Induction of therapeutic gene silencing in leukocyte-implicated diseases by targeted and stabilized nanoparticles: A mini-review

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1. Introduction

RNA interference (RNAi) is an endogenous cellular mechanism of gene silencing. RNAi is carried out by double-stranded RNA (dsRNA) that suppress the expression of specific genes with complementary nucleotide sequences either by degrading specific messenger RNA (mRNA) or by blocking mRNA translation. RNAi can be also activated exogenously by expressing short hairpin RNA (shRNA) with viral vectors, or by incorporating synthetic small interfering RNAs (siRNAs) directly into the cell cytoplasm [1],[2].

siRNAs are chemically synthesized double stranded RNAs (dsRNAs) of 19–23 base pairs with 2-nucleotides unpaired in the 5'-phosphorylated ends and unphosphorylated 3'-ends [3],[4]. Inside the cell cytoplasm, siRNAs are incorporated into RNA induced silencing complex (RISC), a complex that separates the strands of the RNA duplex and discards the sense strand. The antisense RNA strand then guides RISC to anneal and cleave the target mRNA or block its translation [2]. By recycling the target mRNA, the RISC complex incorporating the anti-sense strand may show a therapeutic effect for up to 7 days in dividing cells and for several weeks in non-dividing cells. Furthermore, repeated administration of siRNAs can result in stable silencing of its target [5].

The combination of knocking down a gene of interest and hence treating various diseases by addressing otherwise 'undruggable' targets, the elimination of clinical safety concerns associated with viral vectors and the smaller chances for interference to the endogenous microRNA machinery (which could happen due to saturation of enzymes or transport proteins), emphasizes the potential of siRNAs to serve as a new platform for therapy in personal medicine[6].

Despite this promise, utilizing siRNAs as therapeutics is not a trivial task. For example, due to the large molecular weight (~13 kDa) and the net negative charge, the efficiency with which naked siRNAs molecules cross the plasma membrane and enter the cell cytoplasm is usually very low [2],[7]. When injected intravenously, in addition to rapid renal clearance and susceptibility to degradation by RNAses, unmoldified naked siRNAs are recognized by Toll-like receptors (TLRs). This often stimulates the immune system and provokes interferon response, complement activation, cytokine induction, and coagulation cascades [8,9]. Beside the undesired immune activation, those effects can suppress gene expression globally, generating off-target and misinterpreted outcomes [7],[10]. In addition, the RNAi carriers themselves can activate a robust immune response [11]. Therefore, there is a clear need for appropriate delivery systems for siRNAs, all of which have to utilize cellular mechanisms for internalization, release (from the carriers and escape from the endosomes), and accumulation of the siRNAs in the cell cytoplasm and RISC activation. This mini-review will present the recent progress in this emerging field, focusing on the in vivo applications with particular emphasis on the strategies for siRNAs delivery to leukocytes.

2. In vivo delivery of siRNA

Local delivery of siRNAs has been demonstrated in various animal models [12–15] and is employed in several ongoing clinical trials.
Based on local injections of naked or cationic lipid/polymer-formulated siRNAs, this method of treatment, although having demonstrated very promising outcomes, is suitable only for mucosal diseases or subcutaneous tissues (see Table 1).

Systemic delivery of siRNAs is the most challenging task in this field. While cellular and local delivery strategies have to deal with the need for internalization, release, and accumulation of the siRNAs in the cell cytoplasm, delivery strategies for systemic treatment of an entire animal enforces additional hurdles such as the siRNAs’ interaction with blood components, entrapment within capillaries, uptake by the reticuloendothelial cells, degradation by RNAses, anatomical barriers (such as the liver, spleen and filtration by the kidneys), immune stimulation, extravasation from blood vessels to target tissues, and permeation within the tissue.

Systemic delivery of naked siRNAs may occur by the hydrodynamic method (Table 1). This method involves rapid injection of a large volume of siRNAs in physiological solutions (about 10% of the body weight administered within 5–10 s) [16,17]. Hepatocytes are the main target of this approach. Different studies were done with this method, demonstrating functionally knockdown of specific genes in animals’ liver [16–19]. Nevertheless, due to volume overload side effects, the hydrodynamic method is not appropriate for human therapeutic use.

