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siRNA and RNAi Optimization

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Abstract.

The discovery and examination of the post-transcriptional gene regulatory mechanism known as RNA interference (RNAi), contributed to the identification of small interfering RNA (siRNA) and the comprehension of its enormous potential for clinical purposes. Theoretically, the ability of specific target gene downregulation makes the RNAi pathway an appealing solution for several diseases. Despite numerous hurdles resulting from the inherent properties of siRNA molecule and proper delivery to the target tissue, more than 50 RNA-based drugs are currently under clinical testing. In this work we analyze the recent literature in the optimization of siRNA molecules. In detail, we focused on describing the most recent advances of siRNA field aimed at optimize siRNA pharmacokinetic properties. Special attention has been given in describing the impact of RNA modifications in the potential off-target effects such as saturation of the RNAi machinery, passenger strand-mediated silencing, immunostimulation and miRNA-like off-target effects as well as to recent developments on the delivery issue. The novel delivery systems and modified siRNA provide significant steps towards the development of reliable siRNA molecules for therapeutic use.

Introduction

The breakthrough of RNAi phenomenon[1, 2] has incited the discovery of the core proteins of RNA-induced silencing complex (RISC)[3, 4], the identification of the silencing trigger molecule[5] and the design of synthetic siRNA duplex[6]. Later on, the thrilling idea of siRNA target-specific signature, due to the necessity of perfect identity with its cognate messenger RNA (mRNA), had to deal with the silencing of unintended targets[7]. Furthermore, because of the inherent siRNA limitations (poor bioavailability, instability against nucleases, hydrophilicity and polyanionic nature), the development of RNAi-based therapeutics had suffered a serious slowdown. However, the ease of customization, the possibility of treating "undruggable" diseases and shortening the drug discovery process, have made siRNA-based therapeutics appealing agents for future tailored medicine (Figure 1).

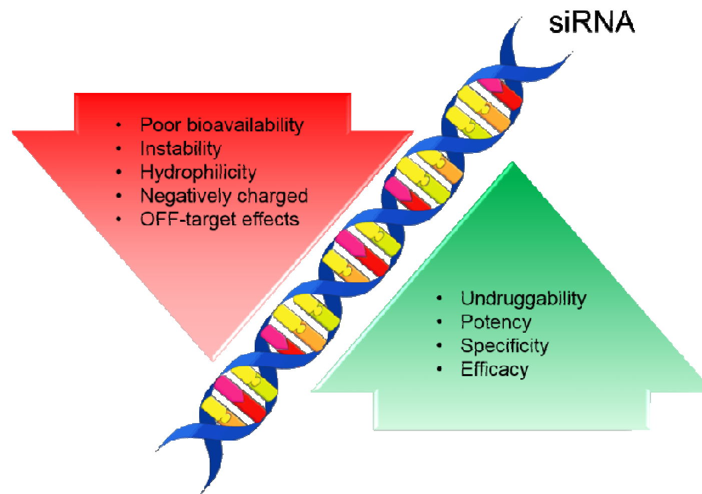

Figure 1. Benefits and drawbacks of siRNA-based therapeutics. In addition to important unwanted off-target effects such as the up- and down-regulation of thousands of unrelated genes, during the attempt of develop RNAi-based therapeutics some intrinsic siRNA limitations have been identified. Upon entering the bloodstream the siRNA molecules are promptly degraded and eliminated, the action of ribonucleases and reticuloendothelial system (RES) compromises its bioavailability under physiological conditions. Furthermore, the siRNA hydrophilicity, high molecular weight and polyanionic nature restricts its passive diffusion across cellular membranes. Even though all these serious issues, the siRNA properties can be adjusted in terms of gene silencing abilities (potency, specificity and efficacy). The ability of siRNA molecule to target any disease-related gene offers a valuable option to block the production of all those undruggable proteins, not-responding to traditional therapy, at the messenger RNA level.

Theoretically, the siRNA nucleotide sequence should determine the specificity, potency and efficacy of siRNA-mediated gene silencing, but many factors influence its reliability. For example, the specificity, the ability to knockdown the target gene without interfering with the expression of other genes, is basically compromised by partial complementarity between the siRNA sequence and unintended targets. To ensure exact recognition of the target mRNA, chemical/structural modifications supported by bioinformatics tools have been extensively employed[8, 9]. But despite the great deal of efforts, the specificity remains one of the most challenging tasks for siRNA optimization. In addition, the mRNA accessibility affects the siRNA efficacy. The presence of secondary/tertiary structures on mRNA transcripts hinders the hybridization between the siRNA-programmed RISC and the target site sequence[10, 11]. Since excess amounts of siRNA saturate the RNAi machinery, the design of powerful siRNA molecules is indispensable. More powerful siRNA duplexes have been successfully synthesized using several chemical modifications.[12]. Finally, the implementation of siRNA activity for clinical applications should be addressed by the design of carriers able to deliver the silencing cargo into specific tissue. To enhance the siRNA bioavailability, its stability into biological *milieu* and overcome the physiological barriers after systemic administration, many delivery strategies have been evaluated[13]. Hence, the achievement of an optimized siRNA design strategy is possible, only taking into account all these issues. Here, we presented an overview focusing on the

recent progress in siRNA field aimed at optimize siRNA pharmacokinetic properties. Furthermore, we outlined the recent advancements in delivery strategies.

