Chapter 8

Structure - Functions Relations in Small Interfering RNAs

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http://dx.doi.org/10.5772/53945

1. Introduction

RNA interference is an evolutionary conserved mechanism of specific gene silencing induced by double stranded RNA homologous to the target mRNA. Small interfering RNAs (siRNAs) are widely used for the control of gene expression in molecular biology and experimental pharmacology. Currently, siRNAs are successfully used for the validation of potent drug targets for anti-cancer therapy. However, application of siRNAs as therapeutics is limited by their sensitivity to ribonucleases, poor cellular uptake and rapid size-mediated renal clearance. These challenges must be overcome to develop a successful siRNA-based drug. Many of these limitations could be resolved with the use of chemical modifications improving the siRNA properties.

This review examines recent data regarding principals of the design of siRNA for the silencing of therapeutically relevant genes. A particular focus will be made on chemical modifications and their impact on siRNA potency, nuclease resistance and duration of the silencing effect. The types of chemical modifications, their location in siRNA structure influence siRNA properties in different modes: modulation of the interaction with RNAi proteins, the thermal stability and thermoasymmetry of the duplex and the sensitivity to the degradation by ribonucleases.

Special attention will be paid to the design of siRNA for the silencing of thermodynamically unfavorable targets: mutant and chimerical genes. In this case the utilization of computer algorithms for the selection of active siRNA cannot be applied. Modification of siRNA structure aimed at the correction of the thermoasymmetry by incorporation of nucleotide substitutions, blocking incorporation of the sense strand in the RISC complex by truncation of one overhang or inactivation of the sense strand can be successfully used. Mismatches in the central part of the duplex can facilitate the cleavage and dissociation of the "passenger" (sense) strand, whereas selective chemical modification protects the non-perfect duplex from accelerated degradation.

Peptides, steroids and other hydrophobic lipid groups can be attached to siRNA, extending the siRNA circulation time and enhancing direct cellular uptake. The potential of bioconjugation of siRNA with different biogenic molecules in altering the bioavailability and distribution of siRNAs following *in vivo* delivery will be discussed. The combination of these approaches can lead to the development of siRNAs with therapeutic value.

2. Mechanism of RNAi

RNAi phenomenon was found during transfection of dsRNAs in *C.elegans* [1] and is inherent in different organisms (flies, vertebrates, higher plants [2, 3, 4]). RNAi mechanism was initially examined in details in *Drosophila melanogaster*, but later it was found that the mechanism is highly conservative between the organisms. There are two stages of RNAi (fig. 1): at the first stage (phase of initiation) specific ribonuclease Dicer binds to and cleaves long dsRNAs yielding short (21-23 nt) duplexes with 2-overhanged nucleotides at the 3′-ends (or siRNAs); at the second stage (effector phase) siRNAs molecules incorporate into multiprotein complex (RISC – RNA-induced silencing complex). One of siRNA strands ("passanger") undergo cleavage and dissociation from the complex upon RISC activation, the other strand ("guide") remains in the complex. Activated complex RISC* specifically binds to RNA target and cleaves it (fig. 1) [5, 6, 7, 8, 9, 10] . Long dsRNAs (> 30 bp) activate the innate immune response in the mammalian cells (except for non-differentiated or low differentiated cells) resulting in non-specific RNA degradation by RNase L, activation of

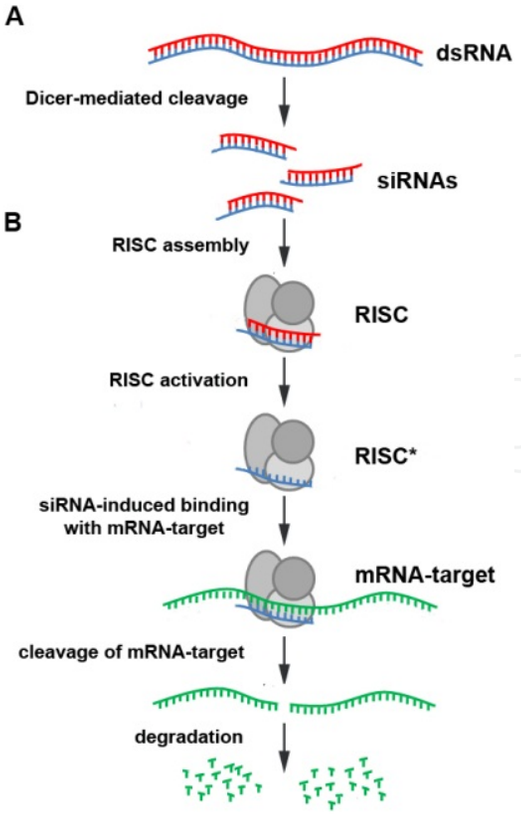


Figure 1. Scheme of RNAi. A. Initiation phase B. Effector phase.

protein kinase PKR that inhibits translation. The expression of numerous genes, including genes encoding interferons and cytokines, alters [11, 12]. Thus, sequence-specific decrease of target mRNA level is not observed. It was found that RNAi in mammalian cells can be induced by chemically or enzymatically (*in vitro*) synthesized siRNAs or endogenously expressed siRNAs 19-21 bp in length. These siRNAs mimic the products of long dsRNAs processing by Dicer and can be involved directly into the effector phase of RNAi mechanism passing the phase of initiation (fig. 1) [13, 14, 15, 16, 17].

The selection of the "guide" strand is a key step determining the efficacy of RNAi induced by synthetic siRNAs. At the first step of RISC* assembly the intermediate complex RLC (RISC Loading Complex) consisting of Dicer, siRNA and R2D2 (*D.melanogaster*) or TRBP [18, 19, 20] (Homo sapiens) forms (fig. 2 A). R2D2 protein along with its human analog contains two dsRNA-binding domains and a Dicer binding domain.

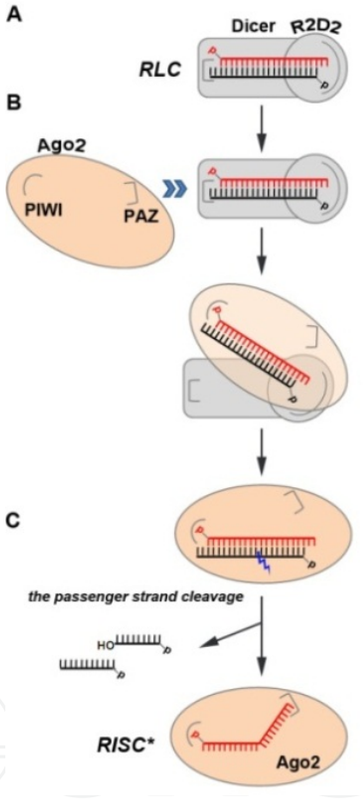


Figure 2. RISC* assembly. **A.** RLC-complex, consisting of Dicer, dsRNA-binding protein, R2D2 and siRNA. **B.** Interaction of Ago2 with RLC. **C.** RISC* formation.

Dicer contains dsRNA-binding domain and PAZ-domain (PIWI/Argonaute/Zwille), that has an affinity to 3'-overhangs of siRNA [21, 22]. It was detected that R2D2 preferably binds with more thermodynamic stable flank of the siRNA duplex, whereas Dicer interacts with less stable siRNA flank [20, 23, 24, 25]. The orientation of siRNA relative to the protein complex Dicer-R2D2 determines the positioning of siRNA in the complex with Ago2 (a catalytic part of RISC*) (fig. 2 B). Ago2 is a protein of Argonaute family, containing PAZ and PIWI domains. The structure of PIWI domain similar to that of RNase H determines endoribonuclease activity

of PIWI [26, 27]. Ago2 cleaves the one of the siRNA strand (“passenger” strand), another strand (“guide” strand) remains into the RISC* and guides the target RNA recognition and cleavage (fig. 2 C). The structure of the complex containing siRNA and Ago2 effects the selection of the strand (sense or antisense) that incorporates into RISC*, hence, determines the efficacy of mRNA-target cleavage. Evidently, the antisense strand that is homologous to the sequence of mRNA-target has to be “guide” strand in order to cleave mRNA. Ago2 replaces the Dicer-R2D2 dimer (fig. 2 B), interacting with Dicer via PAZ domain [28] whereas the phosphate group at the 5′-end of the strand from the Dicer side (“guide” strand) interacts with PIWI domain that contains Mg2+ ion and basic amino acids.

The nucleotides located at 2 - 6 positions of the “guide” strand stabilizes the interaction between siRNA and Ago2 [29, 30, 31]. The presence of the phosphate at the 5′-end of the “guide” strand is essential for RISC* assembly [32, 33]. This orientation of siRNA with respect to catalytic PIWI-domain provides the cleavage of the complementary strand (“passenger” strand) between 9th and 10th nucleotides that facilitates the strand dissociation (fig. 2 C) [34, 35]. Ago2 and the “guide” strand are the main components of RISC* [36], however, other proteins could also interact with RISC* [10]. Thus, the difference between thermodynamic stability of the duplex termini (thermodynamic asymmetry) determines the orientation of Dicer-R2D2 dimer and as a result, the structure of Ago2-siRNA complex. Therefore, thermodynamic properties of siRNA play a key role in the strand selection [20, 23, 24, 25] providing the preferential selection of the strand with low thermodynamic stability at the 5′-end of the duplex as the “guide” strand [37, 38].

It was shown, that the perfect match between 2 - 12 nt of the “guide” strand (corresponding to one turn of dsRNA helix) and the target mRNA is essential for effective recognition and binding of RISC* with mRNA target [39]. The mechanism of RNA-target cleavage (occurs between 10^{th} and 11^{th} nt relative to the 5′-end of the “guide” strand [40]) and the following dissociation are similar to that of the “passenger” strand (described above). Cleaved target RNA and the “passenger” strand are degraded by ribonucleases after dissociation, whereas RISC* becomes available for acting in catalytic mode [7, 9, 17, 41]. Unlike antisense oligodeoxyribonucleotides (ODN), whose efficacy of action is determined by the efficacy of ODN hybridization with mRNA-target (fig. 3) [42], the formation of the duplex between the "guide" strand of siRNA and mRNA- target occurs due to the helicase activity of RISC* and virtually independent from the hybridization properties of the oligoribonucleotide [43]. Silencing of gene expression by siRNAs is observed at the much lower ON concentrations than in the case of antisense ODN (IC50 for siRNAs is 100 – 1000 times lower, than IC50 for antisense ODNs) [43, 44].

Another mechanism of RISC activation - “bypass” route - employs the dissociation of the siRNA strands without preliminary cleavage of the sense strand by Ago2 [35]. This type of RISC activation is inherent in miRNAs, containing unpaired bases in the central part, critical for Ago2 endoribonuclease activity (fig. 4). Moreover, the “bypass” rout was observed for siRNAs with chemically modified nucleotides surrounding the cleavage site (between 9th and 10th nt of the “passenger” strand [45]) impeding Ago2 action [34, 35, 46]. Silencing of gene expression proceeds via the arrest of mRNA translation, since RISC* binds to mRNA-

target together with translation suppressor proteins (for example, helicase RCK/p54 from DEAD box family) [47]. Complex of mRNA and proteins is deposited in "P-bodies" (processing bodies - distinct foci in the cytoplasm involved in mRNA turnover) [48]. In "P-bodies" mRNA decapping followed by ribonuclease cleavage occurs. In the other cases, mRNA complexed with proteins can be deposited in "P-bodies" for a long time without degradation (fig. 4). Later, mRNAs can escape from "P-bodies" and be involved in translation machinery or can be degraded in a described above manner [10].

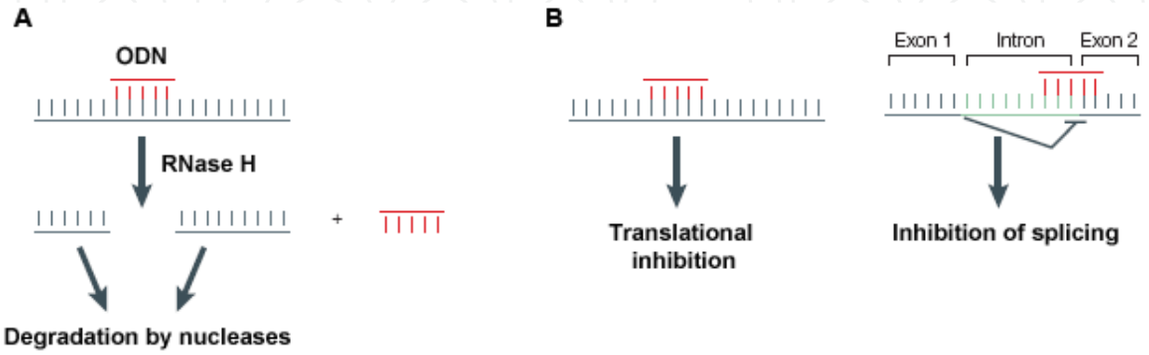


Figure 3. Silencing of gene expression by antisense ODNs: **A.** The degradation of the duplexes: ODN / mRNA or ODN / pre-mRNA by RNase H. **B.** Inhibition of mRNA translation or pre-mRNA splicing by ODNs those are not RNase H substrates [43].

The "bypass" mechanism of RISC activation is less efficient than the "classical" one, since in this case RISC* does not act in a catalytic mode. The efficiency of RISC* assembly also is limited by the low rate of ATP-dependent dissociation of intact siRNA strands [35, 33, 39].

Thermodynamic stability siRNA duplex is an additional parameter determining the efficiency of RISC* assembly, hence, the efficiency of RNAi. Earlier developed algorithms for the selection of active siRNA sequences suggest selection of mRNA targets with approximately 50% GC-content. In the later studies, the percentage of G+C nucleotides in the structure of effective siRNAs varied from 30 to 50% [49, 50]. It was shown that low thermodynamic stability of the central part of the duplex (from 9th to 14th nt counting from 5′-end of the antisense strand) arising from AU-rich sequences or the presence of mismatches is a hallmark of active siRNAs [37]. The average difference between Gibbs energy of central parts of active and inactive siRNAs was found to be about 1.6 kcal/mol. Unlike thermodynamic asymmetry, unstable center of duplex is not a sufficient criteria for selection of active siRNA [37].

Structural features of mRNA-target also effects the RNAi efficacy [51, 52, 53, 54]. The availability of the mRNA sequence for binding with siRNA (the absence of the hairpins in the secondary structure or overlapping with the binding regions of regulatory factors) as well as in the case of the ribozymes and the antisense ODN [55] can influence the silencing efficacy. In order to prove this, siRNAs targeted to mRNA sequences with different binding availability were synthesized [53]. It was shown that siRNAs targeted to the region of the initiation of translation or the 3′-end of mRNA-target were inactive. Whereas siRNAs targeted to the regions forming the hairpins displayed low and average silencing activity. As expected high-performance siRNAs were those targeted to unstructured regions of mRNA [51, 52, 54].

Thus, the analysis of the secondary structure of mRNA-target and thermodynamic properties of siRNA duplex play important role in the selection of active inhibitors of gene expression.

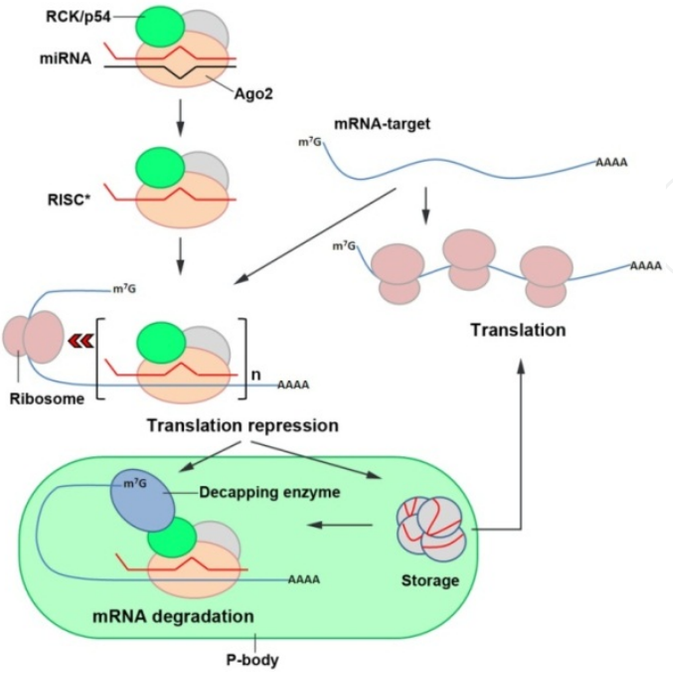


Figure 4. Scheme of RNAi with the "bypass" rout of RISC* assembly (miRNA-like action). RISC* binds with mRNA-target (n is equal 1, if the antisense strand is fully complementary to mRNA) [10].