Naked siRNAs could also be utilized for targeting the kidneys. When systematically administered, large amount of naked siRNAs are excreted by the glomerulus (which excretes any molecule with molecular weight less than 40 kDa) and reabsorbed in the proximal tubule. The accumulation of free siRNA in the kidney is 40 times higher than in any other organ, an ideal propriety for selective gene therapy. Studies in rat models for renal injury indicated functional silencing of p53, a major pro-apoptotic gene, and renal protection, both in single and multiple injections administration [20]. A product based on these studies, QPI-1002, is being developed by Quark Pharmaceuticals for systemic delivery of p53-siRNA in acute renal injury and delayed graft function.

Because of the rapid renal clearance, utilizing naked siRNAs systematically is relevant only when the target organ is the kidney. Otherwise, strategies for systemic delivery of siRNA must rely on carriers. Those carriers should be made from fully degradable materials (to avoid undesired and probably toxic accumulation of the delivery system components in the body) and should act on specific cells or tissues while avoiding collateral damage.

Systemic siRNA delivery strategies could be divided into two major classes: passive and active (targeted) delivery. Passive delivery exploits the inherited tendency of nanoparticles to accumulate in organs of the reticuloendothelial system (RES) also known as the mononuclear phagocytic system. The RES, part of the immune system, consists of phagocytic cells located in reticular connective tissue, primarily monocytes and macrophages. These cells accumulate in lymph nodes, the spleen and kupffer cells in the liver and take up foreign particles recognized as non-self such as viruses, bacteria and parasites of different types, sizes, shapes and charge. Therefore, major attempts have been made to develop siRNA delivery systems for treating different liver diseases. Active (targeted) delivery is based on specific recognition of a cell surface marker utilizing antibodies, ligands or ligand mimetic that direct the nanocarriers to specific target cells and tissues.

3. Passive siRNA delivery systems

Stable nucleic acid-lipid particles (SNALP) are non-targeted liposomes with a diameter of about 100 nm. SNALPs have low cationic lipid content that encapsulates siRNAs and are coated with a diffusible polyethylene glycol-lipid (PEG-lipid) conjugate [21,22]. The PEG-lipid coat stabilizes the particle during its formation and provides a neutral and hydrophilic exterior that prevents rapid systemic clearance. The lipid bilayer containing a mixture of cationic and fusogenic lipids enables the internalization of the SNALP and an endosomal escape while releasing the siRNAs payload. A distribution study demonstrated that 28% of the siRNAs carried by the SNALPs were accumulated in the liver. Functional study of SNALPs encapsulated ApoB-siRNA showed significant reduction in ApoB mRNA levels. Despite the presence of cationic lipids known to trigger toxicities [23], mice and non-human primates did not reveal any adverse effects except an elevation in liver enzyme release in the serum. Based on these results, a phase I clinical trial is conducted nowadays to test the ability of SNALPs to deliver ApoB-siRNAs for reduction in cholesterol level as a proof-of-concept. SNALPs encapsulating siRNA against the polymerase gene of the Zaire strain have been shown to protect guinea pigs from lethal challenge of the Ebola virus [24].

Considering the significant toxicities that have been associated with cationic liposomes, neutral in charge liposomes are very promising carriers for systemic delivery of siRNAs. 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) non-pegylated liposomes encapsulating siRNA against different molecules expressed on melanoma and ovarian cancers inhibited tumor growth in human xenograft models [25,26]. The accumulation of these carriers in the cancerous tissues is based on the permeability and retention (EPR) effect (increased permeability of the blood vessels in tumors caused by rapid and defective angiogenesis, and dysfunctional lymphatic drainage that retains the accumulated liposomes).

