REVIEW

Progress in RNAi-mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia

Breanne Landry[1], Juliana Valencia-Serna[2], Hilal Gul-Uludag[2], Xiaoyan Jiang[3,4], Anna Janowska-Wieczorek[5], Joseph Brandwein[6] and Hasan Uludag[1,2,7]

Leukemias arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, apoptosis, and survival of the transformed cells. With the advent of RNA interference (RNAi) and the short interfering RNA (siRNA) as its pharmacological mediator, it is becoming possible to modulate specific targets at will. This article summarizes current attempts to utilize RNAi reagents for therapy of leukemias, focusing on acute and chronic myeloid leukemia. We first present unique aspects of RNAi-mediated therapy, followed by a brief background on the delivery technology of RNAi reagents. The need for leukemia-specific delivery of siRNA is discussed by describing approaches that targeted agents to leukemic cells. Pharmacokinetics and biodistribution of RNAi agents are then presented, highlighting the critical issues pertinent to emerging siRNA therapy. Efforts to deliver specific RNAi therapies are then summarized in the context of expected clinical outcomes, focusing on limiting leukemic cell survival, sensitizing malignant cells to chemotherapy, mobilization of leukemic cells, and eradication of leukemic stem cells. We conclude with a perspective on the future of RNAi therapy, emphasizing the technological requirements and mechanistic challenges for clinical entry.

Limitations of Current Leukemia Therapies and Promise of RNAi

Leukemic cancers arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, and apoptosis as well as survival of malignant cells. Approximately 350,000 people worldwide are diagnosed with leukemia annually, leading to ~250,000 deaths each year. An overall 5-year relative survival rate of 56.0% (between 2003 and 2009) is estimated for various leukemias combined.[1] The front-line therapy in leukemia is chemo (drug) therapy,[2,3] including broad-spectrum cytotoxic agents against fast-proliferating cells and small-molecule inhibitors targeting specific signal transduction pathways, so called molecular therapies.[4] The molecular pathogenesis of some leukemias, such as chronic myeloid leukemia (CML), is relatively clear; aberrant juxtaposition of BCR (breakpoint cluster region protein) and ABL1 (Abelson murine leukemia viral oncogene homolog 1) genes constitutively activates a tyrosine kinase (p210$^{BCR-ABL}$), whose signaling initially leads to a chronic phase of myeloid cell expansion, while the expanded cells undergo differentiation in peripheral blood. A range of highly specific tyrosine kinase inhibitors (TKIs) has been introduced for clinical use over the

last decade and significant improvements in patient survival have been achieved. For acute myeloid leukemia (AML), however, no new drugs have been introduced in recent years and clinical therapy has relied on "traditional" broad-spectrum cytotoxic drugs, where the leukemic cells display a differential sensitivity to drugs. The therapeutic index in this case is relatively small, and significant side effects at efficacious doses typically limit therapy at advanced disease.

Leukemic cells generally respond well to drug therapy at the onset of treatment, but the drugs lose their effectiveness over a period of 6–12 months in a significant fraction of patients. It is now well recognized that the resistance to broad-spectrum drugs is inevitable, but recent evidence also indicated that even the most advanced molecular drugs can lose their efficacy.[5] In CML, development of resistance to current front-line therapy imatinib and failure to reach a complete cytogenetic response occurred in 24% of patients within 18 months.[6,7] The inherent plasticity of the cells combined with diverse resistance mechanisms allow malignant cells to naturally adapt to drug assault. Additionally, the high relapse rate in leukemia patients has been attributed to existence of a rare population of leukemic stem cells (LSC) capable of evading drug therapies.[8,9] With better understanding of molecular changes in leukemic transformations, treatments

[1]Department of Chemical & Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada; [2]Department of Biomedical Engineering, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta, Canada; [3]Terry Fox Laboratory, British Columbia Cancer Agency, University of British Columbia, Vancouver, British Columbia, Canada; [4]Department of Medical Genetics and Department of Medicine, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada; [5]Center for Innovation, Canadian Blood Services, Division of Hematology, Department of Medicine, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta, Canada; [6]Division of Hematology, Department of Medicine, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta, Canada; [7]Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada Correspondence: Hasan Uludag, Department of Chemical & Materials Engineering, Faculty of Engineering, 2–021 RTF, University of Alberta, Edmonton, Alberta T6G 2G6, Canada. E-mail: hasan.uludag@ualberta.ca

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that target tumor-specific changes are expected to lead to more effective therapies as the normal cells transform into malignant cells.

To this end, a highly specific leukemia therapy can be developed by exploiting RNA interference (RNAi) to silence the aberrant protein(s) responsible for the disease.[10,11] While current small molecular drugs rely on a specific binding mechanism, whether be an active enzyme site or DNA major/minor grooves, RNAi targets a particular mRNA for destruction (or translational blockage) by binding to specific regions in the mRNA. Unlike point mutations that can abolish drug activity, silencing aberrant proteins with RNAi is less prone to resistance development. The mechanism of action for RNAi reagents is similar to previously employed antisense oligonucleotides (AS-ODN) targeting mRNAs (Table 1), except that RNAi employs endogenous mRNA regulatory machinery to suppress protein production. Furthermore, RNAi can target aberrantly expressed isoform(s) of the protein (as in BCR-ABL fusion protein), unlike drugs that abolish activity of the target nonspecifically (as in both ABL and BCR-ABL proteins). RNAi for leukemia has reached clinical trials in two cases. In the NCT00257647, a viral vector, simian virus 40 (SV40), was utilized to deliver siRNA to CML patients against a fusion gene, but there are no published outcomes from this study. The other trial was a nonviral liposomal siRNA tested in one CML patient. A strategy to combine two or more drugs with nonoverlapping target resistance profiles could delay the emergence of drug resistance.[12] However, new point mutations could still be expected to induce resistance to drug combinations,[13] given the plasticity of LSC. FLT3 inhibitors (midostaurin, AC220 and sorafenib), for example, experience resistance development as a result of secondary FLT3-ITD mutations.[14]

The current review provides a comprehensive summary of RNAi efforts for leukemia therapy. We focus our analysis on myeloid leukemias, specifically AML and CML, where RNAi effort is mostly concentrated (but also provide information on other leukemias as appropriate). RNAi is a therapeutic option for all leukemias but we want to explore the critical issues in-depth that should be applicable to all leukemias (not just myeloid leukemias). We review the important aspects involved in utilization of RNAi reagents, with a particular focus on siRNA since it is likely to reach clinical testing ahead of other related reagents. Delivery of RNAi agents with nonviral carriers and factors affecting therapeutic efficacy have been emphasized. Where appropriate, experience with other types of RNAi reagents is summarized to generate a better sense of possible future progress. Finally, we provide the authors perspective on the future of RNAi in leukemic diseases, and identify hurdles and solutions to clinical deployment of RNAi technology.

Technology of Nonviral RNAi Delivery

The endogenous RNAi mechanism for post-transcriptional gene silencing is triggered by transcription of long pieces of double-stranded RNA (dsRNA) that are subsequently cleaved into smaller (21–23 nucleotides) microRNAs by Dicer.[15] For a pharmacological RNAi intervention, a plasmid encoding for short hairpin RNA (shRNA) or a double-stranded siRNA, to bypass the shRNA transcription and processing steps, have been employed.[16,17] The use of siRNA is more practical in hard-to-transfect primary cells and, in addition, it represents a more physiological mechanism to regulate gene expression as compared to AS-ODN[18] (Table 1). The siRNA is incorporated into the RNA-induced silencing complex (RISC), where Argonaute proteins cleave the sense strand of siRNA for release from the RISC. The activated RISC, which contains the antisense strand of siRNA, selectively seeks out and cleaves or represses the complementary mRNA.[15,16,19] While the activated RISC complex can move on to cleave additional mRNAs, it also gets diluted during cell division,[15] so that repeated siRNA administration may be necessary to achieve a persistent effect. The large, hydrophilic, and anionic siRNA cannot cross plasma membrane and an effective carrier is needed to enable internalization and protection from almost immediate degradation by serum nucleases (Figure 1). Electroporation is a common method to deliver siRNA in culture by creating pores in cell membrane. While helpful to implement RNAi in culture,[20–22] such a method cannot be employed in vivo.[23,24] Viral vectors have been alternatively used both in in vitro and in vivo studies including the clinical trial NCT00257647.[25–28] Although viral vectors are a prospective pursuit for leukemia, they present a significant safety risk due to their ability to integrate into a host's genome and/or cause significant immune responses,[26,29] and will not be further addressed in this review. Cationic biomolecules are safer for clinical deployment; they are capable of complexing and condensing anionic siRNA into spherical, stable nanoparticles (NPs) suitable for cellular uptake. Similar delivery systems can be employed for siRNA and AS-ODN since the molecular composition of siRNA is similar to AS-ODN.

Functional carriers for RNAi agents

Carriers specifically explored for siRNA delivery in leukemic cells include cationic lipids, oligomers of cationic amino acids and other moieties, cationic polymers and various nano-structured materials (Table 2). Once the siRNA reaches the leukemic cell, it must gain entry through the cellular membrane, escape endosomes (if so entrapped) and get effectively released into the cytoplasm. The binding and engulfment of siRNA NPs at the plasma membrane require

Table 1 Different types of gene regulators used for leukemia therapy

Class of Compounds	Characteristics	Source	Examples in clinical leukemia therapy
Antisense oligonucleotides	Double-stranded DNA or modified form	Synthetic	GTI-2040 (ribonucleotide reductase), SPC2996 (Bcl-2), LY2181308 (survivin)
Short interfering RNA	Double-stranded, base-matched RNA	Synthetic	BCR-ABL siRNA
Short hairpin RNA	Double-stranded, base-mismatched RNA	In situ expressed	Not available
MicroRNA	Double-stranded, base-mismatched RNA	Synthetic or in situ expressed	Not available

While AS-ODNs have reached clinical testing, only one siRNA, and no shRNA or microRNA were tested in clinics for leukemia therapy.

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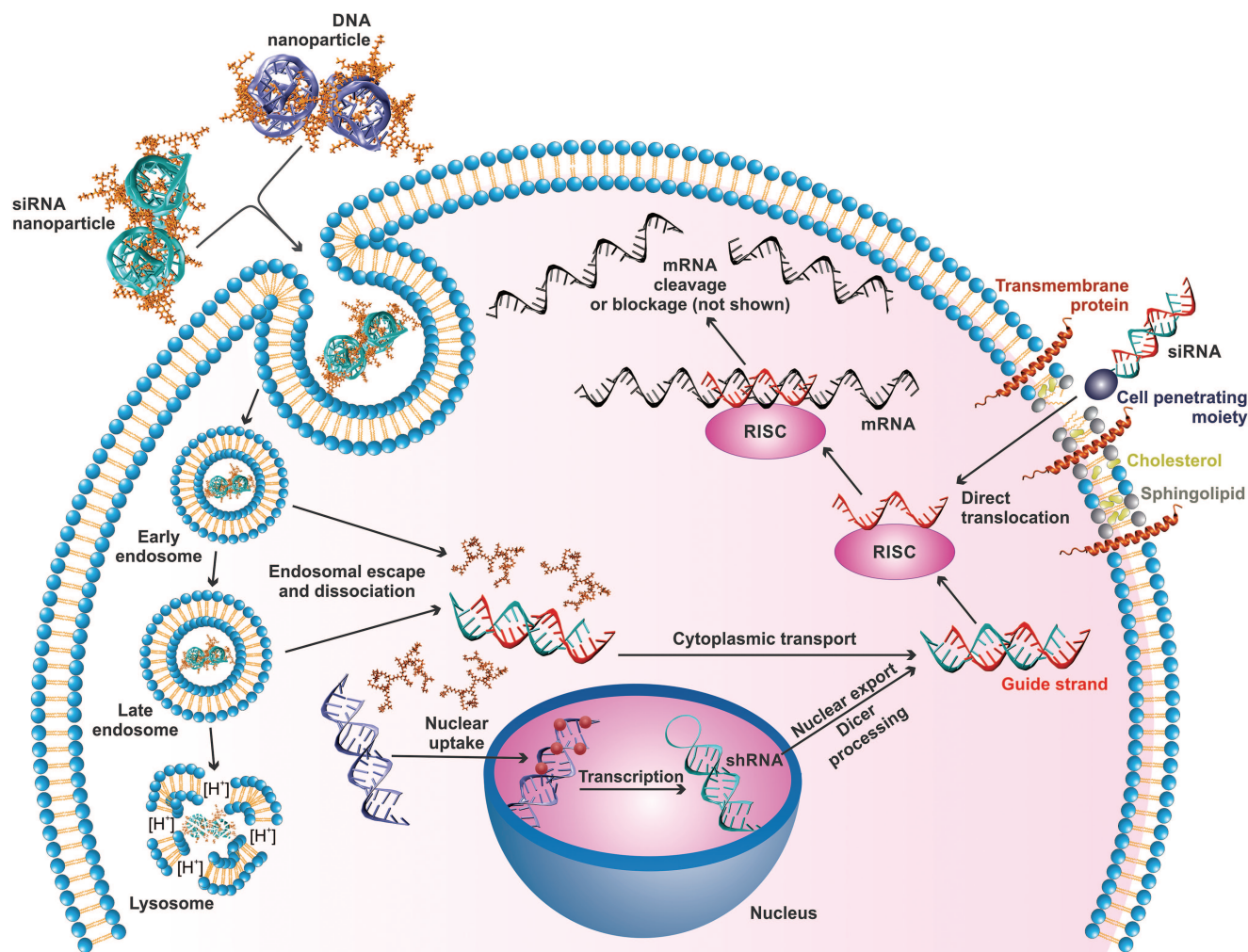Landry et al.

