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siRNA delivery: from basics to therapeutic applications

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1. ABSTRACT

The chance to selectively intervene and stop the development of any gene-dependent disease in different organs and pathologies makes siRNA an ideal therapeutic agent. However, serious issues should be addressed before the real therapeutic use of siRNA. The poor pharmacokinetic properties of siRNA, its short half-life, its low *in vivo* stability, its fast elimination by kidney filtration and its low transfection efficiency complicate the use of siRNA as a therapeutic molecule. In this review, we will describe the latest and most advanced approaches and strategies undertaken to address these limitations and improve siRNA delivery and its gene silencing efficacy as well as the prospects for its therapeutic applications.

2. INTRODUCTION

Gene therapy is a promising strategy for the treatment of several human diseases as an advantageous alternative therapy. Small interfering RNA (siRNA) is a short double-stranded RNA 21-to-23 nucleotides in length (1). It behaves as a mediator of the RNA interference phenomenon by silencing a specific gene expression by the cleavage of a target mRNA at the post-transcriptional level in the cytoplasm (2, 3). siRNA is believed to be a potentially powerful tool for specific gene silencing in gene therapy. However, the real practical use of siRNA for clinical purposes is hindered by its very low stability in biological surroundings and the lack of simple and efficient delivery systems for the siRNA (4). Thus, siRNA delivery

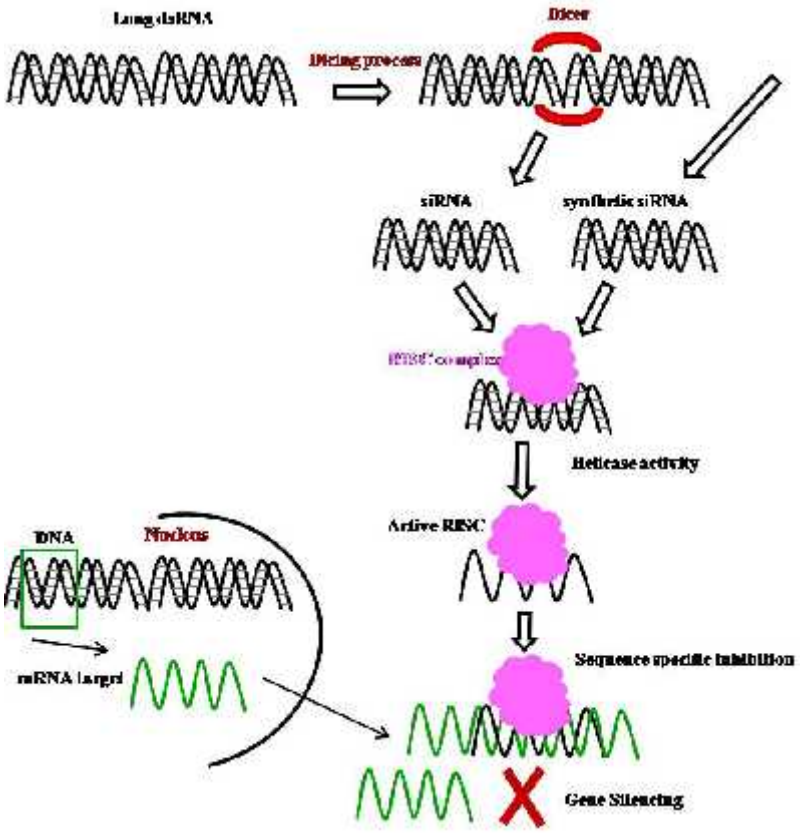

Figure 1. Mechanism of RNA interference. Long dsRNAs are processed by dicer into 21–23-nt siRNAs. Processed or synthetic siRNAs assemble with cellular proteins to form an RNA-induced silencing complex (RISC). During the RISC assembly, one strand (sense or passenger) is eliminated, while the other strand (antisense or guide) produces an active RISC, which eventually triggers the sequence-specific mRNA degradation.

systems must be properly designed to improve the stability of siRNA upon *in vivo* administration, deliver siRNA specifically to the targeted tissue site, and facilitate the cellular uptake of siRNA into target cells (1, 5). Naked siRNA has low stability when administered intravenously, degrades rapidly, and possesses poor cellular uptake. Additionally, the major limitation for the use of siRNA, both *in vitro* and *in vivo*, is the inability of naked siRNA to passively diffuse through cellular membranes due to the strong anionic charge of the phosphate backbone and consequent electrostatic repulsion from the anionic cell membrane surface.

To address these problems, several kinds of nanosized cationic delivery systems have been studied which are based on polyelectrolyte complexes resulting from the electrostatic interaction between the negatively charged siRNA and cationic polymers or lipids (6). It has also been shown that the direct conjugation of aptamers, lipids, peptides, proteins, or polymers to siRNA can improve its *in vivo* pharmacokinetics, increase half-life, and increase delivery efficiency (7-13). Therefore, the primary success of siRNA applications depends on suitable chemical modifications and vectors to enhance siRNA delivery.

2.1. Origins of the "revolutionary" discovery and mechanism of RNA interference (RNAi)

In the late 1980s and early 1990s, some plant biologists working with petunias found out that introducing numerous copies of a gene that codes for deep purple flowers led surprisingly to plants with white or patchy flowers instead of an even darker purple color, as expected (14, 15). Somehow, the introduced genes had silenced both themselves and the plants' own 'purple-flower' genes. Similarly, in 1994 when plants were infected with an RNA virus that had been genetically engineered to contain fragments of a plant gene, the plant's gene itself became silenced (16)**.** At that time the explanation was not fully understood until the revolutionary finding of RNA interference from the work of Fire and Mello who demonstrated in 1998 that the injection of dsRNA into Caenorhabditis elegans triggers suppression of gene activity in a sequence-specific manner. This process was named RNA interference (RNAi) (17) (Figure 1). Their discovery revealed a new mechanism for gene regulation, which plays a key role in many essential cellular processes, such as self-protection against viral attacks (18) and self-regulation of processes to control protein levels in response to various environmental conditions (19, 20).

Over time, this mechanism (see references (21, 22)) has become a potent tool for modulating gene expression in several fields such as functional genomics, drug validation, and transgenic design (14, 23).

2.2. "Anti-mRNA strategy"

Consideration of RNAi mechanisms imply application of a sort of "anti-mRNA strategy". This strategy is developed through the use of different approaches based on different siRNA productions: 1) chemical synthesis, 2) *in vitro* transcription and 3) endogenous RNAi production. Each method posseses advantages and disadvantages. The following paragraph paragraph provides a brief overview of these three approaches.

2.3. Chemical synthesis

Chemical synthesis is the most direct way of creating siRNAs. It has several advantages such as the ability to control the amount and purity of siRNA, the easy characterization and scale-up and the chance to easily introduce chemical modifications to enhance stability and make it specifically targetable (2). On the other hand, a drawback of using chemically synthesized siRNA is that the most effective target sequence is unpredictable (24). In fact, the gene silencing efficiency may vary depending on sequence of the transcripts that are targeted and on the tissue targeted. However, it is possible to obtain an efficient gene silencing by simultaneously delivering a tool composed of different segments of the same transcript (25).

2.4. *In vitro* transcription

As an alternative to the expensive production of chemically synthesized siRNA molecules, many efforts have been made to produce them by *in vitro* transcription by using T7 RNA polymerase. This approach takes advantage of a synthetic DNA template containing the T7 RNA polymerase promoter region followed by the needed RNA sequence. This sequence can be produced using a DNA synthesizer and then amplified by a PCR.

The T7 polymerase binds to the promoter sequence, triggers the transcription, and then moves along the template strand towards the 5'end extending the RNA transcripts. To stop the elongation either a termination region on the DNA or a runoff transcription are used. Transcription of the PCR fragments by this polymerase produces both sense and antisense RNAs that then spontaneously anneal and form a long double stranded-RNA (26, 27). To cleave the long dsRNAs, both recombinant human Dicer and Escherichia Coli RNase III are used.

Nevertheless, this approach is limited by specific sequence requirements related to the T7 that makes the last guanosine of the T7 promoter the first ribonucleotide incorporated into the RNA during the transcription, thus limiting the number of sequences that can be targeted using siRNA molecules generated by this method (26, 28).

2.5. Endogenous RNAi production

Even though it could appear to be the best option to achieve a perfect match with the target gene, the application of chemically synthesized siRNAs is limited because of the low-to-moderate transfection efficiency and the short-lasting effect on gene expression. Furthermore, a single transfection with siRNA sometimes is often insufficient to provide an efficient and functional depletion of proteins with long half-lives.

To overcome these limitations, expression cassettes have been designed to work as expression vectors using short harpin RNA (shRNA) that resemble pre-microRNAs (pre-miRNA) and undergo processing by Dicer. Like synthetic siRNAs, they are designed to pair perfectly with the target mRNA to induce RNAi. Nevertheless, unlike the synthetic siRNAs, these shRNAs are produced using viral expression or plasmid vectors and can induce either transient or long-term gene silencing (29).

2.5.1. Viral expression vectors

Viral vectors encoding shRNA including retroviral, adenoviral, and adeno-associated viral vectors (AAV) are being developed. Typically, these vectors use a Polymerase III promoter, such as U6, H1, or transfer RNA promoters. One of their strong points is their ability to be introduced (and thus express the shRNA) into non-dividing cells, where plasmid vectors cannot be introduced into the nucleus (30). Retroviral vectors have been demonstrated to mediate an efficient and stable siRNA expression (31, 32). Lentiviral vectors efficiently integrate into the genome of non-dividing cells, such as pancreatic islets, hematopoietic stem cells, and terminally differentiated cells. However, lentiviral vectors have sometimes shown insertional mutagenesis. Hence, the use of adenoviral vectors is being studied since these vectors do not integrate into the host genome but efficiently transduce both dividing and non-dividing cells (33). Lack of pathogenicity, and ease of production make AAV vectors an interesting tool for organ-directed shRNA expression. Thus, an evaluation of different AAV serotypes for organ directed shRNA expression is required to selectively target the RNA expression. As an example, AAV-8 vectors expressing shRNA can transduce almost 100% of hepatocytes after intravenous injection into mice (34).

