GTMB Vol 18, 14-25, 2018

siRNA delivery technology for cancer therapy: promise and challenges

Review Article

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Key words: Small interfering RNAs (siRNA), Cancer therapy, Delivery systems, Nanoparticles
Abbreviations: Small interfering RNAs (siRNA)

Received: 1 June 2018; revised: 30 September 2007
Accepted: 10 June 2018; electronically published: 10 June 2018

Summary

Cancer is a leading cause of death worldwide. Despite the significant progress made in our understanding of cancer biology, which has led to the development of better diagnostic and treatment methods, overall cancer mortality remains high. A major reason for this is the inability to target cancerous cells selectively with the current therapeutic agents without adverse effects on healthy tissues. Surgical resection, radiation therapy, and chemotherapy are the current therapeutic strategies for cancer. Chemotherapy has many limitations, including difficult administration owing to the poor solubility of chemotherapeutic agents in aqueous solutions, its inability to target cancer cells selectively, its toxicity to healthy tissues, and cancer cell resistance, which hinder its effectiveness. The field of nanotechnology provides promising methods to address these challenges. RNA interference (RNAi) is an evolutionarily conserved mechanism in which double-stranded RNA (dsRNA) molecules silence post-transcriptional expression of homologous target genes (1). The phenomenon of RNAi was first described by Fire et al. in plants in the late 1980s and then they discovered the ability of double stranded RNA (dsRNA) to gene silencing in Caenorhabditis elegans in 1998 (2). Emergence of new tools in the field of RNAi applications led to demonstration of similar processes in mammalian cells in 2001 (3). siRNA molecules are 21 to 23 base pairs (bp) dsRNA which are mediators of RNAi ,to silence the expression of target genes. When dsRNA enters exogenously into the cell or organism in a short form (21–23 bp) or in the form of long dsRNA molecules, they processed by the endogenous enzymatic machinery after introduction into the cell (Fig. 1). First, long dsRNAs cleaves into shorter fragments of ~20 nucleotide (siRNAs) by the cytosolic enzyme Dicer, leaving 2-3-nucleotide 3′ overhangs and 5′ phosphate and 3′ hydroxyl groups (4, 5) Double stranded siRNA is split into sense (passenger) strand and antisense (guide) strand. The sense strand is degraded by an endonuclease of the AGO2–RISC enzyme complex, while the antisense strand guides the RISC to find complementary target mRNA sequences. Unlike miRNAs, siRNAs bind sequences with perfect or nearly perfect complementarity and induce the cleavage of targets by post-transcriptional gene silencing instead of translational suppression (6, 7). Because they can efficiently silence target gene expression in a sequence-specific manner, siRNAs became indispensable tools to study the function of single genes (6, 8).

I. Introduction

Cancer is a leading cause of death worldwide. Despite the significant progress made in our understanding of cancer biology, which has led to the development of better diagnostic and treatment methods, overall cancer mortality remains high. A major reason for this is the inability to target cancerous cells selectively with the current therapeutic agents without adverse effects on healthy tissues. Surgical resection, radiation therapy, and chemotherapy are the current therapeutic strategies for cancer. Chemotherapy has many limitations, including difficult administration owing to the poor solubility of chemotherapeutic agents in aqueous solutions, its inability to target cancer cells selectively, its toxicity to healthy tissues, and cancer cell resistance, which hinder its effectiveness. The field of nanotechnology provides promising methods to address these challenges. RNA interference (RNAi) is an evolutionarily conserved mechanism in which double-stranded RNA (dsRNA) molecules silence post-transcriptional expression of homologous target genes (1). The phenomenon of RNAi was first described by Fire et al. in plants in the late 1980s and then they discovered the ability of double stranded RNA (dsRNA) to gene silencing in Caenorhabditis elegans in 1998 (2). Emergence of new tools in the field of RNAi applications led to demonstration of similar processes in mammalian cells in 2001 (3). siRNA molecules are 21 to 23 base pairs (bp) dsRNA which are mediators of RNAi ,to silence the expression of target genes. When dsRNA enters exogenously into the cell or organism in a short form (21–23 bp) or in the form of long dsRNA molecules, they processed by the endogenous enzymatic machinery after introduction into the cell (Fig. 1). First, long dsRNAs cleaves into shorter fragments of ~20 nucleotide (siRNAs) by the cytosolic enzyme Dicer, leaving 2-3-nucleotide 3′ overhangs and 5′ phosphate and 3′ hydroxyl groups (4, 5) Double stranded siRNA is split into sense (passenger) strand and antisense (guide) strand. The sense strand is degraded by an endonuclease of the AGO2–RISC enzyme complex, while the antisense strand guides the RISC to find complementary target mRNA sequences. Unlike miRNAs, siRNAs bind sequences with perfect or nearly perfect complementarity and induce the cleavage of targets by post-transcriptional gene silencing instead of translational suppression (6, 7). Because they can efficiently silence target gene expression in a sequence-specific manner, siRNAs became indispensable tools to study the function of single genes (6, 8).