3. Chemical modifications: The influence on RNAi efficacy

Currently, the development of RNAi-based drugs is an important aim in pharmacology. The optimization of siRNAs structure is required for biomedical applications; chemical modifications could be successfully used for this purpose. Different types of chemical modifications were widely used in antisense ODNs before the discovery of RNAi [42]. The data collected in these experiments can be applied for optimization of the properties of siRNAs. There are two types of factors affecting both antisense ODNs and siRNAs efficacy: systemic factors that act on the level of the organism and intracellular factors defining the activity of the therapeutics in the cells. The first type of factors includes: i) fast (approx. 5 min) siRNA elimination from organism resulted from its hydrophobicity and size of the molecule (approx. 14 kDa) [56, 57], ii) high nuclease sensitivity of siRNA [58, 59, 60, 61, 62] and iii) inefficient or/and non-specific delivery into target cells [63]. Intracellular factors are related to RISC assembly and the efficiency of binding to and cleaving the target.

3.1. Chemical modifications of the nucleotides

3.1.1. Modifications of ribose (furanose) ring

All ribose modifications can be divided in two main groups: the modifications related to the replacement of hydrogen atoms in furanose by different groups and structural modifications of the furanose cycle.

3.1.1.1. Substituents in furanose

The 2′-OH group of the ribose is the main target for modification, since this group is involved in the phosphodiester bonds cleavage by endoribonucleases via trans-etherification mechanism [64]. Thus, the modification of 2′-OH defends siRNA against ribonucleases [62]. The size of the substituent is an important characteristic determining its tolerance by RNAi machinery. The small groups (2′-O-methyl (2′-O-Me), 2′-fluoro (2′-F) etc.) (fig. 5 A) virtually do not disturb the conformation of siRNA duplex and are better tolerated than bulky groups: 2′-O-methoxyethyl (2′-O-MOE), 2′-O-allyl (2′-O-allyl) etc. The replacement of 2′-OH by electrophilic groups also stabilizes C3′-endo conformation of the ribose (fig. 6) which corresponds to A-helix geometry of the duplex obligatory for effective RNAi [58]. This sugar conformation arrange for the axial location of the substituents reducing total energy of the system and increasing the affinity to the complementary RNA target. As a result the duplex melting temperature (ΔTm) increases approximately 1 ^{0}C per modification [65].

2′-O-methyl modification is one of the widely used 2′- modifications for the enhancement of nuclease resistance of siRNA [59, 62] and impeding the induction of the interferon response in eukaryotic cells [64, 66, 67, 68, 69, 70]. Obviously, the number and location of the modified nucleotides in the duplex are crucial for the silencing. The increase of the number of modifications was shown to decrease the silencing activity of siRNAs; totally modified siRNAs frequently had no activity [58, 59, 71]. However, in a number of experiments the activity of siRNA with the totally modified sense strand was compatible with that of unmodified analog [72, 73]. This can be related to functional unequivalence of siRNA strands [74, 75]. The introduction of 2 - 4 2′-O-Me modifications in the both strands of siRNA is well tolerated. Moreover, in the case of selective modification of the nuclease-sensitive sites within siRNA the increase of the duplex nuclease resistance and prolonged silencing were detected [59].

The C3′-*endo* conformation could be stabilized by introduction of 2′-F-modified nucleotides in siRNA (fig. 5 A). Since fluorine atom is more electronegative than oxygen atom, the increase of the binding affinity between siRNA strands and target RNA (ΔTm 2 – 4 ^{0}C per modification) is observed [65]. Remarkably, the silencing activity of 2′-F-modified siRNAs virtually does not depend on the number of modifications in contrast to 2′-O-Me-modified siRNAs. The silencing activity of siRNAs with 2′-F-modifications in the sense and the antisense strands was compatible with that of unmodified analogs [76]. The replacement of pyrimidine nucleotides in the duplex with 2′-F-analogs was well tolerated by RNAi machinery both *in vitro* [46, 77, 73] and *in vivo* [78]. The silencing activity of siRNA containing 2′-F modifications located in the site of Ago2 cleavage and unmodified analog was similar [79], as well as long-term silencing effects *in vivo* of modified siRNAs and unmodified analogs [78]. It should be noted that epimers of 2′-F-nucleotides (2′-fluoro-β-D-arabinonucleotides, FANA (fig. 5 A)) with DNA-like C2′-*endo*-conformation of furanose cycle [80] (fig. 7) introduced in siRNA structure also improve the nuclease resistance of the duplex. It was found that partial modification of both strands with FANA or total modification with FANA of the sense strand of siRNAs ensure the A-geometry helix [81] and effective RNAi. These modifications were shown to alter the duplex thermodynamic stability insignificantly [76].

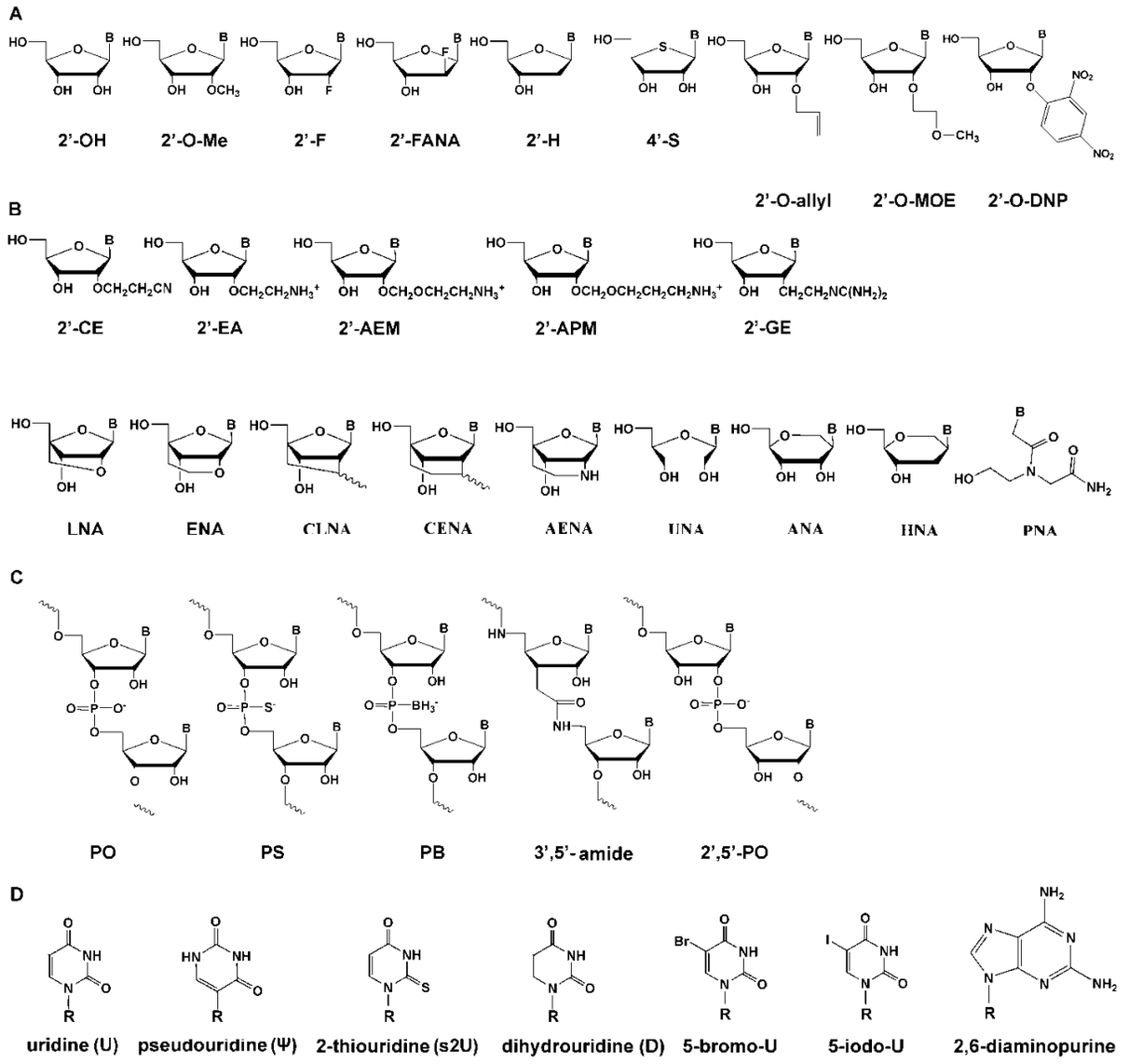


Figure 5. Analogs of nucleotides and nucleosides used for siRNA modification. **A.** Substituents in furanose. **B.** Structural modifications of furanose cycle. **C.** Backbone modifications. "B" – base (**A** – **C**). **D.** Nucleobase modifications. "R" – ribose residue.

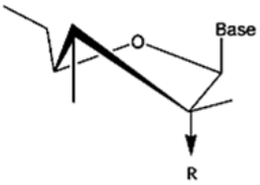


Figure 6. C3′-*endo* conformation of the ribose with the 2′-substituent. R – electron-acceptor group.

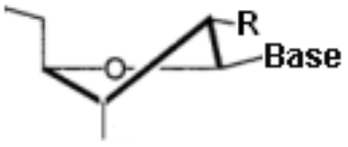


Figure 7. C2′-*endo* (DNA-like) conformation of ribose with the substituent (R) in 2′-position.

On the contrary, the replacement of ribonucleotides of siRNAs with deoxyribonucleotides characterized by C2′-*endo*-conformation of the furanose cycle (fig. 5 A) reduces their affinity to mRNA-target (ΔTm -0.5 ^{0}C per modification) (fig. 7) [65]. Mainly, deoxyribonucleotides are used for the defense of siRNA overhangs from exonucleases [13, 59]. However, the modification of the other duplex regions does not impede effective RNAi [40, 82]. The introduction of deoxyribonucleotides in the duplex region responsible for the recognition of the target mRNA, so called "seed" region (about 8 nt. from the 5′-end of the antisense strand [29]) results in the increase of siRNA specificity since lesser stability of DNA/RNA hybrids as compared with RNA duplex [82]. siRNAs with the totally modified antisense or sense strands had no activity regardless of preferable for RNAi A-helix geometry of the duplexes [32].

Introduction of the bulky groups in the 2′-position of the furanose causes the conformational disturbance of the siRNA duplexes resulting in the decrease of siRNA silencing activity [76]. However, the modification of duplex termini and overhangs with 2′-O-methoxyethyl (2′-O-MOE) or 2′-O-allyl groups (fig. 5 A) was tolerant [46, 83, 84]. The random replacement of 2′-OH in the both strands of the siRNA (up to 70 %) by 2,4-dinitrophenyl ethers (2′-O-DNP) (fig. 5 A) causes the increase of the silencing activity. Since the thermodynamic stability of the modified duplex was comparable with that of unmodified duplex [85] (that indicates the similar hybridization properties [86]), the observed effect may be related to the increase of the nuclease resistance and cellular uptake of the modified siRNA [85, 86, 87]. In order to increase the nuclease resistance of the siRNA duplexes, other chemical modifications - 2′-aminoethoxymethyl (2′-AEM), 2′-aminopropoxymethyl (2′-APM), 2′-aminoethyl (2′-EA), 2′-cyanoethyl (2′-CE), 2′-guanidinoethyl (2′-GE) etc.- may be used (fig. 5 A). It was shown that totally modified siRNAs were inactive, whereas the silencing activity of the partially modified siRNAs depends on the location of the modifications in the duplex structure [88].

Introduction of the modifications at the 4′-position of the furanose results in the slight increase of the RNA-duplex termostability (ΔTm 1 ^{0}C per modification) [89, 90]. Usually the 4′-thio (4′-S) ribonucleosides (fig. 5 A) are used for modification of siRNA, especially, at the termini [91, 92, 93]. The replacement of the several ribonucleotides at the 5′-end of the antisense strand [91] or four ribonucleotides at the sense strand termini and at the 3′-end of the antisense strand [93] by 4′-S- analogs only slightly decrease the silencing activity of siRNAs, whereas the modification of the central part of the duplex significant inhibits RNAi [91, 92]. The introduction of the 4′-S-modifications in siRNA structure increases nuclease resistance of the duplex and improves the pharmacokinetics of siRNA. Unfortunately, this modification simultaneously increases the binding of siRNAs with blood serum components and increases their cytotoxicity [65].

3.1.1.2. Structural modifications of the furanose ring

This type of modifications includes bicyclic derivatives of the nucleotides (LNA, ENA, CLNA, CENA, AENA et al.), acyclic nucleotides (UNA, PNA) or nucleotides containing pyranose ring (ANA, HNA) instead of ribose (fig. 5 B). LNA and ENA (2'-O,4'-C-methylene-

and 2'-O,4'-C-ethylene bicyclic nucleotide analogs are the frequently used bicyclic derivatives which provide the significant increase of the duplex thermostability (ΔTm up to 10 ^{0}C per modification) [94, 95]. The ribose in LNA and ENA is fixed in C3′-*endo*-conformation (fig. 6) due to the methylene or ethylene "bridge" between 2′- and 4′-positions, respectively.

Experimental data on the activity of LNA- and ENA-modified siRNAs are contradictory. In some cases modifications of the 5′- and/or 3′-ends of the both siRNA strands were well tolerant [65, 96, 97, 98], whereas in the other studies ENA-analogs at the 3′-end of the sense strand of siRNAs reduced or abolished the silencing activity [74]. The difference in activity may be connected with the difference in the thermodynamic asymmetry of the modified siRNA [37, 38]. The replacement of the several ribonucleotides with LNA in the antisense strand was shown to be well tolerant [71, 96], whereas extensive modification was tolerant only in the case of sisiRNA with segmented sense strand [99] (see section 1.3.5).

The thermodynamic stability and nuclease resistance of the siRNA could be increased by introduction of HNA and ANA nucleotides with pyranose ring (hexitol nucleic acid and altritol nucleic acid, respectively (fig. 5 B)) instead of ribose [100, 101]. The modified duplex contaning these analogs adopts the A-helix geometry [100], however, the location of the ANA- or HNA-analogs in siRNA structure is critical for silencing activity. Remarkably, the introduction of the ANA- or HNA-analogs at the 3′-termini of one or both strands maintain or increase the silencing activity [102, 103]. The presence of ANA-analogs at the 5′-end of the antisense strand substantially decreased the siRNA activity. It was suggested that modifications at the 5′-end of the antisense strand impede the action of cellular kinases due to steric obstacles [103]. The decrease of the phosphorylation efficacy of the 5′-terminal nucleotide of the antisense strand is critical for efficient interaction of siRNA with Ago2 PIWI domain followed by mRNA-target cleavage [32, 33]. siRNAs with HNA or ANA-analogs of nucleotides in the central part of the sense strand display the effective gene silencing, whereas, similar modifications of the antisense strand substantialy decreased the siRNA activity [88, 103].

In contrast to siRNA duplex stabilizing modifications, the presence of acyclic nucleotide analogs (UNA) (fig. 5 B) in the siRNA increase the conformational flexibility of the duplex and decrease its thermodynamic stability (ΔTm -5 to -8 °C per modification). The location of the UNA-analogs in siRNA was shown to play a key role, since one modification can result in the significant reduction or augmentation of the silencing activity of siRNA [99, 104]. It was suggested that the replacement of ribonucleotides at the 3′-end of the sense strand increases the duplex thermodynamic asymmetry and acquires antisense strand incorporation into RISC* [104, 105]. It was found experimentally that the introduction of 1 - 3 UNA nucleotides at the 3′-end of the sense strand, including the 3′-overhangs, increase to some extent the siRNA activity [106, 107]. Quite the opposite, the modification of the first and the second positions of the "guide" strand abolishes the 5′-end phosphorylation and reduce siRNA silencing activity.

Peptide nucleic acids analogs (PNA) containing N-(2-aminoethyl)-glycin polyamide backbone could be used for modification of siRNA. It was shown that in the antiparallel

PNA/RNA duplex RNA adopts the right-handed helix characterized by anticonformation at the N-glycosidic bond and C3′-*endo*-conformation of the ribose [108], with the similar to the RNA-like A-helix geometry [58]. PNAs are stable in the human blood serum as well as in the cellular extract; they are resistant to protein kinase A and several types of peptidases [109]. It was found that the introduction of PNA-analogs in the sense and / or in the antisense strands of the duplex increase nuclease resistance of siRNA. The increase of the siRNA silencing activity was observed when modifications were introduced in the sense strand. In the other cases the activity of PNA-containing siRNA was comparable with activity of the unmodified siRNA [110]. Due to the functional inequivalence of siRNA strands the modifications in the sense strand a better tolerated than modifications in the antisense strand.

