proliferation)
Tyner et al. (2008)	EPHA4, JAK1, JAK3, LTK, LYN, PTK2, PTK2B PTK6, PTK9, and SRC (all cytosolic kinases except EPHA4 and LTK)	siRNA screen of tyrosine kinase proteins in AML cells	Electroporation (1,000 nM)	Decreased viability

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Voisset et al. (2010)	FES, FER	Investigation of FES and FER in AML	Electroporation (0.4–0.8 nmol in 0.2–0.5 ml; 800–4,000 nM estimate)	Decreased proliferation (FER) and decreased survival (FES) in FLT3-ITD+ AML but not in non-mutated tested cells
Walters et al. (2006)	JAK2	To determine kinases that cause STAT5 phosphorylation in AML	Electroporation (unknown)	Decreased proliferation and viability in AML HEL cells but not in CML K562 cells. Decreased phosphorylation of STAT1/3/5 and Erk1/2. JAK1, JAK3 and TYK2 had no effect
Walters et al. (2006)	JAK3	To identify activated tyrosine kinases in AMKL cells without FLT3 and KIT mutations	Electroporation (unknown)	Decreased proliferation, inhibition of STAT5 tyrosine phosphorylation, increased apoptosis in AMKL. JAK2 and TYK2 had no effect
Dos Santos et al. (2008)	Lyn (a Src family kinase)	Lyn is highly activated. PP2 (SRK inhibitor) caused decreased proliferation and increased apoptosis	Electroporation (3 μg/100 μl for 2×10^6 cells, 2,143 nM estimated)	Decreased leukemic colony formation, linked to mTOR pathway
Okamoto et al. (2007)	Lyn (a Src family kinase)	Lyn and FLT-ITD interactions in AML	Electroporation (3 μg)	Decreased proliferation in FLT3-IDT+ 32D cells. Decreased STAT5 phosphorylation

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Common mutations and related targets				
Gessner et al. (2010)	AML/MTG8 (transcription factor)	AML/MTG8 fusion gene found in AML. (Also studied MLL/AF4 found in ALL)	Electroporation (unknown)	Reduced clonogenicity, induction of replicative senescence (also decreased TERT expression and increased telomere shortening)
Caligiuri et al. (2007)	c-CBL (mutation)	Identification and study of c-CBL and CBL-b mutations in AML	Electroporation (unknown)	Decreased cell proliferation
Wang et al. (2011)	CIP2A (oncoprotein)	Determine role of CIP2A (involved in cancers) in AML	Electroporation (unknown)	Decreased proliferation, decreased clonogenic activity and increased differentiation
Wang et al. (2011)	FLT3 (AML mutation)	FLT3 over-expressed/mutated in AML	sc-29528 reagent, Santa Cruz (in vitro unknown, 5 × 100 μg/kg every 72 h IP for in vivo without reagent)	Arrested in G0/G1 phase, decreased proliferation in vivo and in vitro, increased apoptosis
Walters et al. (2005)	FLT3 (AML mutation)	FLT3 over-expressed/mutated in AML. Developing multiple methods for inhibiting FLT3 due to need for better specificity and resistance development	Electroporation (1 μg/1 × 10^7 cells)	Decreased proliferation, increased apoptosis, and increased sensitivity to MLN518 (a FLT3 inhibitor)
Spirin et al. (2011)	KIT (c-kit oncogene)	siRNA and shRNA studies target c-kit (over-expressed / mutations in AML)	Lipofectamine 2000 (50–200 nM)	Effects were not studied for siRNA transfections. (shRNA studies)