I. Challenges with siRNA-based therapeutics

1- Off-target effects: The first barrier is the off-target effects of siRNAs which are designed to knockdown specific target genes. However, recent studies have shown that they may also silence an unknown number of non-target genes through partial sequence complementarity to their 3 UTRs (1).

2- Efficacy: The second barrier is the efficacy of siRNAs, which is the target selection process is extensional, requiring a thorough mining of databases and pathways (9). Efficacy for different parts of the same mRNA sequence varies widely among siRNAs. and only a limited fraction of siRNAs has been shown to be functional in mammalian cells (10). Among randomly selected siRNAs, 58–78% induces silencing with greater than 50% efficiency and only 11–18% induces 90–95% silencing (11).

3- Delivery: siRNA delivery to target tissues is prevented by many barriers at different levels. SiRNAs are unstable under physiological situation. The intracellular trafficking of siRNAs through blood lead to degradation by nuclease in the serum. SiRNAs delivery by different reagent begins in early endosomes. The early endosomes fuse with sorting endosomes which finally transport their content into late endosomes. Endosome is then relocated to the lysosomes (acidic pH 5.0~6.2), and consist various nucleases that degrade siRNA (12). After injection of siRNA into the blood, it is easily degraded by endogenous nucleases, easily filtered from the glomerulus, rapidly excreted from the kidney, taken up by phagocytes and aggregated with serum protein (13). The major nuclease in plasma is a 3 exonuclease, although cleavage of internucleotide bonds can also happen. The susceptibility to degradation by nucleases is a main problem, leading to a short half-life from several minutes to 1 h in the plasma, potentially limiting the use of siRNAs (14, 15). In addition to cellular membrane uptake, plasma nuclease degradation and renal clearance, a major barrier to systemic delivery of siRNA is uptake by the component of reticuloendothelial system (RES). The RES is composed of phagocytic cells, such as circulating monocytes and tissue macrophages which is to remove foreign pathogens, cellular debris and apoptotic cells (Fig 1) (16) However, some chemical modifications can significantly protect siRNAs from nuclease degradation without interfering with the siRNA silencing efficiency and enhance the stability and uptake of naked siRNAs Some modifications such as 2 –o-methyl modifications have been shown to reduce susceptibility to endonuclease activity and to abrogate off-target effects (17). Further, linkage of phosphorothioate (PS) or hydrophobic ligands (e.g., cholesterol, polyethylene glycol [PEG]) increased protein binding and extended serum half-life (18, 19). Besides these, nanocarriers are important tools, providing protection against both rapid renal clearance and nuclease degradation during delivery of siRNAs to target tissues (20). Undesirable physicochemical properties such as negative charge as well as large molecular weight and size reduce the passive diffusion of siRNAs via the cell membrane, which makes endocytosis the major pathway for internalization. (1).

4- Immune response and toxicity: RNAi is a mechanism involved in the innate immunity to protect cells from invasion by nucleic acids of pathogens such as viruses and bacteria. Several studies demonstrated that siRNAs have been known to result in the activation of innateimmune responses by inducing interferon expression, even at low concentrations (21). SiRNAs activate Protein kinase R (PKR) and several toll-like receptors (TLR) signaling pathways; all evolutionary conserved mechanism combact to invade viral pathogens. siRNA may be involved to activate TLR3 signaling in a sequence independent manner.. Certain siRNAs induce the production of proinflammatory cytokines through TLR 7 on human plasmacytoid dendritic cells (pDCs) and likely via TLR 8 on human monocytes in a sequence-dependent manner. Some particular sequence motifs such as 5′-UGUGU-3′ (22) or 5′-GUCCUUCAA-3′

(23) as well as some secondary structures and uridine content of the sequence were recognized as being important for immune stimulation by these pathways. Therefore, Chemical modifications of siRNA such as 2′-O-methylation 2′-deoxy-2′-fluoro groups, locked or unlocked nucleic acids, or phosphorothioate linkages are required to prevent recognition by the innate immune system. Therefore, not only chemical modifications of the siRNA are needed, but additional delivery materials are also essential to eliminate other barriers in the body. Hence, immunostimulatory effects of therapeutic siRNAs must be tested prior to clinical applications (24).