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Figure 1 Main processes involved in nonviral delivery of RNAi reagents (siRNA and plasmid DNA encoding for shRNA) into a cell. The carriers form nanoparticulate complexes with siRNA/DNA that are conducive for passage through cell membrane. Alternatively, chemically modified forms of siRNA can penetrate through cell membrane due to membrane-compatible cell, penetrating moiety and the small size.

effective interactions to overcome the thermodynamics barriers to membrane poration.[30] The lipid composition of the membrane as well as its dynamic nature influences internalization and may contribute to the difference in silencing among different cell types.[31,32] The highly dynamic lipid rafts[33,34] may further "nucleate" interactions with siRNA NPs, leading to different type of affinities along the membrane.[30] Creating cationic NPs capable of interacting with surface proteoglycans has been one approach to enhance siRNA uptake. Cationic single wall carbon nanotubes, for example, were used to silence cell-cycle regulator cyclin A_2 in CML K562 cells;[35] a significant (~70%) reduction of cell numbers was obtained as a result of enhanced apoptosis. When cationic carriers are utilized for delivery, increasing the carrier:siRNA ratio (often referred as the N/P -amine/phosphate- ratio) often improves delivery as a result of increased charge of the complex.[36,37] The cellular uptake of siRNA (binding and internalization) is generally observed to occur within a few hours for both targeted and untargeted carriers, and less (e.g., ~1 hour) for liposomes in AML cells[38] and albumin-coated cell-penetrating peptides (CPPs) in ATLL cells.[39] Interestingly,

a high peak delivery (96%) was achieved with a targeted peptide system at ~2 hours with a rapid decline thereafter.[40] siRNA silencing was not demonstrated with this system and the reason of the rapid decline was not discussed, but could indicate siRNA release (affecting measurable fluorescence levels) or perhaps even exocytosis. siRNA delivery studies, performed with lipid- polyethylenimine (PEI) carrier libraries in CML cells and breast cancer cells confirmed the lower delivery percentage in CML cells. These results initiated further formulation alterations to achieve more comparable delivery in the CML cells.[41]

Cationic CPPs[26,39,40,42,43] composed of 20–30 amino acids with membrane translocation activity were alternatively employed for siRNA delivery. Their polycationic nature enables them to interact electrostatically with phosphate backbones of nucleic acids, while also allowing them to effectively bind to cell membranes. A Tat-derived CPP (amino acids 49–57 of HIV-1 Tat protein) covalently attached to membrane-active peptide (Tat-LK15) was used to complex electrostatically with nucleic acids and deliver them to K562 cells.[26] The combination of these peptides increased the transfection

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Table 2 Nonviral, noncommercial carriers developed for siRNA-based therapy of leukemia

Ref.	siRNA carrier	Carrier design rationale	siRNA targets (cell)	Delivery[a] In vitro	Delivery[a] In vivo	Silencing[b] (nmol/l) In vitro	Silencing[b] (nmol/l) In vivo	Therapeutic effect[c] (nmol/l) In vitro	Therapeutic effect[c] (nmol/l) In vivo
Multiple leukemia types									
45	Lipid NP; Cationic lipids (with alkylated DMA) + neutral lipids + PEG coating	Alkylated DMA key for improved transfection (increased particle order and stability). Protamine, HA, peptides (PPAA and INF7) enhanced transfection.	KIF11, FLT3 (AML (THP-1, KG-1, Molm13, Mv4-11, HEL), CML (K562), Molm13 in vivo.)	X	X	√ (10–500)	√!	√ (10–30)	X
73,120	Modified siRNA; TLR9 antagonist CpG	Not a carrier. Targeted delivery for siRNA. Does not protect against serum nucleases.	STAT3 (AML (MV4-11, patient), MM (KMS-11, patient), TLR9+ hematopoietic cells, human PB blood cells (monocytes, T cells, NK cells, B cells, mDCs, pDCs)^	√	√	√ (500 in MM)	√	√!	√
42,43	Peptide; CPP PepFect6	Characterized and tested amphipathic and arginine-rich CPPs for siRNA silencing. Amphipathic PepFect6 was the most promising CPP. (Comprised of stearyl-TP10 peptide with trifluoromethylquinoline moieties for endosomal escape, effective with serum proteins). Electrostatically formed NP.	Luciferase (reporter gene) (AML (SKNO-1)); HPRT1; (CML (K562) and ALL (Jurkat)) ^	√; X	X; X	√ (50–200); √ (12.5–100)	X; √!	X; X	X; X
44	Fusion protein; PTD-DRBD	DRBD for binding to siRNA, PTD for cellular delivery. Developed for delivery to primary cell and thus also tested in other cell types/animal models. DRBD avidity to siRNA mediated NP formation.	GFP (THP-1 differentiated to macrophages?) ALL (Jurkat)^	X	X	√ (100–400)	X	X	X
46	Lipid NP; Transferrin ligand + cationic lipid DODMA	Microfluidic formation for controlled mixing parameters during self-assembly.	RRM2 (AML (MV4-11) and CML (K562))^	√	√	√ (100–500)	√	√ (100–1,000)	X
AML									
36,118, 119	Polymeric complex; Caprylic or linoleic acid substituted on 2 kDa PEI	Lipids for cell interactions. Low MW PEI for decreased toxicity while maintaining beneficial properties of PEI.	GFP (reporter gene), CXCR4, SDF-1, CD44 (THP-1, KG-1, KG-1a, patient)	√	X	√ (25–100)	X	√ (25–100)	X
49	Chitosan NP; Chitosan	Chitosan is biocompatible, cationic, and adhesive. Electrostatically formed NP.	VEGF, FLT1 (U937)	X	X	√; Unknown	X	√; Unknown	X
37	Micelle; Amphiphilic diblock copolymers (4.5 kDa PCL with 15.5 kDa PDMAEMA or 5 kDa PEG).	PDMAEMA provides charge for siRNA binding and buffering for endosomal rupture. PEG provides colloidal stability/RES protection. Two polymers allow for cell type optimization through cationic charge and resulting toxicity.	Luciferase (reporter gene), RUNX1/ETO (SKNO-1)^	√	X	√ (500)	X	X	X
38	Liposome; Anti-CD33 Ab + EPC/chol/mPEG$_{2000}$-DSPE w/wo PEI 25 kDa core	CD33 for cell targeting; PEI (electrostatically binding to siRNA) increased liposome encapsulation of siRNA however PEI encapsulation did not improve silencing results.	AML1/MTG8 fusion protein (SKNO-1, Kasumi-1)	√	X	√ (600–2,500)	X	√ (30–125)	X

Table 2 Continued on next page

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Ref.	siRNA carrier	Carrier design rationale	siRNA targets (cell)	Delivery[a] In vitro	In vivo	Silencing[b] (nmol/l) In vitro	In vivo	Therapeutic effect[c] (nmol/l) In vitro	In vivo
CML									
41	Polymeric complex; Palmitic acid substituted on 1.2 kDa PEI	Lipid for enhanced cell interactions. Low MW PEI for decrease toxicity. Electrostatically formed complexes.	GFP (reporter gene), BCR-ABL (K562)	√	X	√ (36–100)	X	√ (50–100)	X
50	Chitosan NP; Chitosan	Chitosan for siRNA delivery. Tested in multiple cell types including CML. Electrostatically formed NP.	BCR-ABL (K562)^	X	X	√ (50)	X	X	X
26	Peptide; CPP (HIV-Tat (49–57)) + membrane lytic peptide (LK15)	Peptides for cytoplasm delivery and endosomal escape. Shows studies with shRNA and siRNA. Electrostatically formed NP.	BCR-ABL (K562)	√	X	√ (24–729 Est.)	X	√ (1,428–2,142, Est.)	X
35, 147	Carbon Nanotubes; Ammonium functionalized SWNT	SWNT provides high siRNA loading. Ammonium provides positive charge to bind siRNA. Electrostatically formed.	Cyclin A_2	X	X	√ (25)	X	√ (25)	X
47	Liposome; Transferrin- PEG$_{2000}$-DSPE + Chol/DSPC/ DODAP/C16 mPEG 2000 Ceramide	DODAP (+ve at pH 4/neutral at physiological pH) and optimized buffer concentration provides high siRNA encapsulation.	BCR-ABL (K562 and LAMA-84)	√	X	√ (500–2,000)	X	√ (500–2,000)	X
ALL and ATLL									
40	Peptide; Minibody (anti-JL1) conjugated to oligo-9-Arg- peptide CPP	Specific mini-body against JL1 (specific to leukemic cells and not mature hematopoietic cell). CPP for internalization. Electrostatically formed.	No target (FITC scrambled siRNA) (CCRF-CEM, Jurkat)	√	√	X	X	X	X
51	NP; Chitosan + TPP	Chitosan is biocompatible and adhesive. (Adhesion properties may promote tumor survival). Electrostatically formed NP.	Hsp70 (Jurkat)	X	X	√ (50)	X	√ (50)	X
39	NP; Albumin coated CPP complex	Albumin coating for stabilization and prevention of flocculation. Melittin derived P5RHH peptide for endosomal escape. Electrostatically formed NP.	p65 and p100/52 (NfκB) (F8)	√	√	√ (50–200 Est.)	X	√ (50–200 Est.)	X

Various carrier formulations were categorized based on the type of leukemia they were tested in.
[a]Delivery reported if measured by fluorescent siRNA. [b]Silencing of a target if reported with indicated siRNA concentration (nmol/l). [c]Therapeutic effect of silencing a protein target, if reported with indicated siRNA concentration (nmol/l). "√" indicates study was performed and significant results obtained. "*X*" indicates study was not performed or significant results were not obtained. In all cases, a "√" is only indicated when the studies were performed in leukemic cells. In some cases, delivery, silencing and/or therapeutic effects were demonstrated in nonleukemic cells, the results of which are not shown. "!" Indicates study was done in non-leukemic cells. "^" Indicates testing was also done in other cell types.
AML, acute myeloid leukemia; CPP, cell penetrating peptide; C16 mPEG 2000 Ceramide, *N*-palmitoyl-sphingosine- 1-(succinyl(methoxypolyethylene glycol) 2000); Chol, cholesterol; CML, chronic myeloid leukemia; DC-Chol, 3b-(N-(N,N-dimethylaminoethane)-carbamoyl) hydrochloride; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DODMA, dioleyloxy-N,N-dimethyl-3-aminopropane; Egg PC, Egg phospha-tidylcholine; HA, hyaluronic acid; MW, molecular weight; NP, nanoparticle; PEG, polyethylene glycol; PCL, polycaprolactone; PDMAEMA, poly((dimethylamino) ethyl methacrylate); PEI, polyethylenimine; PPAA, poly(propylacrylic acid) peptide; RES, reticuloendothelial system; TPP, tripolyphosphate; Trf, Transferrin. "Est" indicates concentration was estimated from the data provided.