1. Dissecting the siRNA: chemical modifications and siRNA structure.

1.1 How Argonaute splits the siRNA molecule

Argonaute proteins are the fundamental components of the small-RNA-driven gene silencing mechanism. In human, eight Argonaute proteins have been identified; four of them belonging to Argonaute subcategory (hAgo 1-4) and the other four to the PIWI subcategory (hPIWI 1-4). Among them, only Ago2 possesses the ability to cleave the target mRNA[14]. Following the discovery of the core protein of RNAi pathway, intensive studies have defined its domain composition. The hAgo2 has revealed cradle shaped architecture and highly conserved domains: MID, PIWI, PAZ and N-lobe[4] (Figure 2). The MID interacts with the first nucleotide (5'-phosphorylated) at the 5'-end of the siRNA guide strand[15]. The PIWI motif, harboring the RNase-H like catalytic core of Ago2, is responsible for the endonucleolytic cleavage of the target mRNA[16]. The PAZ domain is a hydrophobic cavity able to recognize the 3' terminal dinucleotide overhang of the siRNA strand[17]. Finally, the N-lobe acting as a molecular wedge, is strictly required for duplex unwinding and maturation of RISC[18]. Thanks to the 3' terminal unpaired dinucleotide structures and 5' phosphate group, the Argonautes can efficiently identify the siRNA/miRNA (microRNA) molecules among other small RNAs[19]. Moreover, the RISC is able to discriminate between double stranded (ds) RNA and dsDNA molecules, only the formers are powerful triggers of gene silencing. Unlike dsDNA, that adopts a B-form conformation (right-handed helix, 10bp per helical turn and 20 Å in diameter), dsRNA displays an A-helix motif and one helical turn containing 11 bp with a diameter of 23 Å. The wider major groove of the A-form helix is needed for stable interactions with RISC and target mRNA[20].

Upon RISC assembly and the degradation of the passenger strand, Ago2 functionally classifies the guide strand into 5 discrete sections: (i) anchor; (ii) seed; (iii) central; (iv) 3' supplementary and (v) tail[21] (Figure 2).

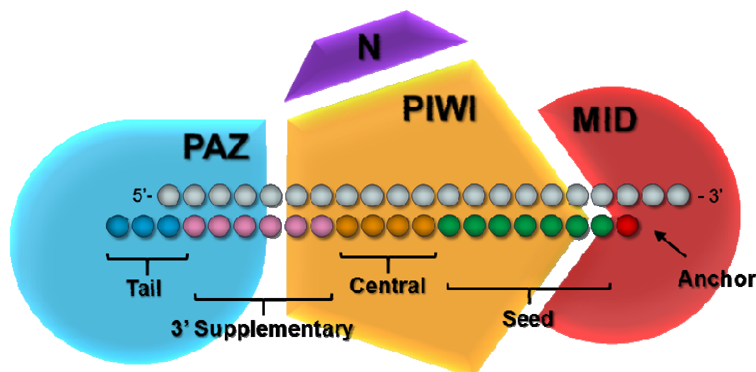

Figure 2. Ago2 diagram and siRNA molecule. Top strand is the passenger or sense, bottom strand is the guide or antisense. The passenger strand during RISC maturation is nicked and degraded, whereas the guide strand is retained and serves as complementary sequence for the identification of the target mRNA.

1.1.1 The anchor site (1)

The first nucleotide of the guide strand (Figure 2 in red) is stably buried into the MID domain of Ago2 protein and is not actively engaged in target recognition[22]. Moreover, the presence of phosphate group at 5' *terminus* is essential for siRNA efficacy[23, 24], the phosphorylation of the 5'-end is also required for strand loading and proper Ago2-mediated cleavage.