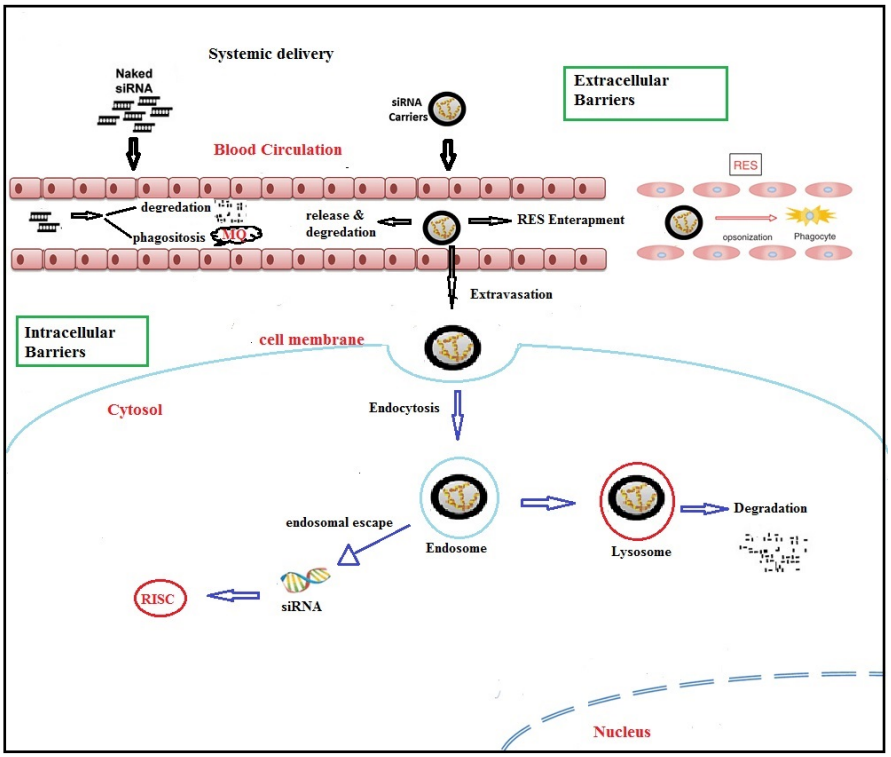

Figure 1: Barriers encountered by systemic siRNA delivery. Extracellular barriers to distribution of siRNA and carriers to the target organs include the degradation of siRNA and carriers, opsonization and phagocytosis by mononuclear phagocyte system and entrapment in reticuloendothelial system (RES). Intracellular barriers include extravasation and penetration in extracellular matrix which are dependent on the physiological structure of the target tissue reticuloendothelial system (RES) and cellular internalization are dependent on the surface properties of siRNA and carriers (e.g., charge, size, pegylation, and specific binding antigen). The crucial barriers for delivering siRNAs to its site of action are the endosomal entrapment and lysosomal degradation of siRNA and carriers. (Reproduced with permission from reference (16).

II. Delivery: local vs. systemic (delivery of siRNA therapeutics: barriers and carriers)

The site of action of siRNA therapeutics is the cytosol. The barriers to siRNA delivery are multiple and depend on the targeted organs and the route of administration. In general, systemic delivery of siRNA poses greater barriers compared to local delivery For example, intravitreal or intranasal routes of siRNA against respiratory syncytial virus, either naked or encapsulated in polycationic liposomes, was almost equally impressive in reducing the viral infection (25). Several excellent reviews have outlined the physical and immunologic barriers to siRNA delivery to the eye, skin, lung, and brain (26-28). Figure 2 shows barriers to systemic siRNA delivery to travel from the site of administration to the site of action. After delivery into the bloodstream, the siRNA undergoes an initial distribution to organs via the circulatory system. Within an organ, siRNA extravasates the intravascular space within a blood vessel to enter the interstitial space. After entering the tissue interstitium, the siRNA is transported across the interstitial space to the target cells. After reaching the target tissue, siRNA can be internalized through endocytosis, in this process siRNA being encapsulated in endocytic vesicles that fuse with endosomes. After entering the cell, the siRNA undergos endosomal escape and be released from its carrier into the cytosol in order to be loaded onto RISC (29).

In spite of the therapeutic promise of RNA therapeutics, effective and safe drug delivery remains a major challenge. Therefore, these therapeutic trends have unfavorable physicochemical properties, including negative charges, large molecular weight and size, and instability. As soon as Naked siRNA is dispensed to the blood, the innate immune system is stimulated, readily degraded by serum endonucleases and is effectively removed by glomerular filtration, resulting in a short plasma half-life of <10 min (30). These problems will have to be solved by common chemical modifications to the siRNA backbone and the use of nano-sized carriers (31).

Since naked siRNA is rarely applied in systemic delivery, this section discuses on siRNA-loaded carriers, Such as nanospheres, nanocapsules, liposomes, micelles, microemulsions, conjugates, and other nanoparticulates.

Owing to similar physicochemical properties between DNA and siRNA, DNA carriers have also been applied to siRNAs. These vehicles for gene delivery divided into two categories, i.e., viral and non-viral. (31). In addition, low toxicity is the most important part of siRNA delivery and due to the unacceptable levels of the viral vectors toxicity; therefore, several synthetic non-viral vectors have been developed to offer alternatives to viral vectors for nucleic acid delivery applications. Non-viral siRNA vectors typically associated with a biodegradable and positively charged vector (e.g., cationic cell penetrating peptides, cationic polymers , dendrimers, and cationic lipids), conjugation of siRNA with a variety of small molecules (e.g., cholesterol, bile acids, and lipids), polymers, peptides, proteins (e.g., antibodies), as well as aptamers (e.g., RNAs) , and encapsulating siRNA in nanoparticulate formulations improves the stability, cellular internalization, or cell-specific active targeting delivery

(Fig. 2). Modification of the RNA backbone improves the stability of the siRNA in serum and is tolerated without any significant affecting its RNAi efficiency. The selection of siRNA carrier systems depends on the siRNA properties , the type of target cells, as well as the delivery routes for in vivo application (32).

