

Structural Diversity Repertoire of Gene Silencing Small Interfering RNAs

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Since the discovery of double-stranded (ds) RNA-mediated RNA interference (RNAi) phenomenon in *Caenorhabditis elegans*, specific gene silencing based upon RNAi mechanism has become a novel biomedical tool that has extended our understanding of cell biology and opened the door to an innovative class of therapeutic agents. To silence genes in mammalian cells, short dsRNA referred to as small interfering RNA (siRNA) is used as an RNAi trigger to avoid nonspecific interferon responses induced by long dsRNAs. An early structure–activity relationship study performed in *Drosophila melanogaster* embryonic extract suggested the existence of strict siRNA structural design rules to achieve optimal gene silencing. These rules include the presence of a 3' overhang, a fixed duplex length, and structural symmetry, which defined the structure of a classical siRNA. However, several recent studies performed in mammalian cells have hinted that the gene silencing siRNA structure could be much more flexible than that originally proposed. Moreover, many of the nonclassical siRNA structural variants reported improved features over the classical siRNAs, including increased potency, reduced nonspecific responses, and enhanced cellular delivery. In this review, we summarize the recent progress in the development of gene silencing siRNA structural variants and discuss these in light of the flexibility of the RNAi machinery in mammalian cells.

Introduction

RNA INTERFERENCE (RNAi) is an evolutionarily conserved mechanism of posttranscriptional gene silencing by double-stranded (ds) RNAs (Hannon, 2002). Originally discovered by Fire and Mello in *Caenorhabditis elegans* (Fire et al., 1998), long (typically 300–1000 bp) dsRNAs introduced into cells or organisms effectively trigger RNAi to specifically inhibit target gene expression in a wide range of organisms. The RNAi pathway is initiated upon cleavage of long dsRNA into ~21-nucleotide (nt)-long small interfering RNA (siRNA) by a ribonuclease III enzyme called Dicer. This siRNA duplex subsequently gets assembled into an RNA-induced silencing complex (RISC), in which one strand (sense or passenger strand) is eliminated and the other (antisense or guide strand) recognizes and cleaves the complementary mRNA with the help of Argonaute-2 (Ago-2) and other auxiliary RISC proteins. Because of the outstanding potency and specificity compared with other loss-of-function technologies, RNAi-mediated gene

silencing has rapidly become a fundamental tool for gene function studies (Fraser, 2004) and a promising therapeutic modality for a variety of diseases (Lares et al., 2010).

However, in contrast to other organisms, the initial effort to use long dsRNAs to trigger RNAi in mammalian cells was largely unsuccessful, because of the strong induction of interferon and the activation of protein kinase R (PKR), produced as a consequence of an antiviral response to the long dsRNA molecules. This undesired response results in the nonspecific degradation of mRNAs and inhibition of protein synthesis (Stark et al., 1998; Caplen et al., 2000; Ui-Tei et al., 2000). Successful silencing of specific genes via an RNAi mechanism in mammalian cells was first reported by the Tuschl group, who demonstrated that chemically synthesized siRNA, a structural mimic of the Dicer cleavage product of long dsRNA, could trigger efficient and specific target gene silencing in mammalian cells without generating undesired interferon responses (Elbashir et al., 2001a, 2001b).

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The same group also performed a structure–activity relationship study to define the structural features of potent siRNAs (Elbashir et al., 2001c). Using *Drosophila melanogaster* embryo extract as a model experimental system, they investigated the gene silencing activity of various dsRNA structures, ranging in length from 19 to 25 nt, with different overhang structures. From this experiment, they found that there was a strict limit to the siRNA duplex length for optimal gene silencing activity; 19-bp-long duplexes showed optimal gene silencing, whereas duplexes shorter or longer than 19 bp were significantly less potent or inactive. Their results also led them to emphasize the importance of overhang structures; duplexes without overhangs (blunt-ended) or with 5' overhangs were less potent than duplexes with 2-nt-long 3' overhangs. Therefore, they concluded that a 19-bp RNA duplex with 2-nt 3' overhangs at both ends, often referred to as the “19 + 2 structure,” is the most potent siRNA structure for gene silencing, and this structure was adopted as the standard in the RNAi field.

