



Immunomodulation of hematological malignancies using oligonucleotides based-nanomedicines



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ABSTRACT

Hematological malignancies are a group of diseases characterized by clonal proliferation of blood-forming cells. Malignant blood cells are classified as myeloid or lymphoid cells depending on their stem cell origin. Lymphoid malignancies are characterized by lymphocyte accumulation in the blood stream, in the bone marrow, or in lymphatic nodes and organs. Several of these diseases are associated with chromosomal translocations, which cause gene fusion and amplification of expression, while others are characterized with aberrant expression of oncogenes. Overall, these genes play a major role in development and maintenance of malignant clones. The discovery of antisense oligonucleotides and RNA interference (RNAi) mechanisms offer new tools to specifically manipulate gene expression. Systemic delivery of inhibitory oligonucleotides molecules for manipulation of gene expression in lymphocytes holds a great potential for facilitating the development of an oligonucleotides-based therapy platform for lymphoid blood cancer. However, lymphocytes are among the most difficult targets for oligonucleotides delivery, as they are resistant to conventional transfection reagents and are dispersed throughout the body, making it difficult to successfully localize or deliver oligonucleotides payloads via systemic administration. In this review, we will survey the latest progress in the field of oligonucleotides based nanomedicine in the heterogeneous group of hematological malignancies with special emphasis on RNA based strategies. We will describe the most advanced non-viral nanocarriers for RNA delivery to malignant blood cells. We will also discuss targeted strategies for cell specific delivery of RNA molecules using nanoparticles and the therapeutic benefit of manipulating gene function in hematological malignancies. Finally, we will focus on the *ex vivo*, *in vivo*, and clinical trial strategies, that are currently under development in hematological malignancies - strategies that might increase the arsenal of drugs available to hematologists in the upcoming years.

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1. Introduction

Hematological malignancies are a group of diseases characterized by clonal proliferation of blood-forming cells that collectively represent 9% of all cancers and affect people of all ages. Malignant blood diseases are classified as myeloid or lymphoid depending on their stem cells of origin, and as acute or chronic based on the clinical course. The myeloid lineage normally produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells. The myeloid neoplasms are a group of diseases that primarily develop and expand in the bone marrow and can home to peripheral hematopoietic tissues. Myeloid neoplasms include myeloproliferative neoplasm (chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL), polycythemia vera (PV), primary

myelofibrosis and essential thrombocythemia), myelodysplastic syndromes and acute myelogenous leukemia (AML). The lymphoid lineage produces B lymphocytes, T lymphocytes, natural killer, and plasma cells. Lymphoid neoplasms are characterized by lymphocyte accumulation in the blood stream, the bone marrow, or in lymphatic nodes and organs. Lymphoid neoplasms include acute leukemia of uncertain lineage, mature B-cell neoplasms, acute B and T leukemias (ALL), B and T lymphoblastic leukemia/lymphoma, chronic lymphocytic leukemia (CLL), lymphoma, and multiple myeloma (MM). In adults, AML and CLL are the most common types of leukemia. Leukemia is the most commonly diagnosed cancer in children aged 0 to 14 years, accounting for up to 35% of all cancers, 77% of which are ALL [1]. Although hematological malignancies represent >60 distinct disease types, each having particular clinical features, treatment pathways, and outcomes, these diseases are related in the sense that they may all result from acquired mutations to the DNA of a single lymph- or blood-forming stem cell. Several of these diseases are associated with chromosomal translocations, which cause gene fusion and amplification of expression, while others are

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characterized by aberrant expression of oncogenes. Overall, these genes play a major role in development and maintenance of malignant clones.

The current treatment of most hematologic malignancies is still based on various combinations of chemotherapeutic agents. However, the treatment landscape for hematologic malignancies is evolving rapidly, as recent insights into the genetic signatures of disease continue to appraise the development of targeted therapies in the form of specific small molecules and antibodies against various epitopes expressed on malignant cells. These potent therapies are more effective and potentially safer than standard chemotherapy because they target specific proteins and mutated gene products while leaving other cells unharmed.