Thus, virtually all ribose modifications provide the increase of the nuclease resistance of siRNA. However, the optimization of their number and location in the duplex structure (depending on the type of modification) is required to make them tolerable in the process of RNAi.

3.1.2. Backbone modifications

This type of modifications includes the replacement of phosphate group (PO) with phosphorothioate (PS) or boranophosphonate (PB) groups, the replacement of the 3′,5′-phosphodiester bond with 2',5'-bond or the amide bond instead of the ester bond (fig. 5 C). All of these modifications increase the nuclease resistance of siRNA, however their impact on the efficacy of RNAi is varied [76]. PS-modification is known to reduce the melting temperature of the duplex and oligoribonucleotide binding affinity [71]. However, the experimental data related to the activity of PS-modified siRNAs are discrepant. The comparable silencing activity of the PS-modified siRNAs and their unmodified analogs was shown in the studies [83,111], whereas in the other studies the silencing activity of the PS-modified siRNAs was lower, than that of their unmodified analogs [58, 73, 112]. The modification of the central part of the duplex decreases siRNA activity [113]. Remarkably, siRNAs with blunt 3′-ends containing totally PS-modified one or both strands were active [73]. However, high binding affinity of the phosphorothioate analogs to serum albumin, IgG, IgM, lactoferrin and the cellular membrane receptors is disadvantageous [114]. It results in the development of toxic effects both *in vitro* and *in vivo*, even siRNAs containing alternate PS-analogs were toxic in cell cultures [83, 111]. Introduction of boranophosphonate analogs (fig. 5 C) increases nuclease resistance of the duplex as compared with PS-containing and unmodified siRNA [65] and increases RNAi efficacy. It was found that an optimal position for PB-modifications is the terminal region of the sense strand of siRNA, whereas the modification of the central part of the duplex resulted in the significant decrease of silencing activity [65]. This effect is likely connected with the inhibition of the sense strand cleavage by Ago2 during RISC* formation. The replacement of the 3′,5′- by 2′,5′-phosphodiester bond or 3′,5′-amide bond (fig. 5 C) increases the nuclease resistance of the duplex and is tolerant when located in the sense strand [115] or the 3′-overhangs of siRNA [116].

Thus, the most promising modification of the siRNA backbone is the boranophosphonate modification, since it provides the increase of the nuclease resistance of the duplex and its silencing activity and is not accompanied by the development of toxic effects. However, the price of PB-analogs limits their usage.

3.1.3. The chemical modifications of the nucleobases

In contrast to the other types of modifications, the modifications of heterocyclic bases (nucleobases) have no influence on the nuclease resistance of RNAi. Earlier it was found that the presence of modified nucleobases in the antisense ODN altered their hybridization properties [42]. It is known that the replacement of the uridine by 5-bromo- or 5-iodouridine (fig. 5 D) improves their ability to interact with adenine owing to the increase of the acidity of the imine and results in the stabilization of the base pair [117]. The replacement of the adenine by 2,6-diaminopurine results in the formation of the additional H-bond, hence, in the stabilization of the nucleotide base pair [58]. At the start, it was suggested that the introduction of these analogs in the antisense strand of siRNA will enhances its binding affinity to mRNA and increases the RNAi efficacy. Unexpectedly, the decrease of silencing activity of modified siRNA in comparison with unmodified ones was observed. This effect is possibly related with the decrease of the dissociation rate of siRNA duplex on the step of RISC* formation [58]. The replacement of uridine by 2-thiouridine (s^2U) or pseudouridine (ψ) (fig. 5 D) provides the increase of the duplex thermodynamic stability due to the stabilization of the ribose in the C3′-*endo*-conformation (fig. 6) [118, 119]. Dihydrouridine (D) contains non-aromatic ring that does not participate in staking interactions (fig. 7) which resulted in the destabilization of siRNA [120], but one substitution with D at the 3′-end of the sense or the antisense strand does not change siRNA silencing activity. The replacement of U by Ψ and s^2U at the 3′-end of the sense strand reduces the silencing activity, whereas introduction of the same modification at the 3′-end of the antisense strand creates favorable thermodynamic asymmetry and provides siRNAs with higher activities than the parent siRNA. Thus, these modifications could be applied for the design of thermoasymmetric siRNAs [120] (see section 3.3).

3.2. Chemical modification of siRNA termini

This type of modifications includes the modification of the nucleotides at the 3′-overhangs and the 5′-ends of the duplex. These modifications could be used for the solution of different problems: i) to increase the nuclease resistance of siRNA towards exoribonucleases (inverted 3′-3′ or 5′-5′ deoxyriboses [61, 121] (fig. 8), dideoxycytosine [122], ENA-analog of the thymidine [74] etc.), ii) to facilitate asymmetric RISC* assembly (the replacement of the 5′-OH by 5′-OMe group in the terminal nucleoside [123]) and ii) to provide the efficient accumulation of siRNA in cells (attachment of lipophilic molecules [57, 124, 125], folic acid [126], peptides [14, 127, 128], aptamers [129]). The fluorescent residues [111] and biotin [130] are widely used as a termini modification for siRNA detection.

The type of the applicable terminal modification has to be selected experimentally. In many cases the modification of the 5′- and 3′-ends of the sense (“passenger”) strand is well

tolerated [59, 131, 111, 130], however the data on modification of the antisense ("guide") strand are discrepant. It is known that the phosphorylation of 5′-OH groups of the antisense strands of synthetic siRNAs by the kinases [33, 122] is essential for interaction with PIWI-domain of Ago2 and for correct selection of the "guide" strand [29, 30, 31]. Therefore the replacement of the 5′-OH group by the 5′-OMe in the sense strand blocks its phosphorylation and the incorporation into RISC* as a "guide" strand [123]. On the other hand, the same modification of the antisense strand resulted in the decrease of siRNA silencing activity [59, 131, 122]. However, the attachment of the fluorescein residue to the 5′-phosphate of the antisense strand via hexamethylene linker is well tolerated [111], suggesting that this modification does not prevent the interactions of the 5′-terminal phosphate of siRNA with PIWI-domain. The 3′-end of the antisense strand recognized by PAZ-domains of Dicer and Ago2 [21] is less sensitive to modification [14, 33, 59]. It was shown that the attachment of puromycin or biotin to the 3′-end of the antisense strand [130] or the replacement of the 3′-terminal ribonucleotide by ddC, or the attachment of the aminopropyl linker via phosphodiester bond [122] virtually does not change the silencing activity. However, the introduction of the 2-hydroxyethylphosphate, ENA-analog of thymidine [74] or fluorescent dyes [111] at the 3′-end of the antisense strand abolished the silencing. The replacement of the terminal nucleotides of siRNA in both strands by 5′-5′- or 3′-3′-dioxyribose (fig. 8) results in the increase of the nuclease resistance and does not reduce the silencing activity [59, 61].

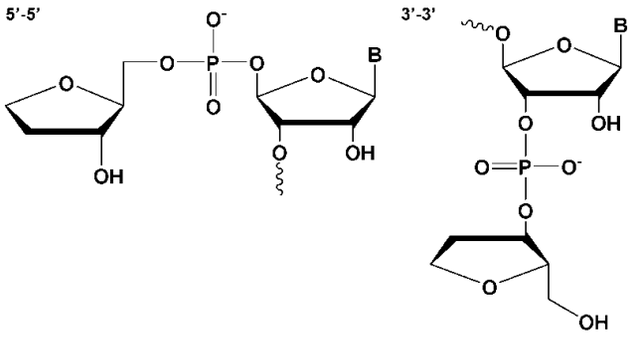
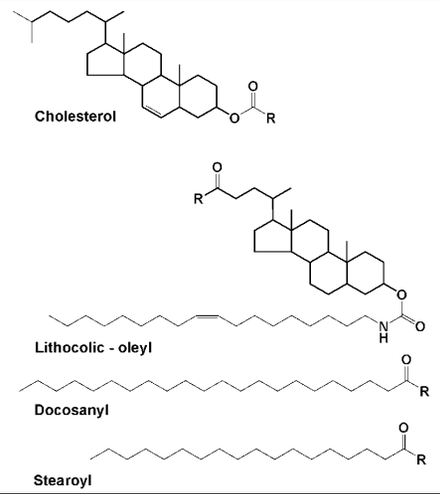
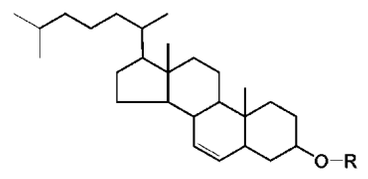
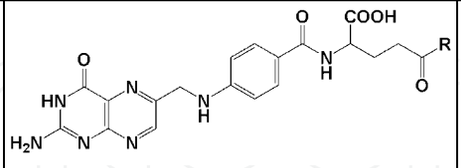
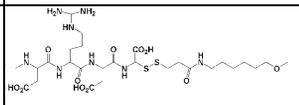


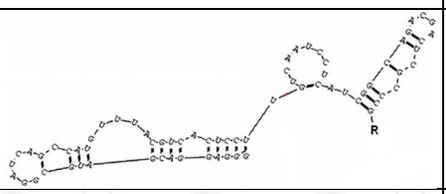
Figure 8. Inverted deoxyribose applied for the protection of 5′- or 3′-termini of siRNA.

3.2.1. Bioconjugates

Anionic siRNA cannot effectively pass through the electrostatic and hydrophobic barriers of the cellular membrane to enter the cytoplasm and to induce RNAi. Conjugation of siRNAs with the lipophilic molecules (cholesterol, derivatives of the oleic, lithocholic and lauric acids), peptides, antibodies, aptamers and other compounds is an effective tool to overcome this problem [132, 133]. The cholesterol was suggested as the first candidate for conjugation, since the natural mechanisms of cholesterol transport exist in mammalian organisms. Apolipoprotein B (ApoB) expressed in the liver and intestine cells is involved in the assembly and secretion of the lipid-protein particles known as very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) and in the transport and metabolism of the cholesterol. Since ApoB located on the cellular surface is specific to LDL-receptors, responsible for the delivery of the lipoproteins into cell [132, 134], it was suggested that

LDL-receptors could deliver the cholesterol-conjugated siRNAs inside the cells via receptor-mediated endocytosis. It was proved experimentally that LDL-receptors bind with the LDL-particles preliminary associated with cholesterol-conjugated siRNA or the conjugate of siRNA and the oleic or lithocholic acids, however the mechanism of their penetration into cell is not well-defined [125] (Table 1). Moreover, the involvement of the transmembrane

siRNA conjugate [1)]	**Modification** [2)]	**Linker, site of attachment to siRNA**	**Cell or target organ**	**Target gene**	**Silencing efficacy, %**	**Ref.**
Lipo-siRNA		trans-4-hydroxy proline linker at the 3′-end of the sense strand	mouse liver	*Apo-B-1*	55-25	125
Chol-siRNA		aminocaproic acid-pyrrolidine linker at the 3′-end of the sense strand	mouse liver	*Apo-B-1*	50	57
		-(CH_2)-S-S-$(CH_2)_6$- at the 5′-end of the sense strand	mouse lungs	*P38 MAP kinase*	40	127
		6-aminohexyl at the 5′-end of the sense strand	β-Gal/Huh-7 cells	*β-Gal*	55	124
Folate-siRNA		 at the 5′-end of the sense strand	HeLa cells	n.d. [3)]	n.d.	126
TAT-siRNA	YGRKKRRQRRR	N-terminal Cys-HN-$(CH_2)_3$- at the 3′-end of the antisense strand	HeLa cells	*GFP CDK9*	60 – 70	14
		C-terminal Cys –S-$(CH_2)_6$- at the 5′-end of the sense strand	Lungs of mouse	*P38 MAP kinase*	40	127
Penetratin-siRNA	CRQIKIWFQNRRMKWKK	N-terminal Cys-S-$(CH_2)_6$- at the 5′-end of the sense strand	COS-7 cells	*F.luciferase*	60	128
		C-terminal Cys –S-$(CH_2)_x$- at the 5′-end of the sense strand	Lungs of mouse	*P38 MAP kinase*	20	127

Transportan-siRNA	CLIKKALAALAKLNIKLLYGASNLTWG	N-terminal Cys-S-$(CH_2)_6$- at the 5′-end of the sense strand	COS-7 cells	*F.luciferase*	50	128
Aptamer-siRNA		at the 5′-end of the sense strand [4]	LNCaP and PC-3 cells	*Bcl-2 PLK1*	80	129
Antibody-siRNA	Antibodies to insulin receptor	biotin-tetraethylene glycol at the 3′-end of the sense strand	HEK 293 cells	*F.luciferase*	> 90	141
	Antibodies to transferrin receptor	biotin-tetraethylene glycol at the 3′- or 5′-end of the sense strand	Brain of mouse		69 – 81	142

[1] Lipo – lipophilic residues, Chol – cholesterol residue.
[2] siRNA with linker are denoted «R».
[3] In this investigation the biological activity of the conjugate was not measured (n.d.).
[4] The conjugate of siRNA and aptamer synthesized by *in vivo* transcription (without linker).

Table 1. Bioconjugates of siRNAs

protein SID-1 in the transport of the lipophilic conjugates was also shown [135]. It was suggested that SID-1 facilitates the dsRNA penetration into cells forming the channels for diffusion or mediating the interactions with other proteins [125, 135]. The successful use of the cholesterol and the derivatives of lithocholic, oleic and lauric acids for siRNA modification was demonstrated *in vitro* [124] and *in vivo* [57] (Table 1). The conjugate of siRNA and cholesterol, attached to the 5′-end of the sense strand, is able to penetrate into the human liver cells in the absence of the transfection agents. siRNA bearing cholesterol in the sense strand inhibits *ß-Gal* gene expression more effectively than siRNAs with the cholesterol in the antisense strand or in the both strands. In the presence of the transfection agent the activity of the unmodified siRNA was comparable with that of the conjugates [124]. The accumulation of siRNA-Chol was detected in liver, heart, kidneys, fatty and pulmonary tissues after intravenous injection of the radioactively labeled conjugates (containing cholesterol at the 3′-end of the sense strand) in mice. It should be noted that cells of these organs express LDL-receptors at high level. Other examples of cholesterol-modified siRNAs with high penetration ability are presented in the Table 1.

The conjugation of siRNAs with the peptides could also improve their cellular accumulation (Table 1). 11 amino acid cationic peptide derived from cell-permeable Tat protein and responsible for its nuclear localization was used in a number of studies [14, 127]. It was found that the attachment of this peptide (TAT-peptide) with additional cysteine to the 3′-end of the antisense strand of siRNA results in its effective accumulation in HeLa cells and effective silencing of the target genes *EGFP* and *CDK9* [14]. The endocytosis was suggested to be the mechanism of the conjugates penetration into cells [136, 137].

The addition of the cell penetrating peptides (transportan and penetrantin) to the 5′-end of the sense strand of siRNA improves the penetration ability and the silencing activity of anti-*EGFP* and anti-*GL2* siRNAs in COS-7, C166-GFP, EOMA-GFP and CHO-AA8-Luc Tet-Off

cells [128] (Table 1). It was suggested that penetration of the conjugates does not occur via pino- or endocytosis, but is mediated by diffusion through plasma membrane [128]. Besides the increase of transfection efficacy, the attachment of the peptides to siRNAs may enhance their specificity. Bioconjugates of siRNAs with peptides inhibiting the RISC assembly and containing specific sequence cleavable by cell-specific peptidases are promising agents for cell-specific gene silencing [138]. The efficient inhibition of the exogenous *GFP* expression was observed in choriocarcinoma Jeg-3 cells after electroporation of the conjugate of siRNA and a peptide with the "LEVD" sequence recognized by caspase-4, whereas in caspase-4 deficient HEK 293 cells the conjugate was inactive [138].

Another approach to the enhancement of siRNA cellular accumulation is the delivery of siRNA conjugated with antibodies or aptamers [132]. The aptamers are structured synthetic nucleic acids with size less than 15 kDa (Table 1), they can be chemically modified for the defense from the nucleases [139]. The penetration of these conjugates occurs via specific interactions with receptors on the surface of the target cells, providing the effective cellular accumulation of siRNAs [132]. The conjugates of anti-*BCL2* or anti-*PLK1* (polo-like kinase 1) siRNA and PSMA-receptor specific aptamer, effectively penetrate into prostate cancer LNCaP cells and induce effective gene silencing [129]. Injection of these conjugates into tumor expressing PSMA-receptors results in tumor regression [140]. It was shown also that the conjugates of siRNA and anti-transferrin or anti-insulin monoclonal antibodies attached to siRNA via streptavidin-biotin linker effectively reduces the exogenous *F.luciferase* expression in HEK 293 cells and rat glial cells implanted in the brain [141, 142] (Table 1).