Even though the MID pocket provides adequate space to the accommodation of the natural bases, the presence of uridine or adenine is preferred over guanine and cytosine[15, 25]. The interactions between the MID cleft and the different natural bases are essentially not-specific and the identity of the interacting MID-nucleotide does not disturb the overall protein structure. Detailed studies on nucleobase recognition by the MID domain have revealed the presence of a well-arranged water network able to mediate contacts between several residues (Met 437, Lys 440, Ile 477 and Asp 480) and the N6 of the adenine[26]. Furthermore, Schirle and co-workers have suggested that the identity of the first nucleotide of the guide strand is not decisive for the initial recognition of the target mRNA but is crucial for increasing the stability between the Ago2 and the mRNA after seed-pairing nucleation.

Computational screenings of MID pocket and *in vitro* tests have also identified new 5' end modifiers able to enhance siRNA potency[27]. For example, bulky adenosines analogues have demonstrated to accommodate poorly into the MID pocket, their introduction at the 5'end of siRNA molecules were detrimental for RNAi activity in comparison with the natural adenosine. On the other hand, siRNAs modified with triazole derivatives, fitting better into the 5'-nucleotide binding pocket, have revealed stronger silencing activity with respect to unmodified siRNA molecules.

1.1.2 The seed region (2-8)

The stretch from position 2 to 8 (Figure 2 in green), well known as the seed, is responsible for the initial base-pair formation during the nucleation step of target recognition[28]. Proper seed pairing is important for both miRNA and siRNA target recognition[29], but is also critical for serious unintended miRNA-like OFF-target effects[30]. SiRNA molecules with different AU or GC content in the seed region have revealed opposite performance in terms of seed-matched target(s) silencing. The presence of four to seven A/Us in the seed stretch has proved to effectively reduce the seed-off target silencing while sustaining high activity for target gene[31]. Thus, the reduction of seed thermodynamic stability can diminish the siRNA seed-dependent off-target effects[32]. Moreover, the modification of the seed sequence with UNA (Unlocked Nucleic Acid) moieties has demonstrated to reduce the siRNA off-target potential. The presence of UNA modification locally destabilizing the RNA-RNA hybridization impacts more heavily on partial target recognition, responsible for the miRNA-off target effect, rather than on fully complementary target binding. For instance, the introduction of one UNA unit at 3'-end of the seed stretch has dramatically reduced the silencing mediated by partial complementarity without affecting the siRNA potency[33].

Even though strong interactions can be prevented using AU-rich seed siRNAs or UNA seed modified siRNA, it was demonstrated that 3' supplementary region actively contributes to OFF-target effects, neutralizing the influence of 5' base pairing[34, 35].

The modification of guide seed with 2'-O-methyl-RNA moieties (Figure 3), especially at position 2 of the guide strand, has also resulted in reduced silencing of off-target transcripts[36]. Of note, position 2 of the guide strand also plays an important role in ON-target recognition, 2'-O-methoxyethyl-RNA modification (Figure 3) has demonstrated to be detrimental for adequate target binding and Ago-mediated cleavage[23]. To diminish the stability between guide seed and mRNA, the seed region was also replaced with DNA units[37]. Since the DNA/RNA hybrid is less stable than RNA homoduplex, weaker interactions help to hinder the target duplex nucleation in absence of supplementary interactions beyond the seed sequence. Because the seed portion needs to be arranged within the Ago into a perfectly ordered A-form helix[38], the DNA/RNA helical deviation from A-form geometry is most likely detrimental for target recognition and seed-mediated activity. Further studies, on correlation between helical distortion and proper target binding, remarked upon the importance of A-form duplex geometry for the Ago-mediated mRNA initial recognition. Increasing numbers of mismatches along the seed stretch, entailing local distortion of the duplex structure, lead to a general "decoupling" between the guide and the mRNA. Central seed mismatches (4 – 5 positions) heavily weigh on Ago processivity more than those closer to the seed ends[21]. Furthermore, single wobble base pair (G:U) within the seed sequence has demonstrated to be a good strategy able to efficiently abrogate the off-target silencing[39, 40]. Thus, both duplex stability and structural parameters contribute to the effectiveness of guide-mediated off-target silencing.