Silencing genes using inhibitory oligonucleotides is an attractive therapeutic strategy for the treatment of hematological malignancies. This approach includes small interfering RNAs (siRNAs), micro RNA (miRNA), anti-miRNA oligonucleotides (AMOs), and antisense oligonucleotides (ASOs). siRNAs are produced by cleaving long double-stranded RNA (Dicer substrate) by the endonuclease Dicer into molecules of 21 to 23 nucleotides in length. siRNAs are double-stranded RNAs with an antisense active strand that is exactly complementary to a sequence anywhere in the target mRNA and a sense strand [2]. Inside the cell cytoplasm, siRNA associates with an Argonaute (AGO) protein within the precursor RNAi-induced silencing complex (pre-RISC) which cleaves the sense siRNA strand. The mature RISC contains the antisense strand which directs the complex to the target mRNA for post-transcriptional gene silencing [3]. miRNA is recruited to the RISC and regulates gene expression of protein-coding genes through diverse mechanisms. The interaction of miRNAs with the 3' untranslated region of protein-coding genes is considered as the main mechanism, which usually leads to a decrease in protein output either by mRNA degradation or by translational repression [4]. AMOs act by blocking the interactions between miRNA and their target mRNAs through competitive binding, therefore neutralizing specific miRNAs. ASOs are single-strand, chemically modified oligonucleotides that bind to complementary sequences in target mRNAs and reduce gene expression both by RNase H-mediated cleavage of the target RNA and by inhibition of translation by steric blockade of ribosomes [5].

Since the discovery of RNAi in mammalian cells, RNAi has become an important tool in understanding gene expression and function in many types of cells. The main advantage of using this strategy is that it can silence in principle any gene with high specificity and selectivity, including “undruggable” target genes, which are uniquely expressed in different types of hematological malignancies, translocated genes, overexpressed genes, as well as mutated genes. Therefore, RNAi molecules may represent the future medicine of targeted therapeutics for hematological malignancies.

Despite this promise, utilizing RNAi for therapeutics is not a trivial task. For example due to their large molecular weight, the net negative charge and their hydrophilicity, the efficiency with which naked RNA molecules cross the plasma membrane and enter the cell cytoplasm is very low. Furthermore, RNA molecules are easily excreted from the kidney and extremely susceptible to nuclease degradation in plasma [6,7]. In addition, RNA molecules bind to innate immune cell receptors responsible for nucleic acid recognition: the transmembrane toll-like receptors, and the cytoplasmic sensors retinoic acid-inducible gene I (RIG-I)-like helicase family receptors [8]. Activation of innate immune cells may lead to immuno-toxicity by the secretion of IFN and proinflammatory cytokines [9,10]. To avoid innate immune recognition and increase therapeutic RNAi molecule stability, engineered molecules that include nucleotide chemical modifications have been developed, such as short interfering ribonucleic neutrals (siRNNs), whose phosphate backbone contains neutral phosphotriester groups [11] and conversion of one nucleotide to phosphorodithioate (PS2) and 2'-O-Methyl (2'-OME) MePS2 [12]. Encapsulation of modified RNAi molecules in nanocarriers provides additional benefits; as nanocarriers can condense their charge and could be surface decorated with targeting

ligands to endow these carriers the ability to deliver the payloads to specific cell types. To this end, the use of nanotechnology strategies might be highly beneficial [13].

Nanotechnology offers a variety of conjugate and particle-packaging methods that utilize the special characteristics of nucleic acids and could mediate the delivery of oligonucleotides directly into blood cancer cells. While most of these developments were able to silence genes in various blood cancer cell lines *in vitro*, only few of them demonstrated *in vivo* gene silencing, in disperse mouse model of leukemia/lymphoma, or *ex-vivo*, in patients' primary cells and in clinical trials. In this review, we will survey the latest progress in the field of oligonucleotide based nanomedicine in the heterogeneous group of hematological malignancies. We will describe the most advanced non-viral nanocarriers for oligonucleotides delivery to malignant blood cells and special emphasis will be made on the *ex vivo*, *in vivo*, and clinical trials, which are currently under development.

2. The requirements from oligonucleotides delivery systems for site-specific targeting to malignant leukocytes

The major obstacle preventing clinical translation of this powerful technology from revolutionizing treatments of hematological malignancies is the difficulty to introduce oligonucleotides into malignant blood cells. The field of systemic drug delivery into tumor cells has emerged extensively during the recent years. However, the science of delivery into dispersed population of blood cells remains a challenge. Seemingly, systemic drug delivery to leukocytes should be less challenging in comparison to solid tumors, as there are fewer barriers to cross. Upon targeting solid tumors, the intact drug should cross into the blood vessels surrounding the tumor, then pass through extracellular matrix, bind and internalize into the tumor cell and enter into the cell cytoplasm. However, due to the fact that blood cells and especially leukocytes are notoriously hard to transfect and are spread not only in peripheral blood but also in the bone marrow, lymph nodes, and lymphatic organs, there is an unmet need for developing designated delivery strategies to leukocytes [14].