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Balusu et al. (2011)	NPM1 (molecular chaperone—proteins and nucleic acids)	Common mutation in AML	Electroporation (100 nM)	Chemosensitizes (ATRA and cytarabine), decreased cells in S-phase, induced differentiation, increased apoptosis (NPM1 mutant+ AML)
Fernandes et al. (2010)	Rho, Rac, Cdc4 (Rho family GTPases)	Understanding AML Casitas B lineage lymphoma (CBL) mutations	Electroporation (unknown)	Decreased proliferation (CBL mutation+ AML)
Geletu et al. (2007)	Ubc9 (small ubiquitin-related modifier conjugation)	To identify target proteins of C/EBPalphap30 (mutation that occurs in 10 % of AML)	Electroporation (500 ng)	Prevents differentiation block caused by C/EBPalphap30 (co-transfected) when CD34+/U937 cells go through granulocytic differentiation
Various others				
Fiskus et al. (2006)	EZH2	Effect of EZH2 on AML cells	Electroporation (100 nM)	Co-treatment with LBH589 (inhibitor) decreased colony formation (HL-60 and U937) and increased differentiation (U937)
Gao et al. (2009)	hnRNP K	Role of hnRNP K in drug induced suppression and apoptosis induction	DharmaFECT-4 (100 nM)	Induced apoptosis
Miyazaki et al. (2010)	HO-1	Involvement in AML	Electroporation (unknown)	Reduced cell survival with and without cytarabine
Schepers et al. (2005)	HSP27 (Heat shock protein family, stress response)	Involvement in AML	Oligofectamine 25 nM	Increased VP-16 mediated apoptosis but not CD95/Fas mediated apoptosis

(continued)

Table 2 (continued)

References	Target (role/pathway)	Rationale	siRNA carrier (concentration)	Outcome
Muranyi et al. (2010)	ILK (PI3K-dependent signalling pathway)	Investigation of ILK and FLT3 as targets in AML (inhibitors used for FLT3 suppression)	Accell modified siRNA (unknown)	Decreased leukemic colony formation
Muranyi et al. (2009)	ILK (PI3K-dependent signalling pathway)	ILK role in AML. Possible benefit in targeting both ILK and FLT-3	Electroporation (50 μg per 5×10^6 cells)	Decreased colony formation, increased cell death
Wang et al. (2010)	MMP-2, MT1-MMP, and TIMP-2	Role of MMP-2, MT1-MMP, or TIMP-2 in AML extramedullary infiltration	Lipofectamine 2000 (400 nM estimated)	Decreased invasion
Lu et al. (2008)	NRP-1 (VEGF receptor)	Involvement in AML	Lipofectamine 2000 (unknown)	Decreased proliferation and chemotaxis of leukemic cells
Powell et al. (2009)	OPN (Ser585-survival pathway, cytokine and chemoattractant))	Investigated Ser585-survival pathway. OPN is a secreted protein and therapeutically accessible	Unknown (50–150 nM)	Increased cell death and decreased survival in AML blasts and leukemic stem and progenitor cells
Yang et al. (2012)	S100A8 (autophagy)	To determine S100A8 role in autophagy in AML	Lipofectamine RNAiMAX (unknown)	Increased chemosensitivity, increased arsenic trioxide induced apoptosis, decreased autophagy

siRNA studies which reported significant anti-survival effects in AML cells were selected from a PubMed 'AML siRNA' keyword search. The targets were segregated based on their physiological mechanism (or action)

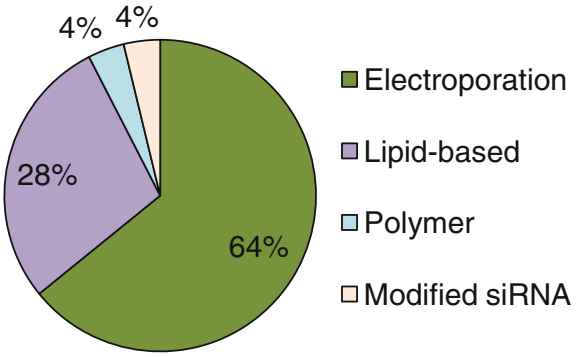

Fig. 2 Different delivery approaches used for siRNA delivery in AML models. The delivery approaches were obtained from the studies summarized in Table 2

