Chapter 5

Noncanonical Synthetic RNAi Inducers

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Abstract

This review focuses on current strategies of development of noncanonical synthetic RNA interference (RNAi) inducers with structural modifications for promoting better gene silencing with low risk of side effects. A particular focus is on longer RNA duplexes 25–30 nucleotides (nt) in length that mimic Dicer substrates to improve interaction of RNAi inducers with RNAi machinery. Various design strategies of efficient Dicer substrate small-interfering RNA (siRNA) are described. It was found that the length, chemical modifications, and overhang structure influence the gene silencing activity and RNA-induced silencing complex (RISC) assembly. Special attention is paid to the long double-stranded RNA duplexes that induce effective gene silencing in Dicer-dependent or Dicer-independent mode. Some structural variants of shorter siRNAs, including hairpin and dumbbell siRNAs and fork-siRNA (fsiRNA) with several nucleotide substitutions at the 3′ end of the sense strand, are also analyzed. These structural modifications provide efficiently increased gene silencing of targets with unfavorable duplex thermodynamic asymmetry. Recent data remove the length and structure limits for the design of RNAi effectors, and add another example in the list of novel RNAi-inducing molecules differing from the classical siRNA, which is discussed in this chapter.

Keywords: RNAi, siRNA, fsiRNA, dsiRNA, tsiRNA, structural modifications, mechanism of action

1. Introduction

RNA interference is a conserved mechanism of a sequence-specific posttranscriptional gene silencing triggered by double-stranded RNAs homologous to the silenced gene [1, 2]. Long double-stranded RNA(dsRNAs) are cleaved in the cell by RNase III class endonuclease Dicer into short fragments 21–22 nucleotides (nt) in length with 2–3-nt 3′ overhangs at both ends [3, 4]. These fragments (small-interfering RNAs, siRNAs) enter RNA-induced silencing complex (RISC) and associate with core proteins belonging to Argonaute (AGO) family [5]. AGO

unwinds the duplex and cuts one of the strands in the middle, and then this strand (designated as "passenger") dissociates from the complex and is degraded by cellular ribonucleases. The other strand (designated as "guide") remains in activated RISC and recognizes the cellular mRNA complementary to the guide strand. The configuration of the complex determines which strand remains in the complex and which strand leaves and degrades. Active RISC complex containing the guide strand binds to the complementary mRNA and induces its cleavage. When the cleaved mRNA is released, RISC is recycled for a new round of cleavage [4, 6]. The details of the RNAi mechanism are well reviewed in literature [7–9].

Synthetic small-interfering RNAs have become an advanced and powerful tool for specific gene silencing and could be considered as promising class of therapeutics for the treatment of diseases associated with overexpression of specific genes [10–12]. However, therapeutic applications of canonical non-modified siRNAs are limited by their sensitivity to ribonucleases, possibility of unfavorable guide strand selection, and activation of innate immune system by siRNA containing immunostimulatory motives in the sequence, which can lead to poor gene silencing efficiency [13, 14]. Different structural variations of the RNAi inducers together with chemical modification were developed to overcome these problems.

This review focuses on current strategies of development of siRNA structural modifications for promoting better gene silencing with low risk of side effects, with particular focus on longer siRNA duplexes 25–30 nt in length that mimic Dicer substrates (Dicer substrate siRNA (dsiRNA)) [15–20]. Special attention has been paid to the long double-stranded RNA duplexes, which induced effective gene silencing and did not require Dicer-mediated processing of the substrate into smaller units: trimer RNA (tsiRNA) with 63 nt in length and tripartite-interfering RNA (tiRNA) with 38 nt in length [21, 22]. Applications of some structure variations of shorter siRNAs and the potential of different synthetic RNAi inducers in different applications have also been reviewed and summarized.

2. Dicer substrate interfering RNAs

Long dsRNAs homologous to the targeted mRNA were successfully used for silencing of gene expression in nonmammalian species [1, 4]. Early attempts to use long dsRNAs in mammalian cells triggering of RNAi failed due to activation of innate immune system by dsRNA [15]. Although activation of innate immunity may be beneficial for the therapy in some cases, uncontrolled induction of the interferon response results in global changes in gene expression profile and, in some cases, in cells death [23–25]. It was found that chemically synthesized 21-mer RNA duplexes with 2-nt 3′ overhangs at both ends, which directly mimic the products produced by Dicer, efficiently suppressed gene expression in mammalian cells [4, 26]. These duplexes, referred to as canonical siRNAs, are widely used in biomedical research [11]. Later, it was found that RNA duplexes, smaller than 30 nt in length but longer than siRNAs, were significantly more efficient than canonical siRNAs and did not induce interferon response in a variety of cell lines [15]. It was established experimentally that 27-mer duplexes possess maximal silencing activity, longer duplexes demonstrated reduced silencing activity, and 40–

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45-mer duplexes were inactive. At the same time, 27-mer duplexes, named as Dicer substrate siRNA (dsiRNA), were efficiently cleaved by Dicer producing a variety of 21-nt-long distinct products. High potency of 27-mer duplexes initially was explained by the formation of siRNA pool containing functional siRNAs with extremely high silencing activity. Some of 27-mer duplexes were significantly more potent at nanomolar or picomolar concentrations than the specific 21-mer siRNA selected according to the current computational algorithms [15]. However, further experiments demonstrated that none of the synthetic 21-nt siRNAs, included in the corresponding set to all possible products of Dicer processing of 27-mer duplexes, demonstrated the same level of silencing activity as 27-mers at low concentrations [15].

Based on the earlier observations that Dicer participates both in the cleavage of dsRNAs and in the incorporation of the products of cleavage into RISC complex in *Drosophila melanogaster*, it has been suggested that Dicer could participate in direct loading of siRNA into RISC and in RISC assembly [16, 27]. It has been experimentally proved that dsiRNAs form the RISC loading complex (RLC) in vitro more efficiently than the canonical 21-mer siRNA duplexes [18]. Because Dicer does not form complexes with 21-base pair (bp) duplexes, it was assumed that Dicer facilitates RLC formation after dsRNA cleavage without dissociation from the cleavage product. These findings become a basis for the development of a new class of RNAi inducers [16, 17, 28].

The silencing activity of dsiRNA depends on its structure. At the first step of recognition, PAZ (Piwi Argonaut and Zwille) domain of Dicer predominantly "anchors" two ribonucleotides on 3′ overhangs because those blunt 27-mer duplexes are not good substrates for Dicer. PAZ domain plays a vital role in the orientation of bound RNA in the active site of the enzyme and determines the cleavage position on RNA for AGO protein. Unlike 21-mer siRNA, where two-base 3′-deoxynucleotide overhangs are often used regardless of their complementarity to the target mRNA sequence (mostly dTdT), the overhang sequences are important for the properties of dsiRNA. Incorporation of deoxynucleotides at the 3′ ends of dsiRNA strands has an adverse effect on dsiRNA processing [19]. The sequence of 3′ terminal overhangs could control dicing polarity and strand selection into RISC. Thus, Dicer preferentially binds with purine/purine (GG, AA) nucleotides [19]. Protruding nucleotides added to the 3′ terminal of the antisense strand facilitate its preferential loading into RISC [19]. Hence, asymmetric duplexes with one 2-nt 3′ overhang and DNA residues on the blunt end of the duplex provide a single favorable PAZ binding site and reduce heterogeneity of cleavage products (Figure 1) [16–18, 29, 30].