3. Systemic delivery of inhibitory oligonucleotides to malignant leukocytes

3.1. Antisense oligonucleotides (ASOs) and siRNA-CpG

ASOs are single-strand, chemically modified oligonucleotides that bind to complementary sequences in target mRNAs and reduce gene expression both by RNase H-mediated cleavage of the target mRNA and by inhibition of translation by steric blockade of ribosomes [5]. Several attempts have been made to treat hematological malignancies with unformulated ASOs constituting unmethylated CpG motifs as an integral part of their design or with siRNA molecules chemically conjugated to CpG sequences (CpG-siRNA) (Fig. 1). CpG oligonucleotides are efficiently internalized by antigen-presenting cells. Upon internalization, these motifs are recognized by the endosomal TLR9, which is expressed on B cells and plasmacytoid dendritic cells and regulates different immune responses [15]. TLR9 are also present on primary malignant blood cells such as MM, CLL and AML [16–18]. Conjugation of CpG oligonucleotide to siRNA therefore creates a molecule capable of both delivering siRNA and activating therapeutic antitumor immune responses [19]. This immune stimulation may be beneficial in immunosuppressive illnesses such as blood cancers. Zhang, Q. et al. [18], synthesized CpG-siRNA STAT3 to target both malignant MM and AML cells and innate immune cells. Using systemic delivery of CpG-siRNA STAT3 they demonstrated reduced level of STAT3 mRNA and anti-leukemic effects both *in vitro* and *in vivo* using subcutaneous mouse MM and AML xenograft models, without significant toxic side effects. A recent study utilized CpG-siRNA STAT3 for the systemic treatment of AML and demonstrated a regression of disseminated orthotopic AML in mouse models, which require

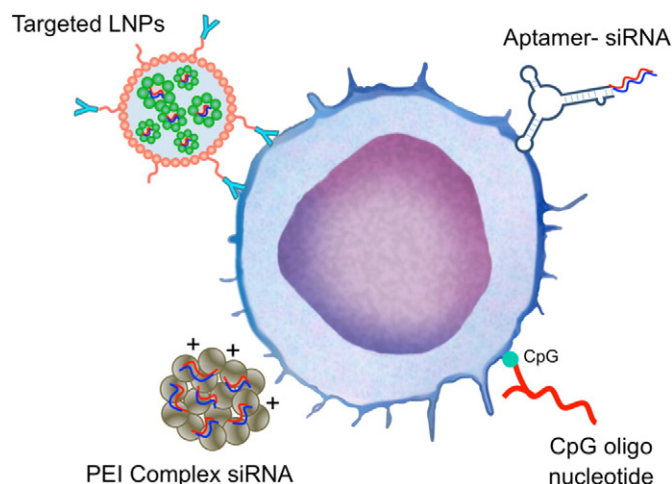


Fig. 1. The most advanced strategies to deliver RNAi to malignant blood cells. Antisense oligonucleotides comprised of CpG motifs as an integral part of their design. Aptamer-siRNA are selective to malignant blood cell antigens and link to siRNA molecules. Polyethylenimine nanoparticles that contain siRNA molecules (PEI complex siRNA). Lipid nanoparticles encapsulated with siRNA molecules and coated with specific targeted ligands for malignant blood cell antigens (targeted LNPs).

host's effector CD8 + T lymphocytes but not TLR9-positive antigen-presenting cells. Interestingly, the investigators reported that CpG-Stat3 siRNA has a direct immunogenic effect on AML cells *in vivo* by up-regulating major histocompatibility complex class-II, co-stimulatory and pro-inflammatory mediators, while also down-regulating co-inhibitory PD-L1 molecule. Multiple administrations of unformulated CpG-Stat3 siRNA conjugates generated tumor remission of disseminated AML in 60% of mice. Nevertheless, the systemic administration of naked CpG-siRNA might lead to unwanted immune responses, which can result in severe adverse reactions and treatment failure [20].