2.5.2. Plasmid vectors

shRNA, siRNA, and miRNA can be produced from plasmid vectors. These plasmids need promoters that are dependent on RNA polymerase, Pol II or Pol III. Different strategies are used to create expression cassettes for generation of shRNA, siRNA, and miRNA. The first strategy is used to generate sense and antisense strands as a single transcript joined by a short loop of 4–10 nucleotides. Thus, the transcript forms a hairpin structure that can be processed by a dicer into a functional siRNA. The second strategy is used to produce sense and antisense strands as two independent transcripts that hybridize to form functional siRNA duplexes in cells. The third strategy gives miRNAs complementary to the target gene (30, 35). However, the production of shRNA expression vectors

raises some serious technical issues. First, it is difficult to sequence constructs that contain a hairpin region because of their tight palindromic structure. Second, between 20% and 40% of constructs become mutated within the hairpin region (36-38).

However, besides the advantages mentioned above, one has to consider some disadvantages. The plasmid- and viral-vector-based expressions of shRNAs by RNA Pol III U6 and H1 promoters sometimes fail to produce the needed levels of gene silencing for cell survival and growth. Additionally, gene silencing for longer periods may result in non-physiological responses. However, this drawback can be solved by generating inducible regulation of RNAi (39, 40). An example of successful regulation of gene silencing is described in (41). Furthermore, , Matsukura *et al.* (42) have applied the same system to a U6 promoter for *in vivo* transcription of shRNA.

2.6. Advantages and limitations in the use of the siRNAs

Ideally the chance to selectively intervene and stop the development of any gene-dependent disease in different organs and for many pathologies makes siRNA the ideal therapeutic agent. In fact, its potential efficacy in the treatment of infections, cancer, neurodegenerative diseases, antiviral diseases, hematological diseases, pain and inherited genetic disorders is likely to lead to several novel medical applications in the near future (see references in (43-47)).

However, to arrive at a therapeutic application of siRNA, several issues still should be addressed. Among them, one can mention the poor pharmacokinetic properties of siRNA, such as its short half-life (intravenous administration of naked siRNA in rats showed a half-life of 6 min and a clearance of 17.6 mL/min), its low *in vivo* stability (since they can be degraded by endogenous RNases), its fast excretion by the kidney filtration because of their small molecular mass (13), its low transfection efficiency, and poor tissue penetration in addition to some biological restrictions and side effects (such as off-target effects and the activation of the immune system (5, 48). Additionally, one of the biggest limitations of its therapeutic potential is represented by the lack of an efficient delivery system to target and deliver the siRNA.

2.7. Over the biological barriers

The full therapeutic efficiency of siRNAs requires overcoming of some biological barriers that stand between the initial administration of these potential drugs and their final activity within cells (49). The understanding of the biological processes underlying these barriers can support the design of more effective delivery systems.

Many strategies (described further in this chapter later) have been studied to overcome the main barriers hampering the delivery of siRNA, such as chemical modifications (to improve the resistance to nuclease digestions when siRNA is in the blood stream) and various polymeric and lipid nanosized delivery systems for an enhanced intracellular siRNA delivery. In this paragraph, we will mention two of the key barriers which affect the delivery, internalization and final activity of siRNA molecules: a) the function of reticuloendothelial system, liver and spleen; and b) the endosomal escape.

2.7.1. Function of reticuloendothelial system, liver and spleen

In addition to circulating nucleases and renal clearance, a major barrier for an effective *in vivo* delivery of oligonucleotides as drugs is the clearance mediated by the reticuloendothelial system (RES). The RES is composed of phagocytic cells, including circulating monocytes and tissue macrophages, whose physiological function is to clear the body from unkonwn pathogens, remove cellular debris that are generated during the tissue remodeling, and clear cells that have undergone apoptosis (50). Phagocytic cells of the RES, particularly the abundant Kupffer cells in the liver and splenic macrophages, also detect and phagocytose antisense and siRNA oligonucleotides, as well as nanoparticle carriers (51) that may be used to enhance their delivery. Macrophages are most abundant in the liver (Kupffer cells) and spleen, tissues that also receive high blood flow and exhibit fenestrated vasculature. Thus, it is not surprising that these organs accumulate high concentrations of antisense and siRNA oligonucleotides after their systemic administration. In addition, uptake by the RES has also toxicological relevance, since *in vivo* toxicity often correlates with the capture and long term deposition of antisense drugs in RES organs, causing harmful side effects including renal tubule degeneration, splenomegaly and increased level of liver transaminases (52). Of note is that uptake by the RES also plays a critical role in the biodistribution of oligonucleotides associated with nanocarriers.

2.7.2. Plasma membrane and endosomal escape

On the other side, the plasma membrane is the main barrier for the siRNA uptake and internalization before it triggers the entire RNAi machinery. In fact, despite its very small size, the high hydrophilicity and negative charge prevent siRNA easy passage through biological membranes. Hence, effective siRNA delivery approaches need to overcome this limitation by improving the cellular uptake. The electrostatic complexation of siRNA molecules with cationic polymers and lipids mask somehow their net negative charge. Furthermore, the net positive charge of these nanoparticles facilitates binding to negatively charged cell membranes with subsequent internalization (53). Alternatively, siRNAs can be delivered to specific cell types through the direct conjugation or association with molecules, including Abs, ligands and aptamers, that recognize specific antigens on the surface of target cells. Upon binding to the cell, these cell-type-specific delivery reagents and the associated siRNAs are taken up by the receptor-mediated endocytosis and deposited into endosomes. Whether they are delivered by cationic lipids, nanoparticles or cell-type-specific delivery reagents, the intracellular trafficking of siRNAs begins in early endosomal vesicles (Figure 2).

These early endosomes subsequently fuse with sorting endosomes, which in turn transfer their contents to

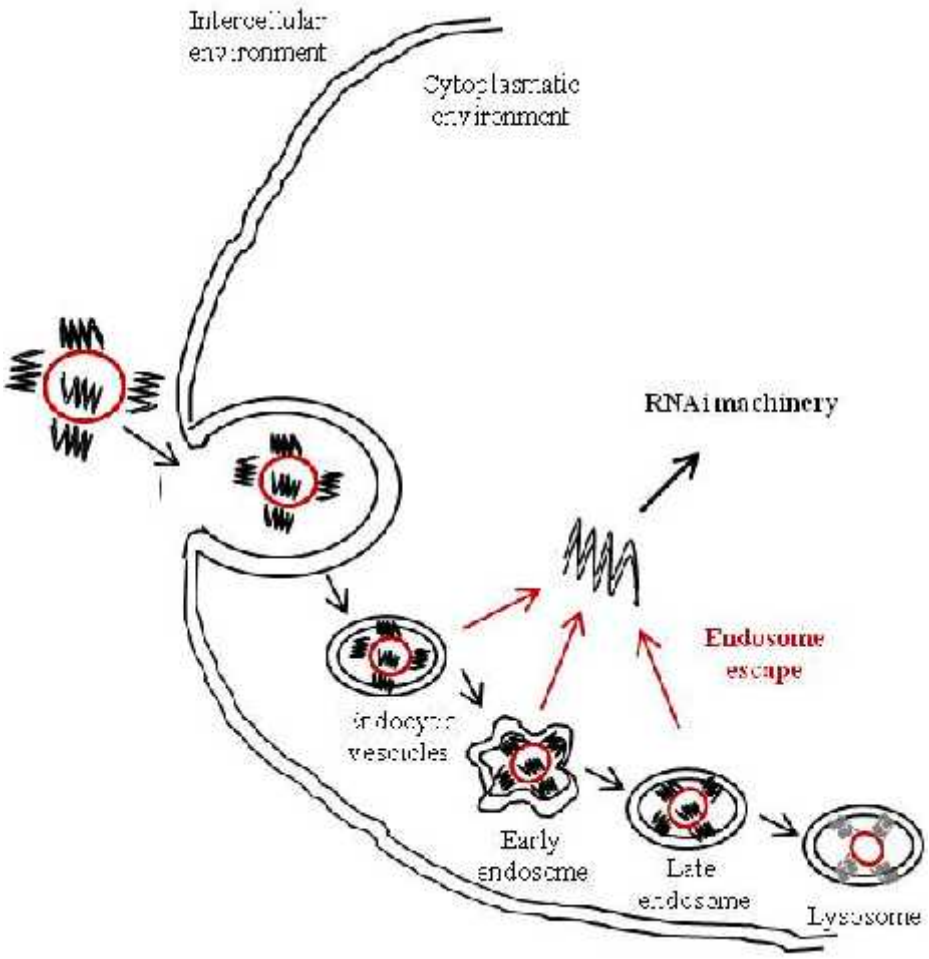

Figure 2. Intracellular trafficking and endosomal escape of a siRNA delivery carrier. The siRNA delivering system interacts with cell-surface and is uptaken into cells by endocytosis. The endocytic vesicles fuse to form the early endosome. The contents of the early endosome are trafficked to the late Endosome to the lysosome, where the hydrolysis of proteins and nucleic acids takes place. To direct target-gene silencing, the siRNAs need to escape from the endosome into the cytoplasm, where they integrate in the RNAi machinery and direct the cleavage of the complementary mRNA.

the late endosomes (54).). Late endosomal vesicles are acidified (pH 5-6) by the membrane-bound proton-pump ATPases. The endosomal content is then relocated into the lysosomes, which are further acidified (pH ~4.5) and contain various nucleases that promote the degradation of the siRNAs. To avoid the lysosomal degradation, siRNAs (free or complexed with the carrier) must escape from the endosome into the cytosol, where they can associate with the RNAi machinery. Endosomal escape represents a major barrier for an efficient siRNA delivery. Currently, some of the approaches to address this issue are represented by the use of fusogenic lipids, pH-sensitive carriers, photosensitive molecules, polymers with high buffering capacity, and membrane-interacting peptides (54).