The stability of dsiRNA in physiological fluids is extremely an important factor for its applications in vivo [31]. Although dsRNAs are more stable in comparison with single-stranded RNAs and 21-bp siRNA, they still rapidly degrade in the serum [32]. It was found that bonds with 3′ pyrimidine nucleotides are cleaved faster than bonds with 3′ purines. Kubo and his colleagues demonstrated that degradation rate of dsiRNAs correlated with the amount of pyrimidines at the 3′ end [31]. At the same time, degradation rate of dsiRNAs also correlates with the presence of AU-rich domains that might be related to low thermal stability, easy dissociation, and faster cleavage by both endo- and exonucleases. Chemical modifications can improve nuclease stability and reduce off-target effects [33–36]. Fluorescein modification of 3′

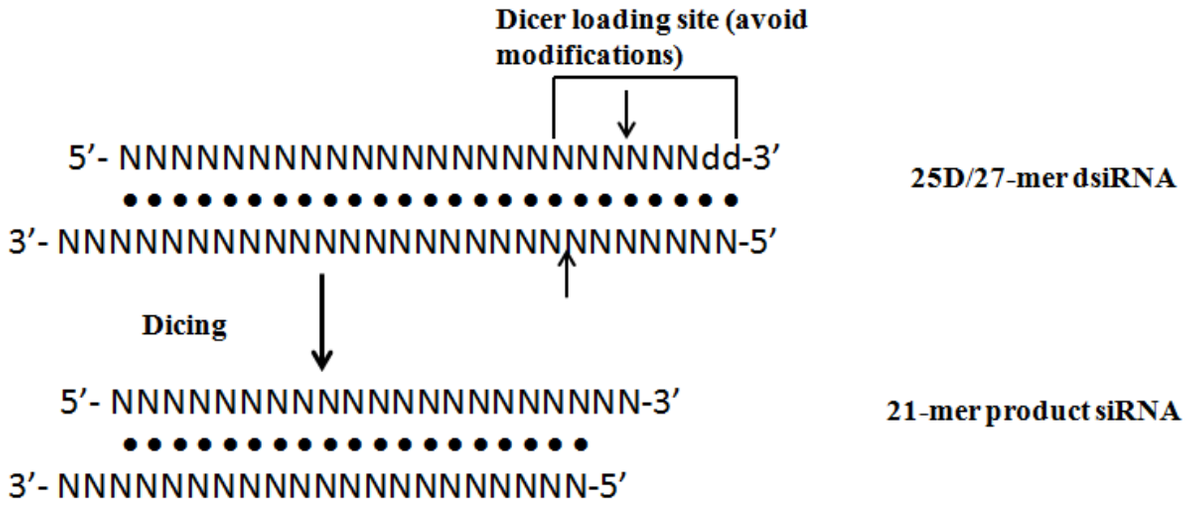

Figure 1. The scheme of dsiRNA design (according to [17]). N – ribonucleic acids, d - deoxyribonucleic acids, short arrows – the site of Dicer cleavage.

end of 27-mer RNA duplexes significantly reduces RNAi activity because 3′ ends are important for interaction with Dicer and should be available for the proper recognition [15]. On the other hand, dsiRNAs with chemical modifications of the 5′ end possess high nuclease stability and RNAi activity in the cell-cultured medium. Thus, 5′-end amino-modified dsiRNA demonstrated improved RNAi activity and stability in the cell-cultured medium [31].

Incorporation of 2′-O-methyl modifications is an efficient and inexpensive method to improve nuclease resistance of synthetic RNA duplexes [33, 37–40]. However, dsiRNA duplexes with modifications of all or the majority of nucleotides in both sense and antisense strands are practically inactive, because extensive modification blocks the cleavage of duplex by Dicer [17]. Limiting the modifications to incorporation of only 9–11 modified bases into the antisense strand and avoiding modifications in the site of Dicer cleavage prevents this undesired effect [17]. Mass spectrometry analysis of in vitro dicing reactions showed that modified duplexes produce a mixture of 21- and 22-nt species, whereas unmodified duplexes are processed only into 21-mer species. If modifications were spaced further away from the dicing site, a preferential accumulation of 21-mer species was observed [17]. However, it was noted that in some cases, usually, a good modification pattern may decrease the silencing activity of dsiRNA. This phenomenon has been related to the newly unidentified sites in the sequence context of some chemically modified dsiRNAs, contributing to impairment of dsiRNAs silencing effect [17]. These observations could explain differences in the efficiency of various dsiRNAs and made possible the creation of the modification patterns compatible with dicing.

Accumulation of experimental data revealed that 27–30-bp dsRNAs, including dsiRNA and, in some cases, even 21-bp siRNA, could stimulate innate immune system and induce interferon response in certain cell types [23]. Toll-like receptors (TLRs) 7 and 8 appear to be the main molecules responsible for the immune recognition of siRNA and dsiRNA, whereas toll-like receptor 3 recognizes longer than 30-bp dsRNA [41, 42]. Activation of innate immune system through TLRs results in the production of interferon α, tumor necrosis factors α, and inter-

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leukins IL6 and IL12 [42]. Immunostimulatory properties of siRNA are sequence dependent; TLR7 and TLR8 receptors recognize GU-rich sequences of siRNA [43]. Moreover, several immunostimulatory motifs of siRNA enriched in GU nucleotides were identified [44, 45]. It is recommended to avoid these motives in siRNA and dsiRNA design; unfortunately, not all immunostimulatory motifs have been discovered that complicate the design procedure. Earlier, it was demonstrated that chemical modifications involving 2′ position of the ribose ring in siRNA could block the immune response [46]. Incorporation of 2′-O-methyl U and G bases into siRNA significantly reduced immunostimulatory activity of siRNA in vitro and in vivo, containing immune-stimulating motives in the sequence [41]. Moreover, the effective suppression of immunostimulatory activity could be reached by using only a small percentage of modified nucleotides (<10%). Collingwood and his colleagues [17] applied this approach to dsiRNA and demonstrated that limited 2′-O-methyl modifications of uridine and guanosine into antisense strand of dsiRNA efficiently prevent induction of innate immune response in different cell lines.

Another option to reduce nonspecific effects of dsiRNA is to use enzymatically produced pools of Dicer substrate RNA [20]. Dicer from the protozoan parasite *Giardia intestinalis* was used to obtain enzymatically produced dsiRNAs. It cuts long dsRNA into fragments from 25 to 27 nt in length. The sequences-related side effects were decreased in the pool of enzymatically produced dsiRNAs due to the low concentration of individual dsiRNAs with undesirable sequence.

In the cases when silencing of more than one gene is required, the transfection of siRNA mixture is used. Co-transfection of different siRNAs may result in different knockdown efficiency of individual targets due to competition between siRNAs for RISC loading depending on the thermodynamic asymmetry of the duplexes [30]. Therefore, preliminary testing is required to assess the degree of competition between various siRNAs. Competition between RNAi inducers aimed at different mRNAs could be avoided by using Dicer substrate RNA. Entry of dsiRNAs into RNAi pathway is not limited by RISC loading step, and discrimination of canonical siRNAs based on RISC incorporation is reduced. These beneficial properties of dsiRNAs can provide an effective tool for targeting multiple mRNAs.