The attachment of the folic acid to siRNA is a promising approach to increasing cellular uptake of siRNA. These conjugates can penetrate into virtually all human cancer cells, since expression of the expression of the folate receptors on the surface of the cancer cells is substantially higher than in normal cells [143]. The efficient accumulation of fluorescein-labeled siRNA with folate attached to the 5′- or 3′-end of the sense strand via disulfide bond was observed in solid tumor in mice [126] (Table 1). In the other studies [144, 145] the delivery of siRNA into cells was performed with folate-containing structures. These experiments (*in vivo* and *in vitro*) verified the advantage of siRNA modification by folate: specific delivery into cancer cells and high silencing effect of these siRNAs was observed.

The introduction of biomolecules in siRNA is one of the promising approaches of non-viral delivery. In contrast to other non-viral methods (cationic lipids and polymers, high-pressure injections) the advantages of conjugation include the cell-specificity and the absence of toxic effect [63, 146, 147, 148, 149]. The employment of cholesterol- and folate-contaning siRNAs *in vivo* is less specific, since LDL- and folate-receptors are expressed by different cells [125, 150], however, this approach is could be usefully applied when strict cell or tissue selectivity is not required.

3.3. Combination of chemical modifications

The application of different modifications simultaneously is a widely-used approach for the optimization of siRNA properties. Two main strategies are considered in order to increase

nuclease resistance of siRNA. The first one is based on the extensive modification of siRNA. In this case optimal design of the duplex (i.e. selection of modifications) could be created experimentally by the analysis of the data on nuclease resistance and biological activity. For example, different types of modifications were used in totally modified siRNA, displaying high activity in mice infected with Hepatitis B virus. The sense strand of this siRNA contains deoxyribonucleotides instead of the purine ribonucleotides, 2′-F-modifications and 5′-5′- and 3′-3′-terminal deoxyribonucleotides instead of the pyrimidine bases; the antisense strand contains alternating 2′-F / 2′-O-Me groups; one PO-bond between 3′-terminal nucleotides was replaced by PS-bond [61] (Table 2). Other examples of siRNA containing different combinations of chemical modifications are presented in the table 2. However, the extensive modification of siRNA often is not well-tolerated by RNAi machinery and rather often leads to the reduction or blocking of the silencing activity [40, 59, 99]. Therefore, minimization of the number of modifications in siRNA structure is required. It was shown, that duplexes with limited number of LNA-modifications could be active and as resistant to nucleases as the totally modified 2′-O-Me / 2′-F-siRNAs, which were inactive [40, 59, 99, 99] (Table 2). Since siRNAs are degraded mainly by endoribonucleases [59], it was suggested that the increase of the thermostability will increase the nuclease resistance [99]. On the other hand, it is known that the pattern of siRNA degradation in the presence of serum is similar to the cleavage by RNase A [62]. Therefore, the selective modification of the nuclease sensitive sites of siRNAs, mapped in the presence of serum, represents an alternative approach to the extensive modification of siRNA. This rational approach of siRNA modification allows to minimize the number of modified nucleotides in the duplex and as a consequence, to maintain its silencing activity [76]. The optimization of siRNA design also includes the defense of the 3′-terminal nucleotides against exonuclease cleavage (see section 1.2.2).

The combinations of chemical modifications are used for optimization of other siRNA properties. In order to achieve *in vivo* both the efficient cellular uptake and their high nuclease resistance, the 2′-O-Me-modified or/and PS-modified siRNAs conjugated with cholesterol were used [57, 151]. In order to increase the thermoasymmetry of the duplex, the nucleotide analogs with opposite effect on the siRNA thermostability can be used simultaneously. For instance, LNA- and UNA-analogs introduced in siRNA simultaneously was shown to enhance the silencing activity of the siRNA due to the increase of thermoasymmetry of the duplex: duplex stabilization of the duplex by LNA-analogs at the 5′-end and destabilization by UNA-analogs at the 3′-end of the sense strand [99].

The increase of the RNAi efficacy was also observed after stabilization of the 3′-end of the antisense strand of siRNA by s^2U or ψ analogs and destabilization of the 5′-end by introduction of **D** at the 3′-end of the sense strand [120]. It should be noted that the decrease of the thermostability of the duplex at the 3′-end of the sense strand is more preferable in comparison with the stabilization of the duplex at the 5′-end of the sense strand if the duplex stabilization is not needed [99].

Thus, in order to optimize siRNA design by chemical modifications the following principles should be considered: modifications should provide 1) the RNA-like A-helix geometry of the duplex; 2) the access of the terminal 5′-OH group of the antisense strand for

Structure of the modified siRNA analog: 5' – sense strand – 3' 3' – antisense strand – 5' [1)]	$\tau_{1/2}$, h	Target	Silencing efficacy, %	Ref.
B_**GGA***CUUCUCUC***AA***UUUUCU***TT**_B **TsT***CCU*GAAGAGAG*UU*AAAAGA	48 – 72	RNA of hepatite B virus	70	61
p-*GGGUAAAUACAUUCUUCAU* *CCCAUUUAUGUAAGAAGUA*-p	> 7	Endogenous gene *PTEN*	90	77
A A G G A G A U C A A C A U U U U C ATT TTUsUsCsCsUsCsUsAsGsUsUsGsUsAsAsAsAsGsU	48	Endogenous genc *Survivin*	75	72
GCGGAUCUUGAAGUUCACCUU *UUCGCCUAGAACUUCAAGUGG*	> 18	Endogenous gene *GFP*	90	152
GA**C**GUAAA**C**GGC**C**A**C**AAGU**TC**U U**CG**CUGCAUUUGCCGGUGUUCA	- [2)]	Endogenous gene *eGFP*	87	99
GACGUAAACGGCCACAAGUUC *CGCUGCAUUUGCCGGUGUUCA*	-	Endogenous gene *eGFP*	26	99

[1)] Bold letters – deoxyribonucleotides, underlined letters – 2'-O-Me, italics – 2'-F-analogs, B_ – 5'-5'-, _B – 3'-3'-inverted deoxyribose, underlined bold letters – LNA-analogs, s – phosphorothioate bond between nucleotides.

[2)] "-" data is not available.

Table 2. Extensive and selective modification of siRNA

siRNA property	The role of chemical modification	Examples of modifications
Thermostability	To increase the thermostability of siRNA by providing the optimal conformation of nucleotides for base pairing[65, 99, 120].	2'-O-Me, 2'-F, 2'-F-ANA, 2'-O-MOE, 2'-O-DNP, 4'-S, LNA, ENA et al.
Thermoasymmetry	To increase the thermoasymmetry of the duplex by the decrease of thermostability at the 3'-flank of the sense strand or/and the increase of the thermostability at the 5'-flank of the sense strand of the duplex [99, 105, 107, 120].	UNA, LNA, nucleobase modifications
Nuclease resistance	To increase the duplex nuclease resistance by introduction of the chemically modified analogs of nucleotides in its structure[153,76].	Virtually all modifications
Duration of circulation in the bloodstream	To increase siRNA affinity to serum proteins and the time of circulation [57, 65].	PS, 4'-S, Chol
Cellular uptake	To provide the effective mechanisms of siRNA penetration: receptor-mediating endocytosis, diffusion via cellular membrane or other ways (if the mechanism is unknown)[153, 86, 126].	Chol, 2'-O-DNP, folate
Target cellular uptake	To increase the specificity of uptake by the attachment of the molecules with high binding affinity to surface of the cells of particular organs and tissues[154].	Peptides, antibodies, aptamers
Specificity of action	To increase the specificity of the silencing by: 1) the increase of duplex thermoasymmetry[99, 105, 107, 120]; 2) alteration of binding affinity of the antisense strand of siRNA to mRNA-target[155, 99, 82]; 3) the absence of receptors providing the activation of immune response [64]; 4) blocking the phosphorylation of the 5'-OH group of the sense strand of siRNA [123].	1) see above; 2) UNA, 2'-O-Me, deoxyribonucleotides 3) 2'-O-Me 4) 2'-O-Me

Table 3. Influence of the chemical modifications on siRNA properties

phosphorylation; 3) low thermostability of the 5′-end of the antisense strand, hence, the modification has to increase the favorable thermoasymmetry or has no effect on the duplex thermostability.

The information on the influence of chemical modifications on siRNA properties is summarized in the table 3.

4. The impact of the siRNA structure on the efficiency of RNAi

The investigation of the structures of natural siRNAs formed by dsRNA processing with Dicer and endogenous miRNA revealed the common siRNA structures. "Classical" small interfering RNA resembles duplexes 18 - 23 bp in length [18, 22, 156, 157] with 2 nt overhangs at 3'-ends [40, 45] (fig. 9).

However, it was clearly demonstrated, that the silencing activity of structurally similar duplexes with different sequences varies significantly. As it was mentioned above thermodynamic asymmetry of the duplex and to a lesser extend the structure of mRNA target determines the siRNA activity [44, 45, 57, 58, 60]. Possibility to optimize the duplex structure was demonstrated in a number of publications. Introduction of nucleotide substitutions in siRNA, resulting in a formation of non-canonical base pairs or mismatches [38, 158, 159] is a perspective approach for the optimization of the thermodynamic properties of the duplex. The other approach utilize the inactivation of the sense strand of siRNA (for example, segmentation) to guarantee the incorporation of the antisense strand into activated RISC* independent from the thermodynamic properties of the duplex [99]. The silencing activity of siRNA can be increased by the lengthening of the duplex and converting siRNA into Dicer substrate DsiRNA [160, 161] or by the modifications of the overhands affecting duplex thermodynamic asymmetry [162]. Single stranded analogs of siRNA can be also used as inducers of RNAi, in this case the problem of the strand selection does not exist [40, 77]. All mentioned approaches will be described in details below.

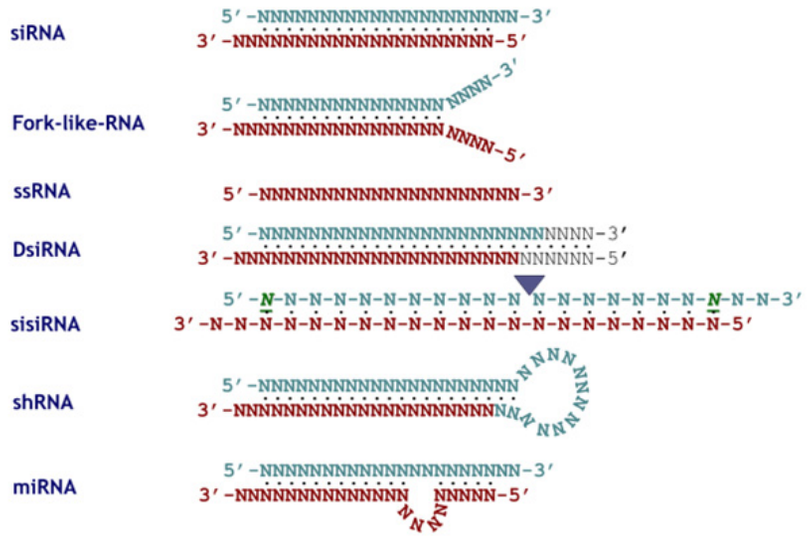


Figure 9. Structural repertoire of siRNA. The triangle indicates single stranded nick.

4.1. The influence of nucleotide substitutions on the silencing activity of siRNA

Thermodynamic properties of siRNA play a key role on the stages of RISC activation and mRNA target cleavage, determining the efficiency of strand dissociation, strand selection and mRNA cleavage. Since that, nucleotide substitutions affecting thermodynamic stability of the duplex could change the silencing activity of siRNA. The nature of the changes depends on the type of substitutions and the location of the substitutions in siRNA structure. The data on the impact of nucleotide substitutions in the silencing activities of siRNAs reported in the literature are summarized in Table 4.

The kinetics of mRNA cleavage directed by anti-*F.luciferase and anti-sod1* siRNA, containing nucleotide substitutions in one of the strands of the duplex were exanimated in details in [38]. It was shown, that the substitution of cytosine with uracil at the 5′-end of the sense strand of anti-*F.luciferase* siRNA, resulting in U:G pair formation (stabilized by two hydrogen bonds)[163]), reduced the target mRNA cleavage rate. At the same time, the rate of the antisense RNA (complementary to the first target) cleavage increased substantially. The analysis of the binding of siRNA strands with RISC* revealed, that single nucleotide substitution at the position 1 of the sense strand reduce the incorporation of the antisense strand in the complex, whereas the efficiency of the sense strand incorporation into RISC* increased (fig. 10 A).

Nucleotide substitution at the 3′-end of the sense strand of anti-*sod1* siRNA resulting in the formation of A:G mismatch (fig. 10 B), caused the opposite effect: the rate of mRNA cleavage increased, while the rate of antisense mRNA cleavage decreased. This result indicates that the efficiency of the sense strand incorporation into RISC* decreases. The observed effect could be caused both by preferential incorporation of the antisense strand into RISC complex, and by non-perfect complementarity between the sense strand of siRNA and its target (antisense mRNA), but the results of the additional experiments with the substitution of G by C at the 5′-end of the antisense strand (fig. 10 B) verified the key role of preferential RISC loading [38]. Thus, it was clearly demonstrated, that single nucleotide substitutions at the flank regions of siRNA could change the thermodynamic asymmetry and to determine the preferential incorporation of one strand into RISC*: destabilization of the duplex at the 3′-end of the sense strand increase the activity of siRNA , the destabilization of the opposite end of the duplex, reduce the silencing activity [38].

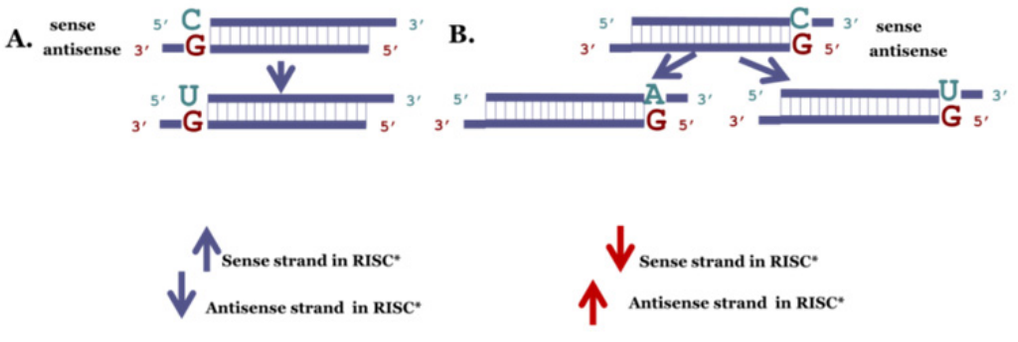


Figure 10. The scheme of the experiment described in [38]. **A.** Substitution at the 5′-end of the sense strand of anti-*F.luciferase* siPHK. **B.** Substitution at the 3′-end of the sense strand (left) or at the 5′-end of the antisense strand (right) of anti-*sod1* siRNA.