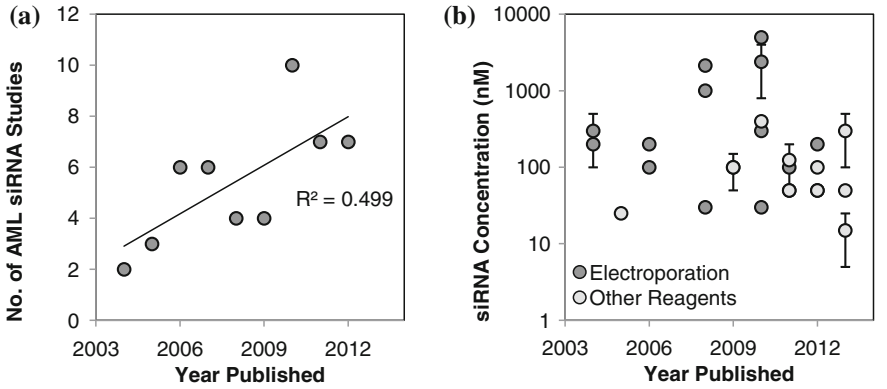

Fig. 3 **a** Number of studies published between 2004–2012 involving siRNA delivery in AML (original data from Table 2). **b** Effective in vitro siRNA concentrations utilized (if reported) in the studies outlined in Table 2. For clinical purposes, one would like to have an effective concentration less than 50 nM. Error bars are displayed to indicate the siRNA concentration range used in a given study

et al. 2009; Lodish et al. 2013). The heterogeneous lipid composition and distribution of hundreds of lipid species present in cell membrane influences the degree of lipid diffusion in the membrane as well, as the thickness and shape (architecture) of the cell membrane. These characteristics are not conserved among the cells and are dependent on the cell type, cellular activity and the constant changes in signaling with the external environment (Lodish et al. 2013; Janmey and Kinnunen 2006). The heterogeneity of these components and the affinity among some of them, such as the affinity between cholesterol and sphingolipids, lead to the formation of clusters along the membrane that are known as lipid rafts (Brown and London 2000; van Blitterswijk 1988). These clusters have their own charge, which can make a siRNA nanoparticle more or less interactive with specific regions on cell surface, leading to different type of interactions along the membrane. The successful integration of the siRNA nanoparticles with the membrane will depend

on the ability to interact to this area by charge affinity or to move to another area of more affinity (Nel et al. 2009). These factors make the cell membrane a dynamically uneven surface with unstable characteristic features for interaction with siRNA nanoparticles (Nel et al. 2009).

6.1 Differences in Delivery Between Suspension-Growing Versus Attachment-Dependent Cells

The delivery of siRNA nanoparticles is challenging when the target cells are suspension-growing (i.e., attachment-independent) cells. The interaction of suspended particles with cells growing in suspension is expected to be different from cells that are attached to other cells or to tissue culture plastic. The siRNA nanoparticles need to be designed in such a way that they are able to bind to suspended cells and promote their entrance into the cells for delivery of nucleic acids. Uptake of siRNA nanoparticles in suspension-growing cells such as leukemic cells has been studied and it has been found that the delivery of nucleic acid moieties is more difficult to achieve in these cells (Valencia Serna et al. 2013).

Initial studies performed in Uludag lab compared siRNA uptake between the adherent breast cancer MDA-MB-231 cells and suspension-growing K562 CML cells with a generally-effective PEI2LA. A 15-fold reduction in siRNA uptake was found in K562 cells in comparison to MDA-MB-231 cells, showing a considerable reduction in the siRNA delivery efficiency. In addition, despite a 29-fold increase in the siRNA uptake with these carriers (in comparison to non-carrier groups), silencing of the green fluorescent protein (GFP) in GFP-positive K562 cells was proven to be ineffective (Valencia Serna et al. 2013). The relatively small amount of siRNA may have not reached its target (RISC for degradation of the mRNA), possibly due to incomplete internalization or endosomal entrapment of the particles (Valencia Serna et al. 2013). Similarly, Lorenz et al. evaluated the interaction of polymeric particles with different cell types and found that when the amount of amino groups of the particles was increased, a greater amount of particles interacted with cell membranes (Lorenz et al. 2006). Moreover, although it was found that the interaction between the cells and nanoparticles was the same with all the cell lines tested, whether attachment-dependent (HeLa and mesenchymal stem cells (MSC), or suspension-growing (KG1a as a model for CD34+ hematopoietic stem cells and Jurkat as model for T cells) cells, the internal location of these particles differed among the cells: particles that interacted with MSC and HeLa (attachment-dependent) cells were located in intracellular compartments, most likely located inside endosomes; while particles that interacted with KG1a and Jurkat (suspension-growing) cells were found at the cell membrane or periphery of the cells (Lorenz et al. 2006), suggesting that these particles were not internalized in the cells and were not able to overcome the cell membrane barrier. An active endocytosis was perhaps limited in the latter cells. Zhao et al. (1996) also