Currently, siRNAs have become a powerful tool for effective suppression of expression of target genes in vitro and in vivo applications. Moreover, several compounds are already used in clinical trials. However, the examples of Dicer substrate RNAs usage in vivo are fewer in number. Several studies use dsiRNA to silence therapeutically relevant genes in vivo (Table 1). Frequently, cancer-related genes and genes of viruses [50, 51, 56–59] are chosen as targets for dsiRNAs [47–49]. Several researchers used *TNFα* gene as a target for the treatment of inflammatory and autoimmune diseases [52–54]. Murine models are the most popular animal models among various studies that used dsiRNA in vivo [47–54]; however, there are studies where other animal models, for example, rats, were used [53, 55]. An exciting example of dsiRNA application was described by Doré-Savard and his colleagues, who demonstrated, for the first time, the efficient suppression of target genes in central nervous system (CNS) of rats by dsiRNA [55]. In this study, 27-mer dsiRNAs were used to reduce expression of neurotensin receptor-2 (NTS2) involved in ascending nociception. dsiRNAs were formulated with cationic

lipid i-Fect and used in intrathecal spinal cord injection. Extremely low doses of dsiRNA (0.005 mg/kg) efficiently silenced NTS2 mRNA and protein levels for 3–4 days. It is known that administration of high doses of non-modified siRNA increases the risk of activation of innate immune system, especially when siRNA is used together with cationic lipids. Low doses of highly active dsiRNAs could minimize this adverse effect. No apparent toxicity and other off-target effects were found during the experiment [55]. The dose–response experiments performed in another study [28] also show that 27-mer Dicer substrate RNA provide improved gene silencing when used at lower concentrations [28]. The silencing activity of canonical 21-mer siRNAs was compared with that of dsiRNA at 1 and 5 nM concentrations. The 27-mer dsiRNA displayed more potent gene silencing at 1 nM concentration, while at 5 nM concentration, the difference in silencing was less pronounced [28].

Experimental system	Structure	Target (gene)	Disease	Concentration/dose	Biological effect	Reference
MDA-MB-435 cells	25D/27-mer	*cdc20* (mouse)	Breast cancer	20 nM	>80% cell growth inhibition	[47]
Mice				2 μg/mouse	Tumor growth inhibition after second injection	
Huh7.5 cells	25D/27-mer	5' UTR and coding regions of hepatitis C virus: *NS3, NS4B, NS5A, NS5B*	Hepatitis C virus infection	5 nM	99.5% inhibition in luciferase assay	[57]
PC-3 cells	25D/27-mer	*HSP27* (human)	Prostate cancer	50 nM	>50% reduction of both mRNA and protein	[48]
Mice		*Hsp27* (mouse)		3 mg/kg	>50% reduction of both mRNA and protein	
HAE cells obtained from bronchi and lungs	25D/27-mer 2′OMe	*N* gene of respiratory syncytial virus (RSV)	Respiratory syncytial virus infection	250 nM	> 100-fold decrease of viral titer	[57]
Hela cells	25D/27-mer 2′OMe	*CTNNB1* (human)	Liver cancer	1 nM	>90% mRNA level reduction	[49]
Mice		*Ctnnb1* (mouse)		5 mg/kg	Significant reduction of tumor weight	
AY-27 cells	25D/27-mer	*Mki-67* (rat)	Bladder cancer	10 nM	50% mRNA reduction	[53]
LLC-MK2 cells	25D/27-mer	*N, D,L* genes of human	Human metapneum	0.65 nM	50% reduction in plaque assay	[50]

Experimental system	Structure	Target (gene)	Disease	Concentration/dose	Biological effect	Reference
		metapneumovirus (hMPV)	ovirus infection			
Mice				4 mg/kg	Reduction of virus titers in lungs of infected mice	
RAW264.7 cells	25D/27-mer	*Tnf* (mouse)	Inflammatory diseases (sepsis model)	5 nM	>50% reduction of the number of TNFα positive cells	[52]
Mice				10 mg/dose	4-fold reduction of the number of TNFα positive peritoneal macrophages	
keratocytes from rabbit corneal stroma	25/27-mer	*JKAMP* (rabbit)	Corneal wound healing	10 nM	70% - *JNK1* mRNA level reduction 50% - *JNK2* mRNA level reduction	[58]
CCRF-CEM cells	25/27-mer	*TNPO3* *CD4* (human) *Tet/rev* (viral)	HIV-1	50 nM	50% *TNPO3* mRNA level reduction 75% *CD4* mRNA level reduction 60% *Tet/re* mRNA level reduction	[51]
Mice				0.15 mg/kg	Prolonged antiviral effect	
Kupffer cells	25D/27-mer	*Tnf* (rat)	Inflammatory diseases	10 nM	80% reduction of TNFα level after LPS stimulation	[53]
Rat				100 μg/kg	50% reduction of TNFα level in blood	
CHSE-214 cells (fish)	25/27-mer	*N* gene of hemorrhagic septicemia virus (HSV)	Hemorrhagic septicemia virus infection	15 nM	99% mRNA level reduction	[59]
Murine peritoneal macrophages	25/27-mer 2′OMe	*Tnf* (mouse)	Rheumatoid arthritis	50 nM	66% protein level reduction	[54]
Mice				5 μg/dose	Block the development of inflammation after second dose	
NTS2 cells	25D/27-mer	*Ntsr2* (rat)	Pain states	10 nM	>90% mRNA level reduction	[55]

Experimental system	Structure	Target (gene)	Disease	Concentration/dose	Biological effect	Reference
Rat				0.005 mg/kg	86% and 62% mRNA level reduction in lumbar dorsal root ganglia and in spinal cord, respectively	

Table 1. Application of dsiRNA for silencing of disease-related genes (summarized from PubMed). 25/27-mer – dsiRNAs with 25 - base sense strand and 27 – base antisense strand; 25D – 2 bases at the 3′-end are substituted with DNA; 2′OMe – 2′ – O methyl modifications as described in [17].

In another study, potent 2′-O-methyl modified dsiRNAs targeted to β-catenin were designed [49]. It is known that β-catenin acts as the transcription factor and its overexpression causes the development of several types of cancer, including liver cancer. At the first step, large-scale screening of 488 dsiRNAs for in vitro mRNA knockdown activity was performed to choose the most efficient dsiRNAs for targeting β-catenin. Then, the absence of immunostimulatory activity attributed to selected dsiRNA was confirmed using the assay based on the ability of an oligonucleotide to induce the production of antibodies to the PEGylated components of the lipid nanoparticles containing oligonucleotides. dsiRNA was administered to mice intravenously twice a week during 3 weeks after implanting Hep 3B tumor cells. dsiRNAs induced strong β-catenin mRNA knockdown and efficient tumor inhibition. Other examples of dsiRNAs applications as potential therapeutics for inhibition of the disease-related overexpressed genes in vivo and in vitro have been summarized in Table 1.