Target, model system	Type of siRNA [1)]	Substitutions [2,3)]	Silencing efficiency, %	Reference
***P.luciferase*,**	1	-	98	158
(+2/+ 23),		$G_1G \rightarrow U_1U$	94.5	
plasmid, transfected into	2	$A_{19} \rightarrow U_{19}$	99.5	
Hela cells		$U_{18}A \rightarrow A_{18}U$	99.8	
		$A_{17}UA \rightarrow U_{17}AU$	99.8	
		$C_{16}AUA \rightarrow U_{16}UAU$	99.6	
		$U_{18}A \rightarrow A_{18}U$, $G_{12} \rightarrow U_{12}$	99	
***P.luciferase*,**	1	-	50	
(+21/+ 43),		$A_1C \rightarrow U_1A$	8	
plasmid, transfected into	2	$C_{19} \rightarrow U_{19}$	76	
Hela cells		$G_{18}C \rightarrow U_{18}U$	78	
		$U_{17}GC \rightarrow A_{17}UU$	75	
		$C_{16}UGC \rightarrow U_{16}AUU$	70	
		$G_{18}C \rightarrow U_{18}U$, $G_{12} \rightarrow U_{12}$	78	
***Lamin A/C* ,**		-	67.5	
(+829/+ 851),	1	$A_{18}C \rightarrow U_{18}U$, $A_{12} \rightarrow U_{12}$	79.5	
Hela cells	2			
***Lamin A/C*,**		-	79	
(+608/+ 630),	1	$C_{18}A \rightarrow U_{18}U$, $U_{12} \rightarrow A_{12}$	92	
Hela cells	2			
***Dnm T1*,**		-	77.5	
(+70/+ 89),	1	$G_{18}A \rightarrow U_{18}U$, $G_{12} \rightarrow U_{12}$	84.5	
Hela cells	2			
***Dnm T1*,**		-	82.5	
(+185/+ 203),	1	$U_{18}U \rightarrow A_{18}A$, $G_{12} \rightarrow U_{12}$	89.5	
Hela cells	2			
***R.luciferase*/*P.luciferase* [c] , [4)]**	1	-	86	159
plasmid, transfected into		$A_1C \rightarrow U_1A$	72	
Hela cells	2	$G_{18}C \rightarrow U_{18}U$	95	
***R.luciferase*/ *P.luciferase* [a],**	1	-	92	
plasmid, transfected into		$C_{18}A \rightarrow A_{18}U$	89	
Hela cells	2	$C_1G \rightarrow U_1U$	20	
***F.luciferase*,**		-	60	74
plasmid, transfected into	1	$G_9 \rightarrow C_9$	57	
NIH3T3 cells		$G_{6,9} \rightarrow C_{6,9}$	56	
		$G_{6,9,13} \rightarrow C_{6,9,13}$	30	
		$G_{3,6,9,13,17} \rightarrow C_{3,6,9,13,17}$	15	
***MGC29643*,**	1	-	50	166
(+576/+596)		$C_8 \rightarrow G_8$	10	
Hela cells		$A_{12} \rightarrow U_{12}$	55	
		C_8, $A_{12} \rightarrow G_8$, U_{12}	3	
***GPR39*,**	1	-	50	
(*MGC29643*		$C_{12} \rightarrow G_{12}$	55	
(+576/+596)[a])		$A_8 \rightarrow U_8$	5	
Hela cells		A_8, $C_{12} \rightarrow U_8$, G_{12}	0	
***Jagged-1*,**	1	-	0	165
(+3382/+3402)		$C_{18} \rightarrow U_{18}$	50	
plasmid, transfected into		$C_5 A_{12} C_{15} \rightarrow U_5 G_{12} U_{15}$	0	
HEK 293T cells				

Target, model system	Type of siRNA [1)]	Substitutions [2,3)]	Silencing efficiency, %	Reference
sod1/F.luciferase [c], plasmid, transfected into HEK 293 cells	1	- $G_1 \rightarrow U_1$ $C_{19} \rightarrow A_{19}$ $\mathit{G_1} \rightarrow \mathit{U_1}$ $\mathit{C_{19}} \rightarrow \mathit{A_{19}}$	55 0 70 70 0	164
sod1/F.luciferase [a], plasmid, transfected into HEK 293 cells	1	- $G_1 \rightarrow U_1$ $C_{19} \rightarrow A_{19}$ $\mathit{G_1} \rightarrow \mathit{U_1}$ $\mathit{C_{19}} \rightarrow \mathit{A_{19}}$	75 75 45 40 80	
APP$_{(L)}$/luciferase [5)], plasmid, transfected into HEK 293 cells	2	- $C_{18}C \rightarrow U_{18}U$	0 45	159
APP$_{(S)}$/luciferase, plasmid, transfected into HEK 293 cells	2	- $G_{18}C \rightarrow U_{18}A$	0 80	159
EGFP, (+306/+324) human lung carcinoma H1299	1	- $G_{19} \rightarrow C_{19}$	55 50	24
EGFP, (+396/+414) human lung carcinoma H1299	1	- $G_{19} \rightarrow A_{19}$ $G_{19} \rightarrow C_{19}$ $G_{19} \rightarrow U_{19}$ $\mathit{C_{19}} \rightarrow \mathit{A_{19}}$ $\mathit{C_{19}} \rightarrow \mathit{U_{19}}$ $\mathit{C_{19}} \rightarrow \mathit{G_{19}}$	65 42 40 35 52 45 45	
F.luciferase, plasmid, transfected into Hela SS6 cells	1	- $C_1 \rightarrow A_1$ $A_{19} \rightarrow C_{19}$ $A_{19} \rightarrow G_{19}$ $A_{19} \rightarrow U_{19}$ $G_9 \rightarrow A_9$ $G_9 \rightarrow C_9$ $G_9 \rightarrow U_9$ $A_{10} \rightarrow C_{10}$ other substitutions in the sense strand (2 – 8, 12 – 18 positions)	1 [b)] 0.3 1.15 1.2 1.25 4.5 3.3 2.15 2.3 0.2 – 1.3	36

[1)]

siRNA type	Structure of siRNA	Strand	Length of the strand, nt
1	5'-NNNNNNNNNNNNNNNNNNNNN-3'	sense	21
	3'-NNNNNNNNNNNNNNNNNNNNN-5'	antisense	21
2	5′-NNNNNNNNNNNNNNNNNNN-3′	sense	19
	3′-NNNNNNNNNNNNNNNNNNNNN-5′	antisense	21

[2)] Numeration of the nucleotides from the 5'-end.

[3)] Substitutions in the sense strand are presented in normal letters; substitutions in the antisense strand are presented in *italic*.

[4)] *P.luciferase* [s (a)] – a gene or a fragment of gene encoded sense ([s]) or antisense ([a]) RNA.

[5)] *APP$_{L(S)}$/luciferase* – recombinant gene, containing mutant London ([L]) or Swedish ([S]) type of *APP* allele.

[b)] Silencing activity of structurally modified siRNA is presented as the ration of IC50 (primary siRNA) / IC50 (modified siRNA), where IC50 is the concentration of siRNA, inducing –50 % silencing of the target gene.

Table 4. The influence of the nucleotide substitutions in siRNA on the efficiency of RNAi

The elevation of the thermodynamic asymmetry by single nucleotide substitution at the 1th position of the antisense strand was reported to increase or have no influence on the silencing activity of different siRNAs, targeted to *sod1* / *F.luciferase* mRNA or the complementary sequence (antisense mRNA) [164] (Table 4). In turn, the equal efficiency of the cleavage of *htt* mRNA and *htt* antisense mRNA by thermodynamically symmetrical siRNA suggests the equal probability of sense or antisense strand incorporation into RISC* [38].

Introduction of the nucleotide substitutions at the 3′-end of the sense strand of siRNA duplex resulted in the formation of the mismatches, has led to the creation of the structurally new class of small interfering RNAs – fork-like siRNAs (fsiRNAs) [158]. More effective silencing of the expression of *R.luciferase*/*P.luciferase* gene by fsiRNAs then by classical siRNA targeted to the same sequences was demonstrated in Hela cells (Table 4). The most pronounced augmentation of the activity was observed for siRNA with initially moderate activity [158]. Fork-like siRNA with two nucleotide substitutions at the 3′-end of the sense strand was found to be the most active [11, 158, 159] (Table 4). Introduction of from 2 to 4 nucleotide substitutions at the 3′-end of the sense strand of siRNA targeted *sod1* and *htt* mRNAa correspondingly increased the efficiency of silencing [38].

The increase of the thermodynamic asymmetry of the duplex by the introduction of the mismatches at the ends of the strands cannot guarantee the positive influence on the activity. It is known, that the region of the "guide" strand spanning from 2 to 6 position determines the efficiency of the interaction of siRNA with Ago2 [21, 30, 31]; this fact limits the introduction of substitutions in this region. Nevertheless, C→U substitution in 2^{d} position of the antisense strand of siRNA targeted *Jagged-1* mRNA increased the silencing effect from 0 to 50% in HEK 293T cells transfected with vector, containing cDNA fragment of the target gene [165] (Table 4). This data suggests, that the substitutions in the antisense strand resulted in the formation of non-canonical base pairs is more tolerable than the substitutions resulted in the formation of mismatches. In the other study, single nucleotide substitutions at 19th position of the sense strand or at the 1st position of the antisense strand resulting in the formation of the mismatches as well as non-canonical base-pairs reduced the activity of anti-*EGFP* siRNA in H1299 cells. It was assumed that the reduction of the efficiency of the interaction of TRBP (but not the Dicer) with siRNA was the possible reason of the observed effect [24] (Table 4).

Introduction of nucleotide substitutions in the central part of siRNA duplex could be used for the destabilization the central region and the subsequent elevation of the silencing activity. Nevertheless, this type of structure modification could switch the mode of siRNA action to miRNA-like mechanism [167] (fig. 4). It was demonstrated in experiments on HeLa cells, that substitutions in 9th and 10th positions of the sense strand of siRNA leads to the 2 - 4.5-fold increase of IC50 as compared with IC50 for siRNA with classical duplex 36. Substitutions in the sense strand or in both strands of siRNA [167] reduced the efficiency of RNAi (Table 4).

Thus, the introduction of nucleotide substitutions could be successfully used for the design of siRNA characterized by favorable thermodynamic asymmetry and low thermostability of the central part of the duplex. This approach can be applied for the creation active siRNA

targeted to any desired sequence for silencing of mutated or chimerical genes. However, there is not enough data publish up to date for the formulation of the universal rules for the design of fork-like siRNA and siRNA with destabilized center, the activity of resulted siRNA cannot be predicted and should be determined experimentally.

4.2. The influence of the siRNA overhangs structure on the efficiency of gene silencing

2 - 3 nt overhangs at the 3′-ends of siRNA are a distinguishing feature of both natural siRNA generated by Dicer and synthetic siRNA [40, 45, 162]. The overhangs are important for the interaction of siRNA with PAZ-domens of Dicer and Ago2 [21, 27, 168]. However, the data on the activity of siRNA with blunt ends (with no overhangs) are rather contradictory: in mammalian cells the activity of this type of siRNA did not vary significantly from the activity of siRNA with 2-nt overhangs targeted to the same sequence [59] , controversially, in extracts from drosophila cells blunt ends siRNA were less active [40, 45]. The assumption that observed difference could be accounted for the difference in the mechanism of action of heterodimer Dicer-R2D2 in drosophila and its analogue Dicer-TRBP in humans [59], did not find the experimental support, since it was reported recently, that in HeLa S3 the activity of the blunt ended siRNA was similarly lower (60% silencing) than the activity of classical siRNA (80 % silencing) [162]. Moreover, the presence of 2 nt overhang at the 3′-end of only one of the strands leads to the selection of this strand as a guide strand due to the preferential loading into RISC*[162]. The presence of the mismatches at the 5′-end of the antisense strand resulting in the formation of thermodynamically asymmetrical duplex increases the efficiency of the incorporation of antisense strand, containing 2 nt overhang, into RISC*. Efficient silencing of the target gene expression by fork-like structurally asymmetrical siRNA was achieved in a number of studies conducted on HeLa cells (Table 4, 2nd type of siRNA).

It was found the 1 nt shortening of the overhang (1 nt overhang instead of 2) at the 3′ end in some cases did not affect significantly the silencing activity [40]. The sequential lengthening of the 3′-end overhang in the sense or the antisense strand up to 7 nt induced the substantial reduction of the silencing activity of siRNA [40], probably due to inefficient interaction of this siRNAs with PAZ-domen of Dicer [29]. siRNAs, containing overhangs at the 5′-ends of the duplex were inactive in RNAi [40], and induced side effects related to the PKR-mediated activation of the innate immunity [169].

Therefore, exploitation of the structurally asymmetrical siRNAs, containing 2 nt overhang only at the 3′-end of the antisense strand together with the reduction of the thermodynamic stability of the duplex at the 5′-end region of the antisense strand could be successfully used in siRNA design for increasing the efficiency of RNAi.

4.3. Single stranded analogous of siRNA (ss-siRNA)

Before the discovery of RNAi, it was demonstrated that long (several hundred bases in length) single stranded RNA are capable of sequence specific silencing of the expression of

homologous genes in *C.elegans* [170]. Later, it was assumed that the minor fraction of dsRNA in the preparations of ssRNA obtained by *in vitro* transcription was the agent that silence the expression of the target genes 1. However, the ability of antisense, but not sense ssRNA (22 – 40 nt) to induce gene silencing was experimentally verified in *C.elegans* [171]. It was demonstrated that mRNA degradation induced by ssRNA proceeds via RISC* assembling (fig. 9) [131,122]. The evaluation of the silencing activity of anti-*luciferase* ss-siRNA in HeLa cells demonstrated, that the required concentration of ss-siRNA was 8-fold higher, than the concentration of classical siRNA to reach the similar level of gene silencing. In the other report, it was shown, that the concentration of ss-siRNA essential for the RISC assembling was 10 – 100 higher than the concentration of double stranded siRNA analogous [122]. High sensitivity of single stranded RNA to ribonucleases [62], and less efficient interaction with the RNAi machinery proteins [131,122] were supposed to be the possible reasons of the lower efficiency of RNAi induction by ssRNA.

Thus, the application of non-modified ss-siRNA as inhibitors of gene expression is not beneficial in comparison with double stranded siRNA.

4.4. dsRNA (27-30-mers) as Dicer substrates

During the search of the effective inductors of RNAi, able to silence target gene at low (nanomolar and lower) concentrations, it was found that dsRNAs 25-30 bp in length which are substrates of Dicer (here and after DsiRNA – Dicer-substrate siPHK), are significantly more active in comparison with "classic" or "conventional" siRNAs [161, 172, 173, 174]. To compare interfering activity of DsiRNA and conventional siRNA the duplexes of different length (21-30 bp) and structure (presence or absence of 3′ or 5′ dinucleotide overhangs) targeting *EGFP* were used [174]. The interfering activity of dsRNA was evaluated at concentration ranging from 50 pMol to 50 nMol using HEK293 cells transfected with the plasmid encoded EGFP. It was shown that at subnanomolar concentrations (50 *pM* – 200 *pM*) DsiRNAs were significantly more active than siRNA. Among duplexes tested the DsiRNA 27 bp in length with blunt ends displays the highest interfering activity: this DsiRNA (1 nM) inhibited expression of the target gene by 95% (Table 5) while corresponding siRNA was almost inactive [174]. It turned out, that this DsiRNA (27 bp, blunt ends) is processed by Dicer yielding pull of different siRNAs 21bp in length. Seven siRNAs (21 bp with 3′-dinucleotide overhangs) generated by single-nucleotide shift along the *EGFP* mRNA sequence homologous to DsiRNA were synthesized to answer the question can the synthetic siRNA be as active as this particular DsiRNA. It was shown that at the concentrations 50 pM and 200 pM neither each synthetic siRNA nor pull of synthetic siRNAs silence expression of EGFP as efficiently as corresponding DsiRNA. Interestingly, in the case if DsiRNA was processed by Dicer *in vitro* and then transfected into the cells the silencing efficiency of this processed DsiRNA was similar to the activity of synthetic siRNAs [174]. It has been suggested, than after DsiRNA cleavage Dicer did not dissociated from the complex with DsiRNA and thus governed the mode of interaction of R2D2 (*D.melanogaster*) or TRBP (*H.sapiens*) and, as a consequence, the unequivocal orientation of siRNA within the RISC. This provides for incorporation into the RISC the antisense strand of the duplex.

However, if an equivocal binding of Dicer with dsRNA takes place which results in a formation of a set of siRNA cleavage products, the possibility of formation of incompetent RISC*, containing sense strand of siRNA as a leading strand is increased. Thus, optimization of a structure of DsiRNA is a current task.

It is known, that one of the functions of Dicer within the cell is a processing of pre-miRNA via binding with their 3'-end, containing two hanging nucleotides followed by cleavage of the duplex at a distance of 21-22 nucleotides from the 3'-end [172]. Taking into account that Dicer has a low affinity to DNA, a set of asymmetric dsRNA was synthesized, containing at the 3'-end of the sense or antisense strand two nucleotides overhang, while the opposite end was blocked for Dicer binding by the replacement of two ribonucleotides in the complementary strand by deoxyribonucleotides [161, 172] (Table 5, structures 25D/27 and 27/25D). It was anticipated that such a structure of DsiRNAs provide for an unequivocal binding of the duplex with Dicer.

According to mass-spectrometry the cleavage of these DsiRNA by Dicer (Table 5, structures 25D/27 and 27/25D) yields the same product in both cases [161]. However, the silencing activity of siRNAs derived from these DsiRNAs was different; which possibly is explained by the peculiarities of DsiRNA interactions with Dicer, determined the efficiency of sense or antisense incorporation into RISC*.