efficiency by twofold compared to Tat peptide alone. With a dose of 24–729 nmol/l (our calculation), expression of p210 BCR-ABL was reduced by ~70%, but significant cytotoxicity (*i.e.*, up to ~30% cell death) was also observed.[26] Low over-all charge (due to charge neutralization) has been found to be an impediment for delivery with peptides. Thus, TAT has been alternatively combined with a double stranded RNA-binding domain (DRBD) creating a fusion protein for siRNA delivery where DRBD, due to its high avidity for minor-groove recognition, binds the siRNA and masks the siRNA negative charge. Delivery with PTD-DRBD (100–400 nmol/l siRNA) in GFP-expressing Jurkat T-cells resulted in ~90% reduction of GFP fluorescence (in line with mRNA reduction), while lipo-fection (Lipofectamine 2000 and RNAiMAX) was generally less effective, with reduced protein levels of 40–50%. Similar results were found when targeting CD4 and CD8 in primary
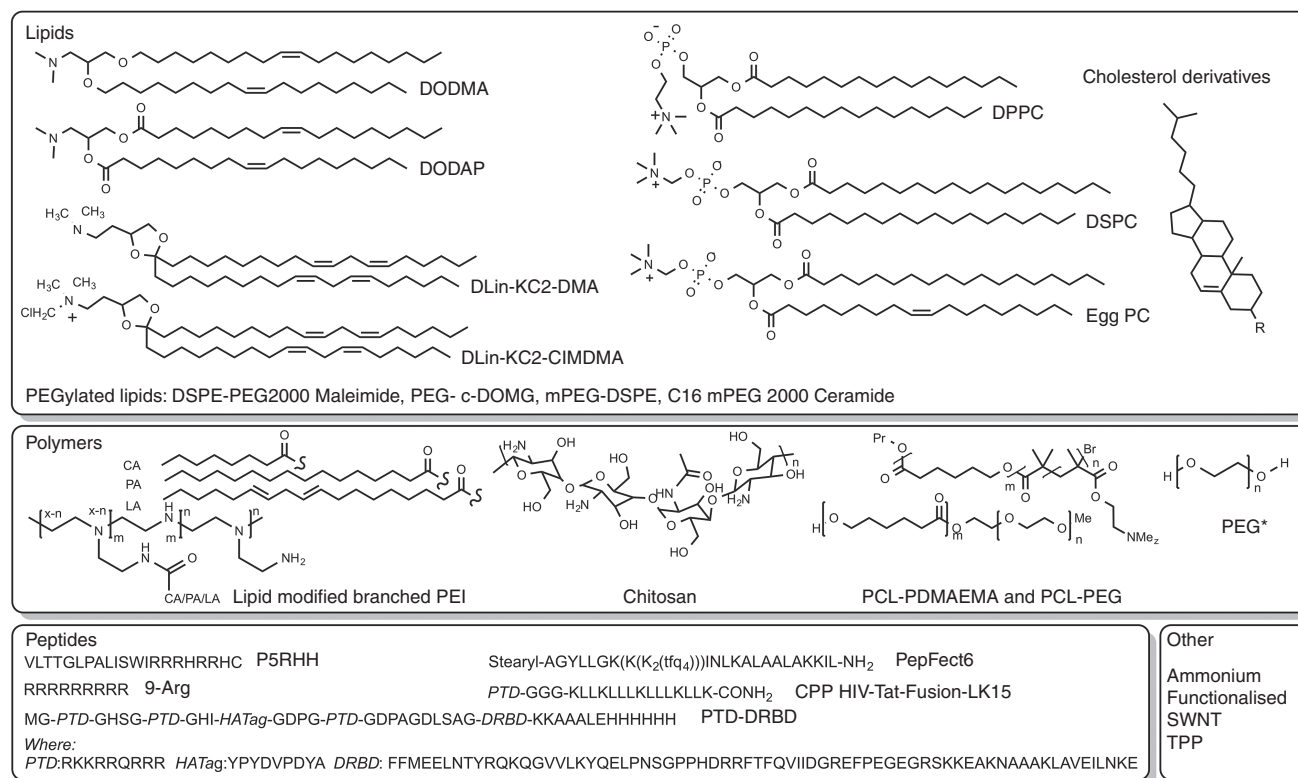
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Figure 2 Chemical structure of carrier components used for siRNA delivery in leukemia. Chemical structures are from studies described in Table 2 with references). CA, caprylic acid; C16 mPEG 2000 Ceramide: N-palmitoyl-sphingosine-1-(succinyl(methoxypolyethylene glycol) 2000); DLin-KC2-DMA: 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-(1,3)-dioxolane; Dlin-KC2-CIMDMA, Alkylated DLin-KC2-DMA; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DODMA, 1,2-Dioleyloxy-N,N-dimethyl-3-aminopropane; DPPC, dipalmitoylphosphatidylcholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphatidylcholine; Egg PC, Egg phosphatidylcholine; LA, linoleic acid; mPEG-DSPE, methoxy-polyethylene glycol (MW 2000) distearoyl phosphatidylethanolamine; PA, palmitic acid; PCL, polycaprolactone; PDMAEMA, Poly((dimethylamino)ethylene methacrylate); PEG, polyethylene glycol; PEG-c-DOMG, R-3-((ω-methoxy poly(ethylene glycol)2000) carbamoyl))-1,2-dimyristyloxl-propyl-3-amine; PEI, polyethylenimine; SWNT, single-walled carbon nanotube; TPP, tripolyphosphate. *PEG is incorporated into carrier structures.

murine T-cells with PTD-DRBD, while no toxicity was found on human umbilical cord vein endothelial cells. About 20% reduction in nonspecific target mRNAs was seen when compared to scrambled siRNA,[44] which was not surprising considering the high siRNA concentrations used. Amphipathic CPPs (TP10, PepFect6, PF14) as well as arginine-rich CPPs (R9, Tat, hLF, and R9-hLF) electrostatically forming siRNA complexes were also attempted for delivery and silencing in SKNO-1 cells.[42] Luciferase reporter silencing was achieved with all peptides, however the amphipathic peptides demonstrated higher silencing ability (60–85% silencing with 50–200 nmol/l siRNA for the best performing CPPs, PepFect6, and Pepfect14), which matched with cellular localization of the amphipathic CPPs being dispersed within the cytoplasm compared to cellular membrane localization of other peptides. The authors highlight physiochemical characteristics, serum protein resistance, polyanion induced decomplexation and cellular delivery (not cell association) to be key for efficient CPP carrier systems as demonstrated in the leukemic cell line.[42]

Lipidic carriers forming solid NP and core-shell liposomes have also proven effective in AML, CML, and acute lymphoblastic leukemia (ALL) cells,[38,45–47] providing significant

in vitro silencing as well as therapeutic outcomes in most cases (Table 2). The lipid components in such NPs were similar to lipids utilized for other cancers,[48] with an overall cationic charge (Figure 2). It was possible to further enhance silencing efficacy in leukemic cells by using modified lipids (DLin-KC2-DMA to DLin-KC2-CIMDMA), in AML and CML cell lines.[45] Another targeted and PEGylated liposomal system utilized PEI within its core, which resulted in better siRNA loading efficiency, but did not improve silencing despite PEI's well known ability to escape the endosome and release siRNA within the cytosol.[38] The lipid carriers may additionally use direct membrane fusion in order to gain entry into cells, bypassing the endocytosis pathway and eliminating one of the bottlenecks during intracellular delivery, namely endosomal escape. While a correlation with endocytosis markers is obtained with recently reported solid lipid NP formulations,[45] the role of membrane fusion remains to be explored in leukemic cells.

Carriers derived from polymers provide ideal control in design and optimization of delivery given the abundance of functional groups. Unlike CPPs, functional groups in polymers could be modified without concern of specific structural motifs, where CPPs may rely for delivery. PEIs that can serve
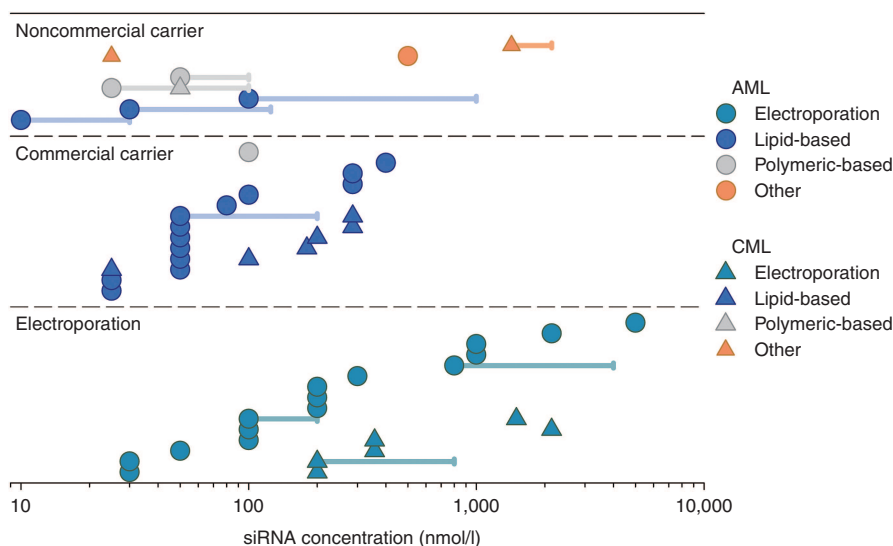
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Figure 3 **Effective *in vitro* siRNA dose ranges for experimental siRNA therapies in AML and CML with nonviral carriers**. The "markers" (circle/triangle) indicate the lowest dosage utilized for siRNA silencing that produced a therapeutic effect on myeloid leukemia cells whereas the "lines" indicate any additional dosage range utilized that also provided a therapeutic effect in the reported study. The dose ranges were obtained from *in vitro* studies that demonstrated therapeutic effects and reported both the siRNA concentration and nonviral carrier utilized in **Supplementary Table S1**. Where necessary, siRNA concentrations were estimated by the authors from the reported amounts and volumes used in specific experiments. AML: Electroporation (refs. 140,141,154,156,163,166,171,172,216–222), commercial/lipid-based (refs. 151,155,159,160,177,183,223–229), commercial/polymeric-based (ref. 230), noncommercial/lipid-based (refs. 38,45,46), noncommercial/polymeric-based (refs. 118,119), noncommercial/other (ref. 73). CML: Electroporation (refs. 20,22,142,150,231,232), commercial/lipid-based (refs. 177,227,228,233–235), noncommercial/polymeric-based (ref. 41), noncommercial/other (refs. 26,35).

as nonspecific carriers in a range of adherent cells[48] have been derivatized with lipophilic moieties to make them effective in leukemic cells (**Table 2**). The "proton-sponge" feature of PEIs that facilitates endosomal escape of nucleic acids[16] presumably aids in effectively liberating internalized siRNAs in leukemic cells. By modifying the amine groups of low MW (1.2–2.0 kDa) PEI, we designed a range of lipid-substituted PEIs. Our studies with AML cells indicated linoleic acid (C18:2) and caprylic acid (C8) substitution to sustain silencing of a reporter (GFP) and the CXCR4 gene;[36] however, the polymers that were effective in CML cells were different and we found a particular polymer (1.2 kDa PEI) substituted with a relatively high amount of palmitic acid (C16) to be most effective. The ability of this polymer to deliver siRNA intracellularly was high, underpinning its relative efficiency. The oncogene BCR-ABL was effectively silenced in CML (K562) cells, resulting in induced apoptosis of target cells.[41] The liposomal agent Lipofectamine 2000 seemed to be equally effective to the polymeric carrier in the K562 model of CML, but this carrier is not recommended for *in vivo* use. Amphiphilic diblock polymers, which form micelles, have been also explored for siRNA delivery.[37] Two diblock copolymers PCL-PDMAEMA and PCL-PEG were utilized in these formulations, so that the components responsible for endosomal escape (PDMAEMA) and protection from reticuloendothelial system (PEG) could be independently optimized. The natural polymer chitosan has also been utilized as an effective carrier due to its perceived biocompatibility,[49–51] but heterogeneity in chitosan structure and ubiquitous activities of its low molecular weight forms may complicate its clinical utility.[52]

Additional functionalization of carriers for siRNA delivery was required in some cases.[29,30,53,54] Bioactive peptides

for endosomal escape (*e.g.*, P5RHH in albumin-CPP complexes[39], LK15 in a fusion peptide,[26] and stearyl-TP10 in CPPs[43]) and other biomolecules for siRNA release (*e.g.*, protamine, HA, and peptides PPAA and INF7 (ref. 45)) were explored. Cationic CPP-siRNA complexes were found to become negatively charged with decreased particle size when measured in the presence of serum, indicating coating with serum proteins. Alternative methods of cellular uptake (such as scavenger receptors) might occur rather than the expected electrostatic/surface proteoglycans interactions.[42] Stability of CD33 targeting liposomes were tested *in vitro* by incubation in 50% human plasma for up to 10 days and showed a loss of binding of 30–40% after one day with no further significant changes.[38] The CPPs and polycationic carriers are expected to suffer from excess protein adsorption in serum, given their repetitive and high charge density, but lipid NPs are also expected to suffer from un-specific protein adsorption due to their hydrophobic surfaces; the difference might be the nature of adsorbed proteins and corresponding fate of the NPs,[55] which is awaiting detailed studies in leukemia models.