Beneficial properties of dsiRNAs make these structures popular inhibitors of target genes. At first, dsiRNAs induce more potent silencing of the target genes at lower concentrations than canonical siRNAs. The next advantage of dsiRNAs is longevity of silencing: In some cases, it lasts up to 10 days. Then, the usage of dsiRNAs enables to minimize off-target effects such as toxicity and heterogeneity of processed products. An additional benefit is the high potency of dsiRNAs in silencing of multiple mRNAs where canonical siRNAs due to competition during RISC loading step appear to be less effective. The main disadvantage of dsiRNA is the higher cost of synthesis in comparison with canonical siRNA. However, low dosage of dsiRNA used in experiments eliminates this drawback. On the other hand, dsiRNAs share with siRNAs the same problems in therapeutic applications. The major challenge lies in the delivery of these structures into desired cells, tissues, and organs. To overcome this problem, various approaches are developed; however, this question has not been completely answered yet. Nevertheless, dsiRNA as potent inducers of RNAi offers promising strategies for efficient therapy.

3. Interfering RNA with noncanonical duplex structure

Different variations of siRNA duplex structures were proposed to improve their silencing activity. Here we will consider three types of the most frequently used siRNAs with structural

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modifications of duplexes: short hairpin RNAs (shRNAs)/microRNA (miRNA) mimics, dumbbell RNAs, and fork-siRNA (Figure 2).

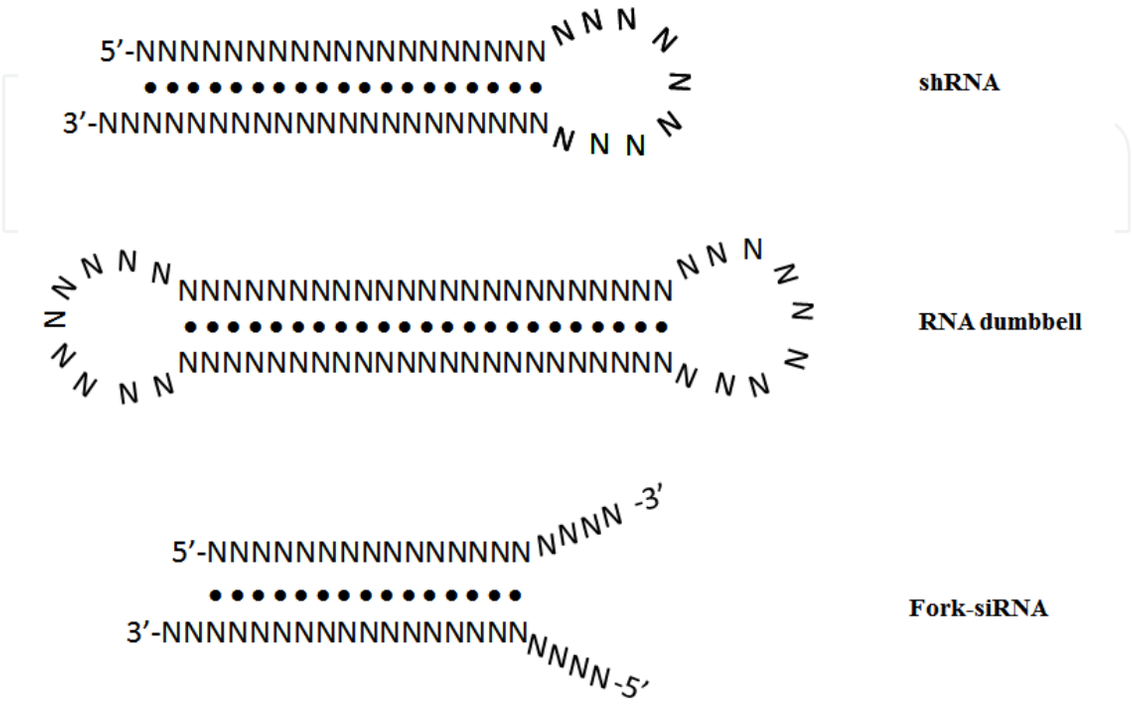

Figure 2. The different types of interfering RNAs with non-canonical duplex structures.

The identification of a large class of endogenous regulatory RNA molecules – microRNAs (miRNAs) arouse interest in constructing the similar synthetic structures for efficient silencing of target genes. miRNA precursors are generated in the cell as long primary transcripts that are cleaved in nucleus by RNase III class nuclease Drosha [60–62]. Then, they are exported to the cytoplasm and cleaved by Dicer, which is active at processing of complex hairpin structures [63]. It is known that Dicer substrates more effectively enter RISC complex than canonical siRNA and induce more potent RNAi [15–17, 28]. Moreover, shRNAs could interact with particular chaperones that promote recognition of shRNA by Dicer [64]. miRNAs form imperfect complementary complexes containing bulges with 3′ untranslated region of the target mRNA, wherein the position of the loops defines the mechanism of action: target cleavage or the block of translation. In the first case, synthetic miRNA mimics have no advantages over shRNA, and in the second case, they do not act in a catalytic mode. Therefore, synthetic miRNA applications are restricted to exploring the miRNA-regulated pathways involved in the natural processes, or development of replacement therapy for the diseases associated with mutation in specific miRNA. The use of shRNA seems to be more promising.

Although long dsRNA hairpins are prepared synthetically, enzymatically, or endogenously expressed, plasmid or viral vectors could be used in nonmammalian organisms. Long RNA hairpins cannot be applied in mammals for the specific gene silencing because they also induce

interferon response in mammals via the same mechanism used by long RNA duplexes [3, 65, 66]. Therefore, length of hairpin RNAs for application in this type of species is limited by 30 bp. shRNAs expressed by different vectors under control of RNA polymerase III and CMV promoters were proved to efficiently trigger RNAi [67, 68].

Applications of viral vector-based expression of shRNAs are limited because of some obstacles such as possibility of insertional mutagenesis, malignant transformation, and host immune response [69]. At the same time, an application of plasmid vectors is safe, but inefficient delivery into cells limits its use only for experimental purposes, where antibiotic resistance genes included in the vector is used for the selection. In contrast to expressed shRNA, synthetic shRNA seems to be more attractive for RNAi-based therapies. It was found that chemically synthesized short hairpin RNAs (shRNAs) with 19–29-base-pair stem, at least 4-nucleotide loop and 2-nucleotide 3′ overhangs are more potent inducers of RNAi than the canonical small-interfering RNAs targeted to the same sequence in mRNA [64, 70–73]. Two main types of shRNAs with opposite positions of the loops were designed (Figure 3). The right loop structures (R-shRNAs) have sense strand at the 5′ end of the hairpin, whereas the left loop shRNAs (L-shRNAs) have antisense strand at the 5′ end of the hairpin (Figure 3) [71–74]. The majority of studies were carried out using R-hand loop structure.