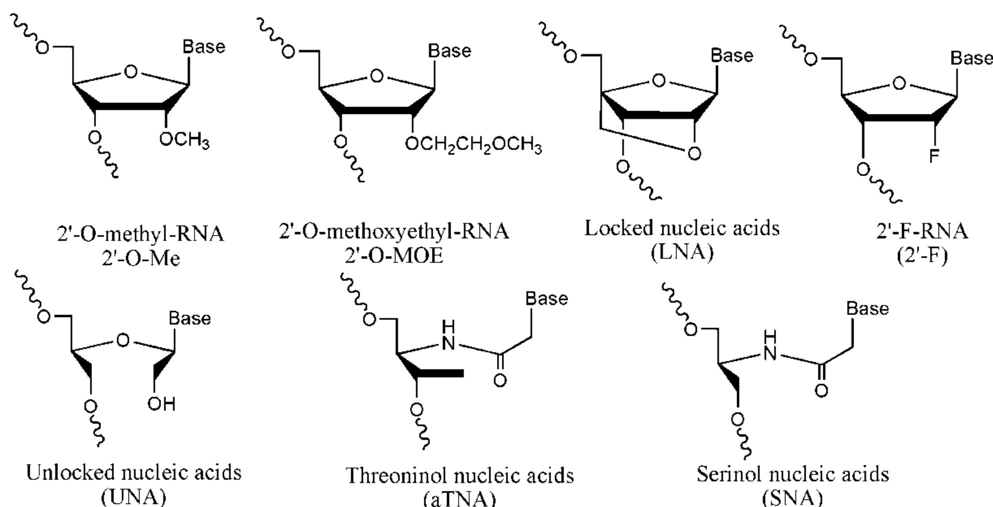

Figure 3. Chemical structures of modified backbones used in siRNA.

1.1.3 The central region (9-12)

Central base pairs (nucleotides 9 – 11) (Figure 2 in yellow) lying within the catalytic part of the Ago2 protein support the cleavage of the target mRNA. Central base pair matches are required for RISC maturation and for the correct conformation of the Ago's active site. Passenger strand is the first RISC target, its cleavage helps the dissociation from the guide strand and the production of the active RISC[41]. Slicer-dependent maturation of the RISC is the main mechanism for the elimination of the passenger strand. Phosphorothioate substitution of the scissile phosphodiester group makes the passenger strand non-cleavable and promotes a slower bypass mechanism in which the duplex unwinding is cleavage-independent[42, 43]. The substitution of central nucleotide pairs with DNA units has demonstrated to lower the silence activity, the deviation from canonical A-form structure of the guide:mRNA duplex likely leads to poor cleavage capability. On the other hand, replacement of central nucleotides either on guide or passenger strand marginally affects the inhibition, probably because hybrid DNA/RNA molecules, assuming a helical conformation between the A- and B-form, are less detrimental for the slicing[44]. Modifications, which prefer C3'-endo conformation, and stabilize the local RNA structure in A-form geometry (i.e. 2'-O-methyl, 2'-Fluoro, 2'-O-methoxyethyl, Pseudouridine, 2'-Thiouridine), have proved to be tolerated at central positions of guide strand[40, 23, 45, 46]. Conversely, more flexible units such as Dihydrouridine and L-threoninol-thymine (Figure 3), increasing the flexibility of duplex geometry inhibit the silence activity[40, 47]. Thus, proper RNA A-form geometry, around the cleavage site, is essential for adequate Ago2-mediated catalysis. The perturbation of Ago2 catalytic efficiency in presence of central mismatches has once more confirmed the importance of proper duplex arrangement within the cleavage site[21].

1.1.4 The 3'-supplementary region (13-18)

The 3' supplementary region (Figure 2 in pink) is responsible for the propagation of duplex formation over the central cleavage region. In binary complex, the nucleotides 14-18 are surrounded by a narrow channel formed between the PAZ and N domains, their base stacking interactions are broken and nearly all Watson-Crick edges point inward. The extensive contacts with the Ago2 protein prevent the hybridization between the guide 3'-half and the complementary target. Conversely, the arrangement of guide-target pairing beyond the 5'-half portion leads to significant conformational changes, the PAZ-N channel gets wider and the guide 3'-half is repositioned forming A-form helix[22]. Stable hybridization between the stretch 14-18 and the target also facilitates the correct positioning of central nucleotides and the cleavage activity[28].