While successful deployment of different carriers is encouraging, their performances, measured as the effective siRNA concentrations (**Figure 3**), are highly variable, with some delivery systems yielding an effective therapy at <50 nmol/l while others requiring ~1,000 nmol/l. This analysis inherently assumes that the best results (*i.e.*, most effective doses) were obtained with the optimized formulation for each carrier (not necessarily the same N/P ratio, carrier concentration, etc.). The absolute level and turnover rate of target mRNA, as well as characteristics of cell models (*e.g.*, surface proteoglycans, proliferation rate, etc.) could contribute to this variability

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Table 3 Ligands used for derivatizating NPs to deliver chemotherapy drugs, plasmid DNA, siRNA (bolded), and microRNA to leukemic cells

Ligands	Carrier derivatized	Payload	Leukemia type	Evaluation (delivery and/ or silencing)	Ref.
Ab antiCD3 or Transferrin	PEI	Plasmid	CML (K562), ALL (Jurkat E6-1), melanoma (H225), murine melanoma (B16F10), neuroblastoma (Neuro2A)	In vitro	90
Ab antiCD3 or antiCD19	Streptavidin-PEI	Plasmid	ALL (Jurkat Clone E6-1 and J.RT3-T3.5), Lymphoma (Granta 519)	In vitro	93
Ab antiCD19	Liposome	Norcantharidin	ALL (Nalm-6 Pre-B, patient), CLL (Raji)	In vitro	256
Ab antiCD20	Lipopolyplex	AS-ODN	CLL (patient, Raji)	In vitro/vivo	94
Ab antiCD33	PLGA (core) with albumin (shell)	Everlimus, sorafenib	AML (KG1a)	In vitro	257
Ab antiCD33	**Liposome w/wo PEI core**	**siRNA**	**AML (SKNO-1, Kasumi-1)**	**In vitro**	38
Ab antiCD33	Liposome	Cytarabine	HL60	In vitro/vivo	92
Ab antiCD34	Liposome	DOX	AML (KG-1a)	In vitro	97
Ab antiCD37, antiCD19, an-tiCD20 or combination	Liposome	Fingolimod	CLL (patient, Raji, RS11846, Ramos, Daudi)	In vitro	258, 259
Ab antiCD2, antiCD3, antiCD5, antitransferrin	Liposome	Methotrexate-γ-aspartate	ALL (Jurkat, Molt-4, CEM)	In vitro	91
Ab antiCD74 (milatuzumab)	Liposome	Dexamethasone	CLL (patient, Raji), ALL (697)	In vitro/vivo	260
Ab antiCD96 or antiCD117 (c-kit)	PEG/Calcium phosphosilicate	Indocyanine green	LSC AML (AML patient cells) or LSC CML (murine 32D-p210-GFP; CML patient)	In vitro/vivo	106
Ab antiJL1	**CPP**	**siRNA**	**ALL (CCRF-CEM, Jurkat, H9)**	**In vitro/vivo**	40
Ab antiJL1	Poly(L-lysine)	Plasmid	ALL (MOLT-4)	In vitro	261
Ab antiIgM	HPMA copolymer (star or classic)	DOX	Murine BCL (BCL1)	In vitro/vivo	262, 263
CLL1 peptide	Micelle	DAUN	LSC AML (A549 expressing CLL1; Cd34+ leukemic patient)	In vitro	74
CLL1	Magnetic NPs	Paclitaxel	CML (K562)	In vitro/vivo	75
Aptamer against PTK7	Polyplexes	pDNA	ALL (MOLT-4)	In vitro	59
Aptamer against PTK7	Hairpin DNA/Au	DOX	ALL (CCRF-CEM)	In vitro	77
Aptamer against PTK7	Single-walled carbon nanotube	DAUN	ALL (MOLT-4)	In vitro	78
Aptamer against PTK7 or KK1B10	DNA core connector/Photo-cross-linked	Intercalating drug (DOX) and AS-ODN	ALL (CCRF-CEM), CML (K562, DOX R K562),	In vitro	79
CpG- oligodeoxy-ribonucleo-tide against TLR9	*****	**siRNA**	**AML (MV4-11, patient), MM (KMS-11, patient), TLR9+ hematopoietic cells**	**In vitro/vivo**	73
Transferrin	**Liposome**	**siRNA or AS-ODN**	**CML (K562 and LAMA-84)**	**In vitro**	47
Transferrin	**Lipid NP**	**siRNA**	**AML (MV4-11) and CML (K562)**	**In vitro/vivo**	46
Transferrin	Liposomal with PEI/MiR core	miR	AML (Kasumi-1; OCI-AML3; MV4-11; patient)	In vitro/vivo	60
Transferrin	Lipopolyplex	AS-ODN	AML (kasumi-1, patient)	In vitro	61
Transferrin	Lipid/protamine	AS-ODN	AML (MV4-11), CML (K562), CLL (Raji)	In vitro	62
Transferrin	PEG-Cyclodextrin/PEG-adamantane/transferrin-PEG-adamantane	Plasmid	CML (K562)	In vitro	63
Transferrin	Liposome	AS-ODN	CML (K562)	In vitro	64
Transferrin	has	Sorafenib	CML (K562; imatinib/dasatinib resistant K562; imatinib refractory patient)	In vitro	65
Transferrin	Liposome	Rhodamine-PE (label)	ALL (CEM, MOLT-3)	In vitro	66
LDL	LDL	DiO (label)	CML (K562, KCL22, patient), AML (HL60, AML3), enriched MNCs, prostate (PNT1A, PC3), non-CML patient (quiescent LSC)	In vitro	69

Table 3 Continued

Ligands	Carrier derivatized	Payload	Leukemia type	Evaluation (delivery and/ or silencing)	Ref.
LDL Peptide	Liposome	DAUN	AML (THP-1 and NB4)	*In vitro/vivo*	70
LDL	Liposome	Hygromycin B	Guinea Pig ALL (L$_2$C)	*In vitro*	71
LFA-1 Peptide	PLGA	Empty/ coumarin-6 (label)	ALL (Molt-3, Molt-4), AML (U937, HL-60)	*In vitro*	264
Folate	Dextran/retinoic acid micelles	DOX	AML (KG-1)	*In vitro*	82
Folate	Liposome	DAUN, DOX	AML (MV4-11, CHO-K1, KG-1, KG-1a), CML (K562), Human Epidermoid Carcinoma (KB), Murine Leukemia (L1210, L1210JF), Folate-Beta transfected cells (CHO-FR-Beta),	*In vitro/vivo*	83–86
Folic acid	Au	FITC (label)	ALL (CCRF-CEM)	*In vitro*	265
Folic acid or transferrin	Polylysine	AS-ODN	AML (HL-60)	*In vitro*	67,68
Alendrondate (bone) and folate (CML)	Lipid carrier	Mitoxantrone	CML (K562)	*In vitro/vivo*	87
Anisamide against sigma receptors	Lipid-coated nanoscale coordinated polymers	Methotrexate	ALL (Jurkat)	*In vitro*	88
Biotin	Single-walled carbon nanotube	Taxoid	Murine Leukemia (L1210FR)	*In vitro*	80
Streptavidin + Biotin-G-CSF, antiCD33 Ab or antiCD7 Ab	Liposome	Cytarabine	AML (Kasumi-1 IMS-M2), CML-BC (MEG-01 and K562), ALL (Jurkat, KOPN- 30)	*In vitro/vivo*	81
Peptide against MMP-2 and MMP-9 receptors	Liposome	Adriamycin, Rhodamine B (label)	AML (U937), Other (CHO, NRK52E, HT1080)	*In vitro*	72
Saccharide against lectins	Liposome	Sarcolysine	AML (HL-60), lung adenocarcinoma (ACL)	*In vitro*	76

While initial studies were focused on delivering chemotherapeutic drugs, the developed systems were subsequently adopted for delivery of polynucleotides. Ab, antibody; ALL, T-cell acute lymphoblastic leukemia; AML, acute myeloid leukemia; AS-ODN, antisense oligonucleotide; Au, gold; BCL, B-cell leukemia; CLL, chronic lymphocytic leukemia; CLL1, C-type lectin-like molecule-1; CML, chronic myeloid leukemia; CPP, cell-penetrating peptide; DOX R, doxorubicin resistant; DAUN, Daunorubicin; G-CSF, granulocyte colony-stimulating factor; HA, hyaluronic acid; HPMA: N-(2-hydroxypropyl)methacrylamide; HSA, human serum albumin; LDL, low-density lipoprotein; LSC, leukemic stem cells; miR, microRNA; MM, multiple myeloma; NP, nanoparticle; PEG, poly(ethylene glycol); PEI, polyethylenimine; PLGA, poly(lactic/glycolic acid); PNA, Triplex-forming peptide nucleic acids; PTK7, protein tyrosine kinase 7; TLR2, toll-like receptor 2. *Not a true NP. Non-NP systems for the table summary were restricted to nucleotide transfection related payloads.

and perceived relative efficiency of the delivery system, but little emphasis has been placed on exploring this variability, which will be ultimately critical to understand patient-to-patient variation in therapeutic responses. For *in vitro* utility, formulations effective in the 10–50 nmol/l range will be desirable. Based on analysis in **Figure 3**, noncommercial carriers appear to be equally effective as commercial carriers, but the difference might be better revealed in animal models, where the data is limited to-date. Improved performance would be anticipated with newly generated carriers, but our previous analysis[56] did not indicate the new carriers improving in efficacy (*i.e.*, lowering the effective doses of siRNA reagents), leading to proliferation of the type of effective carriers possible but not necessarily leading to carriers with improved efficacy. Towards this goal, more effective therapies may rely on "leukemia-seeking" carriers in the future.

Selective delivery to leukemic cells
Most siRNA studies in leukemia focus on downregulating a target protein to elucidate its function or to develop small molecular drugs against this target, rather than employing siRNA as a therapy. Delivery systems are beginning to be tailored for leukemic cells with a focus on conventional drugs so far, but the information gained will guide the siRNA delivery in the future. Understanding NP uptake in hard-to-transfect nonadherent leukemic cells is important; we noted that CML K562 cells displayed a 15-fold reduction in siRNA uptake using the same lipophilic PEI carriers[41] compared to breast cancer MDA-MB-231 cells. Since the amine content in NPs is the primary determinant of cell interactions,[57] less effective uptake by leukemic cells might be due to relatively weak binding of siRNA NPs due to deficient Ca^{2+}-dependent ligands, such as proteoglycans and cadherins.[58] In attachment-dependent cells (*i.e.*, HeLa and mesenchymal stromal cells, MSCs), NPs were found in intracellular compartments, most likely inside endosomes, while in KG1a and Jurkat cells, NPs were located at the cell membrane or periphery,[57] suggesting active endocytosis to be limited in leukemic cells. A recent study, however, noted a good correlation between the caveolae-mediated endocytosis activity and siRNA uptake,[45] indicating endocytosis, no matter to what extent, to be still critical in leukemic cells. Although weak delivery to leukemic cells can be overcome by increasing the dose (or using more carriers), this results in nonspecific cytotoxicity.[59] Effective delivery to leukemic cells might need to rely on cell-targeting ligands that not only concentrate siRNA at leukemic cells but also encourage endocytic uptake. While the NP uptake can occur through multiple pathways during endocytosis,

therapeutic effect of the payload might not necessarily be equal along all pathways.[45]

Employing ligands specific for leukemic cells. Antibody (Ab)-mediated delivery has been used to target surface proteins overexpressed or differentially-expressed on leukemic cells (Table 3). Other ligands were derived from peptides/proteins, aptamers, saccharides, benzamides, and ODNs with targets including transferrin receptor,[46,47,60–68] low-density lipoprotein,[69–71] matrix metalloproteinase receptors (MMP-2/9),[72] toll-like receptor,[73] C-type lectin-like molecule-1 (CLL1 receptor),[74,75] lectins,[76] protein tyrosine kinase 7 (PTK7),[59,77–79] vitamin receptors for biotin,[80,81] folate/folic acid receptor,[67,68,82–87] alendronate (bone),[87] and sigma receptors.[88] Some of the ligands target "endocytosing" receptors on cell surface, while others such as CPPs facilitate uptake without necessarily undergoing endocytosis.[30,89] Combining ligands with different functionalities can further enhance delivery; for example, (i) a JL1-specific Ab with CPPs[40] yielded higher siRNA delivery in JL1-overexpressing ALL cells (~96% JL1^{high}-CEM cells versus ~6% JL1^{low}-Jurkat cells) and *in vivo* to CEM cells located in the bone marrow, and (ii) targeting bone marrow with alendronate along with leukemic cells (with folate) improved therapeutic effect *in vivo*.[87] The NPs may follow different pathways than the targeting ligand and optimization of conjugation chemistry and ideal ligand density is needed,[63] since "more" is not always "better" for affinity and final delivery.[85] Some ligands are very specific for certain leukemias, but others, such as transferrin and folate, function in several types of leukemias, making it possible to develop more generic delivery systems.