ASOs that hybridize with and downregulate targeted mRNAs have shown promise in a range of hematological diseases. Uckun, F.M. et al. [21] used an immunoconjugate of anti-CD19 antibody and ASO targeting one of the most common chromosomal translocations in acute lymphoblastic leukemia (t(1;19)(q23;p13)), which results in the expression of the oncogenic protein E2A-PBX1. Treatment of E2A-PBX1 + leukemia cells with aCD19-ASO resulted in downregulation of E2A-PBX1 transcripts and promoted apoptosis *in vitro* and *in vivo*. Furthermore, continuous infusion of aCD19-ASO for 14 days using a microosmotic pump resulted in double median survival rate of SCID mice challenged with radio chemotherapy-resistant highly aggressive human E2A-PBX1 + B-lineage leukemia compared to controlled treated mice. Recently, Hong, D. et al. [22] reported on a next-generation of ASO targeting human STAT3 (AZD9150). They have evaluated the efficacy of this new ASO in several preclinical tumor models including subcutaneous cell line-derived xenografts, a systemically disseminated xenograft model of large cell lymphoma, and lymphoma patient-derived tumor explant. Systemic administration of AZD9150 resulted in STAT3 mRNA knockdown of ~40%, 50% or 37%, respectively. Human STAT3 inhibition by AZD9150 resulted in significant tumor growth inhibition in the subcutaneous tumor model and reduced tumor burden of the disseminated model with no effect on the mice body weight. Next, they evaluated the safety of AZD9150 in a phase I dose-escalation study, which included 12 advanced lymphoma patients (out of 25 overall patients). Although partial clinical responses have been achieved in several refractory patients to frontline therapies, severe drug-related adverse events occurred in >5% of patients.

Several other types of ASOs are currently under clinical evaluation in various hematological malignancies indications (Table 1). In most of these trials, ASOs were not used as a single therapy but rather added to chemotherapy, radiotherapy or immunotherapy treatments. A modest response observed in phase II clinical study where the response of

relapsed/refractory AML patients to an addition of ASO targeting p53 (Cenersen™) with Idarubicin® with or without Cytarabine® treatment was measured [23]. An addition of an ASO targeting Bcl-2 (Oblimersen™) to Fludarabine® and Cyclophosphamide® treatment was associated with significant increase in complete response versus nodular partial response in a phase III study of relapsed/refractory CLL patients [24]. Nevertheless, the addition of Oblimersen™ to dexamethasone® in a phase III clinical trial for relapsed/refractory multiple myeloma patients did not improve time to tumor progression [25]. These results emphasize both the great potential and the challenges of using non-formulated ASOs in the clinic. Non-formulated siRNAs or ASOs are well tolerated in murine models in high doses; however, in clinical evaluations significantly lower doses result in various adverse events. This issue can be resolved by formulating ASOs and CpG-siRNAs in nanocarriers. Additional advantages of formulation are prolonged nucleotide stability, altered bio-distribution, decreased potential immune activation, and increased specific targeting to the desired cells. All of the above could augment the therapeutic efficacy of in blood cancer patients.

3.2. Aptamers

Aptamers are small structured RNAs or DNAs selected for high affinity binding of target proteins [26]. Aptamers specific to leukocyte antigens can be linked to inhibitory oligonucleotides to produce multifunctional compounds for targeting immune cells and modulation of gene expression (Fig. 1). Several recent preclinical studies have demonstrated a promising ability of aptamer conjugated siRNA chimeras (AsiCs) to promote *in vivo* gene silencing of T-lymphocytes. Wheeler, L.A. et al. [27] showed that pretreatment of humanized mice with CD4-siRNA-CCR5 AsiCs targeted the HIV co-receptor CCR5 in CD4 + T lymphocytes and prevented genital transmission of HIV. Recently, Zhou, J. et al. generated a novel CCR5-siRNA-CCR5 AsiCs capable of specifically targeting HIV susceptible cells (CD4 + T Lymphocytes and monocytes) via CCR5 receptor and inhibiting HIV infectivity via block of the CCR5 expression [28].

AsiCs that induce efficient cell-specific knockdown can be used as an immunomodulating therapeutic for hematological malignancies. Herrmann, A. et al. [29] recently reported the use of this strategy where CTLA4-siRNA-STAT3 AsiCs used to target human T large cell lymphoma in mouse xenograft model. Intravenous administration of CTLA4-siRNA-STAT3 AsiCs demonstrated tumor growth inhibition as well as reduced STAT3 activity. Overall, while there are clearly important challenges remaining; aptamer-siRNA chimera technology has the potential to become an attractive tool for therapeutic application in hematological malignancies.