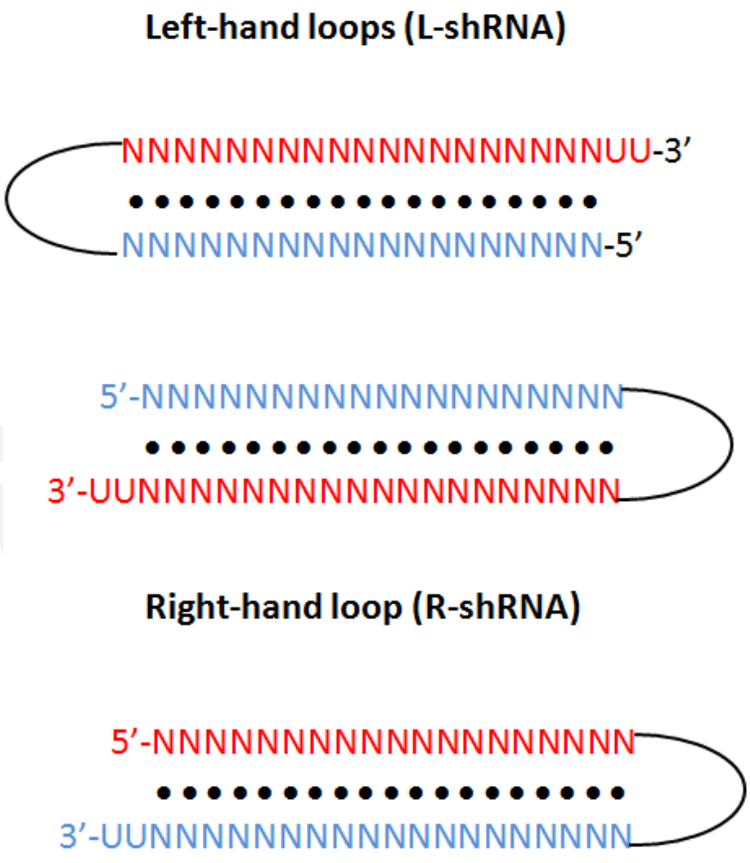

Figure 3. General structures of L-shRNAs and R-shRNA (according to [71]). Red color: sense strand, blue color: antisense strand.

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It was clearly demonstrated that the silencing activity of shRNAs depends on stem length, loop length, sequence, and terminal overhangs [70]. Dicer efficiently cleaves shRNA with certain minimum stem of 19 nt in length forming 22-nt products starting from the free 3′ end of the RNA.

Correct 3′ overhangs increase the efficacy and specificity of processing, whereas blunt-end shRNAs produce a set of products [64]. In the case of endogenously expressing regulatory RNAs, 3′ UU overhangs of miRNA precursors generated by Drosha cleavage determine subsequent proper recognition and processing by Dicer [75]. Synthetic shRNA with similar overhangs and mimicking the products of Drosha preprocessing is used. The presence of a 3′-UU overhang improves silencing activity of 19-mer shRNAs as 3′-UU overhangs might provide additional site for PAZ domain of Dicer [70].

The loop length also plays a crucial role in the silencing activity of shRNAs. Thus, it was found that 29-nt stem and 4-nt loop inhibited the target gene expression more efficiently as compared with shRNA with 19-nt stem and loop [76, 70]. In contrast, when 9-nt loop was used for 19-nt stem shRNA, it demonstrated more potent silencing of target genes than longer shRNA with the 4-nt loop. Brummelkamp and his colleagues also demonstrated that shRNAs with 19-nt stem and 9-nt loops possessed the maximum silencing activity as compared with shRNAs with 7-nt loops, while shRNA with 5-nt loops were inactive [68]. All observed differences in the silencing activities of shRNA, divergent in the length of the stem and loop, were more pronounced under low or intermediate concentrations. The silencing activity of different shRNAs, used at high concentrations, did not depend substantially on the loop length [70].

These results may be explained by the fact that the loop length influences the efficiency of processing by Dicer. [64]. Indeed, 4-nt loops of shRNA with 19-nt stem have poorer conformation flexibility at the junction between the duplex stem and a single strand of the loop. As short loops are set close or at the Dicer cleavage site, the restriction-associated conformational changes made shRNAs stems poor substrates for Dicer. Therefore, shRNAs with short loops and 19-nt stem enter RISC complex in the later stages and remain not processed by Dicer [64]. It was suggested that another single-strand-specific ribonuclease, independent from Dicer, cleaved this type of shRNA. On the other hand, shRNAs with 19-nt stem and longer loops (9–10 nt) are efficiently processed by Dicer [76].

The potency of 19-nt stem shRNAs, targeted to the same sequence, depends on the position of the loop. Right (R)-shRNAs 19 nt in length are significantly less active than left (L)-shRNAs of the same length [71, 72]. However, the position of the loop (left or right) in longer shRNAs did not affect their activity. It was suggested that low potency of R-shRNA form is related to the fact that 5′ end of the antisense strand must be readily available for the efficient binding of AGO2 in the RISC complex. Otherwise, the 5′ end of sense strand would enter RISC and the target mRNA would not be cleaved. [77]. L-shRNAs with a short loop of 1–2 nt in length could be active. Moreover, L-shRNAs without any loop, where sense strand is directly connected with antisense strand, may be also active. In this case, the sense strand is shorter than antisense strand and the loop is formed by 3′ end of the antisense strand [71].

Moreover, the nature of nucleotides in 3′ overhang does not influence the activity of L-shRNA and 3′ overhangs could be substituted for deoxyribonucleotides [71]. The high efficiency of L-shRNAs may be explained by the high energy of binding of antisense strand with AGO2 due to availability of 5′ end of antisense strand in L-shRNA, dominated over the influence of overhangs and loop length. [71].

shRNAs have similar but not identical sequence preferences with siRNAs. Thus, the functional shRNAs have mainly AU nucleotides at position 9 and GC nucleotides at position 11, while these preferences are less significant in functional siRNA. At the same time, the functional shRNAs have the similar thermodynamic asymmetry as functional siRNAs. The computer algorithms for selection of potent shRNAs have been developed [76].

Short hairpin RNAs are a little more resistant to nucleases than siRNAs due to the protection of one end; however, shRNAs still quickly degrade in biological fluids [78]. The elegant method to stabilize non-modified RNA strands was described by Abe et al. [79]. Abe and his colleagues constructed dumbbell-shaped RNA structures and demonstrated their potency as RNAi inducers with stability in the biological fluids [79]. Dumbbell-shaped RNAs were designed by analogy with DNA dumbbells consisted of double-helical stem and closed by two hairpin loops. Dumbbell-shaped RNA structures are used as models for the analysis of local structures in DNA [80]. Local unwinding of duplexes facilitates enzymatic cleavage by nucleases. Two loops at the both ends of dumbbell RNA stabilize the duplex and limit its enzymatic cleavage [79, 81]. Dumbbell structures get processed by Dicer much more slowly in comparison with their linear analogues due to inefficient recognition by Dicer. The rate of processing depends on the stem length, too. For example, dumbbell RNAs with 27-bp-stem length were processed more quickly than the same sequence with 15–19-bp stem length. RNA dumbbells with 23-bp stems and 9-nt loops were found to be the most active. Indeed, 9-nt loops are commonly used in shRNAs as the most effective hairpin loops [67]. The stem length was optimized to keep high potency and reduce interferon response. Silencing activity of these dumbbell RNAs was significantly higher than that induced by linear counterparts and was retained for longer period even at lower concentrations [81]. The introduction of deoxynucleotides into the loop of dumbbell RNAs further significantly increases shRNA stability in biological fluids without loss of silencing activity. Moreover, the loop of dumbbell RNAs can be modified by carriers such as aptamers and peptides [81]. All benefits of dumbbell RNAs make them new potent RNAi inducers. The main disadvantage of these structures is the high cost of their synthesis in comparison with canonical siRNAs. At the same time, the low dosage and prolonged silencing effect can reduce expenses. The detailed scheme of RNA dumbbell synthesis is described by Abe and his colleagues [82].