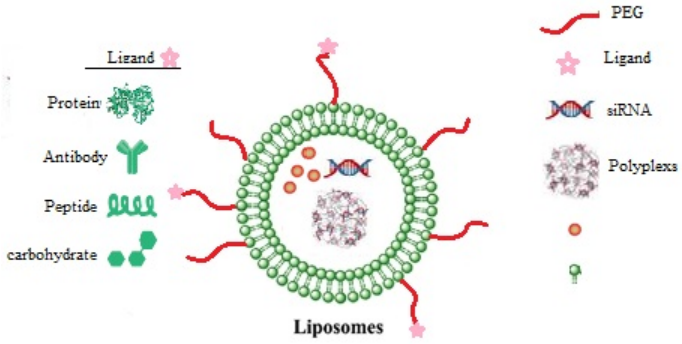

Figure 2. siRNA delivery technologies for systemic applications. Reproduced with permission from ref. (29)

III. Cationic cell penetrating peptides

Cationic cell penetrating peptides (CPP) have been successfully used for the carrying different macromolecules that might vary in size and nature, including proteins (e.g., antibodies), peptides, antisense oligonucleotides, plasmid DNA and nanoparticles (33). In addition to utilizing the traditional endocytic pathways, delivery of CPP-mediated siRNA across the cell membrane in to the cytoplasm is a significant barrier in most primary cells. CPP and siRNA form non-covalent complexes (non-covalent CPP–siRNA) via electrostatic and hydrophobic interactions between positively charged CPPs and anionic nucleic acids, which lead to the formation of complexes with positively charged and different size and stability (34). The main advantage of noncovalent strategy is its simplicity and that the lower concentration of siRNA and CPP is needed to elicit biological response. The lower dose reduces the undesired side effects, like possible toxicity and off-target effects. Moreover, in this approach it does not require a chemical modification of the siRNA, which, in turn, preserves the activity of the siRNA, and protected from the digestion by nucleases both in extra- and intracellular milieu and their half-life is markedly increased (35)

IV. Cationic polymer and dendrimeric carriers

Linear or branched cationic polymers including peptides readily bind and condense DNA and have been used as an efficient transfection reagent for genes, oligonucleotides and siRNA. Structural and chemical properties of these polymers are well established. The positively charged polymers, form polyplexes via electrostatic interactions, with the negatively charged phosphates of DNA (36). This process leads to DNA condensation and protects plasmids from nuclease degradation; facilitate their cellular uptake via endocytosis and resulting in prolonged half-life. In addition, complete encapsulation of siRNA inhibits off-target effects such as immune activation by a toll-like receptor dependent mechanism .Other polymeric vehicles of siRNAs comprise micelles, nanoplexes, nanocapsules, and nanogels (37). The polyplexe charecteristic (e.g., size, surface charge, and structure) depend on the ratio of the positive charges of cationic polymers to the number of phosphate groups of siRNAs. The binary complexes of siRNA with cationic vectors such as lipofectamin (LP), polyethylenimine (PEI), poly-L-lysine (PLL), poly-D,L-lactide-co-glycolide (PLGA), poly(alkylcyanoacrylate), chitosan, and gelatin have been investigated (32).

Dendrimers are highly branched synthetic, monodisperse, and usually highly symmetric, spherical polymers with three-dimensional nanometric structure which is centered on an inner core (~100 nm). The unique structural properties such as flexible structure and molecular size, large number of accessible terminal functional groups, as well as capacity to encapsulate cargos add to their potential as drug vehicles (38).

Polycationic dendrimers like poly (amidoamine) (PAMAM) and poly(propylenimine) (PPI) dendrimers have been reviewed for siRNA delivery in recent years. PAMAM dendrimers have become the most used dendrimer-based siRNA delivery because of the synthesis fesibility and commercial availability. However, PAMAMs were displayed to be cytotoxic, mainly related to apoptosis mediated by mitochondrial dysfunction. Various modifications can reduce the inherent cytotoxicity without compromising gene silencing efficiency. Surface-modified and cationic PAMAM dendrimers penetrate the cancerous cells in vitro and show very low cytotoxicity to the normal cell, even at high concentrations. SiRNA nanoparticles were first formulated with PPI dendrimers , and these nanoparticles showed efficient gene silencing (39)

V. Intracellular siRNA delivery dynamics of integrin-targeted, PEGylated chitosan–PEI hybrid nanoparticles: A mechanistic insight