4. Supramolecular nanocarriers for systemic delivery of inhibitory oligonucleotides into blood cancers

Nanocarriers (NCs) offer effective delivery platforms for the negatively charged oligonucleotides, providing protection against both rapid renal excretion and nuclease cleavage, reduced unwanted immune response (either suppression or activation), having the ability to deliver oligonucleotides in combination with either soluble or insoluble drugs, controlled drug release mechanisms, and improved intracellular penetration [30]. NCs come in different 'flavors' and can be controlled by adjusting their size, surface chemistry and shape. For instance, formulations of NCs that range between 100 and 200 nm have been determined to be optimal for long circulation in the bloodstream since at this specific size range they can avoid uptake by the reticuloendothelial system (RES), also known as the mononuclear phagocytic system. In addition, hydrophilic surface achieved by coating NCs with either Poly(ethylene glycol) (PEG) or hyaluronan (HA) protect them from protein opsonisation – a process which tags the NC for removal from circulation by specialized macrophages [31]. NCs also provide mechanisms for release of the therapeutic cargo, such as activated release that breaks

Table 1

Clinical trials using oligonucleotide based-nanomedicines in hematological malignancies.

Sponsors	Drug	Mechanism	Vehicle	Route	Target	Status	Phase	Condition	Clinical trial ID	Refs
Dicerna Pharmaceuticals	DCR-MYC, DCR-M1711	siRNA	LNP	IV	c-Myc	Recruiting	I	Multiple myeloma;	NCT02110563	
Mirna Therapeutics, Inc.	MRX34	miRNA	Liposome - SMARTICLES	IV	miR-RX34	Recruiting	I	non-Hodgkins lymphoma Primary liver cancer, SCLC, lymphoma, melanoma, multiple myeloma, renal cell carcinoma, NSCLC	NCT01829971	
Bio-Path Holdings, Inc.	BP1001 LDAC	ASOs	Liposome	IV	Grb-2	Ongoing	I	Recurrent adult acute myeloid leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, Ph1 positive CML	NCT01159028	
Senesco Technologies, Inc.	SNS01-T	siRNA pDNA	PEI-based nanoparticles	IV	eIF5A	Ongoing	I/II	Relapsed or refractory B cell malignancies	NCT01435720	[34]
National Cancer Institute (NCI)	Oblimersen sodium, rituximab	ASOs	–	IV	Bcl-2	Completed	II	Recurrent B-cell non-Hodgkin lymphoma	NCT00054639	
National Cancer Institute (NCI)	Oblimersen sodium, cytarabine, daunorubicin hydrochloride	ASOs	–	IV	Bcl-2	Completed	I	Previously untreated patients with acute myeloid leukemia 60 years of age	NCT00039117	
National Cancer Institute (NCI)	Daunorubicin hydrochloride, cytarabine, oblimersen sodium	ASOs	–	IV	Bcl-2	Completed	III	Previously untreated acute myeloid leukemia	NCT00085124	
National Cancer Institute (NCI)	Oblimersen sodium, imatinib mesylate	ASOs	–	IV	Bcl-2	Completed	II	Chronic myelogenous leukemia	NCT00049192	
National Cancer Institute (NCI)	Oblimersen sodium, rituximab, ifosfamide, carboplatin, etoposide, filgrastim, pegfilgrastim	ASOs	–	IV	Bcl-2	Completed	I/II	Relapsed B-cell non-Hodgkin's lymphoma	NCT00086944	
National Cancer Institute (NCI)	Oblimersen sodium, cytarabine, fludarabine phosphate	ASOs	–	IV	Bcl-2	Completed	I	Refractory and relapsed acute myeloid leukemia and acute lymphoblastic leukemia	NCT00004862	
Genta Incorporated	Oblimersen sodium, fludarabine, rituximab	ASOs	–	IV	Bcl-2	Completed	I/II	Chronic lymphocytic leukemia	NCT00078234	
Genta Incorporated	Oblimersen sodium	ASOs	–	IV	Bcl-2	Completed	I/II	Chronic lymphocytic leukemia	NCT00021749	[51]
Genta Incorporated	Oblimersen sodium, gemtuzumab ozogamicin	ASOs	–	IV	Bcl-2	Completed	II	Elderly patients with relapsed acute myeloid leukemia	NCT00017589	[52]
Genta Incorporated	Oblimersen sodium, dexamethasone	ASOs	–	IV	Bcl-2	Completed	III	Relapsed or refractory multiple myeloma	NCT00017602	[25]
Genta Incorporated	Filgrastim, oblimersen sodium, cyclophosphamide fludarabine phosphate	ASOs	–	IV	Bcl-2	Completed	III	Relapsed or refractory chronic lymphocytic leukemia	NCT00024440	[24,53]
University of Maryland	Oblimersen sodium, dexamethasone, thalidomide	ASOs	–	IV	Bcl-2	Completed	II	Relapsed and refractory multiple myeloma	NCT00049374	[38]
British Columbia Cancer Agency	Oblimersen sodium, rituximab, cyclophosphamide, doxorubicin, hydrochloride, prednisone, vincristine sulfate	ASOs	–	IV	Bcl-2	Completed	I	Untreated advanced stage diffuse large B cell lymphoma	NCT00070083	[39]
Santaris Pharma A/S	SPC2996	ASOs	–	IV	Bcl-2	Completed	I/II	Chronic lymphocytic leukemia	NCT00285103	
Eleos, Inc.	Cenersen, idarubicin, cytarabine	ASOs	–	IV	p-53	Completed	II	Refractory and relapsed acute myelogenous leukemia	NCT00074737	
University of Pennsylvania	c-myb AS ODN	ASOs	–	IV	c-myb	Completed	I	Advanced hematological malignancies	NCT00780052	
Abramson Cancer Center of the University of Pennsylvania	G4460, filgrastim, busulfan, cyclophosphamide, autologous bone marrow transplantation <i>In vitro</i> -treated bone marrow transplantation	ASOs	–	IV	c-myb	Completed	II	Chronic myelogenous leukemia	NCT00002592	
Enzon Pharmaceuticals, Inc.	EZN-2968	ASOs	–	IV	HIF-1 α	Completed	I	Adult patients with advanced solid tumors or lymphoma	NCT00466583	
Ionis Pharmaceuticals, Inc.	IONIS-STAT3Rx	ASOs	–	IV	STAT3	Ongoing	I/II	Advanced cancers, DLBCL, lymphoma	NCT01563302	