Another type of RNAi inducer, fork-siRNA, was first introduced by Hohjoh [83]. Fork-siRNAs contain base substitutions in the 3′ end of the sense strand of siRNA, resulting in destabilization of the duplex [83–85]. The effect of fork-siRNAs is explained by the fact that thermodynamic asymmetry of the duplexes determines the orientation of siRNA in RISC. Thermodynamic stability of the terminal regions of the duplex defines which strand is cleaved and dissociated during RISC activation, and another strand remains in the activated RISC and guides target

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mRNA recognition and cleavage [86]. Antisense strand of siRNA must be included in activated RISC for efficient gene silencing, if activated RISC contains the sense strand no silencing occur.

The selection of active siRNAs may be complicated if a target mRNA is mutated or is a chimerical gene. To address this issue, the favorable asymmetry can be achieved by the introduction of several base substitutions at the 3′ end of the sense strand. Mismatches at the 3′ end of the sense strand, resulting in the formation of unpaired or destabilized regions, increase the silencing activity of siRNA with low or moderate concentrations [83]. The number of mismatches in fork-siRNA also plays a crucial role in its silencing activity [85]. Fork-siRNAs with one to two mismatches at the 3′ end possess silencing activity similar to that of canonical siRNAs, indicating that this number of mismatches is not enough for the efficient silencing. Fork-siRNA with four mismatches is the most potent, whereas fork-siRNA with six mismatches possesses reduced silencing activity [85].

An optimal number of mismatches depend on the overall thermodynamic stability of the duplex. Computational algorithms for siRNA sequence selection determine the recommended range of T_m difference between the terminal regions, and four mismatches could work for sequences within the range. On the other hand, mismatches in the 3′ part of the sense strand and long unpaired ends increase the sensitivity of fork-siRNA to nucleases. Consequently, the application of non-modified fork-siRNAs in vivo is limited by the fact that they have reduced stability in biological fluids due to the increased degradation by nucleases [83, 85]. To solve this problem, the algorithm for designing nuclease-resistant fork-siRNAs that contain 2′-O-methyl modifications in nuclease-sensitive sites was developed, which allows obtaining fork-siRNAs whose stability is comparable to that of canonical siRNAs [85].

Thereby, fork-siRNAs may improve unfavorable asymmetry of siRNA with low or moderate silencing activity, especially when the selection of functional siRNA is restricted by the sequence content of the corresponding mRNA. It makes sense to use them for silencing of uneasy or precisely located targets.

4. Short noncanonical RNA

siRNA shorter than canonical siRNA could also induce efficient silencing of target genes in mammalian cells acting via RNAi mechanism [87–90]. Short siRNAs have some benefits as inducers of RNAi such as reduction of immune response and decreased cost of the synthesis [76]. Various strategies have been used to design the minimal length for inducing RNA interference. As A-form helix of RNA plays an essential role for inducing RNAi, Chiu and Rana [91] found minimal length of dsRNA A-form helical structure required to enter active RISC complex. They demonstrated that siRNA with 16 bp in length and 2-nt 3′ overhangs representing ~ 1.5 helical turns efficiently assembles into catalytically active RISC and was sufficient for silencing of target genes. Indeed, 16-mer siRNAs were more potent in comparison to 19-mer siRNAs, while 15-mer siRNAs silenced gene expression at lower efficacy than 16-mer siRNAs, and 14–13-mer siRNAs were practically inactive [87]. It was demonstrated that the mechanism of target cleavage was different: cleavage sites in 16-mer siRNAs were shifted to

3 nt in comparison with 19-mer siRNAs (Figure 4). The 16-bp siRNAs induced the silencing faster than canonical siRNA due to the higher efficacy of RISC formation [87, 91]. Moreover, asymmetric duplexes with 3′ overhang on the antisense strand only demonstrated reduced off-target silencing in comparison with symmetric duplexes of the same length due to preferential incorporation of the guide strand into the RISC complex [91]. Thus, considering the benefits of 16-mer siRNAs, they possess high potential for using in biomedical studies, but new examples of their use are not available.

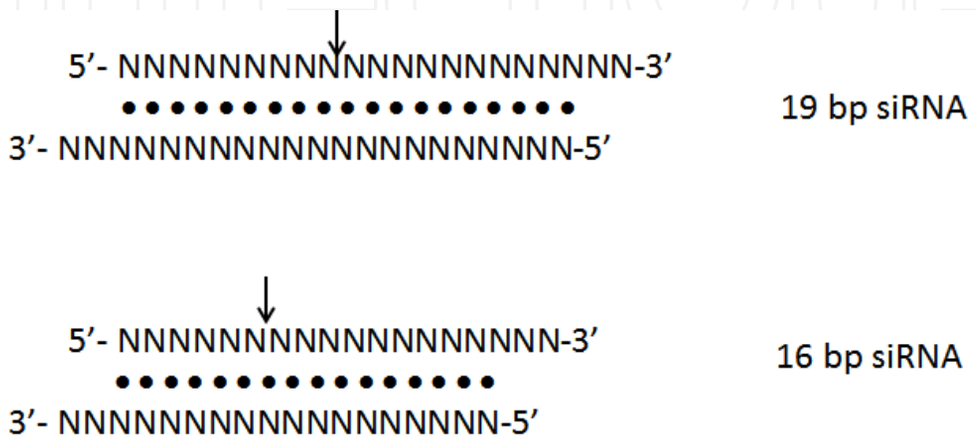

Figure 4. The mechanism of Dicer cleavage for 19 bp siRNA and short siRNA 16 bp in length. Arrows indicate cleavage sites defined by the 5′-end of guide strand (according to [87]).

Antisense siRNA also have been proposed as RNAi triggers. The sense strand of siRNA duplex is degraded and antisense strand remains in the active RISC to target complementary mRNA. Early studies demonstrated that 5′-phosohorylated antisense siRNAs with 22–40 nt in length were able to induce gene silencing in *Caenorhabditis elegans* [92, 93]. Several studies investigated the possibility of using antisense strand of siRNA 19–29 nt in length for transient knockdown of target genes in mammalian cells [88–90]. Antisense siRNA entered the RISC complex and provided mRNA cleavage but with lower efficiency in comparison with canonical siRNA. Antisense siRNA and canonical siRNA possess similar mRNA target position effects, cleavage fragment production, and tolerance to mutational and chemical modifications. However, antisense siRNA modified at 3′ end by fluorescein group or deoxyribose showed reduced silencing activity compared with canonical siRNA, where silencing activity remained at the same level in spite of 3′ modifications [89]. Moreover, the velocity of mRNA degradation induced by antisense siRNA is higher than that provided by canonical siRNA, but the duration of silencing effect is shorter. Thus, it was assumed that antisense siRNA induces RNAi through similar pathways as double-stranded siRNA but enters the pathway at the intermediate stage [89].

The differences in the silencing activity between antisense and canonical siRNAs may be explained by the low intracellular stability of the single-stranded RNA and by low efficacy of association with RISC [89]. Among the advantages of siRNAs, a lower price of synthesis and no side effects associated with the induction of interferon response should be considered [88].

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Partial boranophosphate backbone (BP) modifications were designed to increase the stability and the silencing activity of the antisense siRNA [88]. BP-modified antisense siRNAs possess silencing activity comparable to unmodified double-stranded siRNAs. Partial 2′-O-methyl modification was used for the stabilization of antisense RNA, the activity resulting in single-stranded siRNA was comparable with the activity of double-stranded siRNA when used in high or intermediate concentrations, where in low concentration, canonical siRNAs were more active [85].