1.1.5 The tail region (19-21)

The terminal part of the guide strand spans nucleotides 19 – 21 (Figure 2 in blue) and comprises a dinucleotide-unpaired structure, named overhang, which protrudes from the siRNA duplex and specifically interacts with PAZ pocket[19]. Even though the 3'-overhang does not actively participate to target recognition, its presence is critical for siRNA activity[48]. Along with the interaction between the 5'end and the MID pocket, stable anchoring of 3'-end within the PAZ domain is essential for the proper unwinding and passenger release[28, 49]. During the formation of catalytically competent RISC the passenger strand is nicked and discarded, its elimination permits the propagation of base pairing towards the guide 3'-end and facilitates the displacement of the overhang from the PAZ cleft. Once the cleaved products are released, the PAZ domain again fastens the guide 3'-end. The PAZ motion, according to the "two state" model, has permitted the elucidation of the siRNA-programmed RISC catalysis as a multiple turnover process[50]. The lodging/dislodging motion is regarded to be a decisive factor in boosting siRNA potency. Likely, more affinity for the PAZ cleft, favoring a rapid increase of the fasten/unfasten rate, accelerates the RISC cleavage turnover. So, in view to improve siRNA potency, through the strengthening of the interactions between the 3' guide overhang and the PAZ pocket, numerous overhang modifications (Figure 3) have been tested[51-55]. Of note, the size of overhang modification, affecting the proper accommodation into the PAZ hydrophobic cavity, influences the siRNA activity[56-58]. Because of the extreme RNA instability in biological *milieu*, overhang modifications have been primarily developed to protect the siRNA integrity from the action of nucleases[6]. Thereafter, the detection of siRNA-associated side effects has led to evaluate the ability of overhang-modified siRNAs in promoting the correct strand selection[59]. For example, thanks to different binding affinity of modified overhang, chemically asymmetric siRNAs have demonstrated to improve the loading of the intended strand[60]. Indeed, the enhanced siRNA potency is likely the result of preferential loading of the strand bearing the overhang with better PAZ binding affinity. The favored guide strand incorporation impedes the passenger strand misloading, preventing the passenger-mediated OFF-target effects. Remarkably, the absence of passenger strand overhang, eliminating the structure recognized by the PAZ domain, promotes the loading of the strand bearing the overhang[61]. Furthermore,

overhang-modified siRNAs have extensively employed to induce long-term gene silencing. Enhanced stability towards nucleases has been assumed to endure the silencing effects, even if later studies have clarified that the silencing duration *in vitro* depends mostly on the cellular doubling-time rather than improved stability[62, 63]. Thus, nuclease-stabilized siRNA has more influential impact during *in vivo* delivery of naked molecules[64].

2. Handling the OFF-target effects (OTEs)

The development of siRNA-based therapeutics, straight after an initial burst, has suffered a setback caused by ever-growing issues questioning on their biological safety. Unintended side-effects of siRNA-mediated gene silencing essentially hinge upon: (i) suppression of non-target genes; (ii) saturation of RNAi machinery; (iii) passenger strand mediated silencing; (iv) immune response activation (Table 1).

OFF-target type	Effects	Solutions
Saturation of RNAi machinery	Up-regulation of miRNA-controlled genes	Improve siRNA potency by chemical modifications[12, 58], use highly potent siRNA molecules[59, 60]
Passenger strand-mediated silencing	Down-regulation of mRNA target(s) partially and/or fully complementary to passenger strand	Enhance thermodynamic asymmetry, blunt end siRNA[53], si-siRNA[67], asiRNA[53, 68, 69], aiRNA[70], ss-siRNA[71], block passenger 5'-end phosphorylation[63-65], dual targeting siRNA[73, 74, 75]
Seed-mediated silencing	Down-regulation of partially complementary genes	Avoid common seed complementary sequences[98], AU-enriched seed[99]
Immunostimulation	Induction of inflammatory cytokines and type-I Interferons	Avoid immunostimulatory motifs[78, 79], escape the immune system recognition by chemically modified siRNAs[83]

Table 1. Schematic summary of siRNA-mediated OTEs

2.1 Saturation

The estimated RISC concentration inside the cells ranges between 3 – 5 nM. RISC amount, considering the average volume of a mammalian cell (10^{-13} L – 10^{-12} L), is in the order of about 10^3 – 10^4 molecules[65]. Thus, saturation of the RNAi machinery is theoretically reached with 10 - 100 pM siRNA, which corresponds to 10^3 – 10^4 dsRNA molecules. The transfection of exogenous siRNAs principally leads to competition for RISC binding between the endogenous miRNA pool and the exo-siRNAs. MiRNA displacement influences the miRNA-mediated

silencing and leads to an overall upregulation of miRNA-controlled genes. The preservation of robust silencing effects with lower dose is advantageous in terms of RISC competition. For this reason, the design of potent siRNA molecules fundamentally aims at the prevention of RISC clogging. If only a fraction of cellular RISC pool is actively occupied by the exo-siRNAs, more RISC molecules are available for endogenous pathways.

Besides canonical modified siRNAs (extensively reviewed elsewhere[12, 66]), which demonstrated to be more potent with respect to the unmodified ones, innovative approaches based on new siRNA structural variants have been applied. For instance, Dicer substrate interfering RNAs (DsiRNAs) have demonstrated stronger activity with respect to canonical 21-nt siRNAs. Dicer executing the conversion of DsiRNA precursor into active siRNA, assists the Ago loading and raises the RISC incorporation rate. In addition, adequate strand polarity conferred by DsiRNAs minimizes the incorporation of the passenger strand into the RISC[67]. Another successful example of potent siRNA precursors emerges from the synthesis of dumbbell-shaped circular siRNA: a circular RNA molecule that folds into a central stem region and 2 terminal loop structures. Interestingly, dumbbell-shaped siRNA, with 23-bp stem region and 9-nt long loops, has disclosed powerful improvement on silencing activity with respect to canonical 21-bp siRNA. The lack of loose ends, distinctive of siRNA structure, entails better resistance towards nucleases and reduced activation of innate immune response. Furthermore, the slow Dicer processing of dumbbell siRNA, likely allowing a gradual but constant production of active siRNA molecules, drives long-lasting silencing[68].