compared the oligonucleotide uptake between leukemic and the different types of normal (from bone marrow or peripheral blood) human hematological cells and found that the uptake differed among the hematopoietic cell types: the uptake was highest in myeloid/macrophages, followed by B-cells, T-cells and finally with neutrophils having the lowest uptake level. On the other hand, human leukemic cells were also found to take up more oligonucleotides than normal or residual cells from the same patient and, this uptake was increased or decreased in leukemic and normal cells upon cell growth factor stimulation and cell growth inhibition, respectively. This led the authors to conclude that the uptake in leukemic cells was greater due to their higher cell growth and activation (Zhao et al. 1996). These observations indicated that the uptake of nucleic acid nanoparticles is dependent; not only on the cell type (i.e., attachment-dependent vs. suspension-growing or attachment-independent), but also on the internalization pathway that could be variable among the attachment independent cells. Thus, not only cationic charges seem to be important for high affinity interactions with cell membranes of suspension-growing cells, there might be also a need of specific ligands (as will be discussed later) for particles to display direct interaction with cell membrane components that lead to a complete cell internalization and continuation with the silencing of the targeted mRNA.

Among the explanations for less effective transfection in leukemic or suspension-growing cells, Labat-Moleur et al. (1996) suggested that the poor transfection ability of cationic vectors in lymphocytes, and other non-adherent cells might be attributed to weak interaction of these vectors due to the lack of Ca^{2+}-dependent cell surface extracellular matrix (ECM) ligands, such as, proteoglycans and cadherins, that are only present in adherent cells. Others have explored further the presence of binding proteins in leukemic cells. Rainaldi et al. for example, had cell culture flasks coated with polylysine and added foetal bovine serum to surfaces so that polylysine could immobilize proteins necessary for binding of K562 cells. The expressions of the most common ECM proteins, including fibronectin (VLA-5), vitronectin ($\alpha_v\beta_3$), collagen (VLA-2) and, hyaluronan (CD44), were then evaluated and, fibronectin was found to be the only cell membrane protein that was expressed in K562 cells attached to a surface under these conditions. The authors concluded that facilitated-adhesion of K562 cells onto polylysine did not occur directly between these two, but rather between the fibronectin receptors on the surface of K562 cells and the fibronectin absorbed onto the polylysine-coated surface by the addition of FBS (Rainaldi et al. 2001).

Sun et al. (2013) investigated the expression of CD44 in different cell lines (SHI-1, THP-1, NB4 and K562) and K562 cells were found to differ from the rest of cells lines: the expression of CD44 in SHI-1, THP-1 and NB4 cells (all cell models of acute myeloid leukemia, AML) was shown to be significantly higher than that of K562 cells. Moll et al. (1998) have also shown that K562 cells do not normally express CD44 protein but that these cells can express this molecule when they are stimulated to differentiate towards the myeloid lineage. The expression of CD44 receptors on the cell surface seems to be a characteristic related to the adhesion and migration of AML cells. Suspension-growing and leukemic cells

seems to not express most of the cell membrane receptors that are involved in the cell adhesion. How the lack of these receptors reduces the interaction with the binding of nucleic acids and their carriers still remains to be clarified.

6.2 Structure-Function Relationships in Cellular Delivery

In order for the uptake to take place, particle binding and engulfment at the adhesion site require specific and nonspecific interactions to overcome the resistive forces that hinder particle uptake (Nel et al. 2009). There are two types of strategies that aim to deliver nucleic acid to the suspension-growing cells, mediated by either unspecific binding or specific binding. Here, we review the latest studies and delivery systems that are being used for improving the delivery efficiency in the suspension-growing leukemic cells. The challenges for the optimization of the design of the nucleic acid delivery carriers are additionally discussed.