Overall, in spite of lower silencing activity compared with canonical siRNA, antisense siRNA may be used in specific situations, for instance, to eliminate off-target silencing of genes in the case when the sense strand has substantial homology to nontarget genes [88].

5. Long-interfering RNAs

Long dsRNAs >30 nt in length efficiently silence the expression of target gene in nonmammalian cells [1, 4]. The early attempts to use the similar structures for efficient knockdown of target genes in mammalian cells failed due to activation of interferon response [4, 94]. Later, various design strategies have been developed to prevent the induction of interferon response and construct new potent RNAi inducers [21, 95, 96]. Depending on the architecture of duplexes, all long dsRNAs may be divided into linear and branched structures.

Partial 2′-O-methyl modification effectively prevents the activation of interferon response by Dicer-substrate RNAs [17]; therefore, it was proposed to use similar approach for longer linear duplexes [21]. Longer siRNAs containing the sequence of canonical siRNAs repeated two and three times are called dimer (42 nt in length) and trimer (63 nt in length) small-interfering RNA. Selective 2′-O-methyl modifications were introduced into nuclease-sensitive sites of both sense and antisense strands of dimer and trimer siRNAs, the modifications in the sites of potential Dicer cleavage were omitted. Selectively modified dimer and trimer siRNAs, unlike the unmodified ones, did not induce interferon response in cultured cells. The trimers (called tsiRNA) were significantly more active at lower dose-equivalent (per moles of 21 bp) concentrations than their canonical analogues but the silencing effect develops more slowly [21] and acts in a Dicer-independent mode, presumably via direct RISC loading. Although the Dicer cleavage sites were free from modifications, modifications in flanking regions of tsiRNAs could inhibit the Dicer cleavage. The observed mechanism may be associated with a specific pattern of modification, used by the authors, which cannot be excluded such that the change in the pattern will allow the tsiRNA to be processed by Dicer and act through a canonical mechanism.

Targeting single mRNA by RNAi inducer for therapeutic purposes has several limitations: (1) the presence of mutation in the target site reduces the efficiency of silencing, which is especially important for viral genes, and (2) signal pathways involved in cancer cell growth contain duplications of regulatory elements and bypass regulatory pathways [97–99]. Thus, simultaneous inhibition of several genes seems to be an effective strategy. Co-transfection of several siRNAs may be not effective due to competition between siRNAs [30]. Therefore, long linear synthetic siRNAs targeted two or more genes hold great promise in these cases.

Peng and his colleagues designed long linear siRNA at least 30 nt in length (multi-siRNAs) for dual-gene silencing [95]. To avoid undesired interferon response and improve RNAi potency, 2′-O-methyl modifications and gap in either sense or antisense strands were used. 2′-O-methyl modifications were introduced into every second nucleotide of both strands. The gap divided the complementary strand into two equal segments. It was demonstrated that multi-siRNAs with the gap provided more efficient simultaneous silencing of two target genes in comparison with corresponding single-target siRNAs (Figure 5). Interestingly, the simultaneous silencing of two target genes by long siRNA without gap was ineffective. It was supposed that the gap may provide sites for Dicer or facilitate Dicer processing. Because the Dicer substrates have preference in RISC loading, multi-siRNAs could possess more efficient silencing activity than canonical siRNAs [16, 18]. The experiments demonstrated that silencing effects of multi-siRNA was eliminated when AGO2 was downregulated confirming the action through the same RNAi pathway as canonical siRNAs [95]. However, further experiments are required to clarify the exact mechanism of increased activity of these siRNAs.

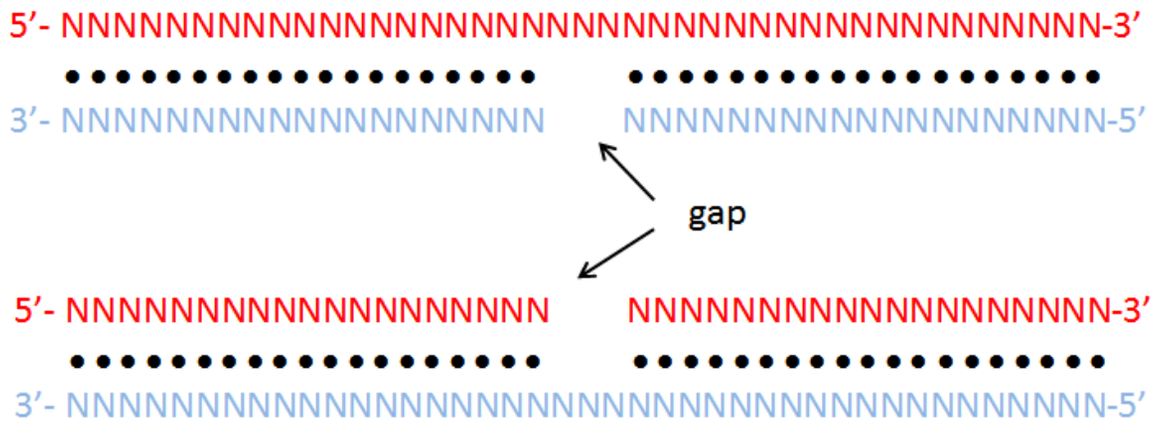

Figure 5. Design of long linear duplexes with gap in either sense or antisense strands. Red color: sense strand, blue color: antisense strand.

Long unmodified siRNAs up to 42 nt in length were used for silencing of gene expression in some specific cell lines without the induction of interferon response [96–101]. For example, direct fusion of two 17- and 19-bp long non-modified siRNAs resulted in efficient silencing of two target genes [96]. Two RNAs were merged "head-to-head" in a way that the 5'-ends of both antisense strands would look outside from the duplex allowing efficient and stereospecific AGO2 binding and efficient silencing of both targets [96]. Heterologous duplexes merged “head-to-tail” of antisense strands demonstrated reduced silencing activity [96]. Similar results were obtained for tandem siRNAs of 40–42 nt in length consisting of 21+21 and 21+23 units [101] as well as 40-nt long duplexes [100]. These results may be explained by the fact that the induction of interferon response depends on the cell type [23]. Indeed, some cell lines may possess reduced immune-sensitivity to the siRNA treatment and the results obtained on the cell cultures cannot be unacceptable for in vivo experiments.

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Another class of long siRNAs are various branched structures (Figure 6). Initially, branched oligonucleotides were applied to study mRNA splicing [102–104]. Then diverse branched structures were used as building blocks for self-assembling nanostructures [105–107]. Moreover, nanostructures of different shapes and sizes have been proposed as an effective delivery system for siRNA, ribozymes, etc. [106, 107]. Recently, it has been demonstrated that branched small RNA structures may also be effective RNAi inducers. Thus, these duplexes can simultaneously inhibit two or more genes and possess improved silencing activity and intracellular delivery properties [96–109]. Different strategies are developed to form branches. Symmetric doubler phosphoramidites are used to construct branches with two or four strands [108]. In another variant, trebler phosphoramidite structure with extended short DNA linker is used as a core for branched small RNA with three arms [110]. Direct annealing was used to design RNAs with three and four arms [111, 109]. However, base pairing close to the junction region may be disturbed and single-stranded nuclease-sensitive region may be formed.