Cationic lipidoid (synthetic lipid-like molecules)—containing liposomes are another siRNAs delivery system that has been shown to induce effective genes silencing (80% reduction in ApoB and Factor VII mice’s mRNAs levels) in the liver. Single intravenous injection of cationic lipidoid-containing liposomes encapsulate ApoB-siRNA resulted in 50% decrease in the protein level 3 days and up to 2 weeks after the treatment. Although no immune response was

<table>
<thead>
<tr>
<th>Type of delivery</th>
<th>Carrier</th>
<th>Target tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local delivery</td>
<td>Naked siRNAs; cationic liposomes</td>
<td>Lungs, vaginal, ocular, solid tumor (intra-tumor injection).</td>
<td>12–15</td>
</tr>
<tr>
<td>Passive delivery</td>
<td>Naked siRNAs; hydrodynamic injection</td>
<td>Liver</td>
<td>16–19</td>
</tr>
<tr>
<td>Systemic delivery</td>
<td>Naked siRNAs</td>
<td>Kidneys</td>
<td>20</td>
</tr>
<tr>
<td>Passive delivery (SNALP)</td>
<td></td>
<td>Liver</td>
<td>21–24</td>
</tr>
<tr>
<td>Passive delivery (neutral liposomes)</td>
<td></td>
<td>Tumors</td>
<td>25–26</td>
</tr>
<tr>
<td>Passive delivery (lipidoid containing liposomes)</td>
<td></td>
<td>Liver</td>
<td>27–28</td>
</tr>
<tr>
<td>Passive delivery (atelectoclagen)</td>
<td></td>
<td>Tumors</td>
<td>29–32</td>
</tr>
<tr>
<td>Targeted (cholesterol–siRNAs)</td>
<td></td>
<td>Liver, gut, kidney and steroidogenic organs</td>
<td>33–35</td>
</tr>
<tr>
<td>Targeted (dynamol polyconjugates)</td>
<td></td>
<td>Liver (hepatocytes)</td>
<td>36</td>
</tr>
<tr>
<td>Targeted (PEI nanoparticles)</td>
<td></td>
<td>Certain cancers and tumor vasculature overexpress αv, integrins</td>
<td>37</td>
</tr>
<tr>
<td>Targeted (antibody–protamine fusion proteins)</td>
<td></td>
<td>Breast cancer cells express ErB2 and melanoma cells expressing gp160</td>
<td>38</td>
</tr>
<tr>
<td>Targeted (aptamer–siRNAs)</td>
<td></td>
<td>Prostate cancer cells and tumor vascular endothelium overexpress PSMA</td>
<td>39</td>
</tr>
<tr>
<td>Targeted (targeted cationic liposomes)</td>
<td></td>
<td>Hepatic stellate cells/solid tumors/dendritic cells</td>
<td>40–42</td>
</tr>
<tr>
<td>Targeted (CDP)</td>
<td></td>
<td>Cancers with T1 receptors upregulation</td>
<td>43–45</td>
</tr>
</tbody>
</table>
indicated, increases in the levels of two liver enzymes may suggest liver toxicity [27,28].

Atelocollagen is a biomaterial consisting of a low-immunogenic fraction of pepsin-digested type I collagen from calf dermis. Rich in positively charged residues (lysine and hydroxylysine), it complexes the negatively charged siRNAs and interacts with the plasma membrane, hence helps to incorporate the siRNAs into the cells. Although these particles have not been modified to target tumors, passive targeting due to the EPR effect, causes the selective accumulation within the cancerous tissues as shown in several studies with different tumor xenografts [29–32].

4. Targeted siRNA delivery systems

siRNAs conjugated to targeting moieties is a common strategy for active delivery. Cholesterol–siRNA conjugate is one example. The specificity of this delivery system is determined by the lipoprotein to which the cholesterol–siRNA conjugates are attached in the circulation. When the conjugates bind low density lipoproteins (LDL), the particles are mainly taken up by the liver due to its LDL-receptors’ expression whereas when they bind high density lipoproteins (HDL), they accumulate in the liver, the gut, the kidney and steroidogenic organs, all of which express scavenger receptor class B, type I (SR-BI) receptors, which bind HDL. [33]. Cholesterol-Proapo-siRNA conjugate as well as α-tocopherol [34] and lathiocholic acid or lauric acid conjugated to Proapo-siRNA [35] reduced serum cholesterol and ApoB mRNA levels in the liver. Another example for this strategy is the dynamic polyconjugates [36]. This system includes membrane-active polymers whose activity is masked until reaching the acidic environment of the endosomes. Thanks to the use of N-acetylgalactosamine, which binds to the asialoglycoprotein receptor, they target hepatocytes. Like the SNALPs, these particles, when carried ApoB-siRNAs, decreased ApoB mRNA levels in the liver.