Soon after the application of siRNAs in functional genomic studies and the development of therapeutics, it was found that siRNAs triggered several unintended nonspecific responses when introduced into cells and animals (Tiemann and Rossi, 2009). These nonspecific responses included off-target gene silencing triggered by the incorporation of the sense strand into the RISC or incomplete base pairing of the siRNA antisense strand with nontarget mRNA, activation of nonspecific innate immune responses by pattern recognition receptors [eg, Toll-like receptors (TLR) and retinoic acid inducible gene I (RIG-I)-like cytoplasmic helicases], and RNAi machinery saturation by excess exogenous siRNA, which inhibits endogenous microRNA processing. These nonspecific responses have limited the use of siRNA as a specific tool for therapeutics and loss-of-function studies.

To circumvent these problems, chemical modifications have been introduced into the classical 19 + 2 siRNA backbone. However, chemical modification of siRNA is often associated with unfavorable side effects such as toxicity and reduced silencing efficiency. Therefore, the design of novel siRNA backbone structural variants that can not only trigger efficient gene silencing but also ameliorate nonspecific responses triggered by classical siRNA structures is an important area of current research.

Several recent studies reported the design and analysis of novel RNAi-triggering structures distinct from the classical 19 + 2 siRNA structure (Fig. 1). These novel RNAi-triggering structures do not conform to the key features of classical siRNA in terms of overhang, length, or symmetry. Importantly, the newly designed siRNA structural variants show improved functionality over classical siRNAs such as more potent gene silencing activity, reduction of nonspecific responses, immune stimulation, or enhanced internalization. In this review, we summarize several recent findings that challenge the dogma surrounding the 19 + 2 siRNA architecture and discuss these findings in view of the flexibility of the RNAi machinery in mammalian cells.

siRNA Without 3' Overhangs

The initial structure–activity relationship study of gene silencing siRNAs using *D. melanogaster* embryo lysates concluded that the presence of 2-nt-long 3' overhangs was an