Target gene / model system	Optimal or the only studied concentration of DsiRNA, nM	DsiRNA structure peculiarities	Level of target gene silencing, %	Ref
EGFP, Plasmid DNA encoded EGFP transfected in HEK 293 cells	50	21+2 [1)] 23-2 23+2 24-2 24+2 25-2; 25+2; 26+0; 27+0; 27+2; 27-2	72 80 98 82 95 ~ 100	174
EGFP, Endogenous gene in NIH3T3 cells	1	27+0 30+0 35+0 40+0 45+0	98 98 97 2 <1	
EGFP, Plasmid DNA encoded EGFP transfected in HEK 293 cells	1	19+2 27+0 27/25D [2)] 25D/27	<1 68 53 95	161
***F.luciferase* 1** [3)], Plasmid DNA transfected in HEK 293 cells	10	27/25D 25D/27	80 97	
***F.luciferase* 2** [3)] Plasmid DNA transfected in HEK 293 cells		27/25D 25D/27	97 96	
***F.luciferase* 3** [3)] Plasmid DNA transfected in HEK 293 cells		27/25D 25D/27	23 78	

Target gene / model system	Optimal or the only studied concentration of DsiRNA, nM	DsiRNA structure peculiarities	Level of target gene silencing, %	Ref
***F.luciferase* 4**[3)] Plasmid DNA transfected in HEK 293 cells		27/25D 25D/27	95 94	
La antigene in HEK 293 cells	10	27/25D 25D/27	30 82	172
STAT1, endogenous gene in HeLa cells	1	25D/27	95	173

[1)] The structures of dsRNA – substrates of Dicer: L+2 – duplex of L bp with two nucleotide overhangs at the 3′- ends, L–2 – duplex of L bp with two nucleotides overhangs at the 5′ ends, L+0 – duplex of L bp with blunt ends where L varied from 19 to 45 bp.

The structure of DsiRNA	Scheme of DsiRNA	Length of the strand (L), bp
L+2	5′– NNNNNNNNNNNNNNNNNNNNNNN–3′ 3′– NNNNNNNNNNNNNNNNNNNNNNN–5′	23 23
L-2	5'–NNNNNNNNNNNNNNNNNNNNNNN–3' 3'–NNNNNNNNNNNNNNNNNNNNNNN–5'	24 24
L+0	5′–NNNNNNNNNNNNNNNNNNNNNNN–3′ 3′–NNNNNNNNNNNNNNNNNNNNNNN–5′	26 26

[2)] Asymmetric DsiRNA (25 bp), containing at the 3′-end of the sense (antisense) strand 2 nt overhang, the opposite end was blocked by replacement of two ribonucleotides with deoxyribonucleotides – **N**.

The structure of DsiRNA	Scheme of DsiRNA	Strand	Length of the strand (L), bp
27/25D	5′-NNNNNNNNNNNNNNNNNNNNNNNNNNN-3′ 3′-**NN**NNNNNNNNNNNNNNNNNNNNNNN-5′	sense antisense	27 25
25D/27	5′-NNNNNNNNNNNNNNNNNNNNNNN**NN**-3′ 3′-NNNNNNNNNNNNNNNNNNNNNNNNNNN-5′	sense antisense	25 27

[3)] The numbers correspond to different sequences within the target gene *F.luciferase.*

Table 5. The influence of DsiRNA structure in its silencing activity

It was shown, that in HEK293 and HeLa cells the DsiRNAs with antisense strand having dinucleotide overhang at the 3′-end were more active than those with dinucleotide overhang at the 3′-end of the sense strand (Table 5) [161, 172]. Thus, it is possible to predict the structure of forming siRNAs upon processing of DsiRNA by Dicer [161, 172], however, the activity of these siRNAs will depend on the thermodynamic parameters of their duplexes. It worth mentioning that in the case of unfavorable context of the target sequence within mRNA DsiRNAs have a significant advantage over conventional siRNA [174].

The silencing activity of a longer dsRNAs with blunt ends (27 – 45 bp) decreases with the increase of dsRNA length. It turns out that this drop of silencing activity correlate well with the drop of efficiency of cleavage of longer dsRNAs by Dicer [174]. The silencing effect caused by DsiRNA was longer in comparison with conventional siRNA: DsiRNAs efficiently silence *EGFP* expression in NIH3T3 cells for 10 days while corresponding siRNA displays silence activity not longer than 4-7 days [174].

Despite the promising results on silence activity of Dicer substrates the optimization of the properties of DsiRNAs by chemical modification is relevant. The influence of 2′-F- and 2′-O-Me modifications within DsiRNA type 25D/27 on the silencing activity and nuclease resistance was studied [173]. When choosing sites of modifications authors try to avoid the region needed for Dicer to execute dsRNA cleavage, despite presence there of nuclease sensitive motives (Fig. 11).

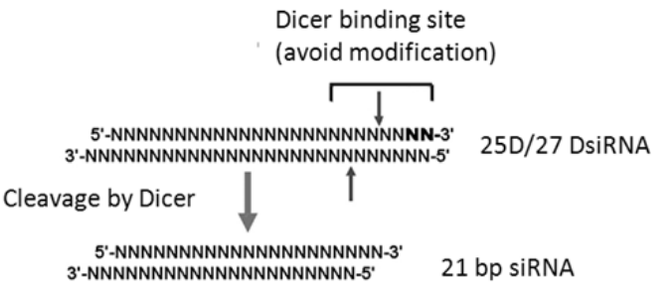


Figure 11. Design of chemically modified DsiRNA. By bold letter **N** deoxyribonucleotides are shown, short arrows – cleavage site of DsiRNA by Dicer.

It was shown, that chemical modification of DsiRNA affects its silencing activity in respect to *STAT1* mRNA only at the lowest concentration used (100 pM), while at higher concentration of DsiRNA (1 and 10 nM) the drop of silencing activity was detected only for extensively modified molecules, containing combination of several 2′-F- and 2′-O-Me nucleotides in a row. DsiRNA containing 11 2′-O-Me nucleotides in the antisense strand was very nuclease resistant: the fraction of intact DsiRNA was found in reaction mixture after 24 h of incubation in the presence of serum. However, such an extensive modification affects pattern of DsiRNA cleavage by Dicer and lead to the formation of two products of 21 and 22 bp instead of one product of 21 bp formed with unmodified DsiRNA. Replacement of one or two 2′-O-Me nucleotides in the vicinity of Dicer cleavage sites by the natural ones facilitated formation of 21 bp cleavage product. The introduction from 1 to 4 mismatches into central part of each strand of anti-*EGFP* DsiRNA significantly reduced its silencing activity [174].

On the other hand, chemical modification of DsiRNA can block induction of the unspecific immune response in mammalian cells, linked with the presence of triphosphate groups at the 5′ ends of DsiRNA or/and immunostimulating motives in the duplex [43,175, 176, 177, 178, 179,180]. So, DsiRNA, which contains in the antisense strand 10 -11 2′-O-Me nucleotides (including 2′-O-Me-cytidine and 2′-O-Me-uridine nucleotides known as the most effective blockers of interferon response activation via endosomal TLR [64]), blocked IFN-α secretion by human PBMC and did not induce immune response in RIG-I-dependent T98G cells opposite to unmodified DsiRNA and 2′-F-modified disPHK, respectively [173].

Full replacement of natural nucleotides by the analogs (2′-O-Me, 2′-F, etc.) is unacceptable as these DsiRNA will not be processed by Dicer [173]. Therefore, upon the introduction of chemical modifications in the structure of DsiRNA one should avoid modifying the nucleotides from 1 to 8 from 5′ end of the sense strand and complementary region of the sense strand (Fig. 11). Moreover, two overhanging nucleotides at the 3′-end of the antisense

strand as well as two deoxyribonucleotides at the 3′-end of the sense strand facilitate the correct orientation of Dicer and selection of the antisense strand of DsiRNA as a leading strand.

4.5. Small segmented RNA

Recently another approach to enhance RNAi efficiency based on modification of siRNA structure was proposed [99]. It was shown that siRNA in which the sense strand is subdivided in two parts of 10-12 nucleotides long, are more active in comparison with conventional siRNA duplexes. The efficiency of this internally segmented siRNA or sisiRNA (small internally segmented interfering RNA) (Fig. 9) was provided by unequivocal incorporation of the antisense strand into RISC*, since the nick in the central part of the sense strand blocked its usage as a guiding strand and facilitate the dissociation of the sense strand from the duplex [99]. Thus, sisiRNA have an advantage over conventional siRNA with unfavorable duplex thermoasymmetry. In addition unlike siRNAs with the classic structure the extensive chemical modification of antisense strand of sisiRNA has only little effect on its silencing activity. As an example, the silencing activity of anti-*EGFP* sisiRNA with antisense strand having 6 LNA-nucleotides or alternative 2′-F / 2′-O-Me-nucleotides and internally segmented sense strand with 6 LNA-nucleotides in each part was significantly higher than that of conventional siRNA bearing the same modifications [99]. Besides, both mentioned sisiRNA display high nuclease resistance. Thus, sisiRNAs as sequence specific inhibitors of gene expression are very promising, because they provide efficient RNAi even at the unfavorable thermodynamic parameters RNA duplex.

According to literary data the structural and thermodynamic peculiarities of interfering RNAs are a major factors providing for efficient gene silencing.

Prediction of silencing activity of any siRNA based on its thermodynamic profile is an efficient method of selection of active molecules. This approach is used in a number of software and algorithms for search and selection of such siRNA [37]. An alternative approach to the problem of creating efficient sequence-specific inhibitors of gene expression is the use of structure-modified siRNAs: fork-like siRNAs, DsiRNAs, or sisRNAs which silencing activity is less dependent on their thermodynamic parameters as compared with conventional siRNA diplex.

5. Conclusion

Small interfering RNAs are not optimal to be used as therapeutics *in vivo*, therefore, development of the approached aimed to improve or/and optimize the properties of siPHK is of significance nowadays. One of the approaches widely used is chemical modification of siRNAs. At the beginning this approach was proposed as a way to increase nuclease resistance of siRNA in the presence of serum, in the cells and bloodstream [61, 62]. The enhancement of nuclease resistance of siRNA by chemical modification not resulting in the drop of its silencing activity represent a complex task, since silencing activity of siRNA is

strongly affected by the nature, number and locations within the duplex of chemical modifications [65, 76, 181]. Despite many works carried out in this area, a general algorithm which allows receiving active nuclease resistant siRNA is not developed yet.

Chemical modification of siRNA can be also used to develop delivery vehicles for siRNA. It is well known that naked or free siRNA is not able to penetrate through cellular membrane and accumulate in the cells. In the case of in vivo conditions, the small size of siRNA molecule promotes its rapid clearance [57]. In other words, the systems for in vivo delivery of siRNAs should provide for efficiency of cellular accumulation, specificity of delivery (if applicable), duration of silencing as well as safety and possibility of systemic administration. Today no one delivery approach encounters these criteria.

On the other hand, the low efficiency of RNAi can be a result of its unfavorable sequence determining thermodynamic properties and/or by stable secondary structure at the target site within mRNA [37, 38, 51, 52, 54]. There are several algorithms to select siRNAs of high-performance [182, 183, 184, 185, 186], however it is impossible to predict precisely silencing activity of any particular siRNA in vivo. In addition, the design of siRNA targeting chimeric or mutant genes represent of complex task since the limitations of target site selection does not allow to use these algorithms for choosing the sequence siRNA. This is why structure modifications of siRNA improving their thermodynamic properties are of interest. The fork-like siRNA [158, 159], able to silence target gene more efficiently that conventional siRNA is a promising approach for creating high-performance siRNA targeted any sequence of the gene. Despite this, the number of works in this field is negligible so further research of properties of siRNA with modified structure are relevant.

To obtain high-performance siRNA often optimization of multiple parameters is required, so using modification of the siRNA structure together with its chemical modification is a promising approach. Thus, advanced research of chemically and/or structurally modified siRNA, as well as development and optimization of the algorithms for modification are important.

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Acknowledgement

This research was supported by the Russian Academy of Science under the programs Molecular and Cell Biology (grant no. 22-1) and Science to Medicine (grant no. 37), the Russian Foundation for Basic Research (grant nos. 11-04-01017-a and 11-04-12095-ofi-m-2011) Ministry of Science and Education of the Russian Federation (grant no. 14.740.11.1058) and the Siberian Branch of Russian Academy of Sciences (grant no. 85).

6. References

[1] Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E. and Mello C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 391, 806-811.

[2] Bosher J. M. and Labouesse M. (2000) RNA interference: genetic wand and genetic watchdog. Nat Cell Biol. 2, E31-36.

[3] Sharp P. A. (1999) RNAi and double-strand RNA. Genes Dev. 13, 139-141.

[4] Vaucheret H. and Fagard M. (2001) Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet. 17, 29-35.

[5] Hammond S. M., Bernstein E., Beach D. and Hannon G. J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature. 404, 293-296.

[6] Aigner A. (2006) Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. J Biotechnol. 124, 12-25.

[7] Aronin N. (2006) Target selectivity in mRNA silencing. Gene Ther. 13, 509-516.

[8] Hannon G. J. (2002) RNA interference. Nature. 418, 244-251.

[9] Hutvagner G. (2005) Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation. FEBS Lett. 579, 5850-5857.

[10] Rana T. M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol. 8, 23-36.

[11] Hohjoh H. (2002) RNA interference (RNA(i)) induction with various types of synthetic oligonucleotide duplexes in cultured human cells. FEBS Lett. 521, 195-199.

[12] Manche L., Green S. R., Schmedt C. and Mathews M. B. (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol Cell Biol. 12, 5238-5248.

[13] Elbashir S. M., Harborth J., Lendeckel W., Yalcin A., Weber K. and Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 411, 494-498.

[14] Chiu Y. L., Ali A., Chu C. Y., Cao H. and Rana T. M. (2004) Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells. Chem Biol. 11, 1165-1175.

[15] Elbashir S. M., Harborth J., Weber K. and Tuschl T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods. 26, 199-213.

[16] Paddison P. J., Caudy A. A. and Hannon G. J. (2002) Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A. 99, 1443-1448.

[17] Preall J. B. and Sontheimer E. J. (2005) RNAi: RISC gets loaded. Cell. 123, 543-545.

[18] Zamore P. D., Tuschl T., Sharp P. A. and Bartel D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell. 101, 25-33.

[19] Chendrimada T. P., Gregory R. I., Kumaraswamy E., Norman J., Cooch N., Nishikura K. and Shiekhattar R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 436, 740-744.

[20] Tomari Y., Matranga C., Haley B., Martinez N. and Zamore P. D. (2004) A protein sensor for siRNA asymmetry. Science. 306, 1377-1380.

[21] Ma J. B., Ye K. and Patel D. J. (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature. 429, 318-322.
[22] Zhang H., Kolb F. A., Brondani V., Billy E. and Filipowicz W. (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. Embo J. 21, 5875-5885.
[23] Grunweller A. and Hartmann R. K. (2005) RNA interference as a gene-specific approach for molecular medicine. Curr Med Chem. 12, 3143-3161.
[24] Kini H. K. and Walton S. P. (2009) Effect of siRNA terminal mismatches on TRBP and Dicer binding and silencing efficacy. FEBS J. 276, 6576-6585.
[25] Liu X., Jiang F., Kalidas S., Smith D. and Liu Q. (2006) Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes. Rna. 12, 1514-1520.
[26] Hutvagner G. and Simard M. J. (2008) Argonaute proteins: key players in RNA silencing. Nat Rev Mol Cell Biol. 9, 22-32.
[27] Song J. J., Smith S. K., Hannon G. J. and Joshua-Tor L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. Science. 305, 1434-1437.
[28] Bernstein E., Caudy A. A., Hammond S. M. and Hannon G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 409, 363-366.
[29] Ma J. B., Yuan Y. R., Meister G., Pei Y., Tuschl T. and Patel D. J. (2005) Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature. 434, 666-670.
[30] Parker J. S., Roe S. M. and Barford D. (2005) Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. Nature. 434, 663-666.
[31] Rivas F. V., Tolia N. H., Song J. J., Aragon J. P., Liu J., Hannon G. J. and Joshua-Tor L. (2005) Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol. 12, 340-349.
[32] Meister G. and Tuschl T. (2004) Mechanisms of gene silencing by double-stranded RNA. Nature. 431, 343-349.
[33] Nykanen A., Haley B. and Zamore P. D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell. 107, 309-321.
[34] Leuschner P. J., Ameres S. L., Kueng S. and Martinez J. (2006) Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep. 7, 314-320.
[35] Matranga C., Tomari Y., Shin C., Bartel D. P. and Zamore P. D. (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. 123, 607-620.
[36] Addepalli H., Meena, Peng C. G., Wang G., Fan Y., Charisse K., Jayaprakash K. N., Rajeev K. G., Pandey R. K., Lavine G., Zhang L., Jahn-Hofmann K., Hadwiger P., Manoharan M. and Maier M. A. (2010) Modulation of thermal stability can enhance the potency of siRNA. Nucleic Acids Res. 38, 7320-7331.
[37] Khvorova A., Reynolds A. and Jayasena S. D. (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell. 115, 209-216.
[38] Schwarz D. S., Hutvagner G., Du T., Xu Z., Aronin N. and Zamore P. D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell. 115, 199-208.
[39] Haley B. and Zamore P. D. (2004) Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol. 11, 599-606.