2.2 Passenger strand mediated silencing

The claim assuring:" the strand with less stable 5'-end is preferentially incorporated into RISC and serves as the guide", holds back the possibility of improper strand incorporation. It has been established that passenger strand could be efficiently loaded as the guide strand, provoking the downregulation of complementary target(s)[69]. Passenger strand-driven silencing compromises siRNA specificity and engulfs the RNAi machinery with the wrong strand. To promote the correct strand election, chemical and structural approaches have been investigated. Since the presence of 5' terminal phosphate is strictly required during RISC assembly and is essential for silencing activity[70], chemical modifications able to block the Clp-1-mediated 5'end phosphorylation, permit desirable strand incorporation or limit passenger strand activity[71, 35, 72, 73]. Moreover, passenger seed substitutions, altering the duplex stability between the modified strand and the target mRNA, prejudice its ability to participate in target cleavage[33, 35, 47, 54]. Hence, strand-blocking properties of chemical modifications are successfully exploited to weaken the passenger strand-mediated silencing. It was also noted, that in the absence of passenger overhang, the activity and the strand selection of blunt siRNAs incline towards the guide strand[61]. Given that the presence of overhang is decisive for PAZ-mediated RISC recruitment, the asymmetrical structure of blunt siRNA promotes the loading of the strand carrying the overhang.

In order to improve the poor inherent siRNA properties (especially the specificity), novel siRNA architectures have been analyzed. For example, new siRNA construct has originated as a result of the development of LNA (Locked Nucleic Acid, Figure 3) modification. Internal segmented siRNA (si-siRNA) consisting of a 22-nt guide strand and 2 segmented (10 + 12 nt) passenger strands has demonstrated to be as effective as canonical siRNA. Furthermore, the three-stranded siRNA composition, combining the LNA stabilization effect on passenger strands and the silencing inability of shorter passenger strand portions, has proven to be an highly specific siRNA architecture[74]. Therefore, passenger shortening can be an intriguing approach to the elimination of passenger strand-mediated silencing. Asymmetric shorter duplex siRNA (asiRNA), asymmetric siRNA (aiRNA) and blunt end siRNA[61, 75-77] have exhibited silencing ability comparable to canonical siRNA structure and less siRNA-mediated off-target effects. Interestingly, the progressive passenger strand trimming from its 5'-end does not compromise RISC assembly and its off-target effects. On the other hand, the shortening of the passenger 3'-end promotes the preferential loading of the opposite strand. Even though the thermodynamic asymmetry of siRNA duplex contributes to guide strand selection, the presence of the 3' overhang substantially weighs on strand preference.

Since active RISC needs only one strand to execute the cleavage of the target mRNA, single-stranded siRNA (ss-siRNA) was able to trigger gene silencing[78]. This strategy completely abolishes the passenger-mediated silencing but has disclosed decreased potency with respect of double stranded siRNAs. Furthermore, 5' unphosphorylated ss-siRNA are not functional, only the chemical addition of a phosphate at 5'-end rescues the silencing activity[79]. In single-stranded fashion, the endogenous phosphorylation is likely hindered, so only pre-phosphorylated ss-siRNA can stably interact with the RISC. An appealing solution to passenger off-target effects came from dual targeting siRNAs, which harbor two functional strands. Since each strand individually recognizes distinct targets, the passenger-driven silencing is virtually eliminated[80, 81]. Fundamentally, the key steps for correct dual-targeting design are the choice of target genes and the calculation of *termini* stability. Bioinformatics analysis on gene candidates facilitates the selection of partial overlapped sequences able to form stable multi-bulged duplex siRNA. Moreover, computation of balanced ends permits unbiased strand incorporation and confers comparable effectiveness to both strands[82].