Table 1 (continued)

Sponsors	Drug	Mechanism	Vehicle	Route	Target	Status	Phase	Condition	Clinical trial ID	Refs
MedImmune LLC	MEDI4736, tremelimumab, AZD9150	ASOs	–	IV	STAT3	Not yet open	I	Adults with diffuse large B-cell lymphoma	NCT02549651	
Aegera Therapeutics	XIAP antisense	ASOs	–	IV	XIAP	Completed	I/II	Refractory or relapsed AML	NCT00363974	[54]

the bonds between the drug and NC or leads to particle degradation or efflux of the drug from the NC. All the above have shown to improve the therapeutic efficacy in comparison to non-formulated oligonucleotides under several parameters such as improved gene silencing and increased therapeutic outcome with minimal adverse effects. Although a variety of NCs are being developed (liposomes, lipid-based particles, polyplex, lipoplex, dendrimers, polymeric nanoconjugates and more) for delivering therapeutic nucleotides to tumors, only several of them have shown to effectively silence malignant blood cells, and only few are in advanced stage of clinical trials [32] (Table 1).

4.1. Polymer-based delivery systems

Several polymer-based particles were recently suggested to deliver therapeutic oligonucleotides to malignant blood cells. The most advanced system which is now under clinical studies (phase I/II) for the treatment of relapsed or refractory multiple myeloma and B cell lymphoma named SNS01-T [33]. SNS01-T are polyethylenimine (PEI) nanoparticles that contain both siRNA and a decoy DNA plasmid (Fig. 1). The siRNA sequence targets the eukaryotic translation initiation factor 5A (eIF5A) while the plasmid DNA expresses a non-hypusinable mutant of eIF5A (K50R), which induces apoptosis under a B-cell specific promoter. Although these positively charged nanoparticles are untargeted, the enhanced tumor cell uptake and relatively low toxicity suggest that SNS01-T preferentially targets malignant cells. Using local and systemic administration methods of the siRNA-DNA chimeric NPs, Francis, S. et al. showed a significant growth inhibition of multiple myeloma tumors and increased survival rate in human myeloma xenograft mouse model [34].

siRNA potency and half-life of the targeted protein are other critical components which play a crucial role in the success rate of RNAi based therapy. New siRNA optimization strategies are needed in order to design more potent siRNA sequences for proteins with long half-life. Gavrilov, K et al. [35] leveraged the siRNA potency of BCR-ABL-TMPRSS2 fusion genes by incorporating terminus modifications and targeted the junction site of the BCR-ABL/TMPRSS2 fusion onco-proteins. They developed a 'slow release' and non-toxic delivery system based on poly lactic-co glycolic acid (PLGA) polymers NPs, which demonstrate a robust killing effect of human CML leukemic cell line. However, further *in vivo* analysis and primary cell based assays are needed in order to test the effectiveness of this modified junction site targeted siRNAs against fusion genes.