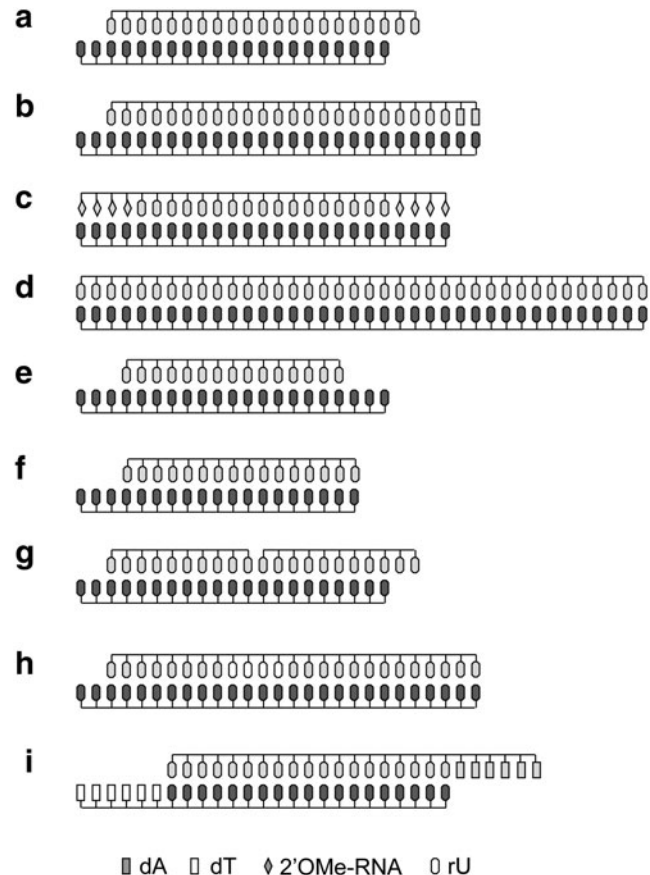


FIG. 1. Structures of siRNA structural variants. *Top strand:* sense (passenger) strand; *bottom strand:* antisense (guide) strand; *ellipsoid:* RNA; *square box:* DNA. (a) Classical 19 + 2 siRNA; (b) Dicer-substrate siRNA (R 25/27D form); (c) Dicer-noncleavable long siRNA (25 bp); (d) 38-bp-long dual-target siRNA; (e, f) asymmetric short siRNAs; (e) aiRNA; (f) asiRNA; (g) sisiRNA; (h) immunostimulatory siRNA; (i) ssiRNA.

essential feature of siRNAs (Elbashir et al., 2001c). Deviations in the overhang length, substitution with deoxyribonucleotides (Hohjoh, 2002), or the addition of chemically modified residues (Harborth et al., 2003) has been shown to reduce the silencing efficiency of siRNAs. All these 3' overhang variations affect the recognition of siRNA by the PAZ domain of Ago-2 (Ma et al., 2004). In contrast, however, many studies have shown that modifications in the length and chemistry of the overhang residues do not affect silencing (Chiu and Rana, 2002; Amarzguioui et al., 2003), raising questions about the proposed limitations in overhang design and the importance of the PAZ domain–siRNA interaction in the human RNAi machinery. Even dsRNAs without any 3' overhangs have been shown to be highly efficient in gene silencing in mammalian cells (Czauderna et al., 2003). The dispensability of the 3' overhang structure was confirmed in another study (Chang et al., 2007). These findings gave a hint that siRNA structural requirement in mammalian cells might be more flexible than those in *D. melanogaster* embryo lysates (Elbashir et al., 2001c) and stimulated several other studies (described later) that demonstrate the structural flexibility of siRNAs in mammalian cells.

Long siRNA Structural Variants

Similar to the overhang structure, it was originally thought that the length of the siRNA duplex was critical for triggering efficient target gene silencing (Elbashir et al., 2001c). However, several recent studies have demonstrated that a great deal of flexibility exists in the length of the siRNA duplex. The first example of efficient gene silencing by siRNA duplexes of different lengths was reported by Kim et al. (2005). This group tested several dsRNA molecules longer than 19 bp and found that 27-bp-long siRNAs could trigger efficient RNAi and were also 10–100-fold more potent at gene silencing than the 19-bp-long siRNAs, depending upon the target sequence. Notably, even though the 27-bp siRNAs were longer than 19-bp ones, they did not induce interferon or activate PKR. The authors of that study found that the 27-bp duplex was processed efficiently by Dicer and that the introduction of chemical modifications that compromised Dicer processing also affected gene silencing activity. Based on this observation, it was hypothesized that the increased potency of 27-bp siRNAs was because Dicer was provided with the substrate duplex rather than the product (19 + 2 siRNA), thus improving the efficiency of siRNA entry into the RISC. Thus, this 27-bp-long siRNA structure was termed “Dicer-substrate siRNA.”

In a follow-up study, functional polarity was introduced into the Dicer-substrate siRNA to define the specific Dicer-cleavage product generated from the 27-bp-long siRNA. Specifically, the “R 25D/27” structure was developed, which consists of a 25-nt sense strand with 2 nucleotides on the 5' end replaced with DNA, and a 27-nt antisense strand, resulting in a blunt-ended antisense 5' end and a 2-nt antisense 3' overhang (Rose et al., 2005). Compared with other Dicer-substrate siRNA structures, the R 25D/27 structure produced defined Dicer cleavage product and showed higher gene silencing activity. The asymmetric overhang structure also resulted in preferential incorporation of the antisense strand into the RISC. A mechanism proposed to explain this observation is that Dicer preferentially associates with the 3' overhang and spatially orients the siRNA cleavage product in the correct position for association of the 5' end of the antisense strand with Dicer, which remains in the RISC.