PEI nanopollexes carrying siRNAs have also induced functional silencing in subcutaneously transplanted tumors. Those particles composed of RGD (Arg-Gly-Asp) peptide coupled via PEG (that is required for greater specificity, longer half-life, and reduced immunogenicity) to polyethyleneimine (PEI, a cationic polymer that in addition to its ability to condense nucleic acids, has a pH-buffering property that disrupts endosomes, thus enabling to reach the cytoplasm). When complexes with siRNAs, some RGD-PEG-PEI molecules form a polyplex, with the positively charged RGD-PEG components exposed on its surface. The targeting ability of this particle is based on the overexpression of α4 integrins, to which RGD peptides bind in certain cancers and tumor vasculature [37].

Antibody–protamine fusion carriers are promising system for systemic siRNA delivery. Protamines are relatively small (5–8 kDa) and highly basic proteins composed of 55–70% arginine residues [38]. Positively charged protamine interacts with the negatively charged siRNAs, hence stabilizes, neutralizes and condenses the siRNAs. ErbB2–protamine fusion protein in complex with siRNA significantly inhibited growth of breast cancer cells [39].

Aptamer–siRNA chimeras are completely RNA-based particles for specific delivery of siRNAs. This approach relies on the fact that structured RNAs are capable of binding a variety of proteins with high affinity and specificity. The chimera includes both a targeting moiety, the aptamer, and an RNA-silencing moiety, the siRNA. The aptamer-siRNA chimeras, have demonstrated specific binding and delivery of siRNAs into xenograft model of prostate cancer. The aptamer portion of the chimeras mediates binding to PSMA, a cell-surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium, whereas the siRNAs reduce the expression of survival genes [40]. Addition advantages are the possibility to synthesize large quantities at a relatively low cost and the smaller size of aptamers compared with that of antibodies (<15 kDa versus 150 kDa), which promotes better tissue penetration.

Different formulations of targeted cationic liposomes served for selective targeting of hepatic stellate cells (which are major cell population involved in the formation of scar tissue in response to liver damage, named fibrosis) or solid tumors. Stellate cells express receptors for retinol binding protein which efficiently uptake vitamin A. Based on these, injection of cationic liposomes coupled to vitamin A and complexed with siRNA to a murine key fibrogenesis factor (gp46) into cirrhotic mice, silenced the specific gene in mice liver and resolved fibrosis [41]. 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomes encapsulating HER2-siRNAs and containing histidine-lysin peptides (to enhance the escape from the endosomes) were decorated with a single-chain antibody fragment (scFv) on the liposomal surface targeting transferrin (Tf) receptors ([which are overexpressed on a variety of tumor cells (including pancreatic, colon, lung, and bladder cancer) owing to increased metabolic rates], have been targeted to tumor xenograft and inhibited its growth [42]. A similar strategy was demonstrated with anisamid-PEG-Liposomes-polyconation-DNA (anisamid–PEG-LPDS), which are unilamellar cationic liposomes coated with PEG-linked to anisamide (a small-molecule compound that binds sigma-receptors) on their surface, and protamine-condensed mixture of siRNAs and a carrier calf thymus DNA in their core. Encapsulating EGF-siRNA, anisamide-PEG-LPDS injected intravenously into tumor-bearing mice, have been shown to increase mice’s sensitivity to chemotherapy [43]. These particles induced a significant increase in serum cytokines levels, and therefore weakens the potential for clinical use.