Overexpression of oncogenic microRNA that are particular to hematological malignancies and correlate with poor prognosis is considered a favorable target for NPs delivery systems of AMOs. MicroRNA 155 is one of these candidates. Babar, I.A. et al. used PLGA NPs with a cell-penetrating peptide, in order to deliver anti-miR-155 molecules in a safe manner to the tumor cells, and showed an anti-tumor effect in a miR-155 cre-lox P inducible lymphoma mouse model [36]. Yet, in these experiments, the particles were injected locally, and continued research will be needed in order to evaluate their effectiveness systemically.

Another non-conventional attempt to deliver siRNA to uneasily transfected human primary T lymphocytes is the use of multi-arm (star) polycationic NPs made of poly 2-dimethylamino ethyl methacrylate (PDMAEMA). The researchers showed >40% gene silencing in these cells [37]. However, only *in vivo* studies will confirm if these star-shaped

NPs can successfully overcome the challenge of transfecting T lymphocytes.

4.2. Lipid-based delivery systems

Lipid based nanocarriers are the first nano delivery systems to make the transition from concept to clinical application [38]. This can be attributed to their attractive biological properties, which include general biocompatibility, biodegradability, isolation of drugs from the surrounding environment, and the ability to entrap both hydrophilic and hydrophobic drugs [30,38]. Multiple properties of lipid based nanocarriers can be altered via surface chemistry including their size, charge, and surface functionality [30].

4.2.1. Liposomes

Liposomal formulations are the veteran drug NCs, with >13 approved clinical products. They are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between different shells of multilayered particles. Liposomes possess many attractive characteristics as they can entrap water-soluble (hydrophilic) pharmaceutical agents in their internal water compartment and water-insoluble (hydrophobic) pharmaceuticals into the membrane [39]. Therefore, liposomes provide a unique opportunity to deliver pharmaceuticals into cells. Furthermore, the size, charge and surface properties of liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation and/or by variation of preparation method.

A novel amphoteric liposome delivery system for nucleic acid named SMARTICLES, developed by Marina Biotech is currently under clinical trial, phase I, dose escalation study for delivery of miR34 (MRX34) in advanced cancer patients with primary liver and hematological malignancies; multiple myeloma and lymphoma. SMARTICLES are composed of unique combinations of lipids having anionic and cationic groups that work together to enable cell uptake, to provide serum stability, and to provide pH-triggered endosomal escape.

An antibody-targeted liposomal delivery system was recently reported to specifically deliver antisense ASO targeting BCL-2 mRNA (G3139), into CD20-positive chronic lymphocytic leukemia (CLL) B cells. The researchers post inserted micelles bound to anti CD20 (Rituximab) into liposomes encapsulating G3139. Using this formulation, they demonstrated that the adverse systemic immunostimulatory responses of the ASO were abrogated. Furthermore, BCL-2 protein levels were significantly reduced, which enhanced Fludarabine-induced apoptosis in CLL B cells and achieved a significant therapeutic effect *in vivo* in an orthotopic B-cell lymphoma xenograft mouse model [40].

4.2.2. Stabilized nucleic acid lipid particles (SNALPs)

A family of lipid nanoparticles (LNPs) known as stabilized nucleic acid lipid particles (SNALPs) are one of the most advanced strategies for siRNA delivery due to their high siRNA encapsulation efficiency and low immunogenic properties and potent gene knockdown in humans [41,42]. Lipid nanoparticles (LNPs) are ionizable particles that contain mixtures of polyethylene glycol-conjugated (PEGylated) lipids, cholesterol and nucleic acids [43]. Ionizable LNPs are neutral in the circulation, where they associate with apolipoproteins (in particular, apolipoprotein E3), which mediates their endocytosis, primarily by

hepatocytes and monocytes [44,45]. The lipids become protonated at low pH in endosomes, which triggers endosomal membrane destabilization and subsequent cytosolic release of some of their nucleic acid cargo. A first-generation LNP, 1,2-dilinolexyloxy-3-dimethylaminopropane (DLinDMA), which potently knocked down liver gene expression in rodents and non-human primates [46], showed limited liver gene knockdown in initial clinical studies and caused some toxicity (complement and innate immune activation). Second-generation LNPs show substantially improved siRNA delivery and knockdown in the liver. Constructed with the anionic lipid dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA), they mediate potent gene knockdown in humans at reduced doses compared with first-generation LNPs [41]. When systematically administered, most SNALPs were trapped by hepatocytes. It is therefore logical to utilize them to treat liver diseases [43], but it is more ambitious to apply this technology for disseminated diseases such as leukemia and lymphoma. Such an effort was recently initiated in a dose escalation phase I study by Dicerna Pharmaceuticals, using a dicer substrate RNAi-based therapy encapsulated in SNALPs that is designed to silence the Myc oncogene (DCR-Myc) in liver tumors and selected solid tumors, but also in multiple myeloma and non-Hodgkin's lymphoma patients.