6.2.1 Delivery Based on Unspecific Binding to Cells

Calcium phosphate transfection method consists of addition of a co-precipitate containing calcium phosphate and nucleic acids to cell culture. The sedimented co-precipitates are then taken up by cells after non-specific binding of the co-precipitates to the cell membrane (Ravid and Freshney 1998). Jordan and Wurm (2004) used calcium phosphate to transfect attachment-dependent and suspension-growing cells, emphasizing some of their transfection differences. They mention that since adherent cells are at the bottom of the plate, they do not interact with the particles present in the medium. To increase this interaction, the particles should be large enough to settle down by gravity, but the bigger the size the lower the transfection efficiency (expected). When the particle is small, it is unlikely to settle, but its inherent transfection ability would be higher due to its smaller size. The suspension-growing cells are more likely to interact with particles because they are suspended in the medium, however the affinity plays an important role for the particles to tightly interact with the cells (Jordan and Wurm 2004).

Cell penetrating peptides (CPPs) can be up to 30 amino acids long and are inherently able to translocate cell membranes. Overall cationic charge of CPPs confers them the ability to interact electrostatically with the phosphate backbone of nucleic acids to form stable nanoparticles, while allowing them to interact with cell membranes as well. Arthanari et al. (2010) used the cationic Tat-derived CPPs (aminoacids 49–57 of HIV-1 TAT protein) covalently attached to cationic membrane active peptide LK15 (Tat-LK15 peptide) for the delivery of siRNA and plasmid DNA encoding for a short hairpin RNA (shRNA) in K562 cells. The authors found that the combination of these two peptides increased the transfection efficiency by two folds in comparison with Tat peptide alone in

several cell lines. With a dose of 1–30 μg of siRNA in 1 ml (24–729 nM based on our calculation), expression of p210 BCR-ABL was reduced to at least ~70 % 48 h post-transfection for all concentrations. High density of positive charges of siRNA nanoparticles led to cytotoxicity ranging from 0 % (10 μg siRNA) to 30 % cell death (30 μg siRNA) (Arthanari et al. 2010).

Wang et al. (2008) prepared siRNA nanoparticles with functionalized single wall carbon nanotubes (f-SWNTs) and siRNA against cyclin A2, which is involved in cell cycle regulation and associated with proliferation in leukemic K562 cells. These cells were treated with particles formed of f-SWNTs and Cyclin A2 siRNA (25 nM), which resulted in a reduction of cell numbers of up to ~70 % 60 h after treatment in comparison with cells treated with f-SWNTs and control siRNA. No significant toxicity was found with cells treated with f-SWNTs alone or in combination with control siRNA. Both a reduction of the *Cyclin A2* mRNA was found 32 h after treatment as well as a 70 % reduction in the colony formation assay 3 weeks post-transfection (Wang et al. 2008).

6.2.2 Delivery Based on Specific Binding to Cells

Among the most effective specific-binding interaction are those ligands or antibodies coupled onto nanoparticles that allow them to interact with complementary molecules (or receptors) on cell membranes (Nel et al. 2009; Wang et al. 2011). These interactions result in either receptor-mediated endocytosis or receptor-mediated direct penetration in the absence of endocytosis, for example when gold nanoparticles and cell-penetrating peptides are used as delivery carriers (Nel et al. 2009). For nanoparticle adherence and engulfment to take place at an adhesion site, ligand-receptor interactions need to overcome the resistive forces that prevent the nanoparticle uptake. Examples of these resistive forces are the memory of the cell membrane to return to its original form and the hydrophobic exclusion of polar surfaces by the cell membrane (Nel et al. 2009).

Antibody-mediated attachment does not necessarily induce internalization of nanoparticles. For example, doxorubicin-loaded liposomes were attached to anti-CD34 monoclonal antibody that targets CD34+ KG-1a cells. The cell-associated level of Dox in KG-1a cells was found to be lower in comparison with free Dox exposure. Since 40 % of the drug seemed to be released after 2 h of incubation for both systems, this targeted-drug delivered system would need to be used in combination with inhibitors of the P-gp efflux pump—whose overexpression in cancerous cells is known to act as a drug resistant method—so that the Dox extrusion is inhibited and the drug can exert its cytotoxic effect inside the cell. The IC_{50} of Dox-loaded CD34+ liposomes was similar to that of free Dox, but 8 times higher than the non-targeted liposome. Based on confocal and flow cytometry studies that show that neither the liposome nor the antibody were internalized by the cells, the authors suggested that the delivery mechanism of this immunoliposome is binding to targeted cells and Dox at the vicinity of the cells, where the drug is consequently internalized as free Dox. The CD34 cell binding did not seem to be capable of triggering the

liposome cell entry (Carrion et al. 2004) and/or the cells were not able to internalize the Dox-loaded liposomes, probably because of the nature of the liposome used.