Cyclodextrin-containing polyconation (CDP) particles have been also successfully used for siRNAs delivery into mice’s subcutaneous tumors. CDP is a polymer with a cyclic oligomeric glucose backbone that when complexes with siRNAs assemblies into a colloidal 50–70 nm particle [44]. To achieve targeting, transferrin-coupled PEG is attached to the surface of the particles exploiting the upregulation of Tf receptors in cancers. However, despite considered less toxic than conventional cationic polymers (such as PEI), safety experiment on non-human primates revealed that in the high concentration tested, injection of these particles induced elevation in blood urea (that might indicate kidney toxicity), mild increase in serum liver enzyme levels and a mild increase in IL-6 levels. Multiple injections of the particles induced antibodies to human-Tf. Despite those disadvantages, the efficacy of Tf-coupled CDP containing siRNAs for melanoma cancer treatment is evaluated nowadays in a clinical trial and early results are very encouraging [45].

5. Targeted delivery systems for leukocytes

Utilizing siRNAs to manipulate gene expression in leukocytes holds great promise for the drug discovery field, as well as for facilitating the development of new therapies platforms for leukocytes implicated diseases such as inflammation, blood cancers, and leukocyte-tropic viral infections. However, due to their resistance to conventional transfection methods and to their dispersing in the body, systemic delivery to leukocytes is even more challenging than the systemic delivery to other organs and tissues.

Kortylewski et al. [46] used siRNAs synthetically linked to a CpG oligonucleotide agonist of TLR9 (Scheme 1A) for targeting myeloid cells and B cells (both are key components of tumor microenvironment) that express this receptor. These particles simultaneously silenced stat3 by siRNA and activated TLRs responses by their agonists. Consequently, they effectively shifted the tumor microenvironment from pro-oncogenic to anti-oncogenic (by causing activation of tumor-associated immune cells and potent antitumor immune responses).

Two studies from the same group presented newly developed siRNA delivery systems for treating viral infections. A single-chain antibody against CD7 (a surface antigen present on the majority of human T cells) that was modified to include a Cys residue for
 conjugation to a 9 Arg peptide is a strategy that utilize arginine as RNAi condensing molecule (Scheme 1B). This conjugate was used for targeted delivery of CCR5 (a chemokine receptor that function as co-receptor for HIV) and Vif/Tat (HIV replication proteins)-siRNAs payloads into T cells, and has been demonstrated to suppress HIV infection in humanized mice without inducing toxicity in their target cells [47]. A similar approach for treating dengue virus infected cells employed DC3 (12-mer peptide that targets dendritic cells)-9dR for targeting, with TNF-α (which plays a major role in dengue pathogenesis) or specific highly conserved sequence in the viral envelope-siRNAs. These complexes significantly reduced virus-induced production of TNF-α and succeeded to suppress the viral replication in monocytes derived dendritic cells and macrophages in vitro. In vivo, treatment of mice with intravenous injection of DC3-9dR-complexes carried TNF-α-siRNAs, effectively suppressed this cytokine production by dendritic cells [48].

Zheng et al. have also developed a strategy for specific delivery of siRNAs into dendritic cells [49]. Their strategy is based on immunoliposomes decorated with monoclonal antibody against DEC-205 (Scheme 1C), a dendritic cells’ specific protein. Those particles containing anti CD40 siRNAs, when injected intravenously to mice, demonstrated selective siRNA uptake in immune organs and functional silencing of CD40 that resulted in immune modulation.

The approach we devised for targeting leukocytes is based on leukocytes’ integrins, which are cell adhesion molecules that mediate cell–cell and cell–matrix interactions [50]. We have developed antibody-protamine fusion proteins utilizing the lymphocyte function associated antigen-1 (LFA-1) integrin, which is expressed in all leukocytes’ subtypes, for selective targeting (Scheme 1D) [51]. The use of LFA-1 for targeting leukocytes is supported by its exclusive expression on leukocytes, its constitutive internalization and recycling activity and its ability to undergo activation-dependent conformational changes. Using those antibody-protamine fusion proteins, we showed selective delivery of siRNAs into leukocytes both in vitro and in vivo. Importantly, neither lymphocytes activation nor interferon response was indicated. Furthermore, by targeting these fusion proteins to the high affinity conformation of LFA-1 that characterizes activated lymphocytes, we demonstrated even more selective gene silencing, which unlike most immunosuppressive therapies, could provide a way to overcome the unwanted immune stimulation without global immunosuppressive effects on bystander immune cells. Additionally, due to the prevalent of aberrant affinity modulation of integrins in a variety of leukocyte-implication diseases [52], [53], targeting the high-affinity conformation of LFA-1 seems to be very promising therapeutic tool [51].