A more promising approach is to construct LNPs that will target specifically malignant blood cells (Fig. 1). In a recent study, we describe a novel strategy to specifically deliver siRNAs to murine CD4⁽⁺⁾ T cells using targeted lipid nanoparticles (tLNPs). The tLNPs were surface-functionalized with anti-CD4 monoclonal antibodies to permit delivery of the siRNAs specifically to CD4⁽⁺⁾ T lymphocytes. Systemic intravenous administration of these particles led to efficient binding and uptake into CD4⁽⁺⁾ T lymphocytes, which was followed by CD45 silencing [47]. A similar strategy was recently described by Weinstein; S.; et al., [48] to specifically deliver siRNAs against cyclin D1 to mantle cell lymphoma cells (MCL) in human MCL- xenografted mice. LNPs coated with anti-CD38 monoclonal antibodies and loaded with siRNAs against cyclin D1 induced gene silencing in MCL cells and prolonged survival of tumor-bearing mice with no observed adverse effects. These results present a novel RNAi delivery system that opens new therapeutic opportunities for treating MCL and other B-cell malignancies.

5. Future outlook

Only limited success has been currently achieved upon careful examination of ASOs in clinical trials for the treatment of hematological malignancies. This may be attributed to the fact that unlike siRNA in which RISC provides a catalytic mechanism and each siRNA molecule can be used multiple times to target mRNA degradation following cell penetration, ASOs utilize a stoichiometric mechanism, with each ASO blocking one mRNA molecule. Therefore, ASOs are only effective at much higher than siRNA doses (200–900 nM vs 10–200 nM) [55]. In addition, most clinical trials are currently utilizing unpackaged ASOs, for which both cell penetration and circulation time may be very limited.

Moreover, it is important to note that even if the target gene is silenced at the mRNA level, it does not necessarily mean a decrease in the protein level that is required for a robust phenotypic change such as blocking the cell cycle (when blocking such proteins) or apoptotic genes that will result in cell death. The targeted protein half-life plays a crucial role in this respect together with a toxicity window, which might be caused by the delivery system itself and can be confused with the specific killing effect of the target cell. The ability to load the siRNA effectively and release the drug (e.g. siRNA) in the specific target cell (e.g. tumor 'neighborhood' cells) in a sustained release manner will influence the therapeutic window dramatically. This requires smart targeted NPs, which will have the capacity to release its cargo in the right target cell on an appropriate release schedule.

We have recently shown that the combination of binding specific targeting moieties (thus creating targeted LNPs, tLNPs) and grafting hydrophilic polymers on the surface of tLNPs (therefore preventing

opsonization) are necessary in order to overcome the recognition of LNPs by liver cells and the mononuclear phagocytic system [47–50]. Therefore, passive targeting systems may not be the best choice to deliver nanocarriers to blood cancer cells. It is also important to consider the expression levels of the receptor of interest on the targeted cells. Robust expression on both diseased and healthy cells reduces the targeting efficiency of the nanocarriers, as therapeutic uptake occurs in healthy cells as well. Though the side effects should be significantly less profound than those of chemotherapy, it is still vital to consider for maximizing targeted therapy.

Recently, Wilhelm, S. et al. published a perspective examining the success of nanoparticle delivery to tumors [56]. In this review, it was stipulated that <1% of nanoparticles actually reach their desired site. This stresses the importance of an efficient active targeting system that will have extremely high specificity for the ligand of interest. Moreover, because many hematological malignancies are cells in constant circulation, molecular targeting becomes increasingly important as opposed to organ localization methods. Thus, we suggest that novel strategies built on selective targeting to hematological malignancies will be developed taking into account the expression level of the specific receptor, its ability to internalize cargo based on ligand binding, and the kinetics of internalization. These strategies necessitate the interaction of hematologists with fundamental cell biologists, immunologists, and material scientists to focus on better understanding current pathology and potential opportunities to develop novel therapies based on receptor expression and biology.

Though these novel strategies increase the complexity of the delivery systems and will need approval for therapeutic use in humans, they may pave the way for improved RNA therapy in hematological malignancies and other leukocyte-related diseases.

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