In the next paragraphs, we will describe strategies that have been implmented to enhance the siRNA stability, facilitate the siRNA to bypass the biological barriers and promote its endosomal release.

3. STRATEGIES FOR AN IMPROVED siRNA STABILITY AND INTRACELLULAR DELIVERY

To address the mentioned limitations and improve pharmacokinetic properties and intracellular delivery of siRNA, a number of strategies including chemical modifications, and the use of non-viral carriers for delivery (8, 55-57) have been suggested.

3.1. Chemical modification of siRNA

We can distinguish between two main categories of chemical modification: a) backbone modifications and b) bioconjugations.

3.1.1. Backbone modifications

To enhance the siRNA stability in biological fluids against the possible attacks by nucleases, the siRNA chain can be chemically modified. Modifications can be made a) on the sugar backbone and b) on the base of the

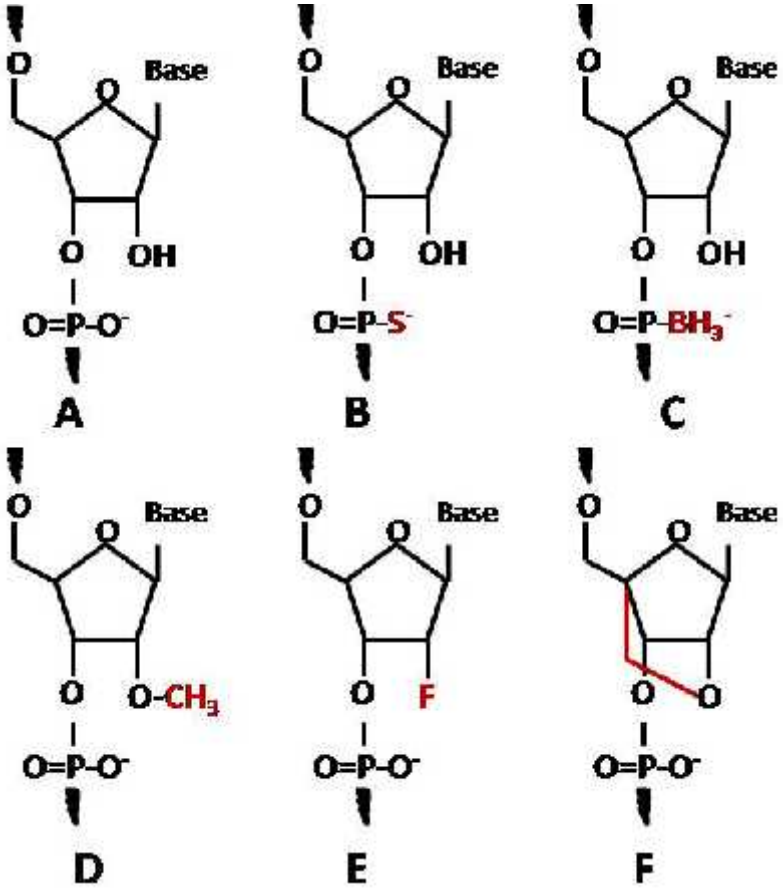

Figure 3. Most common backbone modifications introduced in siRNAs. A: unmodified RNA; B: Phosporothioate RNA; C: Boranophosphonate RNA; D: 2'-O-methyl RNA; E: 2'-deoxy-2'fluoro RNA; F: LNA

nucleotides (Figure 3). These modifications affect the pharmacokinetic profile and reduce the off-target effects without inhibiting the biological activity.

siRNA can be modified on the sugar backbone at the 2'-position of the ribose ring. This kind of derivative showed an enhanced stability against nucleases and favorably reduced the off-target effects (58). However, in the same study it was also observed that the effects of the 2'-O-methyl (2'-OMe) modification on off-target silencing depends on the position.

Chiu and Rana (59) investigated the effects of the 2'-OMe and 2'-deoxy-2'-fluoro modifications and showed that they increase the siRNA plasma stability and *in vivo* efficacy.

Different siRNAs modified at the 2'-OH position and targeting the EGFP gene were tested in HeLa cells. The effects of 2'-OH modifications on the interfering mechanism were studied by replacing uridine and cytidine in the antisense strand of siRNA with 2'-fluoro-uridine (2'-FU) and 2'-fluoro-cytidine (2'-FC), respectively. These modifications increased the siRNA stability upon exposure to HeLa cell extracts, without losing gene silencing activity (59). Additionally, modifications of siRNA with 2'-fluoro pyrimidines did not have any adverse effect on gene silencing and target specificity (60). 2'-F-modified siRNA showed a significant increase in stability in human plasma versus 2'-OH siRNAs. In fact, native siRNA molecules exposed to the plasma were degraded after 4 hs versus a more than 50% stability of the 2'-F siRNA molecules after a 24 h of exposure. Nevertheless, transfecting 2'-F siRNA targeted to GL2 in HeLa R19-Luc cells that express the GL2 luciferase gene showed no significant difference when evaluated on either extension or suppression when comparing the 2'-F and 2'-OH siRNAs (60).

An additional category of siRNA backbone modification is represented by the so-called "locked nucleic acid" LNA. It is a family of locked nucleotides that contain a methylene bridge between the positions 2' and 4' of the ribose ring. Elmen *et al.* (61) reported that this conformation does not affect the interfering mechanism, but it supports the integrity of the siRNA molecule. They stated that the stability of siRNA can be enhanced by conjugating LNA at the 3' ends of the sense strand of siRNA. However, some studies showed that, depending on the extent and location of LNA modifications, they can block not only the induction of IFNs (interferons) but also the gene silencing activity of the siRNA (62).

These kinds of siRNA may be used to increase the half-life of siRNA upon *in vivo* administration by two different mechanisms: (1) by enhancing the resistance of the siRNA molecules against enzymatic degradation, and (2) by supporting the stabilization of the siRNA duplexes.

Some alternative common modifications on the backbone are represented by the "phosphodiester" modifications such as phorothioate linkages (replacing one of the phosphodiester oxygens in the internucleotide linkage with a sulfur atom) and boranophosphonate (where one of the phosphodiester oxygens is replaced by a borane group $–BH_3$).

The degree of modification can be variable and can affect the stability and toxicity of the siRNA molecule. As an example, in the case of phosporothioate modifications, known to reduce the cleavage by nucleases and increase the half-life of ODNs *in vivo*, it was observed that oligonucleotides with extensive substitution linkages can be toxic *in vivo* given their ability to bind to serum proteins (63, 64). Another study by Amarzguioui (29) showed that although backbone modifications do not negatively influence the silencing activity of siRNA, in the case of siRNAs targeting the HTF gene in human keratinocytes, most widely derivatized siRNAs were demonstrated to be cytotoxic resulting in about a 70% cell death.

In the case of backbone modification by boranophosphonate linkages, the charge distribution of boranophosphonate ODNs differs from that of normal phosphate and results in an increased hydrophobicity of the molecule. Boranophosphonate modifications have shown more advantages than phosporothioate modifications. Hall (65) observed that the activity of boranophosphonate siRNAs against GFP in HeLa cells was more efficient than unmodified siRNAs or phosporothioate modified siRNAs, regardless of the base or strand modified. Nevertheless, boranophosphonate subsitutions in the middle of the antisense strand affected the RNAi activity.

3.1.2. Bioconjugations

As in the case of backbone modifications, it has been reported that the direct conjugation of small drug molecules, aptamers, lipids, peptides, proteins, or polymers to siRNA can improve the *in vivo* pharmacokinetic behavior of siRNA (12), since these conjugates can remarkably extend the biological half-life with a simultaneous increase in delivery efficiency to the target tissue while keeping a good gene silencing efficacy.

The two strands of an siRNA molecule play a very important role in triggering the interfering mechanism. Each strand possesses a 3'- and 5'-terminus susceptible to further modifications. However, one has to consider that the antisense strand is the strand with the complementary sequence to a target mRNA. It must be incorporated into the RISC to initiate the RNAi mechanism. Its extremely important functions could be influenced by chemical modification or conjugation of siRNA. Some studies (see references in (59, 66, 67)) indicated that the integrity of the 5′- terminus of the antisense strand is the most important requirement needed to trigger the RNAi mechanism. Hence, the 3′- and 5′-terminus of the sense strand and the 3′-terminus of the antisense strand are considered the potential sites for conjugation with a minimal influence on RNAi activity. However, the 3′- and 5′-terminus of the sense strand are still the most common sites used for conjugation.

Many different conjugates employing several different molecules have been made over the past decade.