Relying on targeting to improve endocytosis. When untargeted lipid NPs were delivered to leukemic cells displaying low (K562 and HEL cells), medium (Molm13 and THP1 cells), or high (Mv4-11 and KG1 cells) propensity for transfection, the levels of endocytosis-related genes, caveolin 1, caveolin 2 and Rab13, were found to correlate to level of transfection.[45] Caveolin 1 and 2 expression were also correlated with transfection difficulty in other adherent and difficult-to-transfect cells.[45] The native endocytosis capabilities can be harnessed by employing ligands that induce endocytosis upon receptor binding on the surface of cells. Transferrin is an iron-binding glycoprotein that binds to its receptor in iron-loaded form for endocytosis. The iron requirement increases in rapidly dividing malignant cells and thus transferrin receptors are often over expressed.[63] In early studies, transferrin-PEI conjugates increased transfection (with pDNA) 10–100-fold in CML (K562) cells and transferrin has been successfully employed in NPs carrying siRNA, miR, AS-ODN, and plasmids with functional release of the payload and therapeutic outcomes in CML, AML, and CLL models.[46,47,60–64,68,90] While transferrin-conjugated liposomes encapsulating a BCR-ABL siRNA provided effective silencing in CML cells, effects on other proteins and cell viabilities were also observed, likely as a result of high concentrations and repeat treatments.[47] Transferrin-targeting lipid NPs also provided efficient delivery and silencing of R2 subunit of ribonucleotide reductase (RRM2) protein (via siRNA) in both CML (K562) and AML (MV4-11) cells.[46] Transferrin-conjugated liposomal NPs with

a PEI/miR-29b core increased uptake and delivery of their payload and resulted in decreased cell and colony numbers in AML cells.[60] The targeted NPs also provided prolonged survival of mice compared with scrambled miR delivered with the same NPs.[60] Transferrin-lipopolyplexes also provided targeted delivery of an AS-ODN (GTI-2040) against RRM2, where targeted delivery greatly improved mRNA and protein suppression in an AML model (kasumi-1 cells) and patient cells, and sensitized the cells to cytarabine.[61] Transferrin targeting with lipid NPs for delivery of AS-ODN GTI-3139 (against Bcl-2) was also successful in suppressing Bcl-2 levels in leukemic cells, resulting in a potential therapeutic effect.[62] While providing support for the potential of leukemia-specific delivery, transferrin-mediated targeting has highlighted the importance of ligand incorporation method in successful targeting,[63] where lysine-mediated attachment of PEG to transferrin provided the least decrease in binding affinity and higher transfection in CML K562 cells.[63]

As an alternative to transferrin, folic acid (*i.e.*, folate) that can cause endocytosis upon receptor binding has been incorporated into polylysine for delivery of AS-ODN against c-myb in AML HL-60 cells[67] as well as for chemotherapy drug carrying micelles, liposomes, and lipid carriers.[82–87] An important consideration of folate is its effect on *in vivo* clearance; folate-functionalized liposomes gave faster clearance possibly due to folate receptor-β expression on phagocytic cells of the reticuloendothelial system.[85] This is not unique to folate and others ligands, such as all-trans retinoic acid[86] and CD33-targeting antibodies[81] also affected the *in vivo* pharmacokinetics of the delivery systems.

Antibody-mediated targeting. Targeting with Abs is especially attractive due to its wide applicability. One can envision incorporating Abs directly into carriers, or using a secondary Ab to target cells already labeled with a primary Ab.[81,91] The former approach is more likely to be amenable for pharmaceutical development. Early efforts have identified functional Abs against CD2, CD3, and CD5 in ALL cells,[90,91] but transferrin-mediated uptake was found to be superior to Ab-targeting in one study.[91] Representative formulations recently explored for leukemia include; (i) a CD33-seeking liposome showed improved delivery and silencing in AML cells (CD33 has little expression in hematopoietic stem cells and nonmyeloid cells[92]), albeit the siRNA concentrations were high and an improvement in efficacy was needed;[38] (ii) a CD3-seeking polyplex was functional in Jurkat T-cells (CD3+/CD19-), while a CD19-seeking polyplex was functional in Granta B-cells (CD3-/CD19+) for plasmid delivery,[93] with good selectivity in a heterogeneous cell population. However, only ~11% of Jurkat cells and ~2% for Granta cells were transfected, indicating difficulties in transfecting nonadherent cells once again, and; (iii) a CD20-seeking lipopolyplex was used to suppress Bcl-2 in CLL with AS-ODN G3139, which suffered from low delivery and immune stimulation when delivered naked, providing reduced immunostimulatory effects and improved Bcl-2 silencing in CLL cells.[94] The complications related to Fc domain-related systemic clearance by macrophages might be circumvented with Fab' fragments of Abs.[92] The accumulated experience with antibody–drug conjugates (ADCs) may independently highlight functional Abs capable

of seeking leukemic cells[95] and hence facilitating leukemia-specific delivery. Although most ADCs were pursued for nonmyeloid leukemias, the promising anti-CD33 ADC bring CD33-based targeting to forefront for design of AML-specific carriers.[96] The undesirable activities of ADCs (as exemplified by clinical experience with the anti-CD33 ADC Gemtuzumab ozogamicin), however, could not be readily translated to other cases, since such activities reflect the combinational affect of the specific Ab and the conjugated drug.

Ab-mediated NP targeting might not always lead to enhanced internalization. In the case of doxorubicin-loaded liposomes attached to an anti-CD34 mAb, the IC_{50} of the delivery system was eightfold higher than nontargeted system in $CD34^+$ AML (KG-1a) without any evidence of increased internalization.[97] This was attributed to local release in the vicinity of cells and rapid transport of doxorubicin through cell membrane. This might be limiting for siRNA therapeutics since locally released siRNA cannot enter cells on its own. If it is the NP that limits internalization (*e.g.*, a particular type of liposome), other types of NPs, such as poly(lactic/glycolic acid) NPs that demonstrated high internalization even without targeting, could be more useful.[98] Alternatively, modified siRNAs capable of entering cells on their own might be required. Chemically-modified siRNAs (*e.g.*, with palmitic acid,[99] cholesterol,[100–102] CPPs,[103] and oligodeoxyribonucleotides[73]) have been described that traverse the cell membrane on their own or via specific receptors. Only the latter agent was explored in leukemia; a TLR9 agonist CpG-oligodeoxyribonucleotide (with STAT3 or Bcl-X$_L$ siRNA) yielded effective silencing in normal TLR9+ hematopoietic cells, KMS-11 multiple myeloma and MV4-11 AML cells, and delivery in multiple myeloma and AML patient cells.[73] *In vivo* intratumoral delivery to MV4-11 xenografts gave delivery to ~76% of tumor cells (100 μg siRNA) and effective silencing of STAT3 and Bcl-X$_L$ (>60%).

Finally, Ab-mediated targeting holds great potential for specific delivery to LSC since they are usually refractory to current drugs. Numerous LSC surface protein targets for monoclonal Ab therapy have also been highlighted (CD25, CD32, CD44, CD47, CD96, and CD123, CLL1)[104,105] and one could foresee their use in NP targeting as well. Using calcium phosphosilicate NPs, a photoactivatable drug (indocyanine green) was delivered to AML and CML LSC by using CD96 or CD117 Abs, respectively, which dramatically improved the efficacy.[106] C-type lectin like molecule-1 (CLL1) was additionally employed, as CLL1 is expressed on AML LSCs and CD38+ progenitor cells but not on CD34+/CD38- hematopoietic stem cells.[74,107] A ligand for CLL1 was also utilized on magnetic NPs to take advantage of receptor-mediated endocytosis in CML K562 cells.[75]

Aptamers for targeting. Aptamers, synthetic ODNs, or peptides with engineered binding affinities and specificities, is another ligand type that attracted recent attention. Anionic aptamers can be electrostatically attached to cationic NPs. An aptamer (sgc-8c), which recognizes protein tyrosine kinase 7 (PTK7) present on ALL cells, was utilized for targeting PEI/plasmid polyplexes and carrying a luciferase reporter plasmid to MOLT-4 cells,[59] hairpin DNA-Au NPs delivering doxorubicin to CCRF-CEM cells,[77] and daunorubicin loaded single-walled carbon nanotubes to MOLT-4 cells.[78] Additionally, PTK7 as well as KK1B10 (for directing to doxorubicin resistant K562 cells) provided targeting for an aptamer-DNA NPs delivering doxorubicin (intercalated with DNA) and antisense oligonucleotides.[79] Given the established history with Abs, clinical deployment of Abs in leukemia-specific delivery may be accelerated (compared to aptamers), but the myeloid leukemias do not seem to have a whole range of targeting Abs, unlike lymphocytic leukemias, so that aptamers may fill this niche if myeloid leukemia-specific aptamers could be generated.

Targeting adhesion receptors with their ligands. There is usually a low level of expression of receptors for attachment proteins in leukemic cells; K562 cells displays only fibronectin receptors (VLA-5) on cell surfaces, but not vitronectin ($\alpha_V \beta_3$), collagen (VLA-2), or hyaluronan (CD44) receptors,[108] but they could be induced to express CD44 upon differentiation into myeloid lineage.[109] Unlike K562 cells, AML cells SHI-1, THP-1, and NB4 cells[110] express significant levels of CD44, which is involved in mobilization of leukemic cells.[111] Although others have explored CD44 for various malignancies by utilizing its endogenous ligand hyaluronic acid (HA),[112] few have focused on leukemic disease. A HA-coated chitosan-triphosphate NP was investigated for delivery to high CD44-expressing macrophages (murine RAW 264.7) and low CD44-expressing K562 cells.[113] Although targeted-NPs were not compared to nontargeted NPs, plasmid transfection efficiency was in proportion to CD44 levels in target cells. Using dual targeting with mannose and HA, beneficial effect of HA was independently shown in macrophages (RAW 264.7) as well as in AML (THP-1) cells.[114] The highly relevant CXCR4, involved in homing to bone marrow microenvironment and survival pathways, was not targeted in leukemic models, but pursued in other systems. A cationic peptide (T22) targeting CXCR4 provided enhanced intracellular delivery to self-assembling NPs in CXCR4+ cells including HeLa and metastatic colorectal cancer model cells (SW1417).[115] In another study, CXCR4 Ab-mediated targeting of liposomes carrying lipocalin-2 siRNA were delivered to CXCR4+ breast cancer cells; CXCR4 Ab was utilized as an additive therapy to lipocalin-2 siRNA, not for demonstrating CXCR4 mediated endocytosis.[116] As CXCR4 and CD44 can serve as therapeutic targets for inhibitors[117] as well as siRNA[118,119] targeting siRNA-bearing NPs specifically to these proteins should improve both potency and specificity of the therapy. A caveat in exploring ligands against adhesion receptors might be the unavailability of already engaged receptors; sufficiently high amount of free receptors must be available for effective targeting to leukemic cells.

siRNA Delivery in Leukemia and Related Models

Relatively few studies have explored siRNA therapy in animal models of leukemia. The studies included subcutaneous and systemic xenograft models and related models that involved siRNA delivery to systemic blood cells (**Table 4**). Experimental studies with intratumoral delivery may act as a bridge to

Table 4 Studies involving siRNA administration in animal models of leukemia and related disorders

