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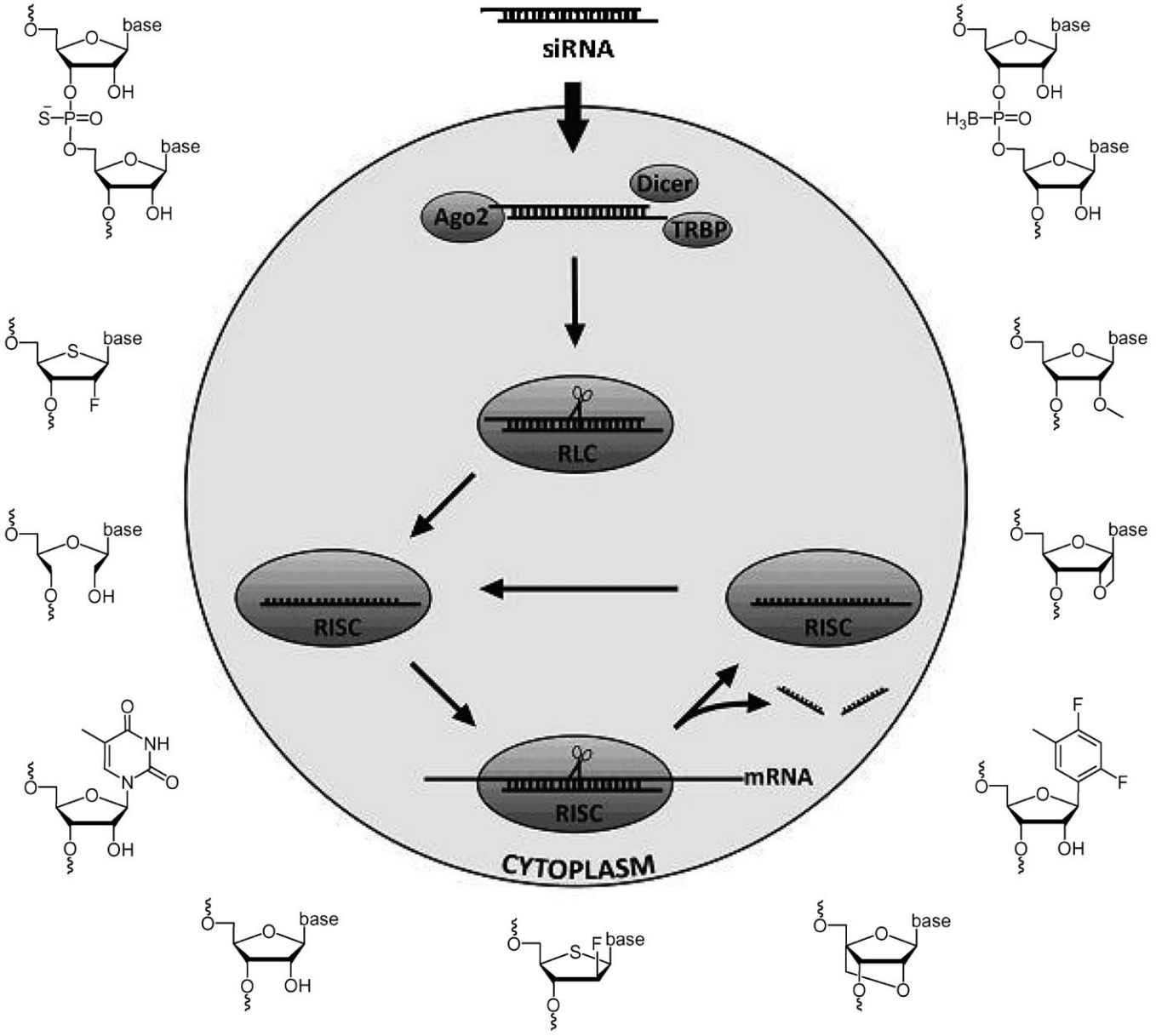
Exploring Chemical Modifications for siRNA Therapeutics: A Structural and Functional Outlook

Siddharth Shukla, Chintan S. Sumaria, and P. I. Pradeepkumar*[a]

Dedicated to Professor Jyoti Chattopadhyaya on the occasion of his 60th birthday.