3.1.2.1. Lipid-modified siRNAs

As already mentioned, chemical modification of siRNA could be used to increase its intracellular delivery, but may affect its specific activity. To overcome this obstacle, Musacchio *et al.* (68) suggested a simple and effective system capable of stabilization, delivery, and subsequent release of free active siRNA within cells. They reversibly modified a double-stranded GFP-siRNA with a phosphothioethanol (PE) portion via the reducible disulfide bond (Figure 4 A) and incorporated the resulting siRNA-S-S-PE conjugate in nanosized PEG-PE micelles. In the mixed siRNA-S-S-PE/PEG-PE micelles obtained, siRNA was well-protected against degradation by nucleases, and was easily released from these nanoparticles in free form in the presence of glutathione (GSH) at a concentration mimicking its intracellular levels. In GFP-C166 endothelial cells, mixed GFP-siRNA-S-S-PE/PEG-PE micelles that delivered a 84 nM concentration of siRNA, down-regulated the GFP production 50-fold more effectively than free siRNA. In addition, siRNA-containing micelles showed none of the cytotoxic side-effects typical for siRNA delivery systems based on electrostatic association of siRNA with cationic carriers. Thus, the reversible siRNA-phospholipid conjugate formulated as mixed micelles with PEG-PE can be an effective, nontoxic system for stabilization and *in vivo* delivery of siRNA.

One of the most common method of siRNA delivery into cells involves the use of nonviral vectors such as cationic lipids and polymers (see next paragraphs). The interaction between siRNA and cationic lipids is due to the formation of electrostatic interactions which form lipoplexes. However, an alternative to the use of cationic lipids is the use of a covalent strategy between the siRNA and lipids. Following this approach, Grijalvo (69) has reported the synthesis by which glycerol lipid-based structures with different polar groups have been efficiently synthesized and introduced into the 3'-terminus of the siRNA sense strand. This represents the first report of oligonucleotides carrying cationic lipids. The amino group of the lipid is used for the generation of guanidinium groups and also for further functionalization of proteins, liposomes, and nanoparticles. No modifications affected the RNAi machinery through silencing TNF-alpha gene expression.

Recently, Chen (70) demonstrated that an siRNA conjugated with lipophilic molecules enhances cellular uptake in cell culture and produces efficient endogenous gene silencing in the liver after systemic administration and

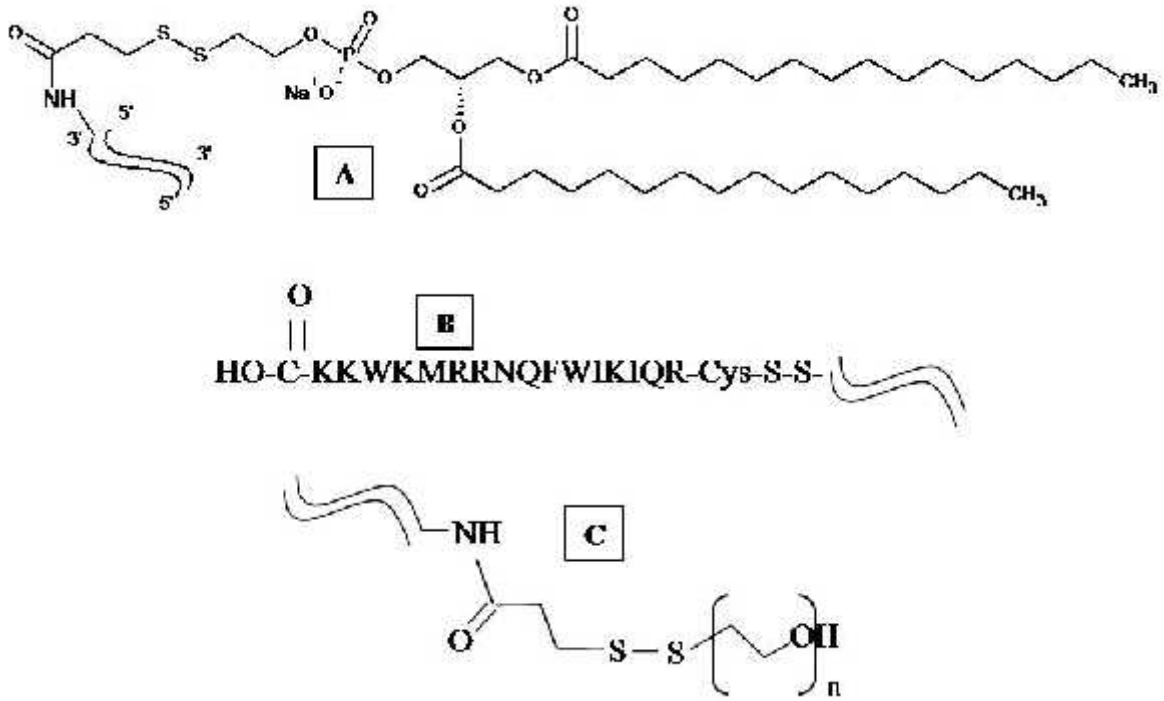

Figure 4. Some examples of siRNA-bioconjugates. A: Lipid-modified siRNA: siRNA-PE conjugate**;** B : CPP-modified siRNA: Penetratin-siRNA**;** C: Polymer-modified siRNA: PEG-siRNA.

as well as in neurons after direct local injection. Specifically, they investigated the *in vivo* delivery of siRNAs conjugated with cholesterol through different linkers by targeting CNPase (2′-3′-cyclic nucleotide 3′-phosphodiesterase) in oligodendrocytes. Cholesterol-conjugated siRNAs administered to the rat corpus callosum by intraparenchymal central nervous system (CNS) infusion showed an improved silencing ability compared with unconjugated siRNA. Furthermore, their siRNA was conjugated to cholesterol with a cleavable disulfide linker that usually appears to be beneficial for improving the potency of silencing of CNPase mRNA in oligodendrocytes *in vivo*. Taken together, these findings indicate that cholesterol-conjugated siRNAs are effective for direct CNS delivery to oligodendrocytes, and that the biocleavable disulfide linker appears to improve the potency of silencing of target mRNA *in vivo*.

In previous works, cholesterol was covalently conjugated to siRNA to study its systemic delivery (13). Cholesterol was conjugated to the 3′-terminus of the sense strand via a pyrrolidone linkage. The cholesterol-siRNA conjugate triggered the RNAi. What is notable is that the conjugate demonstrated a remarkably enhanced cellular transfection efficiency in cultured cells without support from any helpers such as transfection agents. In animal experiments, a significant silencing of the apoliporotein B (apoB) gene, which encodes a protein essential for cholesterol metabolism, was observed in the liver and the jejunum upon i.v. administration of the conjugate. The silencing of the apoB gene was confirmed by a decreased apoB protein level in plasma and thus a reduction in the total cholesterol level. The conjugation of cholesterol to siRNA also improved *in vivo* pharmacokinetic behaviors of siRNA. Additionally, studies concerning the biodistribution of chol-siRNA revealed that it is variously distributed to tissues including the liver, heart, lungs, kidneys, and fat and was detected even 24 h after injection (13).

In addition to cholesterol-siRNA conjugates, we would like to mention studies utilizing the lipidation of siRNA molecules with lipids, such as bile acids (9, 71). They bind to lipoproteins circulating in the blood plasma, such as to high-density lipoprotein (HDL) and low-density lipoprotein (LDL), to lipoprotein receptors, and to transmembrane proteins. Their binding affects the tissue distribution and uptake of the corresponding siRNA conjugates (71). Importantly, modifying the siRNA via “lipidation” implies the modification of the chemical-physical properties of the nucleotides. However, the degree of hydrophobicity, which is directly dependent on the length of the alkyl chain, appeared to be the key factor that determines the affinity of siRNA-fatty acid conjugates for lipoproteins. In fact, those siRNA conjugates with a higher affinity to lipoproteins (the ones with longer fatty acid chains) showed enhanced gene silencing capabilities, supporting the conclusion that lipoproteins facilitate the cellular uptake of the conjugates.

3.1.2.2. CPPs-modified siRNAs

The therapeutic application of siRNA shows a promise as an alternative to small-molecule inhibitors for

the treatment of human disease. However, the major obstacle to its use has been the difficulty in delivering these large anionic molecules *in vivo*. Cell-penetrating peptides (CPPs) or membrane permeant peptides (MPPs) are known to be good vehicle moieties that improve the intracellur uptake of macromolecules such as proteins (72, 73) and nanoparticulate systems (74, 75). They consist of short amino acid chains made mainly of positively charged amino acids, such as arginine and lysine which are responsible for translocation of the peptides through the plasma membrane (76).

One example of such CPPs is represented by the TAT, trans-activator protein, derived from the human immunodeficiency virus type-1 (HIV-1) (77).

The same approach used for proteins or nanosized delivery systems has also been applied to enhance the intracellular delivery of siRNA. The siRNA was chemically modified on the 3′-terminus of its antisense strand with TAT through a heterobifunctional cross-linker (HBFC), sulfo-succinimidyl4-(p-maleimidophenyl)butyrate (78). The TAT-siRNA conjugate confired a significant enhancement in the intracellular delivery of siRNA, and the cellular uptake of siRNA was as efficient as with the use of the helper agent, Lipofectamine. In addition to improved intracellular delivery, the TAT-siRNA conjugate efficiently silenced the target gene. One has to note that, even though the peptide moiety was conjugated to the antisense strand of siRNA via a non-cleavable thioether linkage, this peptidic tail on the siRNA did not affect the interfering process which holds the induction of RNAi in contrast with the assumptions that (as previously mentioned) the 5′-terminus of the antisense strand of siRNA is considered crucial to trigger the RNAi mechanism (66, 67).

Another example employing TATp to efficiently deliver the siRNA intracellularly has been described by Meng S *et al.* (79). They questioned whether or not the siRNA conjugated with TAT (47-57) peptides could enter cultered Huh-7 cells and effectively silence the hepatitis C virus (HCV) infection. For this purpose, the siRNA targeting the 5′UTR of HCV was conjugated to the TAT peptide via the crosslinker sulfosuccinimidyl-4-(p-maleimidophenyl)-butyrate and the conjugates added to the Huh-7 cells. The concentration of HCV RNA was dow-regulated by siRNA-TAT conjugate, and siRNA-TAT mediated the interfering activity that was directly correlated with increasing concentrations of the siRNA-TAT (47-57) conjugate.