Type of NP	Mouse model/treatment goal	Injection route	siRNA dosage /frequency	Silencing (target)	Therapeutic effect	Ref.
Lipid NP	Healthy athymic nude mice/leukemia	IV	80 µg[a] (4 mg/kg)/once × 2 days	mRNA: 45% spleen (KIF11), 37% bone (KIF11), 89% liver (AHSA1)	Y	45
Polyplex; In vivo-JetPEI	IV (MLL-AF4 SEM-luciferase) leukemia in NOD/SCID mice[b]/leukemia	IV	50 µg (2.5 mg/kg[a])/48 hours × 5 weeks	Protein: 62.2% (week 4) 47.0% (week 5) (luciferase)	NS	121
Lipid NP C12-200	Healthy nude mice (COX-7)[b]/inflammatory monocytes	IV	10/20 µg[a] (0.5/1 mg/kg)/24 hours × 3–7 days; other frequencies also used	mRNA: 45.5%; Protein: 36.4% (Splenic Ly-6C^{high} Monocytes) (CCR2); mRNA: 92.6%, 73.1%, 93.8%; Protein: 75.8%, 66.7%, 89.5% (Splenic Ly-6Cl$_{ow}$ monocytes, CD11c+ dendritic cells, F4/80+ macrophages respectively) (CD45)	Y	134,139
Lipid NP; KC2 and C12-200	Rodent myeloid cells and nonhuman primates (male cynomolgus monkeys)/myeloid cells	IV	0.3/1/3 mg/kg/once × 4 days	Significant silencing in monocyte/macrophage lineage in liver/blood/spleen/bone marrow/peritoneal cavity (various targets)[b]	Y	133
Lipid NP LNP201	Healthy Crl:CD-1/ICR mice/in vivo biodistribution study	IV	3 mg/kg/once × 1 day	NSC (Ssb)	NS	137
Lipoplexes (with carrier DNA found to enhance siRNA delivery)	Collagen-induced arthritis in DBA/1 mice/myeloid cells in chronic inflammatory disorders	IV	10 µg (0.5 mg/kg)/72 hours × 4 days; 10 µg (0.5 mg/kg)/96 + 120 hours × 10 days	Protein: 90% (93.8% activated form) (TAK1); Protein: 58.75% (cPLA$_2$α)	Y; Y	135,136
Modified siRNA; CpG-siRNA	SC (MV4-11) in NSG mice[b]; IV (Cbfb-MYH11/Mpl+ AML) Leukemia in C57BL/6 mice and naive mice/leukemia	IT; IV	100 µg (5 mg/kg)/24 hours × 4 days; 5 mg/kg/48 hours × 11 days	mRNA:52% (STAT3); Protein: 61% (STAT3), 65% (BCL-X$_L$); mRNA: 61.9% (STAT3); Protein: 80.0% (activated STAT3)	Y; Y	73,120
Unclear; Carrier	SC (THP-1) tumors in athymic BALB/c nude mice/leukemia	IT and IP	2 µg[a] (0.1 mg/kg)/48 or 72 hours × 13–14 days	NSC	NSC	122,123
Lipid NP Transferrin Targeted	SC (MV4-11) in NOD-SCID mice/leukemia	IV	(2.5 mg/kg)/72 hours (unknown number of days in treatment)	mRNA: 82% (RRM2)	NS	46
Liposome; Ab antiLFA-1 targeted	HIV-seronegative PBMCs in NOD/SCID/IL2rγ[null] and BLT mice/HIV	IV	50 µg ([a]2.5 mg/kg)/up to 10 days with one treatment	Protein: ~50–70% (CD4) in liver, spleen and blood; mRNA: >60% day 3 and ~50% day 10 in PB CD14+ monocytes	Y	138
Peptide; CPP PepFect6	C57Bl/6JOlaHsd mice/(also NMR1 mice luciferase silencing in the liver model, not discussed)	IV	0.25–1 mg/kg followed by 1 mg/kg dosage 24 hours later. Silencing measured at 24 and 72 hours	HPRT1 silencing in main organs	NS	43
Peptide; Minibody (anti-JL1 targeted)	BM (CCRF-CEM) in NOD-SCID mice 24 hours prior to complex injection/leukemia	BM	1 nmole/mouse/2 hours	No specific siRNA utilized	NS	40
Albumin coated CPP complex	Mice with ATLL tumors/leukemia	IV	1 mg/kg	No specific siRNA utilized	NS	39

AML, acute myeloid leukemia; BM, bone marrow injection; CML, chronic myeloid leukemia; IP, intraperitoneal; IT, intratumoral; IV, intravenous; SC, subcutaneous; Y, yes; NS, not shown; NSC, no appropriate control utilized (scrambled siRNA) for assay.
[a]Estimated based on 20 g mouse. [b]Other mice models also used for some assays. Other results are also demonstrated in many of the studies, results shown here are those that relate to leukemic type cells also reported. Days of treatment are counted from first injection to end-point date (last day of analysis for siRNA suppression). Silencing percent are calculated relative to scrambled siRNA to ensure comparability between studies.

systemic studies by providing basic information on cellular uptake, doses for effective silencing and local siRNA clearance kinetics.[73] As leukemic cells mostly exist in blood and bone marrow, it is not surprising that IV injection of NPs (**Table 4**) has effectively delivered siRNA to leukemic or circulating cells where significant delivery was achieved even without specific targeting. An increased delivery to subcutaneous AML (MV4-11) xenografts was achieved after IV injection of transferrin-targeted lipid NPs,[46] thereby demonstrating improved efficacy with specific targeting. Peptide-mediated

delivery (anti-JL1) demonstrated delivery of fluorescence-labeled siRNA to 7.3% of the CEM leukemic cells in bone marrow (which comprised of 3.3% of the total bone marrow cells) after direct injection into the mouse bone marrow with minimal delivery to other bone marrow cells after 2 hours postinjection.[40] Dosage regimes varied widely among the *in vivo* studies (**Figure 4**), ranging from a single treatment (end-point 24 hours later) to 5 weeks of siRNA treatment every 48 hours, while the total siRNA dose ranged from ~0.5 to ~30 mg/kg (first 10 days). The CpG-conjugated
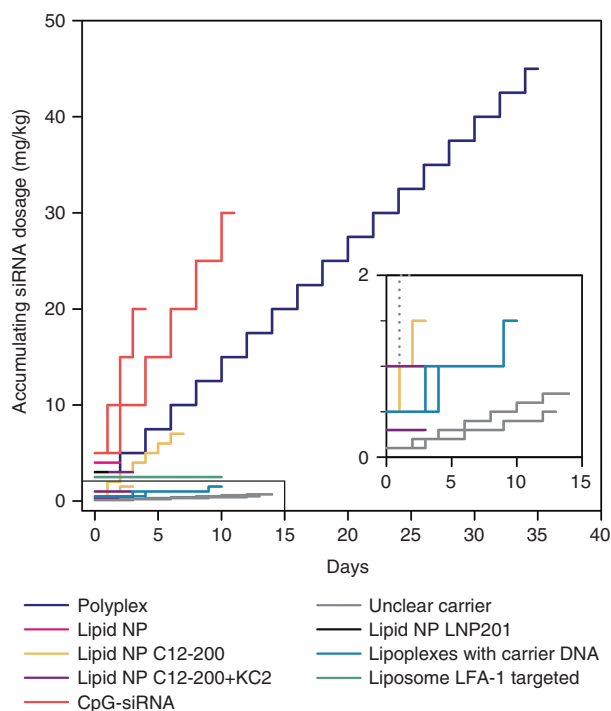
Figure 4 Dosages used for nonviral siRNA therapies in preclinical models. The data were obtained from *in vivo* studies reported in Table 4. Accumulating dosages of the delivered siRNA (mg siRNA/kg body weight) over time until the end of the associated study is displayed in a step-wise graph, where each injection can be visualized (vertical line). Dosages were estimated by assuming 20-g mouse weight when the study reported only siRNA amount (µg) for injection for different delivery systems. Day 0 was taken as the first treatment of siRNA. Note that the range of administered dose varied between ~0.5 and ~30 mg/kg in the first 10 days of administration. The insert is an expansion of the lower left corner of the graph and each line corresponds to a different study with the type of delivery system indicated in the legend. Polyplex (ref. 121), lipid NP (ref. 45), lipid NP C12-200 (refs. 134,139), lipid NP C12-200 +KC2 (ref. [133]), CpG-siRNA (refs. 73,120), unclear carrier (refs. 122,123), lipid NP201 (ref. 137), lipoplexes with carrier DNA (refs. 135,136).

system utilized a large quantity of siRNA; 400 µg over 4 days for intratumoral injection[73] and 600 µg over 6 days for systemic delivery[120], presumably due to rapid extracellular degradation by nucleases. *In vivo* Jet-PEI delivery also utilized a large quantity of siRNA (~900 µg over 5 weeks).[121] Such high siRNA amounts may sometimes be needed for silencing high levels of reporter (luciferase) activity and lower doses are likely to be needed for silencing therapeutic targets. In the lowest reported dose (0.1 mg/kg), it was unclear if the carrier used in the *in vitro* studies was also used in the *in vivo* studies, and efficacy was not compared to scrambled siRNA, making it difficult to assess specificity of the results.[122,123] In the first nonviral clinical siRNA study, BCR-ABL siRNA liposomes were used to treat a Ph1(+) CML patient by IV (10–30 µg/kg) and intratumorally (300 µg) at CML nodules; some evidence of silencing was noted after the first IV treatment but not afterwards.[124] The dosage used for the first human trial was relatively low and it was based on the assumption of (i) siRNAs similarity to AS-ODNs for biodistribution, (ii) reasonable half-life of modified siRNAs, (iii) recommended dosing of an AS-ODN (G3139) being 2–4 mg/kg,[125] and (iv) siRNA

bioactivity being 100–1,000-fold higher than AS-ODNs.[124] It is likely that a higher dosage of BCR-ABL siRNA may be required for a significant effect. To determine possible clinical siRNA dosages for future studies, we can compare AS-ODN preclinical and clinical studies previously done. Clinical AS-ODN studies include LY2181308 AS-ODN study targeting survivin using multiple dosages of 750 mg (7.5–15.0 mg/kg in 50–100 kg patient) with clinical benefits in AML patients,[126] an AEG35156 AS-ODN targeting XIAP with effective dosages used being 110–350 mg/m^2 (2.8–9.5 mg/kg estimated based on the human adult km factor of 37 (ref. 127)) in AML,[128] and an AS-ODN Cenersen AS-ODN study targeting p53 with multiple dosages of 2.4 mg/kg clinical efficacy in AML patients.[129] Preclinical mouse model dosages of AS-ODN models include single or multiple dosages of the AS-ODN LY2181308 ranging between 5–50 mg/kg,[130] AS-ODN AEG35156 ranging between 1–25 mg/kg and AS-ODN G3139 ranging between 5–7 mg/kg.[131,132] The preclinical models (displayed in **Figure 4** and **Table 4**) are comparable to the low end of the preclinical AS-ODN studies described. However, carrier toxicities may limit the siRNA dosage that can be applied. Due to the higher specific activities of siRNAs as compared to AS-ODNs, a more consistent and effective therapeutic response should be achievable at lower doses, as long as the employed carriers do not contribute to treatment toxicities.

Biodistribution and pharmacokinetics

Biodistribution of various NPs was relatively similar, where the highest delivery was always seen at spleen and liver after IV administration,[43,46,120,133–136] and significant silencing was observed in relevant cells and locations (circulation and bone marrow). An exception was albumin-coated CPP complexes which were shown to locate to the ATLL tumor periphery (Cy5.5-labeled siRNA) and minimally locate to the liver and spleen after IV injection. The authors suggest that albumin coating protected the complex from opsonization.[39] As an example, IV delivery of siRNA resulted in uptake in c-Kit+/GFP+ leukemic cells and myeloid immune cells within 3 hours.[120] The highest siRNA delivery was in leukemic and myeloid immune cells in spleen and liver (30–70%), but significant delivery was also seen in bone marrow and lymph nodes. In naive mice, IV CpG-siRNA provided minimal delivery to myeloid progenitor cells and no delivery to hematopoietic cells, limiting possible side-effects.

The systemic half-life of lipid NPs (C12-200) in nude mice was only 8.1 minutes.[134] The liver and spleen retention (in red pulp) was relatively constant starting immediately after injection whereas bone marrow accumulation was detected after 30–60 minutes.[134] After IV administration of lipid NPs, the CD11b+F4/80+ cells (monocytes and macrophages) had high uptake in circulation and spleen, and significant delivery was seen in inflamed ankle joints (arthritis model) and lymph nodes, and minimal delivery to CD3+ T-lymphocytes and B220+ B-lymphocytes. High uptake was seen in monocytes, dendritic cells, and macrophages, and especially splenic Ly-6C^{high} monocytes.[135] In a pharmacokinetic study of transferrin-NPs, the plasma half-life was 10.2 hours, whereas free siRNA had a plasma half-life of only 2.9 hours,[46] clearly reiterating the requirement of a carrier. A lipoplex system designed for delivery to myeloid cells involved in chronic inflammatory

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disorders displayed a high delivery (5–25%) to CD11b+ and CD11c+ cells in circulation/spleen/liver on day 1 and 2 after IV injection (0.5 mg/kg), and low but significant delivery to draining lymph nodes and joints with significant decrease of Cy3-siRNA detection in all areas after 2 days.[135] It was not known whether the decrease reflected actual degradation of siRNA or loss of label. Additionally, low uptake was noted in CD146+ endothelial cells located in the spleen (3%) and liver (10%). Another lipid NP formulation gave higher levels of siRNA in liver and kidney and lower levels in the duodenum.[137] A CPP peptide (PepFect6) was monitored for silencing in main organs (kidney, brain, lung, spleen, liver, and heart) with the strongest silencing seen in the liver, kidney, and lung.[43] Biochemical markers of kidney and liver functions were unchanged with no indication of acute toxicity, suggesting a lack of toxic effect by the CPP treatment. Liposomes with LFA-1 targeting (a ligand relevant for leukemia) demonstrated delivery to human T cells, B cells, and monocytes but not to murine derived CD45+ cells or brain cells with effective silencing of CCR5 (coreceptor for macrophage-tropic strains of HIV) in CD14+ monocytes (2.5 mg/kg).[138]