The natural polycationic chitosan among the polysaccharides has been described in the literature for siRNA delivery many advantages of chitosan include. (i) Its polycationic nature permits for an efficient complexation and condensation of siRNA into nanoparticles via a fast, easy, and gentle process (40, 41) (ii) Chitosan is biodegradable and biocompatible polymer, with mucoadhesive and nuclease resistance properties, which is a crucial factor for in vivo administration (42, 43) (iii) Its chengable chemical structure provides facile chemical modification to equip the polymer with new charecteristics and improve its efficiency (44, 45). However, chitosan has also been used as a carrier for intracellular siRNA delivery and it is limited by low water solubility at pH values above 6.5 and poor colloidal stability in physiologically relevant media (46). Moreover, the transfection efficiency mediated by chitosan is limited owing to its relatively weak buffering capacity and poor endosomal escape (47, 48). Using polyethylene glycol (PEG) grafted chitosan (C PEG) improves the polymer solubility as well as the nanoparticle colloidal stability. Moreover, PEI was included in the

formulation to enhance gene transfection efficiency of the nanoparticles, as previously described (46). Finally, αVβ-3 integrin receptors targeted nanoparticles by conjugating the arginine–glycine–aspartate (RGD) peptide or an RGD peptidomimetic (RGDp) that mimics the RGD motif attach to the distal ends of the PEG chains. αVβ-3 integrins are upregulated in tumor cells and in angiogenic endothelial ce

VI. siRNA bioconjugates

Formulation of siRNAs with a variety of molecules, such as small molecules (e.g., cholesterol, bile acids, and lipids), peptides, polymers, proteins (e.g., antibody), and aptamers (e.g., RNAs) enhance the stability, cellular internalization, or cell-specific active targeting delivery (51). Covalently conjugation of cholesterol to the 3′-terminus of the siRNA sense strand has been done through a pyrrolidone linkage. Conjugation of the cholesterol–siRNA improved stability and, upon intravenous injection, was detected in the liver, heart, lungs, kidneys, and fat tissues at 24 h, silenced gene expression of an endogenous apolipoprotein B (apoB, which encodes a protein involved in cholesterol metabolism) in the liver and the jejunum, decreased plasma apoB protein levels, and reduced the total cholesterol levels. In comparison, naked siRNA intravenous injection did not result in a detectable siRNA activity in tissues at 24 h (32).

VII. Lipid-based carriers

Lipid-based siRNA carrier systems include liposomes, micelles, microemulsions, and solid lipid nanoparticles (52). Liposomes are globular vesicles composed of an aqueous core and phospholipid bilayer, with natural body constituents (e.g., lipids and sterols), and are biocompatible and biodegradable.Moreover, owing to their relative simplicity and well-known pharmaceutical properties, liposomes are popular siRNA vehicles. The amphipathic nature of liposomes allows a wide range of hydrophilic and hydrophobic drugs incorporated into liposomes. Although hydrophilic molecules display greater affinity between the hydrophilic head groups of phospholipid bilayers and the aqueous core of the liposomes, hydrophobic molecules tend to be intercalated into the fatty acyl chains of the lipid bilayer. Several liposomal based anticancer drugs have shown good safety records in humans and one (Doxil) has received FDA approval for human use (32).

The ideal in vivo siRNA carrier system is expected to be safe, non-toxic, biocompatible, biodegradable, and non-immunogenic, and by-pass rapid hepatic or renal clearance. In addition, an ideal carrier system should be able to actively target siRNA to the tumor (20). The enhanced permeability and retention (EPR) effect, typical in the intercellular tumor microenvironment, increases the cellular uptake of nanometric sizes agent and enhances biological action (53, 54). The high cellar tumor density and the stromal compaction can inhibit the movement of drugs into the neoplastic tissue (55, 56). However, the EPR effect for nanoparticles and macromolecules would contribute to drug delivery (55). Non-viral vectors, which are typically based on cationic lipids or polymers, are preferred due to safety concerns to viral vectors. Nanoparticles such as liposomes, micelles, emulsions, and solid lipid nanoparticles have been used as efficient siRNAs delivery. Cationic lipids based delivery systems have been traditionally been the most popular and widely used delivery systems (57). However, strong interaction of cationic lipid carriers with blood components, uptake by the reticuloendothelial system (RES), targeting-related issues, and potential toxicity for lung and other organs were related with the use of these delivery systems (58, 59). Among the non-viral vectors, liposomes are by far the most advanced owing to significant advantages: including efficient interaction with lipidic cell membranes and enhanced endosomal release; resulting in the entrapped siRNA being efficiently delivered. Additionally abundant safe and well-tolerated commercial liposo-mal products for human clinical use offer a large base of knowledge and technical experience. Lipid-based and liposomal delivery vehicles for siRNA molecules have shown their potential by fast entry and growth in clinical trial programs. SNALPs (stable nucleic acid lipid particles) were designed as the most important liposomal-like formulations for siRNA carrying/delivery. Their key portions are a cationic lipid and a ‘‘helper’’ lipid covered by PEG. Results indicated that they can ensure an efficient interaction with siRNAs, endosomal release , and adequate stability for nanoparticle blood circulation (60).