Many investigators prefer to use a cleavable linkage, such as the disulfide linkage between CPPs and siRNAs to minimize a potential negative effect of a covalent bond on the RNAi activity. siRNAs were coupled to the penetratin and transportan peptides to improve their cellular internalizations(11, 80). siRNAs targeting GFP and GL2 were chemically modified with a thiol group at the 5’end of one RNA strand and a 5’ Cy3 on the complementary strand. They were furthermore conjugated to these penetratin and transportan via a disulfide bond (Figure 4 B) that is labile in the reducing environment of the cytoplasm and transfected COS-7, C166-GFP, EOMA-GFP and CHO-AA8-Luc cells. Constitutively expressed luciferase and GFP genes were successfully silenced in experiments (11) with the penetratin- or transportan-siRNA conjugates. Cells were more efficiently transfected with CPP-siRNAs than with siRNAs transfected using Lipofectamine 2000. This suggests that other cell types not efficiently targeted with standard transfection protocols, may also be suitable for specific inhibition of gene expression using these kinds of siRNA-conjugates.

3.1.2.3. Polymer-modified siRNAs

An alternative successful strategy for increasing the siRNA stability is based on polymer bioconjugations (8, 81, 82) . Several examples of this approach have been developed and studied over the past decade.

siRNA was conjugated with PEG (poly(ethylene glycol)) at the four different terminal ends (sense 3', sense 5', antisense 3', or antisense 5') via cleavable disulfide and noncleavable thioether linkages to evaluate their gene silencing efficiencies upon complexation with Lipofectamine 2000. The PEGylation site at the four siRNA termini and PEG molecular weight were not critical factors that significantly affected gene silencing activities. Cleavable siRNA-PEG conjugates showed comparable gene silencing activities to naked siRNA, and exhibited sequence-specific degradation of a target mRNA. Interestingly, non-cleavable siRNA-PEG conjugates were processed by dicer, enabling an RNAi effect in a non-target sequence-specific manner. However, only cleavable siRNA-PEG conjugates significantly reduced the extent of INF-alpha release as compared to noncleavable siRNA-PEG conjugates, suggesting that they can be potentially used for therapeutic siRNA applications (83).

Di- and triblock siRNA/PEG copolymers were synthesized and complexed with cationic SLN (solid lipid nanoparticles) for assessment of their gene silencing efficiency as a function of PEG density (84). siRNA molecules were conjugated with PEG via a cleavable disulfide linkage. AB-type diblock and ABA-type triblock copolymers were successfully prepared by stoichiometric hybridization of siRNA conjugates. The corresponding di- and triblock copolymers were characterized by GPC and gel electrophoresis. The serum stability of the siRNA-copolymers was higher than that of the naked siRNA.

For therapeutic applications of siRNA, serum stability, enhanced cellular uptake and ease of endosomal escape are key points to be considered. However, an additional key issue is also the selection of an optimal carrier. Thus, in order to “wrap” and deliver the siRNA with a specific model carrier, further siRNA modifications are needed which modify its chemical-physical properties. A GFP- siRNA (green fluorescent protein) was conjugated to a six-arm PEG derivative via a reducible disulfide linkage (6PEG-siRNA) and conjugated with a cell penetrating peptide, Hph1, to enhance its cellular uptake properties (6PEG-siRNA-Hph1) (85). Then, the 6PEG-siRNA-Hph1 conjugate was electrostatically complexed with the cationic self-crosslinked fusogenic KALA peptide

(cl-KALA) to form a multifunctional polyelectrolyte complex micelles for gene silencing. The resultant siRNA complex formulation showed superior physical stability and resistance to enzymatic degradation, and the 6PEG-siRNA-Hph1/cl-KALA complexes exhibited enhanced GFP gene silencing efficiency in MDA-MB-435 cells in serum containing conditions.

In another example, VEGF-siRNA vascular endothelial growth factor was conjugated to PEG via a disulfide linkage to prepare polyelectrolyte complexed micelles (PECMs) by condensation with a cationic fusogenic peptide (KALA) (86). The siRNA-PEG conjugate (Figure 4 C) exhibited enhanced resistance to degradation by nucleases. The anionic siRNA-PEG conjugate and cationic KALA, when mixed in an aqueous phase, spontaneously formed nano-sized PECMs (<200nm) with an inner core of a charge-neutralized siRNA/KALA complex surrounded by a PEG corona. VEGF-siRNA induced a VEGF sequence-specific gene inhibition in PC-3 prostate carcinoma cells. The extent of gene silencing was gradually increased with an increasing nitrogen-to-phosphate (N/P) ratio and concentration of siRNA-PEG/KALA PECMs. These results suggest that the formulation of siRNA-PEG/KALA PECMs could be widely applied for intracellular delivery of various therapeutic siRNAs.

To consider hepatocytes and hepatic stellate cells (HSCs) as targets, sense strands of siRNA were conjugated to Gal-PEG and mannose 6-phosphate poly(ethylene glycol) (M6P-PEG) for targeted delivery of siRNAs.(87). These siRNA conjugates were purified by ion exchange chromatography and verified by gel retardation assay. To evaluate their RNAi effects, the validated siRNA duplexes targeting firefly luciferase and transforming growth factor beta 1 (TGF-β1) mRNA were conjugated to Gal-PEG and M6P-PEG, and their gene silencing efficiencies were determined after transfection of HepG2 and HSC-T6 cells. The disulfide bond between PEG and siRNA was cleaved by dithiothreitol, leading to the release of intact siRNA. Both Gal-PEG-siRNA and M6P-PEG-siRNA conjugates could silence luciferase gene expression by about 40% without any transfection reagents, while the gene silencing effects reached more than 98% with the help of cationic liposomes at the same dose. Conjugation of TGF-β1 siRNA with Gal-PEG and M6P-PEG silenced endogenous TGF-β1 gene expression as well. In conclusion, these siRNA conjugates showed potential for targeted delivery of siRNAs to hepatocytes and hepatic stellate cells for efficient gene silencing *in vivo*.

Previously, a remarkably enhanced gene silencing in hepatoma cells was achieved by assembling lactosylated-PEG-siRNA conjugates bearing acid-labile beta-thiopropionate linkages into polyion complex (PIC) micelles by mixing with poly(l-lysine) (88). The PIC micelles with clustered lactose moieties on the periphery were successfully transported into hepatoma cells in a receptor-mediated manner, releasing hundreds of active siRNA molecules into the cellular interior by responding to the pH in the endosomal compartment. Eventually, almost 100-fold enhancement in gene silencing activity was achieved compared to that of the free conjugate for the micelle system, supporting the potential utility of siRNA therapeutics.

3.2. Nanoparticulate carriers

siRNAs on their own are not taken up by most mammalian cells in a way that preserves their activity. Moreover, when applied *in vivo*, siRNA-based approaches are all limited by poor penetration into the target tissue and low silencing efficiency. Hence, one of the main challenges in the therapeutic use of siRNAs is to develop an effective drug delivery system able to selectively deliver to target cells, tissues and organs. Ideally, a delivery mechanism should (a) be capable of binding siRNAs in a reversible manner to ensure subsequent release of the siRNAs in target cells; (b) protect siRNAs from nuclease degradation during transit through the circulation; (c) escape from the endosomal compartment; (d) be biocompatible; (e) and avoid rapid clearance by the liver and kidney (1).

Over the last decade, various nanoparticulate systems such as quantum dot nanoparticles, iron oxide nanoparticles, carbon nanotubes, dendrimers, nanogels, silicon particles, lipid and polymeric complexes (see references (89-96)) have been utilized for the development of multifunctional nanovectors for dual therapeutic and diagnostic purposes.

In the following sections, we will provide an overview of the latest advancements and classify the siRNA delivery systems according to a specific category (Figure 5). We will describe their main therapeutic and diagnostic effects, and their contributions to an improved siRNA efficiency in the treatment of different diseases *in vitro* and *in vivo*.

3.2.1. Dendrimers

Dendrimers are a class of polymeric materials. A dendrimer is generally described as a macromolecule characterized by an extensively branched 3D structure that provides a high degree of surface functionality and versatility. The unique properties associated with these dendrimers such as uniform size, high degree of branching, water solubility, multivalency, well-defined molecular weight and available internal cavities make them attractive for biological and drug-delivery applications (97) as well as for siRNA delivery applications (92, 98).