Another long siRNA structural variant was recently introduced by Salomon et al. (2010). In their report, they described blunt-ended, 25-bp-long, chemically modified siRNA duplexes. One of the chemical modification patterns they evaluated consists of 4 2'-OMe modifications on both ends of the sense strand and an unmodified antisense strand, designated as the “4/4” pattern. The striking finding was that while this modification rendered the 25-bp siRNA completely resistant to Dicer-mediated cleavage, it still triggered efficient target gene silencing. Therefore, although structurally similar, the proposed mechanism of action of Dicer-noncleavable 25-bp siRNA is different from that of Dicer-substrate siRNA. However, no side-by-side comparison data of the gene silencing activity of Dicer-noncleavable siRNA vs. Dicer-substrate 27-bp siRNA duplex were presented in their study. Therefore, a direct parallel comparison awaits further studies.

The finding that the Dicer-noncleaved 25-nt-long antisense strand could execute gene silencing via the RNAi machinery might seem puzzling at first, because the initial X-ray crystallography studies proposed a model in which the MID domain and PAZ domain of Ago-2 bind the 5' phosphate and 3'

hydroxyl ends of the antisense strand, respectively, with an optimal interval of 21 nt (Wang et al., 2008). However, a recent structural study revealed that in a ternary complex of catalytically inactive bacterial Ago-2/21 nt antisense DNA/target RNA, the antisense DNA base-paired with the target RNA only up to the 16th nucleotide from the 5' end, and the 3' end of the antisense DNA was released from the PAZ domain (Wang et al., 2009; Sashital and Doudna, 2010). This new structural finding is consistent with the observation that Dicer-noncleaved 25-nt-long antisense strand can trigger efficient RNAi. Although further structural and biochemical studies are warranted to clearly understand how RNAi is triggered by long antisense strand RNA, these results emphasize the structural flexibility of the RNAi machinery.

Although the Dicer-substrate siRNA did not induce cytokines by triggering the innate immune response in HeLa cells (Kim et al., 2005), a later study by Marques et al. (2006) showed that in cell lines that maintain the dsRNA immune response such as T98G, blunt-ended, 23–27-bp-long siRNAs could trigger the innate immune response, and the level of the immune response increased as the duplex length increased. This innate immune response appears to be sensed by RIG-I, a cytoplasmic RNA-sensing receptor, and can be attenuated by adding 3' overhang structures. Therefore, although the 3' overhang structure might be dispensable for RNAi activity, it is still important for triggering specific RNAi without inducing nonspecific immune responses. In view of this, it is notable that the R 25D/27 structure, which contains a 3' overhang and a blunt-end with DNA modification, not only showed optimal gene silencing activity, but also significantly reduced innate immune stimulation. Thus, the R 25D/27 structure appears to be an optimal structure for Dicer-substrate siRNA.

A key conclusion from an earlier study that defined the optimal siRNA structure for mammalian gene silencing was that dsRNA with a duplex length of 30 bp or longer triggered a potent interferon response, which was avoided by using 19-bp-long siRNA (Elbashir et al., 2001a). Although longer than the classical 19-bp siRNA structure, both Dicer-substrate siRNA and Dicer-noncleavable siRNA are still below the 30 bp length limit. However, Chang et al. (2009a) recently demonstrated that 34–38-bp-long synthetic dsRNA can specifically trigger RNAi-mediated gene silencing without induction of the interferon response or PKR activation. Therefore, the original hypothesis that RNA duplexes longer than 30 bp could not trigger specific RNAi because of the induction of potent antiviral responses needs to be revised. This is an important finding, because the ability to design long dsRNAs for gene silencing will allow researchers to design a variety of complex, multifunctional siRNA structures. Further, using this longer duplex structure, they generated siRNA structural variants that can simultaneously knock down the expression of 2 target genes (termed as dual-target siRNAs or dsRNAs). Once again, these results demonstrate the structural flexibility of gene silencing siRNAs and propose a simple and useful strategy to develop a multitarget gene silencing strategy against diseases with multiple gene alterations, such as viral infections and cancer.