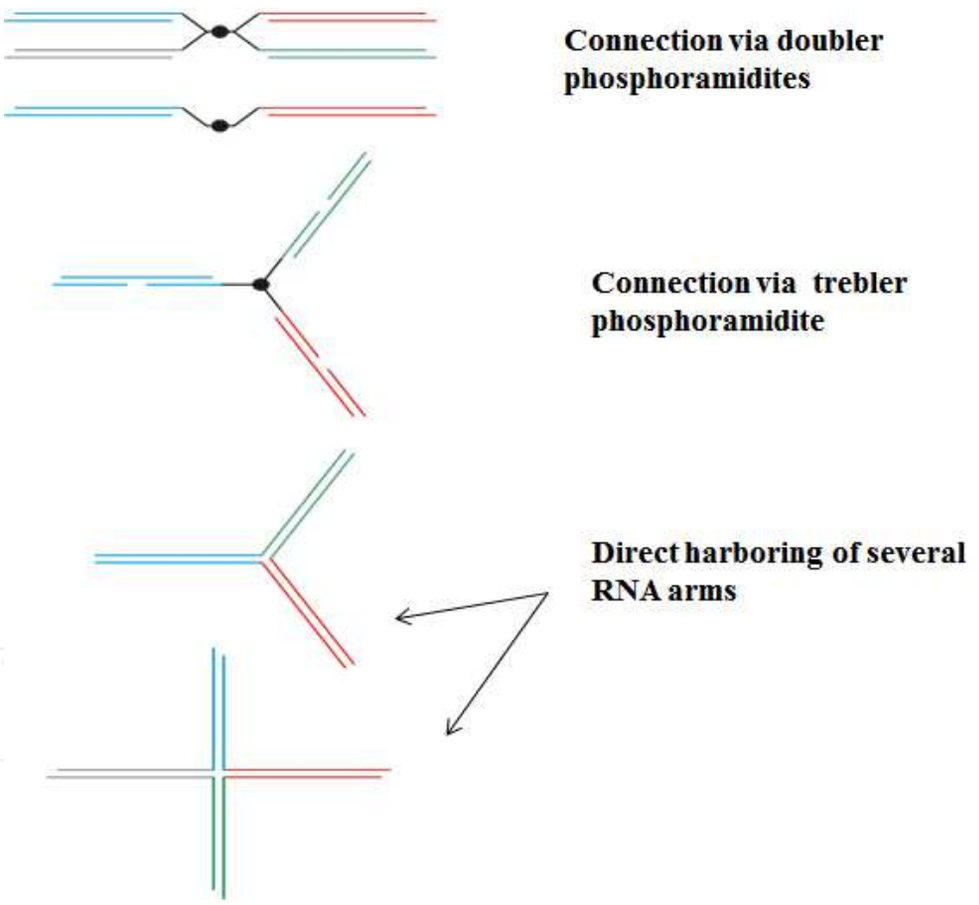

Figure 6. The architecture of various multi-target branched siRNAs. Different colors indicate siRNA units targeted to various genes.

Chang and his colleagues introduced tripartite RNA structure without any linker containing three 19-bp-duplex regions obtained by annealing of three 38-nt single-stranded RNAs [111]. The 5′ end of each antisense strand was directed outside, making seed regions of all antisense

strands accessible for AGO2 loading. Single-stranded regions near the strand junction were defended by 2′-O-methyl modifications affecting six nucleotides. It was demonstrated that tripartite small RNA more efficiently silences the expression of three target genes in comparison with a mixture of corresponding canonical siRNAs due to a more efficient intracellular delivery by Lipofectamine [111]. Specifically, tripartite small RNA was not processed by Dicer possibly due to the influence of 2′-O-methyl modifications introduced into the single-stranded region of tripartite RNA [111]. Similar structures without any modifications, designed by another group of scientists, were efficiently processed by Dicer into 20-nt products [109]. On the other hand, tripartite small RNA without any modifications was unstable in biological fluids and quickly degraded.

Tetramer RNA consisted of four arms 23 bp in length proved to be more stable and also acts in a Dicer-dependent mode. Both trimer and tetramer siRNAs provided prolonged RNAi effect and efficiently inhibited the expression of three or four genes simultaneously. The influence of the structures on the interferon response was not reported [109].

Overall, long linear and branched siRNAs could be efficiently used for simultaneous inhibition of multiple genes. Selective 2′-O-methyl modifications and specific elements of the structure (gaps, nonnucleotide insertions) could reduce undesired interferon response. The application of long RNAi inducers is restricted by the complexity of the design (in the case of branched molecules) and the higher cost of synthesis in comparison with canonical siRNAs; however, recent advances in the synthesis of oligoribonucleotide allows overcoming these problems. Long linear and branched siRNAs could be useful for the development of anticancer and antiviral therapeutics targeting multiple genes.

6. Conclusion

Small-interfering RNAs provide universal and effective method for the silencing of target genes because almost all genes could be targeted by siRNAs. A large number of diseases, associated with hyperexpression of certain genes or expression of their chimeric or mutated variants, could be treated by inhibition of gene expression; therefore, siRNA has a great potential as a new therapeutic drug. Different design strategies have been used to improve properties of siRNAs and reduce off-target effects. Structural modifications can expand the boundaries of siRNA applications.

At present, synthetic siRNAs structurally mimicking the Dicer substrates (dsiRNAs) are widely used as potent RNAi inducers. The use of dsiRNA may prevent the development of undesired toxicity associated with off-target effects of both the inducer and the transfection reagent or any type of carrier due to the lower effective concentrations and the increase in the longevity of silencing. Therefore, application of dsiRNAs is considered to be extremely promising in anticancer and antiviral therapeutics as well as for the treatment of chronic diseases where multiple administrations are necessary to reach the desired silencing effect. Chemical modification patterns compatible with Dicer processing were designed and successfully applied for prevention of undesired stimulation of immune system and for acquiring

nuclease resistance. Single-stranded structured synthetic siRNAs, such as Dicer-processed short hairpin RNA and dumbbell RNA, possess all benefits as Dicer substrates and exhibit additional flexibility in fine-tuning of the stability, kinetics, and silencing duration. Long RNAi inducers, acting in a Dicer-dependent or Dicer-independent mode, effectively silence target genes at low concentrations. Multi-target siRNAs have a great promise in the treatment of complex diseases such as cancer and immune-inflammatory disorders or viral infections [108]. Long linear or branched structures with selective chemical or structural modifications could successfully inhibit the expression of several genes without undesired off-target effects. Currently, however, the complexity and high cost of the synthesis restrict the biomedical application of long small RNAs. Some structural modifications in siRNAs have specific applications. Fork-siRNA are successfully being used for the silencing of genes with restricted selection of sequence content such as chimeric or point-mutated genes.

siRNAs with various structural modifications find a wide application in biomedical research and therapeutics. Some of them have already been used in clinical trials. The great success was achieved in the multi-target therapy that may increase treatment effectiveness. However, the therapeutics applications are limited by the inefficient delivery of these compounds into organs, tissues, and cells. Problem of low bioavailability of siRNA in vivo could be overcome by two ways: the better delivery and the higher activity. Future expansion of the repertoire of RNAi inducers contributes to resolving of both challenges. Although many approaches are developed, more efforts are still needed to improve safety and efficiency of siRNA in vivo.

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