To overcome the known limitations concerning siRNA delivery, Ofek *et al*. (99) assessed a novel polymerized polyglycerol-based dendrimer core shell structure to target the mRNA of tumors *in vivo*. They showed a proof of concept for siRNA delivery *in vivo* using a luciferase-based model. The cationic dendritic nanocarriers exhibited low cytotoxicity and high efficacy in delivering stable and active siRNA using human glioblastoma and murine mammary adenocarcinoma cell lines as model systems. These siRNA-dendrimer polyplexes silenced the luciferase gene otherwise overexpressed in these cells. Additionally, significant gene silencing was accomplished *in vivo* within 24 h of

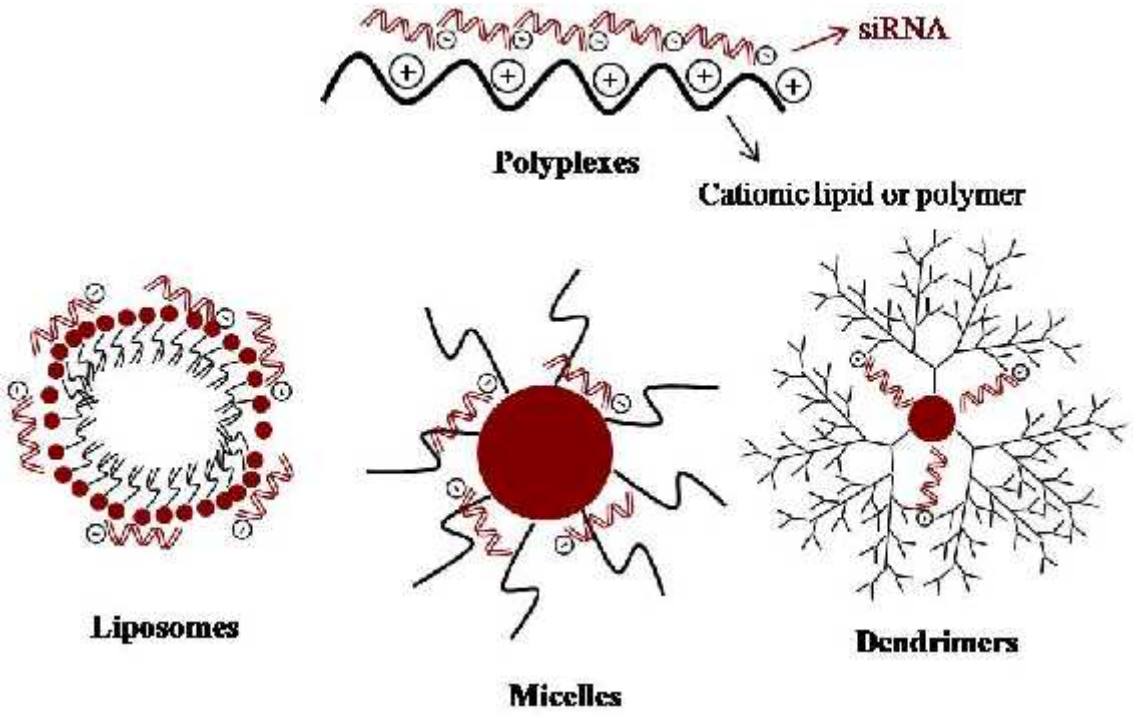

Figure 5. Some examples of the generic structure of nanoparticulate carriers as siRNA delivery systems. Nanocarriers can be additionally variously surface-modified in order to target their siRNA cargo to specific organs or tissues.

treatment with this luciferase siRNA-nanocarrier polyplex, when assessed by non-invasive intravital bioluminescence imaging. Moreover, the siRNA-dendrimers demonstrated very low levels of toxicity. No significant weight loss was observed after intravenous administration of the polyplexes.

A polyamidoamine (PAMAM) dendrimer system, internally cationic and surface neutral, was designed and evaluated for the targeted intracellular delivery of siRNA to the BCL2 mRNA in A2780 human ovarian cancer cells (100). In this study, to improve the siRNA delivery using the hydroxylterminated QPAMAM-OH dendrimer, two independent strategies were investigated: (1) the degree of quaternization for proton sponge or buffering effect; and (2) a targeting ligand as a penetration enhancer. LHRL (luteinizing hormone-releasing hormone) was used as the cancer targeting moiety. Polyethyleneimine (PEI) and polyamidoamine (PAMAM) dendrimers exhibit high transfection efficiency due to buffering or the so-called proton sponge effect that results from the low pKa of tertiary amines (101, 102). It was hypothesized that the substitution of tertiary amines in polyamidoamine (PAMAMOH) dendrimers with permanently charged quaternary amines would obstruct the transfection efficiency of siRNA. Therefore, to increase the transfection efficiency of siRNA, a partially quaternized PAMAM dendrimer (QPAMAM-OH) with approximately 75% of quaternization was synthesized and evaluated as a nanocarrier for siRNA delivery. The evaluation of cellular uptake and transfection efficacy of siRNA delivered by this dendrimer showed that the decreased degree of quaternization enhanced cellular uptake of the QPAMAMOH-siRNA complex and that dendrimers with a lower degree of quaternization (20-30%) delivered siRNA more efficiently when compared with those with a higher degree (70-85%) of quaternization. However, the resultant gene silencing efficacy was very low. Hence, the investigation of down regulation efficiency of siRNA delivered by non-targeted QPAMAM-OH and targeted QPAMAM-OH-LHRH dendrimers demonstrated that only the targeted dendrimer-siRNA complex led to a significant suppression of the expression of the BCL2 gene.

As a modular platform for siRNA delivery for *in vivo* application, we would like to mention the development of PAMAM dendrimer-conjugated magnetofluorescent nanoworms called “dendriworms” (103). This work demonstrated that siRNA-dendriworms can be readily internalized by cells and enable endosomal escape across a wide range of loading doses, whereas dendrimers or nanoworms alone are inefficient. They showed that dendriworms carrying siRNA against the epidermal growth factor receptor (EGFR) reduce protein levels of EGFR in human glioblastoma cells by 70-80%, 2.5-fold more efficiently than commercial cationic lipids when 100 nM siRNA was delivered. Given the efficacy of RNA interference in glioma cells *in vitro*, the effects of siRNA delivery by dendriworms to the brain parenchyma in mice was studied. First, they determined the biodistribution within CNS tissues of healthy CD-1 outbred mice, by formulating 0.5 mg/mL fluorescently labeled dendriworms with 0.115 mg/mL siRNA given by intracranial infusion for a period of 3 days and 7 days using an osmotic pump

delivering at a rate of 0.5 µL/h. Dendriworms were present at the perinuclear region in cells *in vivo* and demonstrated significant suppression of EGFR expression levels *in vivo* in a transgenic mouse model of glioblastoma siRNA (11 µg total siRNA were delivered intratumorally in brain tumor-bearing animals by convection-enhanced delivery using an osmotic pump for a period of 7 days at 0.5 mg/mL of dendriworm and 0.115 mg/mL of siRNA). The magnetic core present in dendriworms should also allow their *in vivo* imaging of dendriworms via MR (magnetic resonance). Collectively, these data establish dendriworms as a multimodal platform that enables fluorescent tracking of siRNA delivery *in vivo*, cellular entry, endosomal escape, and knockdown of target proteins.

3.2.3. Carbon nanotubes

Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure and with novel properties that make them potentially useful in many applications in nanotechnology. Lately, biomedical applications of carbon nanotubes have attracted much attention. Functionalized SWNTs and MWNTs (single and multi walled carbon nanotubes) are conjugated with targeting ligands, including peptides and antibodies for specific cell labeling *in vitro* or tumor targeting *in vivo*. Moreover, CNTs can be loaded with chemotherapy drugs for drug delivery and conjugated with small interfering RNA (siRNA) making them as a further means for the siRNA delivery (91, 104, 105).

CNT have been proposed to offer significant advantages for the intracellular delivery of nucleic acids. Al-Jamal *et al.* (106) recently demonstrated as a proof-of-principle that amino-functionalized multiwalled carbon nanotubes (MWNT) can effectively deliver an siRNA sequence *in vivo* and trigger cell apoptosis that results in human lung xenograft eradication and prolonged survival. A series of polycationic dendron-CNT constructs with a defined tailored number of amino functions (dendron generations) complexed and effectively delivered double-stranded siRNA to achieve gene silencing *in vitro*. A systematic comparison between the amino-functionalized multiwalled carbon nanotubes series in terms of cellular uptake, cytotoxicity, and siRNA complexation was assessed. Significant improvement in siRNA delivery with the dendron-MWNT conjugates and gene silencing was obtained with 2 human cell lines using 2 different siRNA sequences. Thus, through MWNT structure-biological function analysis, novel nanotube-based siRNA transfer vectors can be designed with minimal cytotoxicity, effective delivery and gene-silencing capabilities.

SWNTs have also been used as siRNA delivery vehicles. A novel approach for siRNA cellular delivery using siRNA coiled into carboxyl-functionalized single-wall nanotubes successfully demonstrated non-specific toxicity and transfection efficiency greater than 95%. Three distinct cell types DRG neuronal, neonatal cardiomyocytes and SKHep1 were used to evaluate the potential of short CNTs as transfection agents for clinical therapy. The target mRNA gene was the rat inositol 1,4,5-triphosphate receptor (InsP3R) gene. Cardiomyocytes and dorsal root ganglion (DRG) cells have the common feature of being hard to transfect, which has limited their use in siRNA studies. In SKHep1 cells a higher InsP3R gene knockdown was achieved when cells were exposed to the CNT–siRNA–InsP3R-II complex for 24 and 48 h with a combination of 100 nM siRNA and 0.0250 mg ml^{-1} CNT. CNTs alone did not interfere with InsP3R mRNA levels. Comparable results were found in DRG and cardiomyocyte-transfected cells. In the case of cardiomyocytes, InsP3R gene was additionally suppressed with a higher efficiency when transfected with CNT–siRNA–InsP3R-II complex than with RNAifect transfection agent.

3.2.3. Nanogels

Nanogels are crosslinked particles of sub-micrometer size made of hydrophilic polymers. They have a number of practical applications, mostly in medicine, pharmacy, and lately as siRNA delivery vectors (107-109).