2.3 Immunostimulation

The innate immune system has evolved to support rapid response by the recognition of pathogen hallmarks. Pattern recognition receptors (PRRs) identify pathogen-associated molecular patterns (PAMPs) exclusively present on microorganisms. The innate immune sensors perceive exogenous RNAs as a viral infection, the resulting activation of the defense mechanisms leads to the production of type I interferon and pro-inflammatory cytokines. Sensors designated to the recognition of non-self RNAs belong to TLR (Toll-Like Receptor) and non-TLR pathways[83]. TLRs receptors located at endosomal compartment are the first line of

defense against viral RNA infections. Their almost exclusively compartmentation also dictates the discrimination between host and non-self nucleic acids. TLRs (TLR3, TLR7, TLR8 andTLR9) specifically interact with stimulatory motifs present on double and single-stranded RNA[84]. Even though it was noted that some secondary structures stimulate the activation of TLRs, their activation is mostly sequence-dependent. Indeed, polyuridine tracts and GU-rich sequences, are strong RNA immunostimulatory motifs that should be avoided in tailored-designed siRNA[85, 86]. Among the most important no-TLR receptors, PKR, RIG-I and OAS recognize dsRNA in a sequence-independent manner[87]. DsRNA-dependent protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (OAS) activation restricts viral transcription and translation by blocking cellular protein synthesis and induction of apoptosis[88]. The 5'-triphosphate presence on dsRNA structures triggers RIG-I-mediated antiviral signaling[89]. Many efforts have been made to develop siRNA molecules able to escape the innate immune recruitment. SiRNA chemical modifications, able to mitigate the activation of immune response, permit to identify the basic hallmarks recognized by the immune sensors. Ribose 2'-position modifications, such as 2'-OMe, 2'-F and 2'-H, have demonstrated to strongly reduce the levels of IFNs while retaining optimal siRNA-silencing[90]. New approaches based on minor-groove base modifications have aimed at protection of immunostimulatory hot spot present on miRNA–mimic[91]. Furthermore, adenine substitution over the other nucleotides has beneficial effects on eluding the immune recognition[92, 93]. Although the necessity of neutralizing the immunostimulatory potential of siRNA molecules is fundamental goal to achieve for reliable therapeutic purposes, the siRNA adjuvant effect has been exploited to activate the cellular immunity. A promising approach for cancer and viral therapy came from the development of immunostimulatory siRNAs (is-siRNAs)[86, 94]. These bifunctional molecules, linking potent gene silencing properties to suitable production of IFNs, can effectively control chronic viral diseases such as HIV-AIDS, HBV and HCV infections[95-97]. Furthermore, the application of this new siRNA design can not only overcome the cancer drug resistance, but also strengthen the immune surveillance usually evaded by cancer cells[98-102]

2.4 miRNA-like OTEs

Analysis of multiple microarray datasets has revealed widespread siRNA-driven gene down-regulation [30, 103]. The mechanism underlying siRNA off-targeting resembles that miRNA-based: seed base-pairing interactions between the guide strand and the mRNA (especially in the 3'UTRs), are sufficient to suppress the expression of unintended targets[7]. MiRNA-like silencing produces false-positive data and toxic phenotypes contributing to the problematic interpretation of silencing outcomes and weakening the potential therapeutic benefits of siRNA-based drugs. To mitigate the off-target effects, several modifications such as single nucleotide bulge, UNA and 2'-OMe moieties were introduced within seed sequence. The presence of these modifications has proven to ameliorate the siRNA specificity without reducing the siRNA efficiency[30, 35, 104]. To prevent the miRNA-like silencing, an alternative path comes from the study

of seed complement frequencies (SCFs) in the 3'UTR transcriptome. It is well-described that each siRNA molecule presents specific off-target fingerprint. Fluctuations in off-target signature essentially rely on seed nucleotide sequence mirroring the complementary target abundance in the 3'UTR genome. Thus, fewer off-target silencing and lower false positive scores can be obtained avoiding the introduction within the seed portion of high-frequency seed complements[105]. Since seed-mediated recognition of complementary transcripts is sufficient to determine miRNA-like off-target silencing, the weakening of pairing stability between guide seed portion and target mRNAs optimizes the siRNA stringency[31].