Thus, immuno-targeting with antibodies by themselves does not necessarily correspond to high internalization since the carrier needs to also play an important role with cell membrane interactions and internalization. Guillem et al. constructed immunopolyplexes, using 25 kDa PEI-based polyplexes attached via a streptavidin bridge to biotin-labeled antibodies that target cell membrane proteins. A significant selectivity in delivery was observed: an anti-CD3 immunopolyplex was functional only in Jurkat T-cells (CD3+/CD19-), while an anti-CD19 immunopolyplex was functional only in Granta B-cell line (CD3-/CD19+). However, only ~11 % of Jurkat cells and ~2 % for Granta cells were transfected, showing a dependency of the transfection efficiency on the cell type used. Transfection of a mixture of Jurkat and J.RT3/T3.5 cells (a CD3-/CD19- T-cell line) with anti-CD3 immunopolyplexes showed that >80 % of transfected cells were CD3+, indicating the selectivity of the delivery system in a heterogeneous cell population. Viability studies showed a decrease in cell viability to 50 % for Jurkat cells and to 90 % for J.RT3/T3.5 (Guillem et al. 2002), which shows an association of transfection with significant cytotoxicity in this system. Poor transfection was shown with naked 25-PEI (5 % cells were positive for transfection) in comparison with anti-CD3 immunopolyplex (10 % of cells were positive).

In an attempt to develop a delivery system for T-cell leukemias, more specifically against JL1-positive cells, an antibody-coupled CPP (oligo-arginine$_9$) complex was developed (Lee et al. 2010). Uptake studies showed a higher binding affinity of JL1-CPP nanoparticles to JL1-overexpressing cells than Jurkat cell with low JL1 expression, when a 200-pmol siRNA was used and after 2 h of transfection 96 % of the cell population was already FITC-positive. The uptake was shown to be 5.7 % for JL1- negative H9 cells (Lee et al. 2010). No toxicity studies or silencing experiments were performed. Major limitation of peptide delivery systems is their excessive positive charge and lack of target cell specificity, which may result in non-specific tissue distribution and aberrant immunogenic toxicity (reviewed in Lee et al. 2010).

Therefore, using an antibody seems to increase selectivity and enhance the efficacy of the carrier in the last two cases. However, this targeted systems seems to be limited by the efficacy of the carrier used, therefore, a more efficacious carrier could enhance transfection even further.

6.3 Secondary Effects of siRNA Silencing: Off-Target Effects and Cytotoxicity

Transferrin receptor (TrfR) is a cell membrane-associated glycoprotein that is overexpressed in cancer cells, can be easily accessible and can promote endocytosis once its ligand transferrin (Trf) is bound at the cell surface (Mendonça et al. 2010). Mendonça et al. developed a transferrin receptor (TrfT)-targeted sterically stabilized liposomes encapsulating BCR-ABL siRNA. K562 and LAMA-84 cells

were transfected twice every 2 days with a siRNA concentration ranging from 100 to 2,000 nM. Efficacy experiments showed a dose-dependent reduced viability when BCR-ABL siRNA was delivered to these cells: cell viability was reduced up to ~47 % for LAMA-84 cells and up to ~24 % for K562 cells. However, a dose-dependent toxicity (58 % with 2 μM of siRNA) was also seen with scrambled siRNA with LAMA-84 cells, but to a much lower extent with K562 cells. Levels of *BCR-ABL* mRNA were reduced in a dose-dependent manner up to 1 μM (~60 %) with specific siRNA in LAMA-84 cells while no significant reduction was found with scrambled siRNA. Similar results were found for the oncoprotein as well, except that the scrambled sequence reduced non-specifically the protein levels at the highest siRNA concentration (2 μM) (Mendonça et al. 2010). High toxicity and off-targets effect in this system was probably due to high siRNA dose ($2 \times 1 - 2$ μM of siRNA) and the highly cationic carrier.