Gene Silencing by Short siRNAs

Several recent studies also reported successful gene silencing with siRNA structural variants shorter than 19 bp.

Chu and Rana (2008) tested the activity of dsRNAs shorter than 19 bp in mammalian cells. They claimed that, in human cell lines, 16-bp siRNA with 2-nt 3' overhangs triggered gene silencing of target mRNAs with greater potency than 19-bp siRNA and thus suggested that the minimal requirement for siRNA molecule is a ~ 42 Å A-form helix with ~ 1.5 helical turns (Chu and Rana, 2008). In another study, Li et al. (2009) developed a forward genetic approach to identify nontoxic, highly potent siRNAs from bacterially delivered RNAi libraries. Interestingly, some of the highly potent siRNAs selected against MVP mRNA were only 16 bp in length, and increasing the length of the native sequences dramatically reduced RNAi potency. These results indicate that depending upon sequence features, siRNAs shorter than 19 bp can efficiently trigger target gene silencing in mammalian cells and sometimes even better than the classical 19-bp siRNAs.

Although these studies stated that shorter siRNAs showed greater gene silencing potency than 19-bp siRNAs, some of the claims should be interpreted with caution. For example, Chu and Rana (2008) concluded that 16-bp siRNAs were more effective at gene silencing than the corresponding 19-bp siRNAs. However, a recent study demonstrated that when the short siRNA was made by trimming the 5' end of siRNA duplex with respect to the polarity of the antisense strand, the seed sequence that dictated the target site specificity changed, thus preventing direct comparison of the activities of the 19-bp siRNA and its shorter version. In fact, when short siRNA was made by trimming the 3' end of duplex with respect to the antisense strand (thus keeping the seed sequence identical), the shorter siRNAs were less active than their 19-bp counterparts.

It is possible that although dsRNA shorter than 19 bp could be recognized by and incorporated into the RNAi machinery, the length of the antisense strand must be 19 nt or longer to trigger efficient gene silencing. Based upon this idea, several different asymmetric, shorter duplex siRNA structural variants have been proposed by 3 independent groups (Sano et al., 2008; Sun et al., 2008; Chang et al., 2009b).

In the study by Sano et al. (2008), they reported that the siRNA terminus is a crucial factor in strand selection and RNAi activity. In agreement with the conclusion drawn by Rose et al. (2005), they demonstrated that siRNAs with an asymmetric 3' overhang structure, that is, a 2-nt overhang present only at the 3' end of the antisense strand, showed better antisense strand selection and enhanced efficacy than the symmetric counterparts. Further, they showed that as long as the 3' overhang at the end of the antisense strand was maintained, partial deletion or DNA substitution of the sense strand did not affect gene silencing activity, demonstrating that asymmetric siRNA with a shortened sense strand can trigger efficient gene silencing. Other groups also reported asymmetric shorter duplex siRNA structural designs. Sun et al. (2008) showed efficient mammalian gene silencing using asymmetric RNA duplexes termed aiRNAs, which are composed of a 15-nt-long sense strand and a 21-nt-long antisense strand, resulting in both 3' and 5' antisense overhangs. Chang et al. (2009b) also reported asymmetric shorter duplex siRNA structures, termed asiRNA, composed of a 16-nt-long sense strand and 19–21-nt-long antisense strand, resulting in a 5' blunt end and a 3' overhang with respect to the polarity of the antisense strand. asiRNA duplexes were incorporated into the RISC and mediated sequence-specific cleavage of target

mRNA. These results further support the idea that neither structural symmetry nor a duplex length of 19 bp needs to be maintained for efficient recruitment of the RISC and gene silencing by siRNAs. Nonetheless, it should be noted that asymmetric short siRNA duplexes shorter than 14 bp showed severely compromised gene silencing activity (Chu and Rana, 2008; Chang et al., 2009b), suggesting that a certain minimal RNA duplex length is essential for optimal recognition of siRNA by the RNAi machinery.