[40] Elbashir S. M., Martinez J., Patkaniowska A., Lendeckel W. and Tuschl T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. Embo J. 20, 6877-6888.
[41] Liu J., Carmell M. A., Rivas F. V., Marsden C. G., Thomson J. M., Song J. J., Hammond S. M., Joshua-Tor L. and Hannon G. J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science. 305, 1437-1441.
[42] Braasch D. A. and Corey D. R. (2002) Novel antisense and peptide nucleic acid strategies for controlling gene expression. Biochemistry. 41, 4503-4510.
[43] Dorsett Y. and Tuschl T. (2004) siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov. 3, 318-329.
[44] Bertrand J. R., Pottier M., Vekris A., Opolon P., Maksimenko A. and Malvy C. (2002) Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. Biochem Biophys Res Commun. 296, 1000-1004.
[45] Elbashir S. M., Lendeckel W. and Tuschl T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 15, 188-200.
[46] Prakash T. P., Allerson C. R., Dande P., Vickers T. A., Sioufi N., Jarres R., Baker B. F., Swayze E. E., Griffey R. H. and Bhat B. (2005) Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J Med Chem. 48, 4247-4253.
[47] Chu C. Y. and Rana T. M. (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoS Biol. 4, e210.
[48] Jakymiw A., Pauley K. M., Li S., Ikeda K., Lian S., Eystathioy T., Satoh M., Fritzler M. J. and Chan E. K. (2007) The role of GW/P-bodies in RNA processing and silencing. J Cell Sci. 120, 1317-1323.
[49] Chalk A. M., Wahlestedt C. and Sonnhammer E. L. (2004) Improved and automated prediction of effective siRNA. Biochem Biophys Res Commun. 319, 264-274.
[50] Reynolds A., Leake D., Boese Q., Scaringe S., Marshall W. S. and Khvorova A. (2004) Rational siRNA design for RNA interference. Nat Biotechnol. 22, 326-330.
[51] Brown K. M., Chu C. Y. and Rana T. M. (2005) Target accessibility dictates the potency of human RISC. Nat Struct Mol Biol. 12, 469-470.
[52] Gredell J. A., Berger A. K. and Walton S. P. (2008) Impact of target mRNA structure on siRNA silencing efficiency: A large-scale study. Biotechnol Bioeng. 100, 744-755.
[53] Holen T., Amarzguioui M., Wiiger M. T., Babaie E. and Prydz H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. 30, 1757-1766
[54] Westerhout E. M. and Berkhout B. (2007) A systematic analysis of the effect of target RNA structure on RNA interference. Nucleic Acids Res. 35, 4322-4330.
[55] Amarzguioui M., Brede G., Babaie E., Grotli M., Sproat B. and Prydz H. (2000) Secondary structure prediction and in vitro accessibility of mRNA as tools in the selection of target sites for ribozymes. Nucleic Acids Res. 28, 4113-4124.
[56] Braasch D. A., Paroo Z., Constantinescu A., Ren G., Oz O. K., Mason R. P. and Corey D. R. (2004) Biodistribution of phosphodiester and phosphorothioate siRNA. Bioorg Med Chem Lett. 14, 1139-1143.
[57] Soutschek J., Akinc A., Bramlage B., Charisse K., Constien R., Donoghue M., Elbashir S., Geick A., Hadwiger P., Harborth J., John M., Kesavan V., Lavine G., Pandey R. K., Racie T., Rajeev K. G., Rohl I., Toudjarska I., Wang G., Wuschko S., Bumcrot D., Koteliansky

V., Limmer S., Manoharan M. and Vornlocher H. P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. 432, 173-178.
[58] Chiu Y. L. and Rana T. M. (2003) siRNA function in RNAi: a chemical modification analysis. Rna. 9, 1034-1048.
[59] Czauderna F., Fechtner M., Dames S., Aygun H., Klippel A., Pronk G. J., Giese K. and Kaufmann J. (2003) Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. 31, 2705-2716.
[60] Haupenthal J., Baehr C., Kiermayer S., Zeuzem S. and Piiper A. (2006) Inhibition of RNAse A family enzymes prevents degradation and loss of silencing activity of siRNAs in serum. Biochem Pharmacol. 71, 702-710.
[61] Morrissey D. V., Blanchard K., Shaw L., Jensen K., Lockridge J. A., Dickinson B., McSwiggen J. A., Vargeese C., Bowman K., Shaffer C. S., Polisky B. A. and Zinnen S. (2005) Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. Hepatology. 41, 1349-1356.
[62] Turner J. J., Jones S. W., Moschos S. A., Lindsay M. A. and Gait M. J. (2007) MALDI-TOF mass spectral analysis of siRNA degradation in serum confirms an RNAse A-like activity. Mol Biosyst. 3, 43-50.
[63] White P. J. (2008) Barriers to successful delivery of short interfering RNA after systemic administration. Clin Exp Pharmacol Physiol. 35, 1371-1376.
[64] Judge A. D., Bola G., Lee A. C. and MacLachlan I. (2006) Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. Mol Ther. 13, 494-505.
[65] Manoharan M. (2004) RNA interference and chemically modified small interfering RNAs. Curr Opin Chem Biol. 8, 570-579.
[66] Robbins M., Judge A., Liang L., McClintock K., Yaworski E. and MacLachlan I. (2007) 2'-O-methyl-modified RNAs act as TLR7 antagonists. Mol Ther. 15, 1663-1669.
[67] Judge A. and MacLachlan I. (2008) Overcoming the innate immune response to small interfering RNA. Hum Gene Ther. 19, 111-124.
[68] Takeda K. and Akira S. (2005) Toll-like receptors in innate immunity. Int Immunol. 17, 1-14.
[69] Zamanian-Daryoush M., Marques J. T., Gantier M. P., Behlke M. A., John M., Rayman P., Finke J. and Williams B. R. (2008) Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells. J Interferon Cytokine Res. 28, 221-233.
[70] Kleinman M. E., Yamada K., Takeda A., Chandrasekaran V., Nozaki M., Baffi J. Z., Albuquerque R. J., Yamasaki S., Itaya M., Pan Y., Appukuttan B., Gibbs D., Yang Z., Kariko K., Ambati B. K., Wilgus T. A., DiPietro L. A., Sakurai E., Zhang K., Smith J. R., Taylor E. W. and Ambati J. (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature. 452, 591-597.
[71] Braasch D. A., Jensen S., Liu Y., Kaur K., Arar K., White M. A. and Corey D. R. (2003) RNA interference in mammalian cells by chemically-modified RNA. Biochemistry. 42, 7967-7975.
[72] Choung S., Kim Y. J., Kim S., Park H. O. and Choi Y. C. (2006) Chemical modification of siRNAs to improve serum stability without loss of efficacy. Biochem Biophys Res Commun. 342, 919-927.

[73] Kraynack B. A. and Baker B. F. (2006) Small interfering RNAs containing full 2'-O-methylribonucleotide-modified sense strands display Argonaute2/eIF2C2-dependent activity. RNA. 12, 163-176.
[74] Hamada M., Ohtsuka T., Kawaida R., Koizumi M., Morita K., Furukawa H., Imanishi T., Miyagishi M. and Taira K. (2002) Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of siRNAs. Antisense Nucleic Acid Drug Dev. 12, 301-309.
[75] Parrish S., Fleenor J., Xu S., Mello C. and Fire A. (2000) Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol Cell. 6, 1077-1087.
[76] Watts J. K., Deleavey G. F. and Damha M. J. (2008) Chemically modified siRNA: tools and applications. Drug Discov Today. 13, 842-855.
[77] Allerson C. R., Sioufi N., Jarres R., Prakash T. P., Naik N., Berdeja A., Wanders L., Griffey R. H., Swayze E. E. and Bhat B. (2005) Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. J Med Chem. 48, 901-904.
[78] Layzer J. M., McCaffrey A. P., Tanner A. K., Huang Z., Kay M. A. and Sullenger B. A. (2004) In vivo activity of nuclease-resistant siRNAs. RNA. 10, 766-771.
[79] Muhonen P., Tennila T., Azhayeva E., Parthasarathy R. N., Janckila A. J., Vaananen H. K., Azhayev A. and Laitala-Leinonen T. (2007) RNA interference tolerates 2'-fluoro modifications at the Argonaute2 cleavage site. Chem Biodivers. 4, 858-873.
[80] Ikeda H., Fernandez R., Wilk A., Barchi J. J., Jr., Huang X. and Marquez V. E. (1998) The effect of two antipodal fluorine-induced sugar puckers on the conformation and stability of the Dickerson-Drew dodecamer duplex [d(CGCGAATTCGCG)]2. Nucleic Acids Res. 26, 2237-2244.
[81] Damha M. J., Noronha A. M., Wilds C. J., Trempe J. F., Denisov A., Pon R. T. and Gehring K. (2001) Properties of arabinonucleic acids (ANA & 20'F-ANA): implications for the design of antisense therapeutics that invoke RNase H cleavage of RNA. Nucleosides Nucleotides Nucleic Acids. 20, 429-440.
[82] Ui-Tei K., Naito Y., Zenno S., Nishi K., Yamato K., Takahashi F., Juni A. and Saigo K. (2008) Functional dissection of siRNA sequence by systematic DNA substitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. Nucleic Acids Res. 36, 2136-2151.
[83] Amarzguioui M., Holen T., Babaie E. and Prydz H. (2003) Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. 31, 589-595.
[84] Dorn G., Patel S., Wotherspoon G., Hemmings-Mieszczak M., Barclay J., Natt F. J., Martin P., Bevan S., Fox A., Ganju P., Wishart W. and Hall J. (2004) siRNA relieves chronic neuropathic pain. Nucleic Acids Res. 32, e49.
[85] Chen X., Shen L. and Wang J. H. (2004) Poly-2'-DNP-RNAs with enhanced efficacy for inhibiting cancer cell growth. Oligonucleotides. 14, 90-99.
[86] Liao H. and Wang J. H. (2005) Biomembrane-permeable and Ribonuclease-resistant siRNA with enhanced activity. Oligonucleotides. 15, 196-205.
[87] Ashun M. A., Hu Y., Kang I., Li C. C. and Wang J. H. (1996) Inhibition of murine leukemia virus with poly-2'-O-(2,4-dinitrophenyl)poly[A]. Antimicrob Agents Chemother. 40, 2311-2317.

[88] Bramsen J. B., Laursen M. B., Nielsen A. F., Hansen T. B., Bus C., Langkjaer N., Babu B. R., Hojland T., Abramov M., Van Aerschot A., Odadzic D., Smicius R., Haas J., Andree C., Barman J., Wenska M., Srivastava P., Zhou C., Honcharenko D., Hess S., Muller E., Bobkov G. V., Mikhailov S. N., Fava E., Meyer T. F., Chattopadhyaya J., Zerial M., Engels J. W., Herdewijn P., Wengel J. and Kjems J. (2009) A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. Nucleic Acids Res. 37, 2867-2881.

[89] De Mesmaeker A., Altmann K. H., Waldner A. and Wendeborn S. (1995) Backbone modifications in oligonucleotides and peptide nucleic acid systems. Curr Opin Struct Biol. 5, 343-355.

[90] Inoue H., Hayase Y., Imura A., Iwai S., Miura K. and Ohtsuka E. (1987) Synthesis and hybridization studies on two complementary nona(2'-O-methyl)ribonucleotides. Nucleic Acids Res. 15, 6131-6148.

[91] Dande P., Prakash T. P., Sioufi N., Gaus H., Jarres R., Berdeja A., Swayze E. E., Griffey R. H. and Bhat B. (2006) Improving RNA interference in mammalian cells by 4'-thio-modified small interfering RNA (siRNA): effect on siRNA activity and nuclease stability when used in combination with 2'-O-alkyl modifications. J Med Chem. 49, 1624-1634.

[92] Hoshika S., Minakawa N., Kamiya H., Harashima H. and Matsuda A. (2005) RNA interference induced by siRNAs modified with 4'-thioribonucleosides in cultured mammalian cells. FEBS Lett. 579, 3115-3118.

[93] Hoshika S., Minakawa N., Shionoya A., Imada K., Ogawa N. and Matsuda A. (2007) Study of modification pattern-RNAi activity relationships by using siRNAs modified with 4'-thioribonucleosides. Chembiochem. 8, 2133-2138.

[94] Braasch D. A. and Corey D. R. (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol. 8, 1-7.

[95] Grunweller A., Wyszko E., Bieber B., Jahnel R., Erdmann V. A. and Kurreck J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-O-methyl RNA, phosphorothioates and small interfering RNA. Nucleic Acids Res. 31, 3185-3193.

[96] Elmen J., Thonberg H., Ljungberg K., Frieden M., Westergaard M., Xu Y., Wahren B., Liang Z., Orum H., Koch T. and Wahlestedt C. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res. 33, 439-447.

[97] Hornung V., Guenthner-Biller M., Bourquin C., Ablasser A., Schlee M., Uematsu S., Noronha A., Manoharan M., Akira S., de Fougerolles A., Endres S. and Hartmann G. (2005) Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med. 11, 263-270.

[98] Mook O. R., Baas F., de Wissel M. B. and Fluiter K. (2007) Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. Mol Cancer Ther. 6, 833-843.

[99] Bramsen J. B., Laursen M. B., Damgaard C. K., Lena S. W., Babu B. R., Wengel J. and Kjems J. (2007) Improved silencing properties using small internally segmented interfering RNAs. Nucleic Acids Res. 35, 5886-5897.

[100] Herdewijn P., Allart B., De Bouvere B., De Winter H., Hendrix C., Hossain N., Schepers G., Verheggen I., Wroblowski B. and Van Aerschot A. (1999) Properties of oligonucleotides with six membered base moiety and the hydroxymethyl group mimics

and a 1,4-relationship between the base moiety and the hydroxymethyl group. Nucleosides & Nucleotides. 18, 1371-1376.

[101] Kozlov I. A., Zielinski M., Allart B., Kerremans L., Van Aerschot A., Busson R., Herdewijn P. and Orgel L. E. (2000) Nonenzymatic template-directed reactions on altritol oligomers, preorganized analogues of oligonucleotides. Chemistry. 6, 151-155.

[102] Herdewijn P., Allart B., De Bouvere B., De Winter H., Hendrix C., Hossain N., Schepers G., Verheggen I., Wroblowski B. and Van Aerschot A. (1999) Properties of oligonucleotides with six membered base moiety and the hydroxymethyl group mimics and a 1,4-relationship between the base moiety and the hydroxymethyl group. Nucleosides & Nucleotides. 18, 1371-1376.

[103] Fisher M., Abramov M., Van Aerschot A., Xu D., Juliano R. L. and Herdewijn P. (2007) Inhibition of MDR1 expression with altritol-modified siRNAs. Nucleic Acids Res. 35, 1064-1074.

[104] Kenski D. M., Cooper A. J., Li J. J., Willingham A. T., Haringsma H. J., Young T. A., Kuklin N. A., Jones J. J., Cancilla M. T., McMasters D. R., Mathur M., Sachs A. B. and Flanagan W. M. (2010) Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5'-terminal phosphorylation both in vitro and in vivo. Nucleic Acids Res. 38, 660-671.

[105] Langkjaer N., Pasternak A. and Wengel J. (2009) UNA (unlocked nucleic acid): a flexible RNA mimic that allows engineering of nucleic acid duplex stability. Bioorg Med Chem. 17, 5420-5425.

[106] Kenski D. M., Cooper A. J., Li J. J., Willingham A. T., Haringsma H. J., Young T. A., Kuklin N. A., Jones J. J., Cancilla M. T., McMasters D. R., Mathur M., Sachs A. B. and Flanagan W. M. (2010) Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5'-terminal phosphorylation both in vitro and in vivo. Nucleic Acids Res. 38, 660-671.