Eguchi et al. generated a carrier composed of a TAT-peptide transduction domain (PTD) and double-stranded RNA-binding domain (PTD-DRBD); RNAi induction was evaluated in difficult-to-transfect cells, including T cells. GFP siRNA delivered with PTD-DRBD (siRNA concentration of 100–400 nM) in Jurkat T-cells containing an integrated GFP reporter gene resulted in a reduction of the mean GFP fluorescence to ~10 % and also showed an mRNA reduction to 10 %, while Lipofection (100 nM for Lipofectamine 2000™ and 10–50 nM with Lipofectamine RNAiMAX™) reduced the mean fluorescence to 50–60 % and mRNA levels to ~50 %. Similar results were found when targeting CD4 and CD8 in primary murine T cells with PTD-DRBD with specific siRNAs. No toxicity was found in primary human umbilical cord vein endothelial cells (HUVEC) cells treated with siRNA and PTD-DRBD. About 20 % mRNA reduction or off-target effect was seen when scrambled (negative control) siRNA were used either in Jurkat or HUVEC cells (Eguchi et al. 2009). This system is highly effective in silencing however; off-target effects are probably common when high siRNA concentrations are used.

7 Perspective on the Future of siRNA Delivery in CML

New functional carriers that promote efficient delivery of gene-based agents (i.e., siRNA) in a controlled and non-toxic way, are motivating researchers to find physiological solutions for treatment of CML. A better understanding of the clues that lay behind the uptake and intracellular trafficking of siRNA nanoparticles in the challenging suspension-growing leukemic cells will further help in this endeavor. The effect of carrier characteristics such as molecular size, degree of substitution (or modification) and optimal balance of the lipophilic-cationic moieties should be better understood not only on siRNA delivery efficiency, but also on toxicity, intracellular trafficking and cell specificity. This together with the identification of novel siRNA targets that can be used in combination with classical siRNA targets in CML, such as *BCR-ABL*, to silence gene combinations

involved in the activation of different survival pathways in CML should prove beneficial. The combinational delivery, where multiple targets are silenced simultaneously, is likely going to yield more efficacious therapy, and possibly more specific outcomes. Irrespective of the target, however, non-viral siRNA delivery is more likely to be the clinically acceptable approach, given the relatively safe nature of such a delivery mode. The siRNA therapy could act in conjunction with the current clinically-employed drugs to improve their effectiveness or re-sensitize the cells to current drugs. However, the siRNA therapy could also serve as a stand-alone therapy if LSC could be specifically targeted. There is no reason why the delivery methods used for CML cells could not be applied to other types of leukemias, but this will most likely require a different set of biomaterials effective in a particular types of leukemia. Very little information exists on the molecular details for effective carriers in different leukemias so that this should be a fruitful avenue of exploration in the future.

Since the suspension-growing cells tend to be 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 20–50 nM range in culture for a practical translation to preclinical animal models. It is typical for reported delivery system to employ concentrations beyond this range, including our own work (Valencia Serna et al. 2013). Concerted effort to lower efficacious doses will be beneficial in this regard. In addition to efficacy, specificity is important not to down-regulate targets critical in normal physiology. Given the cationic nature of these nanoparticles, they could theoretically bind to a multitude of cells in vivo. However, 'biochemical' targeting could alleviate this limitation to some extent: only those genes that are aberrantly expressed in CML cells, such as the *BCR-ABL* or other supporting mediators, could be the target of RNAi, so that nanoparticles penetrating 'normal' cells might not lead to silencing important targets. On the other hand, in order to increase the specificity of siRNA delivery, polymers could be coupled with CML-specific ligands, such as antibodies, to deliver the siRNA to only certain cell populations. For example, carriers could be coupled with an anti-CD34 antibody to target at least most of the CML stem cell portion. 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 cell specific cell surface binding molecules.

Finally, little information is available on siRNA delivery to primary cells, either healthy or malignant cells from CML patients. It is critical not only to evaluate the efficacy in human cells, but also evaluating off-target effects of the siRNA delivered and cytotoxic effect of the polymers. While cell lines are preferred (due to practical reasons) to optimize cellular delivery, endocytosis rate and intracellular trafficking pathways are expected to be significantly different from cell lines. Misleading directions could be avoided by employing primary cells early on in development process.

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