VIII. Mechanistic profiling of siRNA delivery dynamics of lipid–polymer hybrid nanoparticles

Poly (DL-lactic-co-glycolic acid) PLGA is one of the most effectively used biodegradable polymeric nanoparticles which are attractive siRNA carriers because of their minimal systemic toxicity, good colloidal stability, and the possibility of obtaining sustained release of their payload (61). However, efficiency of siRNA delivery based PLGA nanoparticles is generally poor as compared to that observed with lipid-based carriers (62). Therefore, incorporation of commonly cationic excipients such as PEI, DOTAP, or polyamine (63) into PLGA nanoparticles has been widely used as a strategy to improve their transfection capability (64). Cationic lipids, e.g., dioleoyltrimethylammoniumpropane (DOTAP), have been successfully combined with PLGA, thus allowing the incorporation of siRNAs in lipid–polymer hybrid nanoparticles (LPNs) by using different preparation procedures (65, 66). Among these, LPNs prepared at a DOTAP:PLGA weight ratio of 15:85 by using a double emulsion solvent evaporation (DESE) method, lead to nano-sized carriers demonstrating i) enhanced siRNA loading efficiency, ii) sustained release, iii) high transfection efficiency in vitro, and iv) promising therapeutic effects in vivo (67-69).

IX. Delivery of oligonucleotides by using lipid nanoparticles

The composition and physicochemical properties of nucleic-acid based therapeutics are sensitive to nuclease-mediated degradation. In addition, high molecular weight and negative charges of nucleic acids result in impermeable to the cellular membrane. Therefore, an efficient delivery system is an obligatory for therapeutic efficacy. To address pharmaceutical strategies such as stability, cellular uptake,

and targeted delivery, a series of delivery carriers have been expanded. Particularly for cell-targeted delivery, nano-sized delivery systems are the most studied systems.

SiRNAs Delivery to target tissues is prevented by many barriers at different levels. siRNAs are easily filtered from the glomerulus and induce rapid renal clearance (70) Together with rapid clearance kinetics, the susceptibility to nuclease degradation is a major problem, resulting in a short half-life (15 min to 1 h) in plasma, thereby potentially limiting the use of siRNAs (71, 72). However, various chemical modifications have been used extensively to protect siRNAs from nuclease cleavage without interfering with the siRNA silencing efficiency (73) and some others including phosphorothioate (PS) modification or hydrophobic ligands (e.g., cholesterol) were shown to increase protein binding, reduce particle aggregation in serum and extend circulation half-life (74, 75). Besides these, nanocarriers are important tools, providing protection against both rapid renal clearance and nuclease degredation during the delivery of siRNAs to target tissues (75). Due to unfavorable physicochemical properties such as sensitivity to enzymatic degradation, negative charge large molecular weight and size complicate passive diffusion of siRNAs via the cell membrane, which makes endocytosis the major pathway for internalization. This procedure adds new limitations at different stages of delivery to molecular targets, including cell penetration via endocytosis and endosomal release into the cytoplasm (70, 76).

X. Co-delivery of HIF-1α siRNA and gemcitabine via biocompatible lipid polymer hybrid nanoparticles for effective treatment of pancreatic cancer

Naked siRNA has a half-life of less than an hour in the bloodstream and is rapidly degraded by nucleases in the plasma or is excreted by the kidney (77). Furthermore, naked siRNAs rarely permeate cell membranes owing to their high molecular weight, hydrophilic properties, and high density of charge (78). In consideration of these barriers to understanding the potential of siRNA based therapeutics safe and effective delivery systems is essential for therapeutics using siRNA in vivo. Two major classes of biomaterials including liposomes and polymers have been employed for siRNA delivery (52, 77, 79). Cationic liposomes are the most popular lipid based carrier used for siRNA delivery. Positively charged lipids can effectively load siRNAs by electrostatic interaction; however, previous studies suggest that cationic lipids are related to severe toxicity strong immunity inflammatory responses (52, 80). Polycations or polycation-containing block copolymers such as poly(vinyl pyridine), poly(L -lysine), and PEI are polymers used for siRNA delivery. Although many different kinds of biodegradable polycations have been investigated, inducing toxicity of carrier is still a challenge for cationic polymers. Generally, to deliver more siRNA, polycations are designed with high charge densities, which are associated with toxicity. Recently, hybrid lipid-polymer nanoparticles for siRNA delivery have emerged as effective vehicles (81, 82). Zhao et al. (2015) employed biocompatible lipid-polymer hybrid nanoparticles to co-deliver siRNA-HIF1a and Gem for pancreatic cancer treatment in subcutaneous and orthotropic tumor models. The cationic copolymer core and a PEGylated lipid bilayer shell designed nanoparticles. The hydrophilic core of the cationic copolymer can encapsulate Gemicitabine, the hydrophilic core of the cationic copolymer ; meanwhile, negatively charged siRNA-HIF1a can be absorbed on the surface of the cationic co-polymer (83). The PEGylated lipid bilayer shell was allocated for the following reasons: (a) the lipid bilayer shell encapsulated siRNAs inside the nanoparticles, which can protect siRNAs from nucleases degradation and recognition by the immune system; (b) the shell can reduce permeation of Gemicitabine and the replacement of siRNAs by other negative charged substances in the bloodstream; (c) unfavorable aggregation owing to a positively charged surface can be addressed by the negatively charge surface of the lipid shell (77, 84); and (d) the PEG on the lipid shell provides “stealth” properties such as inhibition of serum protein absorption (85).