Reduction of Nonspecific Responses by Asymmetric Shorter Duplex siRNAs

Sense strand-mediated off-target silencing occurs because of the incorporation of the sense strand into RISC. This can often lead to unexpected gene silencing (Jackson et al., 2003; Clark et al., 2008). Although it has been shown that siRNA strands with a thermodynamically unstable 5' end are preferentially incorporated into the active RISC (Khvorova et al., 2003; Schwarz et al., 2003), many siRNAs show sense strand incorporation into the RISC irrespective of their sequence features. Because sense strand incorporation into the RISC is due to the symmetric nature of classical siRNAs, siRNA structural variants with asymmetric designs have been shown to successfully ameliorate this nonspecific response. All 3 asymmetric shorter duplex siRNAs described earlier showed reduced sense strand off-target silencing (Sano et al., 2008; Sun et al., 2008; Chang et al., 2009b). It is likely that both an asymmetric terminal structure and a shortened sense strand contribute to the preferential usage of the antisense strand for RNAi.

Besides asymmetric siRNAs, a triple-stranded RNA variant of siRNA called small internally segmented interfering RNA (sisiRNA) has also been shown to reduce sense strand off-target silencing (Bramsen et al., 2007). sisiRNA is composed of an intact antisense strand base-paired with 2 shorter 9–13-nt sense strands, debilitating the sense strand for subsequent RISC association and target gene silencing. Because these segmented sense strands were too short to stably maintain the RNA duplex, locked nucleic acid modifications were introduced to increase the thermal stability of the duplex structure.

Chang et al. (2009b) also showed that asiRNAs could alleviate the saturation of the endogenous RNAi machinery that was observed with the corresponding 19 + 2 siRNA structures. Interestingly, the reduction in the saturation of the RNAi machinery was dependent on the duplex length of the asiRNAs. Because RNAi machinery saturation by exogenously added siRNAs could disturb cellular miRNA biogenesis and potentially lead to nonspecific gene expression changes (Khan et al., 2009) or even cell death (Grimm et al., 2006), the ability of asymmetric shorter duplex siRNAs to alleviate RNAi machinery saturation indicates that they have a great advantage over classical siRNAs with regard to the development of improved functional genomics tools and therapeutic modalities.

The first-in-class siRNA drug to enter clinical trial as a therapeutic molecule was designed to silence vascular endothelial growth factor A (VEGF-A), a gene believed to be largely responsible for vision loss in wet age-related macular degeneration (AMD). Another siRNA targeting VEGF for AMD therapeutics was also under phase II trial. However, while the trials were being perused, a report by Kleinman et al. (2008)

demonstrated that intraocular injection of classical 19 + 2 siRNA structures induced sequence-independent angiogenesis suppression via nonspecific activation of TLR3. In their study, nontargeted siRNA suppressed dermal neovascularization in mice as effectively as VEGF-A siRNA. This study raised serious concerns about the specificity of siRNA drugs and resulted in the termination of phase III clinical trials for bevasiranib.

A computer modeling study suggested a potential interaction between 21-nt siRNA and TLR3, leading to TLR3 dimer stabilization and receptor activation, which explained the remarkable length-based discrimination of dsRNAs by TLR3 (Kleinman et al., 2008). According to this study, dsRNAs shorter than 17 bp did not activate TLR3, as they interacted with TLR3 with a free energy of binding below the threshold for receptor activation. This indicates that specific therapeutics targeting diseases such as AMD can be developed using asymmetric short siRNAs without nonspecific TLR3 activation. Taken together, these findings suggest that asymmetric short siRNA structures are a superior alternative to classical siRNAs for more specific gene silencing with reduced nonspecific effects.