Blackburn (93) described the synthesis of core/shell hydrogel nanogels with a YSA peptide surface modification made via maleimide coupling to the cysteine residue on the C-terminal end of the peptides. The YSA specifically targeted ovarian carcinoma cell lines possessing high expression levels of the Eph2A receptor. Nanogels possess a high degree of porosity permitting a high load capacity. Thus, the investigators were able to develop a scalable synthesis of surface-functionalized, <100-nm diameter, core/ shell nanogels composed of poly(N-isopropylmethacrylamide) (pNIPMAm) (110, 111), an amphiphilic polymer that is strongly hydrated at physiological temperature. The core/shell pNIPMAm nanogel construct was used to encapsulate and deliver siRNA to EGFR gene. EGFR siRNA was encapsulated at a concentration of 16.6 µg of EGFR siRNA/mg of nanogel. The nanogels were then incubated with Hey cells (1 mg of nanogels or 16.6 µg siRNA/5 × 10^5 cells). After a 48 h treatment, a significant reduction in EGFR expression was observed compared to controls (unloaded YSA targeted nanogels, pNIPMAm nanogels, and untreated cells). These nanogels were highly effective in the noncovalent encapsulation of siRNA and enabled cell-specific delivery of the oligonucleotides in serum-containing medium. Cell toxicity and viability assays revealed that the nanogel construct was nontoxic under the conditions studied. Additionally, the results suggested that the endosomal uptake of the nanogels was followed by endosomal escape, resulting in efficient transport/release of the siRNA to the cytosol which was probably driven by an osmotic swelling/deswelling phenomenon fundamental to the phase behavior of gel networks.

Since for the majority of non-viral siRNA carriers studied so far, endosomal compartmentization is the most prominent hurdle, limiting the full gene silencing potential, there is a major interest in methods that enhance endosomal escape of siRNA and improve its intracellular bioavailability. Raemdonk *et al.* (112) proposed an alternatve strategy of nanogel/siRNA delivery for escape from the endosomal compartment based on photochemical internalization (PCI). PCI is a method that employs amphiphilic photosensitizers. It promotes endosomal

escape of several therapeutic molecules, such as chemotherapeutics, proteins and nucleic acids. It has already proven to enhance gene transfer and RNAi silencing with viral and non-viral nanocarriers. After incubation with cells, the photosensitizer predominantly localizes in the endosomal membrane. This results in the production of reactive oxygen species (ROS), predominantly singlet oxygen ($^1O2$) that may catalyze the oxidation of amino acids, unsaturated fatty acids and cholesterol. Due to its short diffusion range (ca. 10–20 nm) and its short lifetime (0.01–0.04 μs), singlet oxygen initiates oxidation reactions mainly in the local production area (113, 114).

This oxidative damage eventually breaks down the endosomal barrier, allowing the transition of internalized nanosized matter into the cytoplasm. Thus, applying PCI at a time-point post-transfection significantly prolonged the knockdown of the target protein only when the siRNA was carried by nanogels and not when a liposomal carrier was used. Combining siRNA nanogels and PCI creates new possibilities for the prolongation of gene silencing by using intracellular vesicles as depots for siRNA followed by well-timed application of PCI becomes critical.

3.2.4. Lipid-based particles

Among various lipid–based particles nanocarrier systems, PEGylated-stealth liposomes may be considered ideal vehicles for siRNA delivery, mainly due to their biological inertness, non-toxicity and protection of siRNAs from nucleases (115). Moreover, the therapeutic efficacy of liposomes can be further increased by their coupling with tumor-specific ligands that enhance their selective interaction with tumors or control the unloading of their cargo within tumors (116). As an example, siRNA-loaded immunoliposomes targeted with anti-transferrin antibody produced specific inhibition of Her-2 expression in animal models of breast cancer and inhibited tumor growth in animal model of pancreatic cancer (117). Surface modification with CPP was also used to deliver siRNA into cancer cells. Liposomes bearing a synthetic arginine-rich CPP are stable and can efficiently transfect lung tumor cells *in vitro* (118). Taken together, targeted siRNA-containing liposomes represent a promising cancer treatment option.

Zhang *et al*. described an interesting approach for siRNA cellular delivery using siRNA encapsulated into liposomes bearing an arginine octamer (R8) attached to their outer surface (R8-liposomes) (95). The authors hypothesized that siRNA-loaded R8-liposomes formulated with a charge neutral ratio of 1,2-dioleoyl-3-trimethylammonium- propane (DOTAP)/siRNA with the addition of the poly(ethylene glycol)-phosphatidylethanolamine (PEG-PE) conjugate for increased *in vivo* longevity could provide an effective transfection system both *in vitro* and *in vivo*. The R8-targeted modified HDM2-siRNA liposomes were tested in lung squamous cell carcinoma (SK-MES-1), non-small cell lung carcinoma (A549), and small cell lung carcinoma (NCI-H446). This system was stable in serum over 24 hs and allowed for a very high transfection efficiency in all three tested lung tumor types. In addition, the system possessed almost no cytotoxicity. The HDM2-siRNAs-containing R8-liposomes were stable during storage at 4 °C with less than 10% release of siRNA after 3 months. R8-lipo-HDM2-siRNA showed biological activity in SK-MES-1 cells in comparisons of HDM2-mRNA levels in the cells treated with 200 nM mock siRNA and HDM2-siRNA over concentrations from 50 to 200 nM for 24 h (untreated cells were used as a control).

One of the latest approaches for targeted drug and siRNA delivery involves the use of phage proteins as carrier moieties (119, 120). Phage display technique offer a unique approach to identification of highly specific and selective ligands that can deliver nanocarriers to the site of a disease. An interesting example of this strategy for intracellular delivery of siRNAs into breast cancer cells through their encapsulation into PEGylated liposomes targeted to the tumor cells with preselected intact phage proteins is described by Bedi *et al.* They applied for the first time the fusion of phage proteins with liposomes for construction of siRNA-loaded nanovehicles that specifically interact with MCF-7 breast cancer cells. As a target, the investigators selected the PRDM14 gene, a member of the family of genes that encode proline-rich domain proteins (PRDM) that may play an important role in breast cancer carcinogenesis (121). The targeted siRNA liposomes were obtained by a fusion of two parental liposomes containing spontaneously inserted siRNA and fusion phage protein pVIII coat protein fused to the targeted DMPGTVLP peptide in MCF-7 cells. These novel phage-targeted siRNA- nanopharmaceuticals demonstrated significant down-regulation of PRDM14 gene expression and PRDM14 protein synthesis in the target MCF-7 cells with delivery at a 40 nM siRNA concentration.

Excitotoxicity is one of the main features responsible for neuronal cell death after acute brain injury and in several neurodegenerative disorders. As an attempt to perform a therapy, siRNA lipoplexes were employed to identify and validate the transcription factor c-Jun, a potential target for successful treatment of excitotoxic brain injury (122). The nuclear translocation of c-Jun and its up-regulation are early events following glutamate-induced excitotoxic damage in primary neuronal cultures. Tf-lipoplexes (50 μl) containing anti-c-Jun or scrambled siRNAs were added to the cells to a final siRNA concentration of 50 nM. An efficient knockdown of this transcription factor occurred using a non-viral vector consisting of cationic liposomes functionalized with transferrin (Tf-lipoplexes). Tf-lipoplexes delivered the anti-c-Jun siRNAs to neuronal cells in culture, resulting in efficient silencing of c-Jun mRNA and protein and let to in a significant decrease of cell death following glutamate-induced damage or oxygen–glucose deprivation. This formulation also induced a significant c-Jun knockdown *in vivo* in the mouse hippocampus of C57/BL6 mice, when 2 μg of anti-c-Jun or scrambled siRNAs was injected at a rate of 0.2 μl/30 s in the right hemisphere (ipsilateral hemisphere) of each animal and resulted in the attenuation of neuronal death. Furthermore, a strong reduction of seizure activity and cytokine production was observed in

animals treated with anti-c-Jun siRNAs. These findings indicate an efficient delivery of therapeutic siRNAs to the brain by Tf-lipoplexes and validate c-Jun as a promising therapeutic target in neurodegenerative disorders involving excitotoxic lesions. This approach prevents the loss of neuronal function in the affected areas and represents a therapeutic alternative to the use of JNK and AP-1 systemic inhibitors, which, although somewhat effective, may lead to serious side-effects in other organs.

3.2.4. Polymer-based particles

A number of polyion complexes (PICs) formed through the electrostatic interaction between the anionic siRNA and cationic polymers have been investigated as micellar carriers of therapeutic siRNA (85, 123-125).

A core-shell-type polyion complex micelle with a disulfide cross-linked core was prepared through the assembly of iminothiolane-modified poly(ethylene glycol)-block-poly(L-lysine) [PEG-b-(PLL-IM)] and siRNA (126). The disulfide cross-links were stable under the non-reductive physiological conditions contributing to the maintenance of micellar structure and then cleaved under reductive conditions following cellular uptake. Disulfides are expected to cleave in the cytoplasm because of high levels of intracellular glutathione (127). The PIC micelles showed a spherical shape of <60 nm in diameter with a narrow size distribution. The micellar structure was maintained at physiological ionic strength but was disrupted under reductive conditions because of the cleavage of disulfide cross-links, which is desirable for siRNA release in an intracellular reductive environment. A series of the disulfide cross-linked micelles composed of PEG-b-(PLL-IM) with various degrees of substitution. PIC-Mx% were evaluated as carriers for siRNA in cultured cells. Huh7 human hepatoma cells were transiently transfected with the reporter genes Pp-Luc and Rr-Luc, followed by the treatment with PIC-Mx% prepared at various N/P ratios containing siRNA against Pp-Luc (126). Effective siRNA transfection was achieved at only the critical N/P ratio of 1.9, where the PIC assembled in the micellar structure. Importantly, stimuli-responsive PIC micelles achieved 100-fold higher siRNA transfection efficacy when compared with non-cross-linked PICs prepared from PEG-b-poly(L-lysine), which were unstable at physiological ionic strength. PICs formed with PEG-b-(PLL-IM) at non-optimum ratios did not assemble into a micellar structure and did not achieve gene silencing following siRNA transfection. These findings showed the feasibility of core cross-linked PIC micelles as carriers for therapeutic siRNA and show that a stable micellar structure is critical for effective siRNA delivery into target cells**.**