3. Delivery

The task of designing potent, highly specific and effective siRNA molecule is only the first checkpoint to cross for the development of siRNA-based therapeutics. Given that siRNA molecule has to reach the cytoplasm to fulfill its function, the tissue delivery becomes a fundamental priority to address[106]. After intravenous injection, siRNA starts to be systemically distributed and eliminated from the blood stream. To be recruited by the cytoplasmic RISC, the siRNA molecule has to overstep several barriers including interstitial/extravascular *milieu* and endolysosomal compartment[107]. Systemically delivered naked unmodified siRNAs, are subjected to the degradation by serum nucleases and elimination by the ReticuloEndotelial System (RES). Moreover, because of its high molecular weight and polyanionic/hydrophilic nature, siRNA molecules cannot easily diffuse across the cell membrane. Thus, the extremely short half-life and the negative physicochemical features of siRNAs have impeded the administration of non-protected siRNA-based drugs. Even though local administration has provided a valid alternative to avoid the systemic route and direct access to an interested region, is only suitable for easily accessible tissues (i.e. eye). Systemic administration remains essential for the treatment of solid tumors, but the poor pharmacokinetic profile of unformulated and unmodified siRNAs renders unfeasible their systemic delivery. In order to optimize the delivery process, carriers should comply with some rules. Prolonged blood circulation time, for example, is attained maintaining the vehicle particle size between 70-200 nm. The obtained stealth properties essentially depend by the escape from phagocytic clearance and rapid renal elimination. Moreover, broad interactions with serum components (i.e. lipoproteins, complement proteins, albumin), which interfere with bio-availability and promote unspecific uptake by liver, spleen and RES (ReticuloEndothelial System), prevent the reaching of the target. The shielding effect of hydrophilic molecules (i.e. PolyEthylene Glycol or PEG) covering the surface of delivery vehicles, is exploited not only for minimizing the interactions with serum proteins but also provide an adequate solution to avoid the activation of the innate immune system[108]. The modification of the internucleotide phosphate linkage, replacing one non-bridging oxygen with one sulfur atom, alleviates the immunostimulation and improves the nuclease stability. Targeted delivery aims to address the siRNA drug toward specific tissue or cell populations with negligible toxicity for other tissues. Passive targeting takes advantage of leakiness in tumor vessels permitting carrier extravasation, whereas active targeting has the intention of limiting the delivery toward a specific target[109]. With the exception of hepatic delivery, passive targeting

is not a feasible approach due to heterogeneity on tumor vascularization and tumor vessel porosity. Diverse conjugation chemistries able to link specific target molecules (i.e. antibody, peptides, aptamers, small molecules) to siRNA cargo, employ the specific ligand-receptor recognition for the uptake of certain tissue or cells[110]. Upon receptor-mediated endocytosis the delivery cargo is incorporated into the endosomal trafficking, final fusion of endosomes with lysosomes acidifies the lumen and liberates the nucleases promoting the degradation of siRNA. The rapid escape from endolysosomal compartment releases the siRNA into the cytosol and permits the association with RNAi machinery. Fusogenic lipids and pH-sensitive carriers (i.e. proton escape) are some of successful strategies used for enhance the endosomal escape while protecting the siRNA from degradation[111].

Of note are also self-assembled RNA superstructures, which consist of hairpin RNA structures condensed in sponge-like spherical fashion (Table 2). Thanks to the use of RNA polymerase it is possible to generate RNAi-microsponges containing about 100000 siRNA copies. The Dicer processes the hairpin RNA precursor and directly liberates active siRNA molecules into the cytosol. Furthermore, the adsorption of polyethyleneimine (PEI) onto the negatively charged RNAi-microsponge, creating a net positively charged outer layer, facilitates the cellular uptake. This self-assembled carrier yielding great siRNA loading, customized siRNA composition, stable protection from degradation, low cytotoxicity and efficient gene silencing is a promising solution to delivery[112]. Additional relevant means for siRNA delivery have come from the discovery of exosomes and HDL particles able to shuttle RNAs between cells[113, 114]. Intriguing novel delivery approaches mimicking materials from endogenous sources has paved the way to the development of exosome-based vehicles (Table 2). Their endogenous derivation, impeding the recognition, the opsonization and clearance by immune system, gains crucial advantages over the other polymer-based and lipid-based carriers. Moreover, the release of the cargo directly into the cytosol, allows the bypass of the endosomal escape[115, 116]. Additionally, starting from the nature and the structure of native HDL, biomimetic lipoprotein carriers have been developed for efficient delivery *in vivo.* Reconstituted HDL-like particles (rHDL), HDL-mimicking nanoparticles are some examples of flexible, not immunological active vehicles able to transfer the siRNA cargo directly into the cytosol (Table 2)[117, 118].

Delivery vehicles	Derivation	Advantages
Microsponges[106]	Enzymatic polymerization	Stealth effects, customization of siRNA composition, stability in biological environment, high-yield siRNA loading
Exosomes[109, 110]	Endogenous	Stealth effects, release of the cargo directly into the cytosol, not immunogenic
HDL-like particles[111, 112]	Endogenous/biomimetic	Stealth effects, bypass the Endosomal compartment, immune-inert formulations

TABLE 2. Most recent delivery strategies

CONCLUSION

Deep understanding of RNAi pathway and nucleic acid delivery has its ultimate goal in reducing the unintended effects without compromising the silencing properties. Each step of design, synthesis and delivery of siRNA-based drugs requires adequate optimization. Rational design of siRNA molecules and tailored engineering of delivery vehicles has promoted giant progress toward clinical application. Modified siRNA provided the design of potent, highly specific and effective molecules, whereas multifunctional carriers have permitted the improvement of siRNA stability during systemic delivery and cell-specific targeting.

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