Silencing efficiency

Significant silencing ranging from 37 to 93% for mRNA and 36 to 80% for protein was reported where leukemic cells typically reside (circulation, bone marrow, and spleen). However, silencing efficiency did not seem to relate to any obvious variable in our analysis of reported studies, such as siRNA dosage or administration schedule, owing to vast number of differences among the studies. Lipid NPs designed for delivery to leukemic cells demonstrated successful KIF11 silencing in healthy blood cells in the spleen (45%) and bone marrow (37%), and separately AHSA1 silencing in liver (89%).[45] Lipid NPs (C12-200 or KC2) demonstrated silencing in monocyte/macrophage lineage cells in the liver, blood, spleen, bone marrow, and peritoneal cavity.[133,134,139] Effective silencing with similar NPs was also demonstrated for the first time in myeloid cells of nonhuman primates in blood, bone marrow, peritoneal cavity, liver, and spleen.[133] Silencing was maintained with repeated siRNA treatments of Jet-PEI polyplexes (every 48 hours for 5 weeks); *in vivo* suppression of luciferase in leukemic cells was evident at 2 weeks after siRNA treatment and showed significant silencing up to 5 weeks.[121] A single injection of a lipid NP formulation (KC2) with CD45 siRNA (2 mg/kg) provided long-term silencing in GFP-peritoneal lavage cells (macrophages) for up to 3 weeks.[133] In another demonstration of long-term silencing, LFA-1-targeted liposomes achieved silencing of CCR5 that lasted for at least 10 days after a single IV injections of siRNA (2.5 mg/kg).[138] Several studies confirmed the RNAi activity by RACE for the cleavage of target mRNAs.[73,133,134]

To probe silencing in circulating monocytes and leukocytes that may relocate after uptake of NPs, mice were injected IV with lipid NPs (KC2) followed by isolation of monocytes/macrophages for *in vitro* culture; maximum silencing was seen at 15 minutes for blood cells, 60 minutes for splenic cells, and 120 minutes for peritoneal macrophages and no silencing for bone marrow cells.[133] With lipid NPs (C12-200), silencing was seen in blood cells sampled after 5 minutes of NP injection followed by 3 days *in vitro* incubation. Silencing
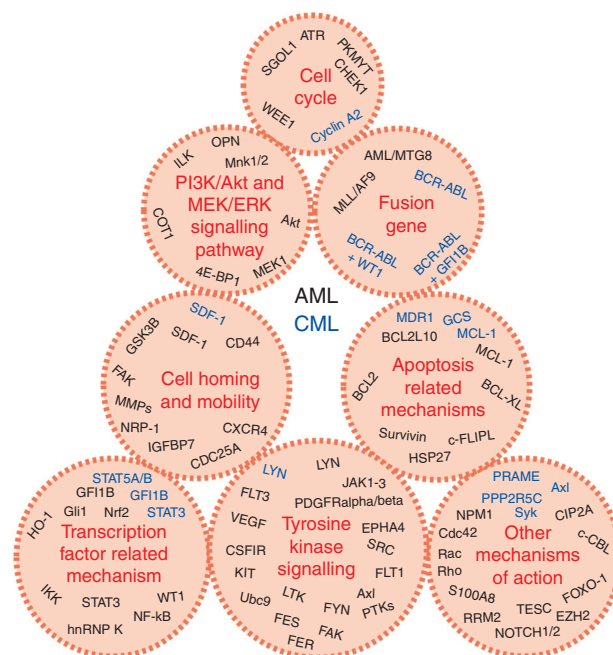

Figure 5 Summary of mRNAs targeted by *in vitro* siRNA delivery that resulted in control of oncogenic characteristics of myeloid leukemias. The data are from studies displayed in **Supplementary Table S1**. Color code for leukemias: black, AML; blue, CML. Targets were grouped based on the role of the protein target: Fusion Gene (refs. 24,26,38,41,47,142,143,224,228,232,234,236,237), transcription factor-related mechanisms (refs. 21,73,120,173,216,217,223,226–228,230,233,238,239), cell cycle (refs. 35,162,240), cell homing and mobility (refs. 118,119,171,172,177,179,181,183,241), apoptosis-related mechanisms (refs. 151–160,176,229,235), PI3K/AKT and MEK/ERK signaling pathway (refs. 164–168,222,242,243), tyrosine kinase signaling (refs. 45,49,123,140,141,219–221,225,231,244–250), and other mechanisms of action (refs. 20,22,46,150,163,169,170,218,251–255).

in the peritoneal macrophages was confirmed to be a result of NPs localizing to peritoneal cavity. IV delivery in nonhuman primates of C12-200 (1 mg/kg) or KC2 (3 mg/kg) NPs followed by blood collection and *in vitro* culture of the cells demonstrated delivery to blood cells within 1 hour of injection as well as effective silencing.

Therapeutic Targets Explored For RNAi in Leukemia

Many studies adopted RNAi for elucidating suitable targets for leukemia therapy without necessarily focusing on clinically translatable siRNA therapeutics. Here, we accentuate the potential targets on myeloid leukemias (**Supplementary Table S1**), which are categorized based on their perceived mechanisms of action (**Figure 5**). Electroporation has dominated siRNA delivery in these studies (52% of listed studies), followed by commercial carriers (33%) while noncommercial carriers were employed to a lesser extent (15%).

Effects on leukemic cell survival

Silencing of chosen targets (**Figure 5**) typically resulted in decreased survival in the form of decreased proliferation and/or viability, increased apoptosis, or increased differentiation

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(**Supplementary Table S1**). Some studies utilized RNAi screens to determine potential targets, which allow comparison among large numbers of targets and possibly "personalize" the therapy. One screen of tyrosine kinases siRNAs highlighted many possible targets (EPHA4, JAK1, JAK3, KIT, LTK, LYN, PTK2 (FAK), PTK2B, PTK6, PTK9, and SRC),[140] as well as targets in patient cells, identifying patient-specific leukemia targets. Decreased cell survival was found in 10 of 30 leukemia patients with kinase siRNAs in one study.[141] In CML, the BCR-ABL kinase has been the main target (**Supplementary Table S1**) and several studies unequivocally demonstrated increased apoptosis as a result of specific BCR-ABL silencing.[24,142] Reducing oncogene levels resulted in changes in other critical mediators, such as antiapoptotic Bcl-X$_L$,[142] cyclin D1,[24] cell cycle inhibitor p27 (refs. 24,142) and transcription factor c-Myc,[143] indicating the possibility of downregulating the survival network by targeting the critical oncogene. shRNA screens highlighted protein bromodomain-containing 4 (Brd4) epigenetic pathway,[144] Syk,[145] and GSK-3α[146] as potential targets.

The preferentially expressed antigen of melanoma (PRAME) was targeted in CML (K562) cells with siRNA.[22] A ~70% knockdown of PRAME mRNA and a complete inhibition of protein expression was achieved, leading to a significant inhibition of proliferation and clonogenic growth, cell arrest at G$_0$/G$_1$ phase, and apoptosis induction. The cell cycle mediator cyclin A$_2$ was another target in K562 cells, whose silencing led to growth inhibition and apoptosis induction.[35] However, a proapoptotic role of cyclin A$_2$ was later elucidated when silencing cyclin A$_2$ in conjunction with doxorubicin treatment; silencing cyclin A$_2$ suppressed doxorubicin-induced growth arrest and cell apoptosis, decreased erythroid differentiation, and promoted megakaryocytic and monocyte-macrophage differentiation in K562 cells.[147] A positive correlation between doxorubicin-induced apoptosis and cyclin A$_2$ upregulation was seen.[147] Cosilencing of a transcription factor, growth factor independence-1B (GFI-1B), which controls development and differentiation of erythroid cells and megakaryocytes,[148] was also investigated as a complementary target to BCR-ABL.[143] A significant loss of cell viability and additive induction of apoptosis was evident with the combination of GFI-1B and BCR-ABL silencing in CML patients.[143] Another target in CML cells is Protein Phosphatase 2, Regulatory Subunit B', Gamma (PPP2R5C), whose expression was significantly decreased in patients undergoing remission.[149] PPP2R5C is involved in induction of p53 dephosphorylation at various residues, which negatively modulates its apoptotic activities, and thus promoting cell survival.[149] Reducing PPP2R5C mRNA levels with specific siRNA in CML cells led to reduced cell proliferation and increased apoptosis.[150]

Sensitizing leukemic cells to chemotherapy

The primary targets found to increase sensitivity to conventional leukemia drugs were antiapoptotic proteins such as Mcl-1, Bcl-2, Bcl-210, Bcl-X$_L$, C-FLIPL, and survivin.[151–160] Additionally, cell-cycle checkpoint proteins had the highest synergistic effects in a genome wide-shRNA/cytarabine and a kinase siRNA/cytarabine screen including CHEK1, HGS, and WEE1 proteins.[161,162] Cell-cycle checkpoint proteins can prevent cells from committing to apoptosis and their silencing could open the door to apoptosis preferentially in leukemic cells over normal cells. WEE1, acting as an intra-S-phase checkpoint, prevents cytarabine induced S-phase arrest and was a promising target for siRNA to sensitize several AML cell lines (TF-1, THP-1, HEL, and MDS-L).[161] Suppression of NPM1, a molecular chaperone and a well known AML mutation, caused inhibition of cell cycle progression and colony growth, increased differentiation and increased chemosensitivity to all-trans Retinoic Acid and cytarabine in mutant-NPM1 expressing AML cells.[163] Signaling proteins in the MEK/ERK (MEK1 (ref. 164), Mnk1/2 (ref. 165), and 4E-BP1 (ref. 166)), and PI3K/Akt pathways (Akt[167] and OPN[168]) also increased drug sensitivity. In one study, cytarabine was found to activate Mnk and MEK/ERK signaling and thus Mnk siRNA and cytarabine cotreatment enhanced suppression of leukemic colony formation.[165] siRNA suppression of TESC, a pH-regulation protein upregulated during sorafenib treatment, was found to increase sorafenib sensitivity.[169] Increased FOXO1 suppression was found to correlate with increased efflux-pump P-glycoprotein (MDR1; P-gp) expression and silencing of FOXO1 restored doxorubicin sensitivity.[170] Interestingly, FLT3 mutation also suppresses P-gp expression, making FOXO1 potentially an additional target for FLT3-negative cells. Suppression of adhesion proteins including CXCR4,[118] whose silencing enhanced cytarabine sensitivity bone marrow stromal cell-attached THP-1 cells and FAK,[171] which increased daunorubicin sensitivity in free KG-1 cells but not as much in fibronectin-attached cells, also increased drug sensitivity. Other drug-sensitizing targets included S100A8 involved in autophagy[153] and transcription factor related proteins HO-1, GSK3β and NF-κB subunit p65.[172,173]

Similarly, tyrosine kinase Lyn has been suggested as a mediator of nilotinib resistance in CML.[174] Lyn was overexpressed in nilotinib-resistant K562 cells and CD34$^+$ patient cells,[20] and an increase in Syk phosphorylation was detected in nilotinib-resistant cells. Using a combination of shRNA and siRNA, inhibition of Syk, Axl, and CDCP-1 increased (or restored) sensitivity to nilotinib, making them potential targets in combinatorial therapy for CML.[20] The upregulated STAT5 was targeted with siRNA to sensitize K562 cells to Imatinib. Similarly, silencing glucosylceramide synthase, which converts the available proapoptotic ceramide into glucosylceramide and increase apoptotic pressure on cells[175,176] was targeted to enhance drug sensitization in doxorubicin-resistant K562 cells.[176]

Effects on mobility and homing

In addition to direct effects on cell proliferation and survival,[118,177–181] suppressing adhesion proteins can diminish homing of cells to protective bone marrow niche. Suppression of CXCR4 (ref. 118), CD44 (ref. 119), ITGB3 (and pathway members),[182] ITGA6 (ref. 178), EVI1 (ref. 178), and ITGB4 (ref. 178) decreased AML adhesion to bone marrow stromal cells (or extracellular matrix coatings such as fibronectin). The CD82 adhesion molecule, overexpressed in AML LSC population (CD34$^+$CD38$^-$), was silenced with shRNA/siRNA in CD34$^+$CD38$^-$ or EOL-1(R) cells, leading to decreased adhesion to fibronectin (by upregulation of MMP-9), increased migration, and decreased engraftment in NOD/SCID mice.[180] Additionally, IGFBP7, a tumor suppressor in solid tumors, was found to be involved in leukemic cell adhesion to endothelial cells, migration, as well as invasion.[179]

siRNA silencing of NRP-1 (a VEGF receptor) decreased chemotaxis.[181] Silencing of MMPs and their activators (*e.g.,* MMP-2, MT1-MMP, and TIMP-2) decreased mobility toward SDF-1.[183] A FAK siRNA also decreased the migration ability in FAK+ AML cells.[171] Ultimately, decreased adhesion and/or mobility toward bone environment are expected to retain the malignant cells in circulation, allowing better response to therapy. Whether the siRNA therapy will be effective to mobilize myeloid leukemic cells from bone marrow niche remains to be shown in a preclinical model.