XI. Preclinical and clinical development of siRNA-based therapeutics

Systemic delivery of siRNAs: Nanocarriers:

Poor cellular uptake, rapid degradation and rapid renal clearance following systemic administration remain an important issue that limits Clinical applications of siRNA-based therapeutics. Nanocarriers not only have great potential to improve cellular uptake but also have great potential to reduce siRNA-related toxicities, prevention of off-target effects, and improvement the biodistribution and pharmacokinetic profiles of siRNA-based therapeutics (86). Nanocarriers are small-sized particles, in the size range 1-300 nm that can carry and deliver drugs, oligonucleotides, peptides, or desired cargos to target tissues. Recently, various nanoparticles have been widely used for siRNA delivery in biomedical applications. Based on surface charge, size, and hydrophobicity, each nanoparticle system has its unique tissue biodistribution, toxicity, and tumor cell uptake profiles. There are several nanomaterials used in the fabrication process including natural or synthetic lipids (e.g., liposomes and micelles), polymers (e.g., chitosan, polylactic-co-glycolic acid, polylactic acid, and PEI), carbon nanotubes, quantum dots, and magnetic nanoparticles (1) which distinguish the attributes of the resulting carrier. Liposomes are microscopic vesicles composed of a phospholipid bilayer that form upon the exposure of a dried lipid film to water. Liposomes present potential benefits for the delivery of siRNAs and have been successfully used in diverse formulations for this reason (87). Liposomes prepared from pure cationic lipids such as DOTAP and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTMA) can effectively take up and condense siRNA and easily interact with negatively charged cell surfaces, thereby facilitating delivery into cells. However, high intracellular stability and resultant failure to release of siRNA contents have limited effects on gene downregulation (88, 89). Challenges observed in the use of cationic liposomes in in vivo mouse models such as dose-dependent hepatotoxicity, pulmonary inflammation, and immune response need to be addressed before their translation into clinical trials (58, 88). Landen et al. 2005 have developed neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)-based nanoliposomes (90).

DOPC-nanoliposomes incorporating siRNAs targeting either EphA2, FAK, neuropilin-2, TMRRS/ERG, IL-8, EF2K, or Bcl-2 were active in orthotropic and subcutaneous xenograft models of various tumors (1) DOPC-nanoliposomes did not cause any detectable distress, toxicity, or immune responses and they were found to be safe after single and repeated intravenous (i.v.) administration of liposomes for 4 weeks (90-92). Additionally, in vivo delivery of naked siRNAs into tumor cells was 10-fold and 30-fold more effective than the delivery of cationic liposomes (DOTAP) and naked siRNAs, respectively (90, 93). Solid lipid-based systems have been developed for systemic siRNAs delivery, utilizing positively charged carriers, including stable nucleic acid-lipid particles (SNALPs) and solid-lipid nanoparticles (SLN) (94, 95) . Although solid lipid based nanoparticles have high serum stability and efficiency in gene silencing, toxicity induced by other cationic lipid-based carriers may pose a challenge (95). Lipidoid particles are lipid like delivery molecules compromised cholesterol and PEG-coated lipids for delivery of specific siRNAs were shown to cause gene silencing at lower doses of siRNA than those required by the original SNALP formulation, resulting in reduced toxicity (95). In addition, siRNA conjugated with lipophilic derivate of cholesterol have been designed for siRNA delivery; however, they require significant improvement with respect to efficacy and safety profiles (74, 96). Polymeric nanocarriers may be useful tools for in vivo applications because of their safety. Nanoparticles made of natural polymers such as chitosan and atelocollagen have been shown to be highly effective for in vivo delivery of siRNAs when administered in mice through intranasal and intravenous administration (97, 98). Synthetic PEI-based nanocarriers show several advantages, including high transfection efficiency and endosomal entrapment. However, due to their cytotoxic effects their use is limited (74, 96). Furthermore, PLGA or polylactic acid (PLA)-based nanoparticles (20), quantum dots (99, 100), and magnetic iron oxide particles (101) have been investigated for siRNA delivery with promising results. The use of nanocarriers is advancement to targeted delivery (active delivery) of therapeutics into tumor cells and/or tumor vasculature. In general, to enhance the delivery of therapeutics into the tumor tissue, high-affinity ligands such as functional peptides, lipophilic molecules, PEG, and aptamers are attached to the exterior surface of nanoparticles Surface functionalization of nanoparticles with proteins such as folate receptor alpha, transferrin receptor, $\alpha V\beta 3/5$ integrin receptors, and prostate specific membrane antigen (PSMA) holds great promise as therapeutic intervention for enhanced tumor delivery of siRNAs (87).