Immunostimulatory siRNA Structural Variants

Although it is generally considered that immune stimulation by siRNAs should be avoided to achieve specific gene silencing, it might be therapeutically beneficial to design immunostimulatory siRNAs that could provoke both an immune response and gene silencing for antiviral and antitumor therapy (Schlee et al., 2006). Indeed, a proof-of-principle study of a bifunctional immunostimulatory siRNA approach to achieve enhanced antitumor activity was recently published (Poeck et al., 2008). In this study, siRNA targeting antiapoptotic *BCL2* mRNA with a 5' triphosphate (3p) modification, which is known to induce the immune response by activating the RIG-I pathway, was used. The *BCL2*-targeting 3p-siRNA induced stronger apoptosis than corresponding siRNA without 3p modification in B16 melanoma cell lines *in vitro* and also in a lung metastasis model *in vivo*.

Gantier et al. (2010) recently presented a rational design for immunostimulatory siRNA backbone structures without any chemical modification. They introduced a 4-nt uridine bulge in the middle of the Dicer-substrate siRNA structure. This structure triggered not only efficient gene silencing but also activation of TLR8, which resulted in the induction of various proinflammatory cytokines. However, although the addition of 4-nt uridine bulge into the 21-nt siRNA structure also potentiated immunostimulation, it reduced target gene silencing, presumably because of the instability of the duplex structure. As this uridine-bulge siRNA structure does not require a special chemical synthesis steps, it could be potentially used for large-scale industrial production of immunostimulatory siRNAs.

Multimeric siRNA Structural Variants with Enhanced Intracellular Delivery

One of the main hurdles in the development of RNAi therapeutics is the efficient delivery of siRNAs to target cells/tissues (Li et al., 2006). A number of lipid- and polymer-based carriers have been developed to facilitate intracellular delivery of nucleic acid drugs, including siRNAs, both *in vitro* and *in vivo* (Aigner, 2007; Whitehead et al., 2009). However, be-

cause siRNAs are less charged than gene-encoding plasmids, their interactions with cationic liposomes or polymers are relatively weaker. Thus, conventional cationic polymer-complexed siRNAs are easily expelled by negatively charged cell surface proteins, compromising the delivery efficiency of siRNAs. Although long dsRNA structures might interact more strongly with cationic delivery reagents than classical dsRNA structures, they could also potentially induce antiviral responses (Stark et al., 1998).

To overcome this limitation, Bolcato-Bellemin et al. (2007) designed gene-like siRNAs with short complementary A6-8/T6-8 overhangs to form transient concatemers, termed ssiRNAs. When complexed with polyethylenimine (PEI), a cationic polymer carrier widely used for gene delivery, the concatemer structure was stabilized and formed a strong complex with PEI. This design increased siRNA-PEI complex stability and protected the siRNA from nuclease attack, resulting in enhanced delivery efficiency (up to 10-fold). Similarly, Lee et al. (2010) and Mok et al. (2010) constructed multimeric siRNA polymers with reducible disulfide bond linkages between the siRNAs; these multimeric siRNA polymers are cleaved into 19-bp siRNA molecules upon internalization. Like ssiRNAs, these multimeric siRNA structures also showed increased silencing efficiency because of the enhanced delivery and protection from nuclease attack afforded by complexation with PEI. These results indicate that multimeric siRNA structures are potential gene silencing therapeutics that have increased delivery efficiency compared with monomeric siRNA structures.

Conclusion and Perspectives

In this review, we have summarized the recent developments in siRNA structural variants and discussed the advantages of these variants compared with classical 19 + 2 siRNA structures. The added benefits of using siRNA structural variants, such as improved gene silencing efficiency, alleviation of off-target gene silencing, reduction in RNAi machinery saturation, reduction in innate immune responses, and enhanced delivery, justifies the efforts made to identify novel gene-silencing siRNA structures. The increasing diversity of siRNA structural variants reported clearly indicates that a great deal of mechanistic flexibility exists in the mammalian RNAi machinery. Further biochemical, structural, and molecular studies into the mechanisms of action of these siRNA structural variants will shed light on the detailed mechanistic features of the RNAi machinery and allow the design of more efficient siRNAs. Advancements in the design and application of siRNA structural variants will not only inform RNAi biology but also provide new tools to combat various diseases with improved safety and efficacy.

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