Eliminating LSCs

LSCs reside in bone marrow and their interactions with bone marrow stroma provide extrinsic factors favoring long-term survival and protection against drugs. Reducing LSC survival is particularly desirable to prevent the residual disease, in addition to enhancing LSC mobilization to peripheral circulation. Treating LSCs specifically is challenging, as they constitute a relatively minor fraction among the leukemic population. NPs delivered to LSCs combined with a cargo that targets LSC-specific proteins (without affecting normal hematopoietic cells) would be ideal. The specific protein signatures of LSC have been recently highlighted. Expression of proteins involved in apoptosis, cell cycle, expression, proliferation, and signaling (as well as activation) is different in LSCs from AML and CD34+ populations, for example, PU.1 (SP1), P27, Mcl-1, HIF1α, cMET, P53, Yap, and phosphorylated-Stat 1/5/6.[184] Other targets include CD32, CD25, WT1 (transcription factor), and HCK (kinase) which are highly expressed in chemotherapy-resistant LSCs and suppression of which does not negatively effect normal hematopoietic cells.[185] The protein Mcl-1 was particularly upregulated in FLT3-ITD AML LSCs, where suppression of Mcl-1 (shRNA) increased apoptosis and suppression of STAT5 (siRNA) downregulated Mcl-1 expression.[186] Additionally, multidrug resistance transporters P-gp, MRP, and LRP were overexpressed in AML LSC population.[187] In fact, increased P-gp expression is distinctive of LSC derived from AML patients,[187,188] as well as LSC associated with CML.[189] This drug transporter appears to protect the LSC particularly from chemotherapy and it might be highly relevant to eradicate the residual AML disease. One can readily envision employing siRNA against drug transporters in combination with conventional chemotherapy. The adhesion molecule CD82 is also overexpressed in AML LSCs, serving as a potential target to prevent LSC harboring in the bone marrow. Additionally, an *in vivo* shRNA screen with a LSC model (MLL-AF9 oncogene expressing granulocyte–monocyte progenitor cells) determined the potential of Itgb3 as a target, whose suppression decreased homing, induced differentiation, and suppressed LSC gene-expression signatures.[182] The adhesion protein, CD44 (ref. 190) was recently targeted successfully with siRNA in primitive KG-1a cells (CD34+/CD38–; an LSC model), more differentiated KG-1 cells and patient cells.[119] Other LSC surface antigens targeted with Abs include CD33, CD44, CD47, CD123, and WT1 (refs. 104,105) and they can be readily targeted with siRNAs as well to explore a therapeutic effect upon down-regulation of these myeloid leukemia-specific ligands.

In the case of CML, most patients harbor residual LSCs and disease typically recurs if TKI therapy is discontinued.[191,192]

TKI treatment alone also does not eliminate LSCs and the possibility of relapse.[143,189,193–195] In addition, CML LSC may not be fully dependent on BCR-ABL kinase activity for survival,[143,196,197] indicating a need to prevent the development of resistant subclones by targeting both BCR-ABL-dependent and independent pathways in LSC. Part of what keeps LSC entrenched in protective bone marrow niche is signaling mediated by stromal cell-derived factor-1 (SDF-1) and cell surface CXCR4.[198] It has been shown that under Imatinib, CXCR4 expression in primary CD34+ cells can be reversibly upregulated, hence allowing these cells to home to bone marrow and remain insensitive to TKIs.[149,199,200] Thus, downregulation of CXCR4 expression along with TKIs therapy could enhance the eradication of LSC. Wnt and β-catenin mediated signaling also seems to play an important role in self-renewal of LSC.[20,199,201] Inhibiting β-catenin with a small molecule, in combination with Imatinib, reduced LSC in a CML mouse model.[198,202] Recently, it has been reported that autophagy can be induced upon Imatinib treatment in CML cells and a combination of Imatinib with chloroquine is more effective than single agents in impairing growth of primitive CML cells *in vitro*.[203] In particular, knockdown of ATG4B, a key cysteine protease, suppressed autophagy, impaired the survival of CML stem/progenitor cells and sensitized them to Imatinib.[204] Furthermore, AHI-1 is a newly discovered oncogene that is highly expressed in primary CML stem and progenitor cells. AHI-1 overexpressing BCR-ABL+ cells had greater resistance to growth inhibition effects of Imatinib and its suppression by shRNA resulted in increased sensitivity to Imatinib. Suppression of AHI-1 in primary CD34+ CML cells also increased Imatinib sensitivity especially in Imatinib-resistant and blast crisis patients who express relatively higher levels of AHI-1.[205] Interestingly, targeting a new AHI-1-BCR-ABL-JAK2 complex by JAK2/ABL dual inhibition was more effective to eradicate TKI-insensitive CML stem/progenitor cells *in vitro* and *in vivo*.[206,207] Similarly, knockdown of JAK2 using a shRNA reduced BCR-ABL and β-catenin expression and induced apoptosis in CML cells.[208,209] Collectively, these studies are clearly pinpointing highly specific targets to which siRNA therapies can be adopted for a cure.

Perspectives on Future of siRNA Therapy in Leukemia

For siRNA therapy to find a place in clinical management of leukemia, functional targets need to be identified that are specific for LSC and its progeny, while siRNA delivery is implemented with effective carriers. Given the diversity of molecular drivers among different types of leukemias, as well as within each subtype, RNAi has the most potential to expeditiously identify suitable therapeutics (siRNA) and personalize the therapy. Such a therapy may offer a more physiologically acceptable intervention based on endogenous mechanisms (unlike chemotherapy), but employing carriers that promote delivery in a controlled and nontoxic way is paramount. A better understanding of mechanisms that lay behind efficient uptake and intracellular trafficking of NPs in leukemic cells are needed, since most of intracellular trafficking studies have employed attachment-dependent cells due to convenience of analysis. While relying on ligand-mediated

delivery is the obvious way to target leukemic cells, there might be promising opportunities in understanding unique features of leukemic membranes, if any. It will be interesting to explore NP features that display preferential penetration into leukemic cells independent of cell surface ligands. It appears that even the NPs designed for leukemic cells function better in attachment-dependent cells,[45] which is consistent with their differences in endocytosis activity. It is likely that membrane-fusing or direct-penetrating NPs might match the penetration rate between the attachment-dependent and attachment-independent cells, and improve the pharmacokinetics profile in leukemic delivery. In this regard, effects of carrier characteristics such as molecular size, degree of modification and optimal lipophilicity-charge balance are routinely elucidated on siRNA delivery/silencing efficiency, but this needs to be simultaneously investigated in the context of toxicity, intracellular trafficking and cell specificity. More importantly, there is a dire need to employ primary patient cells to reveal the details of intracellular trafficking. The lack of further testing in patient cells is a critical issue preventing progress; the routine physiochemical studies and silencing demonstrated in the initial publications are not followed by more detailed studies in clinical samples, and no further studies are published with the developed carrier, indicating a lack of commitment to most carriers. While one wishes to identify carriers suitable for all types of leukemias, current evidence (based on authors' evaluation of polymeric carriers in several leukemia cell lines[36,41,118,119]) suggest that tailoring of carriers will probably be needed for specific types of leukemias and it might even be needed for individual patients. No information exists on patient-to-patient variations in siRNA delivery and evaluating off-target effects of delivered siRNAs and cytotoxic effect of carriers in patient cells is urgently warranted.

The siRNA therapies need to be effective at 20–50 nmol/l range in culture for translation to preclinical animal models and at <10 mg/kg in animal models for clinical translation. It is typical to employ siRNA concentrations beyond this range among the researchers, including our own work.[41] Concerted efforts to lower efficacious doses will be needed, but effective dose of siRNA therapies in leukemic cells did not significantly change over the years, despite the increased diversity (and level of sophistication) in the nature of carriers developed. While one can hope to improve effectiveness with new types of carrier, especially with ones taking advantage of newly discovered mechanisms of macromolecular uptake and trafficking, this is not always the case. An improvement in effective doses, in the opinion of authors, should be expected with each newly developed carrier. Employing more effective siRNAs, such as multimeric, cell-penetrating, or nuclease-resistant siRNAs[210] could be one approach to improving efficacy. Employing microRNAs instead of siRNA may be appealing due to its promise to regulate gene networks (rather than single targets),[211] but early evidence does not indicate superiority in terms of effective doses that need to be delivered. Carriers designed for siRNA delivery specifically to leukemic cells, an under-studied area, will enhance therapeutic efficacies. For carriers found to be promising in culture, there is an urgent need to evaluate them in preclinical animal models to eliminate the ineffective ones quickly and disseminate the relevant data (to

avoid pursuit by others). Polycationic carriers, for example, are perceived to be excessively immune stimulatory so that rapid elimination of stimulatory carriers will better guide the field. With AS-ODN, despite specificity of administered drugs, a wide range of targets in hematopoietic cells, be it stem, progenitor or differentiated, was altered in patients,[212] and the best way to avoid this is to employ low doses of therapies. Given the cationic nature of "typical" NPs, they could bind to a multitude of cells after administration and, to overcome this, noninteracting NPs will be needed by tailoring neutral particles, and/or including sterically-protected surfaces (*e.g.*, PEG).

"Biochemical" targeting could alleviate the physical limitations of delivery by using unique or elevated targets. Targets whose prolonged silencing can be achieved with a single treatment (*e.g.*, CD45 and LFA-1 mentioned before) will be preferable to short-acting silencings; whether this was a feature of the employed siRNA delivery systems or molecular physiology of the target will better elucidate the underpinning of silencing efficiency and the potential of the chosen target(s). Novel siRNA targets[18,213] could prove beneficial for use in combination with established targets, such as BCR-ABL in CML and Flt3 in AML. The combinational siRNA delivery will probably yield more efficacious therapy, and possibly more specific outcomes may emerge. Targeting NPs to overexpressed surface proteins in leukemic cells, which are also therapeutic targets (*e.g.*, CXCR4 whose silencing may lead to reduced proliferation[118]), could provide an effective and highly specific siRNA therapy. Additionally, the siRNA therapy could specifically act on targets causing multidrug resistance (such as P-gp) to prevent drug resistance or act in conjunction with current drugs to improve their effectiveness or to resensitize the cells to current drugs, as demonstrated in many of the described studies. It is, however, preferable for siRNA therapy to be a stand-alone therapy if LSC could be specifically targeted. Identifying a "magic" target, however, might be difficult in myeloid leukemia due to clonal heterogeneity in the disease, where heterogeneous population of subclones are capable of expanding under favorable conditions.[214] It is likely that such a dynamic cell population will affect response to successive siRNA therapies, but this will require longer-term studies than what is reported in the literature. A critical issue is whether resistance to siRNA therapy against a specific target arises in target cells, or whether alternative subclones will emerge resistant to the original therapy. While the response to latter scenario is straightforward, *i.e.*, identifying new targets and designing the right siRNA expeditiously, addressing the former possibility is more ambiguous, since mechanisms of siRNA resistance have not been explored previously. It is not known if RNAi machinery can be modulated in leukemic cells, and if it can be made redundant by the cells under the pressure of siRNA therapy. Given the reliance of cells on RNAi to carry out their normal functions, it is unlikely that the RNAi machinery needed for siRNA action will be dispensed, but functionally equivalent targets could be recruited to overcome critical blockage(s) by exogenous siRNA. With better characterization of clonal heterogenetic at the genetic level, it might be possible (and necessary) to deliver cocktails of siRNA to target different subclones simultaneously at the

onset of therapy, and adjust the composition of such a cock-tail in case of relapse.[215]

Despite these uncertainties, early experience with siRNA-based therapeutic approach has been promising and new, more-effective and less-toxic approaches are expected to emerge for leukemia control. The speed at which new therapeutic agents (*i.e.*, siRNAs) are identified is exceptionally fast as compared to development process needed to identify and assess conventional drugs. Dozens of promising targets have been identified and their therapeutic utility has been validated in a matter of few years. Carriers are inevitably an integral part of this evaluation process; although one is tempted to distinguish highly effective carriers from the summary provided in **Figures 3** and **4**, this might be misleading given the diversity of therapeutic targets, cell models and other experimental variations in the evaluated systems. More reliance on self-assembling small molecular size carriers (unlike nanostructured materials such as carbon nanotubes) is considered prudent, so that dis-assembly and subsequent elimination of exogenous materials are straight-forward. CPPs with tailored structural motifs appear to be most effective considering their small size (since pure polymeric carriers of equivalent sizes are not effective in siRNA delivery), but equivalent size polymeric carriers could be engineered to match their performance if suitable lipidic components are incorporated into them. These carriers, however, lag behind lipid NP formulations in clinical testing, so that rapid clinical entry of siRNA-based leukemic therapies may have to rely on lipid NP formulations. Where leukemia-specific delivery is required, one can rely on Abs given accepted clinical deployment of Abs in cancer therapy as well as their use as drug carriers (so called ADCs). Successful Ab incorporation and effective targeting may also depend on the nature of carrier, so that unique combinations of Abs and carriers might be the optimal solution to the delivery problem. Nevertheless, having so many possibilities for combinational (or modular) carrier design bodes well for a cure of the leukemic disease in the near future.

Supplementary material

Table S1. siRNA targets shown to be beneficial in Myeloid Leukemias.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)