XII. Locally delivered siRNA-based therapeutics:

Local delivery of siRNA is beneficial for diseases, because tissues are externally accessible or locally restricted. To date, locally administered siRNAs have been used in clinical trials for topical diseases mostly including the eye such as age-related macular degeneration (AMD), diabetic macular edema (DME), and glaucoma as well as in those for a small number of other diseases, involving respiratory syncytial virus (RSV) infections, pachyonychia congenita, and pancreatic ductal adenocarcinoma.

XIII. Clinical trial

Today siRNA-based therapies are processing into the clinic especially for diseases requiring locoregional treatments, including age-related macular degeneration, diabetic macular edema, and respiratory virus infection, pachyonychia congenital, hepatitis, human immunodeficiency virus infection, and cancer. (32, 102). In 2004, the first clinical trial involving siRNA was carried out for the treatment of acute macular degeneration (AMD) (103).

Currently, there are some examples of cancer clinical trials using nanoparticle-based siRNA delivery (Table 1). The first clinical trial of the siRNA-mediated effect for human solid tumor was began in 2008; this study targeted ribonuclease reductase (RRM2) using a cyclodextrin based polymer conjugated siRNA (104) These nanoparticles contain a cyclodextrin-based polymer, is an siRNA targeting the M2 subunit of ribonucleotide reductase where siRNA is formulated in self-assembled cyclodextrin nanoparticles with surface pegylation and conjugation with the transferrin ligand (105). These encouraging clinical results indicate that different preclinical cancer models provide proof-of-principle for the transforming of siRNA-based nanotherapies from a research tool into clinic evaluation and future applications (87).

Table 1: Examples of siRNA cancer therapeutics in clinical trial

Target gene	Intervention	Malignancy	Phase
EphA2	Neutral liposome (DOPC)	Advanced solid tumors	I
Fus1	Nanoparticle (DOTAP): Chol-fus1	Lung cancer	I/II
EGFR	Phoshorotioate ODN	Advanced head & neck squamous cell carcinoma	I/II
M2 subunit ribonucleotide reductase (RRM2)	Cyclodextrin nanoparticle, Transferrin, PEG	Solid tumors	I

Polo like kinase I (PLKI)	Lipid nanoparticle (SNALP)	Solid tumors	I
Bcl2 interacting killer (Blk)	BikDD Nanoparticles	Advanced pancreatic cancer	I
HIF-1α	LNA antisense oligonucleotide	Advanced solid tumors or lymphoma	I
Protein kinase N3 (PKN3)	Liposome (Lipoplex, cationic lipid)	Advanced solid tumors	I
VEGF	dendrimer type bio-reducible polymer (PAM-ABP)	human hepatocarcinoma (Huh-7), human lung adenocarcinoma (A549), human fibrosarcoma (HT1080) cells	-

Ozpolat B, et. al, 2014, Davis ME, et al, 2009, 2010.

XIV. Conclusion and future prospects:

Tremendous progress has made in development of RNAi-based therapeutics to enhance further opportunities for successful cancer treatment. siRNA-based therapeutics are highly effective pathways to treat multiple cancers due to the ability to specific silencing the expression of cancer-related genes or to selective regulating the pathways that are involved in cancer progression. Although fundamental progress has been made in the field of in vivo siRNA delivery, there are a number of obstacles and concerns to be overcome before RNAi will be harnessed as a new therapeutic condition. These include strategies to minimize off-target effects, avoiding immune responses, increasing resistance to nuclease degradation competition with cellular RNAi components and effective in vivo delivery to the appropriate cells or tissues by manipulating biopharmaceutical properties. Furthermore, we offer that a delivery route, sophisticated delivery carriers, chemical modification, and modified RNAi foundation are needed to improve targeting efficiency of RNAi in cancer cells.

Recently, nanoparticle based delivery systems hold great potential for successful and safe systemic delivery of siRNA-based therapeutics. Although, this promising technology remains one of the most significant barriers to the widespread use of RNAi therapeutics in systemic delivery system for in vivo application, however neutral lipid-based nanoliposomes have emerged as a highly effective and safe delivery system for systemic use of siRNA therapeutics. Neutral nanoliposomal siRNA-based therapeutics or other safe nanocarriers offer great hope for targeted therapies of various cancers by targeting signaling pathways and oncogenes that promote cell proliferation, cell cycle progression, invasion/metastasis and resistance mechanisms in tumors. Hopefully, small RNA delivery strategies open the door as a conventional treatment for cancer and other human diseases.

XV. References

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