Next, in order to increase payload and achieve more robust targeted gene silencing, we have generated integrin-targeted stabilized nanoparticles (I-tsNP) that successfully deliver siRNAs into specific leukocytes subset involved in gut inflammation (Schemes 1E and 2).

Utilizing this system, we identified cyclin D1, a regulator protein of the entry into, and the progression throughout the cell cycle, as a potential new target for treating inflammation. The I-tsNPs (Scheme 2) have been developed as ~80 nm neutral liposomes that were loaded with siRNAs condensed with protamine. The particles have been coated with hyaluronan (HA), a naturally occuring glycosaminoglycan, for stabilization during siRNA entrapment and prolonged circulation time in vivo. The targeting ability of the particles has been achieved by attaching a monoclonal antibody

Scheme 1. Schematic illustration of targeted delivery systems for leukocytes. (A) siRNA synthetically linked to a CpG (B) schematic illustration of the 9 arginine (9R) strategy either with a single-chain fragment antibody (scFv) (B) or with a ligand such as DC3 (not shown here) (C) immunoliposomes-containing siRNAs (D) scFv-protamine fusion protein loaded with siRNAs (E) integrin-targeted stabilized nanoparticle (I-tsNP) entrapping siRNAs.

Scheme 2. Schematic illustration of the process involved in generating integrin-targeted and stabilized nanoparticles (I-tsNP). Multilamellar vesicles (MLV) are extruded into nanoscale unilamellar vesicles (ULV) then undergone surface-modified with high molecular weight (850 kDa) hyaluronan (HA). The HA-modified particles are coupled to mAb using an amine-coupling method, and then the I-tsNPs are purified by a size exclusion column and lyophilized. The siRNAs entrapment procedure is done by rehydrating the lyophilized I-tsNPs with nucleases-free water containing protamine-condensed siRNAs. Alternatively, condenced siRNAs can be included in the swelling solution when MLV are formed.
against βγ integrin (which is highly expressed in gut mononuclear leukocytes) to HA [54]. Made from natural biomaterials, these nanoparticles offer a safe platform for siRNAs delivery, avoiding cytokine induction and liver damage. Enabling usage of low doses of siRNAs (2.5 mg/kg), this system, in addition to advantages such as high payload capacity (~4000 siRNA molecules per particle) and low off-target effects and toxicities, is economically worthy. We also used the 1-tSNP platform with an LFA-1 integrin-targeted antibody for delivery of CCRS5-siRNAs to human lymphocytes and monocytes. This system has been shown to protect mice from HIV challenge [55]. LFA-1-tSNPs with CCR5-siRNAs did not induce interferon response or TNF-α (inflammatory cytokine) secretion, hence strengthens the potential for clinical use.

In summary, although there is no clinical approved siRNA delivery system yet, we are convinced that in the coming years this situation will be changed. We base this assumption on several advantages of siRNAs delivery platforms: the relative easiness of alternating them in nanoparticles or in active delivery systems, the targeting of siRNAs delivery platforms: the relative easiness of alternating them in nanoparticles or in active delivery systems, the targeting moiety (by replacing the antibody or the ligand decorating the nanoparticle's surface). This opens new avenues for treating wide diversity of diseases as well as adjusting the treatment to the unique molecular signature for a specific patient [6]. Since many leukocytes-impaired diseases, such as blood cancers, inflammation and viral infection can benefit from specific delivery platform carrying RNAi payloads, it is more than possible that these will be available within the next 3–5 years and would help in identifying new drug targets, validating specific genes involved in these diseases and potentially be part of new therapeutic modality to treat these diseases.

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References