pH-responsive complexes have been developed for the delivery of siRNA to sensitize drug-resistant ovarian cancer cells (NCI/ADR-RES) to doxorubicin (96). The electrostatic complexes consisted of a cationic micelle used as a nucleating core, siRNA, and a pH-responsive endosomolytic polymer. Cationic micelles were formed from diblock copolymers of dimethylaminoethyl methacrylate (pDMAEMA) and butyl methacrylate (pDbB). The hydrophobic butyl core mediated micelle formation while the positively charged pDMAEMA corona enabled siRNA condensation. To enhance the cytosolic delivery through the endosomal release, a pH-responsive copolymer of poly(styrene-alt-maleic anhydride) (pSMA) was electrostatically complexed with the positively charged siRNA/micelle to form a ternary complex. Complexes exhibited size (30-105 nm) and charge (slightly positive) properties important for endocytosis were found noncytotoxic and were taken up by >70% of ovarian cancer cells after 1 h of incubation. The pH-responsive ternary complexes were used to deliver siRNA against polo-like kinase 1 (plk1), a gene upregulated in many cancers and responsible for cell cycle progression, to ovarian cancer cell lines. The treatment resulted in <50% reduction of plk1 gene expression in the drug-resistant NCI/ADR-RES ovarian cancer cell model and in the drug-sensitive parental cell line, OVCAR8. This knockdown functionally sensitized NCI/ADRRES cells to doxorubicin at the levels similar to OVCAR8. Sensitization occurred through a p53 signaling pathway, as indicated by caspase 3/7 upregulation following the plk1 knockdown and doxorubicin treatment. This effect could be abrogated using a p53 inhibitor. To demonstrate the potential for dual delivery from this polymer system, micelle cores were subsequently loaded with doxorubicin and utilized in ternary complexes to achieve cell sensitization through simultaneous siRNA and drug delivery from a single carrier. These results showed the knockdown of plk1 leads to sensitization of multidrug resistant cells to doxorubicin. This combination of gene silencing and small molecule drug delivery may prove useful for generating potent therapeutic effects.

In vitro challenges for effective oligonucleotide delivery typically include cellular uptake and escape from endolysosomes. *In vivo* challenges include avoidance of clearance by the liver and spleen and achievement of permeation of the target tissue. In principle, encapsulation can provide protection against oligo degradation and clearance as well as supply a means for cell entry and release into the cytosol (128, 129). Controlled release polymer vesicles or 'polymersomes' (Psomes) seem to possess some of the needed features. Kim *et al* (130) described oligo-loading and functional delivery of a fluorescent-siRNA with nano-transforming polymersomes represented by block copolymers, PEG-polylactic acid (OLA) and inert PEG-polybutadiene (126). OLA Psomes were prepared in a final concentration of 400 µg/ml copolymer and 6 µg/ml siRNA. These degradable carriers were taken up passively by cultured cells, after which the vesicles transform into micelles that allow endolysosomal escape and delivery of either siRNA into cytosol for mRNA knockdown. Quantitation of loading efficiency into the Psomes by spectrofluorimetry revealed up to 30% encapsulation of total added fluorescent-siRNA by OLA Psomes. This encapsulation efficiency appeared similar to that of oligonucleotides in nanocomplexes with the commercially available cationic lipid delivery vehicle, Lipofectamine 2000. The *in vitro* gene silencing by siRNA-Psomes was assessed in human A549 lung cancer epithelial cells (131, 132). The siRNA targeted the mRNA coding for the lamin A/C proteins. The delivery of both fluorescent-polymersomes and fluorescent-siRNA was imaged directly

via the two-color epifluorescence microscopy. Copolymer and siRNA appeared to colocalize in the endolysosomal compartment. The naked siRNA showed no knockdown of the lamin A/C proteins relative to the untreated control cultures – both by imaging and by the plate reader. OLA-Psomes showed a knockdown of 40% that was comparable to Lipofectamine 2000. *In vitro* studies confirmed that polymersome-mediated knockdown appeared as efficient as common cationic-lipid transfection.

4. PROSPECTIVES: THERAPEUTIC APPLICATIONS AND CLINICAL TRIALS

Solutions to issues of formulation, stability, delivery and specificity are crucial for the development of any therapeutic. *In vivo* delivery of siRNA involves basically two approaches: local and systemic administrations. The choice between local and systemic delivery depends largely on what tissues and cell types are targeted and on the selected vector carriers. As additional examples of siRNA delivery to those already described, we can add ocular (133, 134), brain (135, 136), pulmonary (137, 138), muscular (139, 140) and liver (141) delivery.

To date, there are about 20 studies in clinical trials regarding siRNA delivery. Eight out of 20 have already completed their phase I or II trials and are ready to undergo the next steps (source www.clinicaltrials.gov).

Allergan and siRNA Therapeutics carried out a study for the purpose of assessing the safety, tolerability and dose-limiting toxicity of a single intravitreal injection of Sirna-027 (AGN211745) ranging from 100 to 1600 µg. The anatomical changes in the retina, changes in Choroidal Neovascularization (CNV), and changes in visual acuity in patients with subfoveal CNV secondary to Age-Related Macular Degeneration (AMD) were observed. The study enrolled 26 female and male patients at least 50 years old located in the USA (specifically in Maryland and Ohio).

The use of a vector based on a monkey virus called simian virus 40 (SV40) to treat Chronic Myeloid Leukemia (CML) is being studied by the Hadassah Medical Organization in collaboration with the Department of Defense. The method is based on the property of small molecules of nucleic acids (RNA) to specifically repress expression of targeted genes. These siRNA molecules were recently shown to repress, in tissue culture cells, one of two types of the common fusion gene present in CML patients. Those studies showed that treatment with synthetic siRNA inhibited cell growth and increased the sensitivity to Imatinib. An innovative vector has been developed to deliver siRNA molecules into human hematopoietic cells with sufficient efficacy. The viral coat, or capsid, is produced biosynthetically. It was engineered to self-assemble in the test tube around the nucleic acids of choice, and to deliver DNA or RNA into target cells. This vector is safer than other available viral vectors since all of the viral genetic material is excluded from the final product. The vector does not elicit an immune response, thus allowing repeated administration. The study enrolled 25 female and male Israeli patients (at least 18 years old). The criteria of eligibility is to have affected by CLM disease.

Opko Health, Inc. has sponsored a study to assess the safety and efficacy of the small interfering RNA Molecule Cand5 (siRNA molecule that selectively silences the mRNA encoding for VEGF) for the treatment of Diabetic Macular Edema. The purpose of this study was to evaluate the pharmacokinetics, safety and preliminary efficacy of 3 doses of Cand5 (3 mg/eye, 1.5 mg/eye and 0.2 mg/eye). The target population are patients with DME.

Diabetic retinopathy is the leading cause of newly diagnosed blindness in the working age (20-74) population in the United States and is the leading cause of vision loss in diabetic retinopathy. DME is the result of the breakdown of the retinal capillary endothelium in patients with diabetes mellitus (Type I and II). A key factor in the development of DME is the increased permeability of the blood-retinal barrier.

Multiple agents appear to contribute to the disruption of barrier, including vasoactive agents, prostaglandin and vascular endothelial growth factor (VEGF). VEGF acts to promote neovascularization and increases vascular permeability. If the resulting ocular fluid becomes more than the amount that can be removed through the active pump mechanism (retinal pigmented epithelium), fluids continue to accumulate and edema develops. Cand5 is a synthetic double stranded RNA (dsRNA) oligonucleotide. The molecule is a duplex formed by the hybridization of two partially complementary single strand RNAs in which the 3' end are capped with 2 deoxyribose (dT) units. Hybridization occurs across 19 ribose base pairs to yield the Cand5 molecule. Cand5 selectively silences the mRNA encoding for VEGF. The study enrolled 48 female and male patients (at least 21 years old) located in Ohio, US. Volunteers were not accepted for this study; only those patients diagnosed with diabetes mellitus (type 1 or type 2).

Quark Pharmaceuticals concentrated its attention on a study aimed at an evaluation of the dose escalation and safety of the I5NP-siRNA needed to prevent acute kidney injury (AKI) in patients undergoing major cardiovascular surgery. The I5NP is siRNA being developed to protect patients from AKI after cardiac bypass surgery. This first-in-man study tested the safety of I5NP and will consider how long the drug (siRNA) remains in the blood stream after injection. Another aim of this dose escalation study was to determine the optimal dose to be given to study subjects in future studies, even though there were no harmful effects seen in the animals tested. This is a Phase 1, randomized, double-blind, dose escalation, safety and pharmacokinetic study. The study was conducted in several (8 to 10) centers in the United States and Switzerland. 16 patients were enrolled, female and male from 21 to 85 years old. They received a single IV injection of I5NP or placebo following cardiovascular surgery. I5NP was administered 4 hours (+/- 30 minutes) following removal of the cardiopulmonary bypass machine (CBM).

Thus, the data presented suggest the increasing importance, real therapeutic potential and wide applicability that the small interfering RNAs have acquired within the last decade.

5. CONCLUSIONS

Ideally, the chance to selectively intervene and stop the development of any gene-dependent disease in different organs with various pathologies makes siRNA an ideal therapeutic agent. While the silencing of a specific gene has been promising in laboratory studies, there are several limitations to overcome before its therapeutic application can be realized such as stability upon *in vivo* administration, a short half-life, biodistribution, off-target effects and immunostimulation. Several strategies have been studied and implemented to minimize these drawbacks based on chemical modifications of siRNAs and selection of appropriate delivery vehicles. However, improved effectiveness and specificity are necessary to turn siRNA into therapeutics.

In conclusion, RNAi offers great hopes for treating both chronic and acutely acquired diseases as demonstrated by the first results of Phase I and II clinical trials for the delivery of targeted siRNAs.

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