[107] Werk D., Wengel J., Wengel S. L., Grunert H. P., Zeichhardt H. and Kurreck J. (2010) Application of small interfering RNAs modified by unlocked nucleic acid (UNA) to inhibit the heart-pathogenic coxsackievirus B3. FEBS Lett. 584, 591-598.

[108] Brown S. C., Thomson S. A., Veal J. M. and Davis D. G. (1994) NMR solution structure of a peptide nucleic acid complexed with RNA. Science. 265, 777-780.

[109] Demidov V. V., Potaman V. N., Frank-Kamenetskii M. D., Egholm M., Buchard O., Sonnichsen S. H. and Nielsen P. E. (1994) Stability of peptide nucleic acids in human serum and cellular extracts. Biochem Pharmacol. 48, 1310-1313.

[110] Potenza N., Moggio L., Milano G., Salvatore V., Di Blasio B., Russo A. and Messere A. (2008) RNA interference in mammalia cells by RNA-3'-PNA chimeras. Int J Mol Sci. 9, 299-315.

[111] Harborth J., Elbashir S. M., Vandenburgh K., Manninga H., Scaringe S. A., Weber K. and Tuschl T. (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev. 13, 83-105.

[112] Hall A. H., Wan J., Shaughnessy E. E., Ramsay Shaw B. and Alexander K. A. (2004) RNA interference using boranophosphate siRNAs: structure-activity relationships. Nucleic Acids Res. 32, 5991-6000.

[113] Schwarz D. S., Tomari Y. and Zamore P. D. (2004) The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr Biol. 14, 787-791.
[114] Lee M., Simon A. D., Stein C. A. and Rabbani L. E. (1999) Antisense strategies to inhibit restenosis. Antisense Nucleic Acid Drug Dev. 9, 487-492.
[115] Prakash T. P., Kraynack B., Baker B. F., Swayze E. E. and Bhat B. (2006) RNA interference by 2',5'-linked nucleic acid duplexes in mammalian cells. Bioorg Med Chem Lett. 16, 3238-3240.
[116] Iwase R., Toyama T. and Nishimori K. (2007) Solid-phase synthesis of modified RNAs containing amide-linked oligoribonucleosides at their 3'-end and their application to siRNA. Nucleosides Nucleotides Nucleic Acids. 26, 1451-1454.
[117] Saenger W. (1984) Forces stabilizing association between bases: Hydrogen bonding and base stacking. In Principle of nucleic acids structure. Springer-Verlag, New York.
[118] Agris P. F., Sierzputowska-Gracz H., Smith W., Malkiewicz A., Sochacka E. and Nawrot B. (1992) Thiolation of uridine carbon-2 restricts the motional dynamics of the transfer RNA wobble position nucleoside. J. Am. Chem. Soc. 114, 2652-2656.
[119] Davis D. R., Veltri C. A. and Nielsen L. (1998) An RNA model system for investigation of pseudouridine stabilization of the codon-anticodon interaction in tRNALys, tRNAHis and tRNATyr. J Biomol Struct Dyn. 15, 1121-1132.
[120] Sipa K., Sochacka E., Kazmierczak-Baranska J., Maszewska M., Janicka M., Nowak G. and Nawrot B. (2007) Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. RNA. 13, 1301-1316.
[121] Morrissey D. V., Lockridge J. A., Shaw L., Blanchard K., Jensen K., Breen W., Hartsough K., Machemer L., Radka S., Jadhav V., Vaish N., Zinnen S., Vargeese C., Bowman K., Shaffer C. S., Jeffs L. B., Judge A., MacLachlan I. and Polisky B. (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol. 23, 1002-1007.
[122] Schwarz D. S., Hutvagner G., Haley B. and Zamore P. D. (2002) Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol Cell. 10, 537-548.
[123] Chen P. Y., Weinmann L., Gaidatzis D., Pei Y., Zavolan M., Tuschl T. and Meister G. (2008) Strand-specific 5'-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. RNA. 14, 263-274.
[124] Lorenz C., Hadwiger P., John M., Vornlocher H. P. and Unverzagt C. (2004) Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. Bioorg Med Chem Lett. 14, 4975-4977.
[125] Wolfrum C., Shi S., Jayaprakash K. N., Jayaraman M., Wang G., Pandey R. K., Rajeev K. G., Nakayama T., Charrise K., Ndungo E. M., Zimmermann T., Koteliansky V., Manoharan M. and Stoffel M. (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotechnol. 25, 1149-1157.
[126] Thomas M., Kularatne S. A., Qi L., Kleindl P., Leamon C. P., Hansen M. J. and Low P. S. (2009) Ligand-targeted delivery of small interfering RNAs to malignant cells and tissues. Ann N Y Acad Sci. 1175, 32-39.
[127] Moschos S. A., Jones S. W., Perry M. M., Williams A. E., Erjefalt J. S., Turner J. J., Barnes P. J., Sproat B. S., Gait M. J. and Lindsay M. A. (2007) Lung delivery studies

using siRNA conjugated to TAT(48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. Bioconjug Chem. 18, 1450-1459.
[128] Muratovska A. and Eccles M. R. (2004) Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett. 558, 63-68.
[129] McNamara J. O., 2nd, Andrechek E. R., Wang Y., Viles K. D., Rempel R. E., Gilboa E., Sullenger B. A. and Giangrande P. H. (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol. 24, 1005-1015.
[130] Chiu Y. L. and Rana T. M. (2002) RNAi in human cells: basic structural and functional features of small interfering RNA. Mol Cell. 10, 549-561.
[131] Martinez J., Patkaniowska A., Urlaub H., Luhrmann R. and Tuschl T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell. 110, 563-574.
[132] De Paula D., Bentley M. V. and Mahato R. I. (2007) Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. Rna. 13, 431-456.
[133] Gaglione M. and Messere A. (2010) Recent progress in chemically modified siRNAs. Mini Rev Med Chem. 10, 578-595.
[134] Burnett J. R. and Barrett P. H. (2002) Apolipoprotein B metabolism: tracer kinetics, models, and metabolic studies. Crit Rev Clin Lab Sci. 39, 89-137.
[135] Feinberg E. H. and Hunter C. P. (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. Science. 301, 1545-1547.
[136] Potocky T. B., Menon A. K. and Gellman S. H. (2003) Cytoplasmic and nuclear delivery of a TAT-derived peptide and a beta-peptide after endocytic uptake into HeLa cells. J Biol Chem. 278, 50188-50194.
[137] Richard J. P., Melikov K., Vives E., Ramos C., Verbeure B., Gait M. J., Chernomordik L. V. and Lebleu B. (2003) Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem. 278, 585-590.
[138] Poehlmann T. G., Ruediger T., Schaefer H., Koehn S., Imhof D., Seyfarth L., Schubert U. S. and Markert U. R. (2009) Development of small interfering RNA selectively activated in target cells. In RNAi2009: ncRNA: Bridging Biology and Therapy (Media L. P., ed.)^eds.). pp. 16-17, Library Publishing Media, Oxford, UK.
[139] Hicke B. J. and Stephens A. W. (2000) Escort aptamers: a delivery service for diagnosis and therapy. J Clin Invest. 106, 923-928.
[140] Dassie J. P., Liu X. Y., Thomas G. S., Whitaker R. M., Thiel K. W., Stockdale K. R., Meyerholz D. K., McCaffrey A. P., McNamara J. O., 2nd and Giangrande P. H. (2009) Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. Nat Biotechnol. 27, 839-849.
[141] Xia C. F., Boado R. J. and Pardridge W. M. (2009) Antibody-mediated targeting of siRNA via the human insulin receptor using avidin-biotin technology. Mol Pharm. 6, 747-751.
[142] Xia C. F., Zhang Y., Boado R. J. and Pardridge W. M. (2007) Intravenous siRNA of brain cancer with receptor targeting and avidin-biotin technology. Pharm Res. 24, 2309-2316.
[143] Xia W. and Low P. S. Folate-targeted therapies for cancer. (2010) J Med Chem. 53, 6811-6824.
[144] Kim S. H., Mok H., Jeong J. H., Kim S. W. and Park T. G. (2006) Comparative evaluation of target-specific GFP gene silencing efficiencies for antisense ODN,

synthetic siRNA, and siRNA plasmid complexed with PEI-PEG-FOL conjugate. Bioconjug Chem. 17, 241-244.
[145] Zhang K., Wang Q., Xie Y., Mor G., Sega E., Low P. S. and Huang Y. (2008) Receptor-mediated delivery of siRNAs by tethered nucleic acid base-paired interactions. RNA. 14, 577-583.
[146] Jeong J. H., Mok H., Oh Y. K. and Park T. G. (2009) siRNA conjugate delivery systems. Bioconjug Chem. 20, 5-14.
[147] Kawakami S. and Hashida M. (2007) Targeted delivery systems of small interfering RNA by systemic administration. Drug Metab Pharmacokinet. 22, 142-151.
[148] Leng Q., Woodle M. C., Lu P. Y. and Mixson A. J. (2009) Advances in Systemic siRNA Delivery. Drugs Future. 34, 721.
[149] Lv H., Zhang S., Wang B., Cui S. and Yan J. (2006) Toxicity of cationic lipids and cationic polymers in gene d Xia W. and Low P. S. Folate-targeted therapies for cancer. (2010) J Med Chem. 53, 6811-6824.elivery. J Control Release. 114, 100-109.
[150] de Fougerolles A., Vornlocher H. P., Maraganore J. and Lieberman J. (2007) Interfering with disease: a progress report on siRNA-based therapeutics. Nat Rev Drug Discov. 6, 443-453.
[151] Wu Y., Navarro F., Lal A., Basar E., Pandey R. K., Manoharan M., Feng Y., Lee S. J., Lieberman J. and Palliser D. (2009) Durable protection from Herpes Simplex Virus-2 transmission following intravaginal application of siRNAs targeting both a viral and host gene. Cell Host Microbe. 5, 84-94.
[152] Blidner R. A., Hammer R. P., Lopez M. J., Robinson S. O. and Monroe W. T. (2007) Fully 2'-deoxy-2'-fluoro substituted nucleic acids induce RNA interference in mammalian cell culture. Chem Biol Drug Des. 70, 113-122.
[153] Corey D. R. (2007) Chemical modification: the key to clinical application of RNA interference? J Clin Invest. 117, 3615-3622.
[154] Jeong J. H., Mok H., Oh Y. K. and Park T. G. (2009) siRNA conjugate delivery systems. Bioconjug Chem. 20, 5-14.
[155] Jackson A. L., Burchard J., Leake D., Reynolds A., Schelter J., Guo J., Johnson J. M., Lim L., Karpilow J., Nichols K., Marshall W., Khvorova A. and Linsley P. S. (2006) Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA. 12, 1197-1205.
[156] Macrae I. J., Zhou K., Li F., Repic A., Brooks A. N., Cande W. Z., Adams P. D. and Doudna J. A. (2006) Structural basis for double-stranded RNA processing by Dicer. Science. 311, 195-198.
[157] Myers J. W., Jones J. T., Meyer T. and Ferrell J. E., Jr. (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. Nat Biotechnol. 21, 324-328.
[158] Hohjoh H. (2004) Enhancement of RNAi activity by improved siRNA duplexes. FEBS Lett. 557, 193-198.
[159] Ohnishi Y., Tokunaga K. and Hohjoh H. (2005) Influence of assembly of siRNA elements into RNA-induced silencing complex by вилко-siRNA duplex carrying nucleotide mismatches at the 3'- or 5'-end of the sense-stranded siRNA element. Biochem Biophys Res Commun. 329, 516-521.

[160] Kubo T., Zhelev Z., Ohba H. and Bakalova R. (2007) Modified 27-nt dsRNAs with dramatically enhanced stability in serum and long-term RNAi activity. Oligonucleotides. 17, 445-464.
[161] Rose S. D., Kim D. H., Amarzguioui M., Heidel J. D., Collingwood M. A., Davis M. E., Rossi J. J. and Behlke M. A. (2005) Functional polarity is introduced by Dicer processing of short substrate RNAs. Nucleic Acids Res. 33, 4140-4156.
[162] Sano M., Sierant M., Miyagishi M., Nakanishi M., Takagi Y. and Sutou S. (2008) Effect of asymmetric terminal structures of short RNA duplexes on the RNA interference activity and strand selection. Nucleic Acids Res. 36, 5812-5821.
[163] Masquida B. and Westhof E. (2000) On the wobble GoU and related pairs. RNA. 6, 9-15.
[164] Ding H., Liao G., Wang H. and Zhou Y. (2007) Asymmetrically designed siRNAs and shRNAs enhance the strand specificity and efficacy in RNAi. J RNAi Gene Silencing. 4, 269-280.
[165] Patzel V., Rutz S., Dietrich I., Koberle C., Scheffold A. and Kaufmann S. H. (2005) Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. Nat Biotechnol. 23, 1440-1444.
[166] Hu X., Hipolito S., Lynn R., Abraham V., Ramos S. and Wong-Staal F. (2004) Relative gene-silencing efficiencies of small interfering RNAs targeting sense and antisense transcripts from the same genetic locus. Nucleic Acids Res. 32, 4609-4617.
[167] Patzel V. (2007) In silico selection of active siRNA. Drug Discov Today. 12, 139-148.
[168] Lingel A., Simon B., Izaurralde E. and Sattler M. (2004) Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nat Struct Mol Biol. 11, 576-577.
[169] Marques J. T., Devosse T., Wang D., Zamanian-Daryoush M., Serbinowski P., Hartmann R., Fujita T., Behlke M. A. and Williams B. R. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. Nat Biotechnol. 24, 559-565.
[170] Guo S. and Kemphues K. J. (1995) par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell. 81, 611-620.
[171] Tijsterman M., Ketting R. F., Okihara K. L., Sijen T. and Plasterk R. H. (2002) RNA helicase MUT-14-dependent gene silencing triggered in C. elegans by short antisense RNAs. Science. 295, 694-697.
[172] Amarzguioui M., Rossi J. J. and Kim D. (2005) Approaches for chemically synthesized siRNA and vector-mediated RNAi. FEBS Lett. 579, 5974-5981.
[173] Collingwood M. A., Rose S. D., Huang L., Hillier C., Amarzguioui M., Wiiger M. T., Soifer H. S., Rossi J. J. and Behlke M. A. (2008) Chemical modification patterns compatible with high potency dicer-substrate small interfering RNAs. Oligonucleotides. 18, 187-200.
[174] Kim D. H., Behlke M. A., Rose S. D., Chang M. S., Choi S. and Rossi J. J. (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol. 23, 222-226.
[175] Reynolds A., Anderson E. M., Vermeulen A., Fedorov Y., Robinson K., Leake D., Karpilow J., Marshall W. S. and Khvorova A. (2006) Induction of the interferon response by siRNA is cell type- and duplex length-dependent. RNA. 12, 988-993.

[176] Bridge A. J., Pebernard S., Ducraux A., Nicoulaz A. L. and Iggo R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet. 34, 263-264.
[177] Persengiev S. P., Zhu X. and Green M. R. (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). Rna. 10, 12-18.
[178] Sledz C. A., Holko M., de Veer M. J., Silverman R. H. and Williams B. R. (2003) Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. 5, 834-839.
[179] Hornung V., Ellegast J., Kim S., Brzozka K., Jung A., Kato H., Poeck H., Akira S., Conzelmann K. K., Schlee M., Endres S. and Hartmann G. (2006) 5'-Triphosphate RNA is the ligand for RIG-I. Science. 314, 994-997.
[180] Judge A. D., Sood V., Shaw J. R., Fang D., McClintock K. and MacLachlan I. (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol. 23, 457-462.
[181] Shukla S., Sumaria C. S. and Pradeepkumar P. I. (2010) Exploring chemical modifications for siRNA therapeutics: a structural and functional outlook. ChemMedChem. 5, 328-349.
[182] Amarzguioui M. and Prydz H. (2004) An algorithm for selection of functional siRNA sequences. Biochem Biophys Res Commun. 316, 1050-1058.
[183] Matveeva O., Nechipurenko Y., Rossi L., Moore B., Saetrom P., Ogurtsov A. Y., Atkins J. F. and Shabalina S. A. (2007) Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res. 35, e63.
[184] Shabalina S. A., Spiridonov A. N. and Ogurtsov A. Y. (2006) Computational models with thermodynamic and composition features improve siRNA design. BMC Bioinformatics. 7, 65.
[185] Ui-Tei K., Naito Y., Takahashi F., Haraguchi T., Ohki-Hamazaki H., Juni A., Ueda R. and Saigo K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32, 936-948.
[186] Walton S. P., Wu M., Gredell J. A. and Chan C. (2010) Designing highly active siRNAs for therapeutic applications. FEBS J. 277, 4806-4813.