RNA interference (RNAi) is a post-transcriptional gene silencing mechanism induced by small interfering RNAs (siRNAs) and micro-RNAs (miRNAs), and has proved to be one of the most important scientific discoveries made in the last century. The robustness of RNAi has opened up new avenues in the development of siRNAs as therapeutic agents against various diseases including cancer and HIV. However, there had remained a lack of a clear mechanistic understanding of messenger RNA (mRNA) cleavage mediated by Argonaute2 of the RNA-induced silencing complex (RISC), due to inadequate structural data. The X-ray crystal structures of the Argonaute (Ago)–DNA–RNA complexes reported recently have proven to be a breakthrough in this field, and the structural details can provide guidelines for the design of the next generation of siRNA therapeutics. To harness siRNAs as therapeutic agents, the prudent use of various chemical modifications is warranted to enhance nuclease resistance, prevent immune activation, decrease off-target effects, and to improve pharmacokinetic and pharmacodynamic properties. The focus of this review is to interpret the tolerance of various chemical modifications employed in siRNAs toward RNAi by taking into account the crystal structures and biochemical studies of Ago–RNA complexes. Moreover, the challenges and recent progress in imparting druglike properties to siRNAs along with their delivery strategies are discussed.

1. Introduction

Gene silencing in plants was reported back in 1990 by Jorgensen and co-workers.[1] In their attempt to overexpress a pigmentation gene in petunia through the introduction of a transgene, the group observed an unexpected loss of pigmentation in the flowers of 42% of the plants. This phenomenon was reported to be caused by a decrease in messenger RNA (mRNA) levels produced by the homologous natural gene through a process termed "co-suppression".[1] A similar post-transcriptional gene silencing (PTGS) effect termed "quelling" was reported in 1992; the authors observed that the introduction of exogenous genes in *Neurospora crassa* results in the repression of two genes critical for the albino phenotype.[2] In 1995, Guo and Kemphues demonstrated the capacity of single-stranded RNAs (ssRNAs) to induce PTGS in the nematode *Caenorhabditis elegans*.[3] However, the role of double-stranded RNA (dsRNA) in gene silencing was not evident in the effects discovered in plants and *C. elegans*. Clear evidence for the participation of dsRNA in PTGS was revealed in 1998 by Fire, Mello, and co-workers, who reported that an externally introduced long dsRNA could decrease the expression of a complementary mRNA in *C. elegans*.[4] This PTGS mechanism was termed as RNA interference (RNAi). Further research in this field brought to forefront two important classes of gene regulators: small interfering RNAs (siRNAs) and micro-RNAs (miRNAs).[5–7] Research into the therapeutic applications of RNAi gained momentum after the seminal discovery in 2001 by Tuschl and co-workers that synthetic 21-nucleotide (nt) siRNAs could trigger RNAi in mammalian cells.[8] The roles of protein assemblies in gene silencing induced by both siRNAs and miRNAs also came to the forefront. While enzymes belonging to the Dicer family were shown to be important for dsRNA cleavage into small RNAs,[9,10] the Argonaute (Ago) family of proteins were shown to be the core of silencing assemblies.[11] Both siRNA- and miRNA-induced RNAi mechanisms progress by selective incorporation of one of the strands into a protein complex known as the RNA-induced silencing complex (RISC); subsequent accommodation of the target mRNA strand thus silences its expression.[12,13]

The rapid advances in siRNA therapeutics are underscored by the fact that the first clinical trial of an siRNA-based drug started in 2004, just three years after the discovery of synthetic siRNA-induced RNAi. Several siRNA-based drugs are currently under clinical evaluation (Table 1).[14,15] Along with the significant potential benefits come numerous challenges associated with the therapeutic application of siRNAs. Unmodified siRNAs are not stable enough to persist unaided in the bloodstream[16] and can induce an interferon response from the innate immune system.[17] Another problem is their delivery to target cells, while ensuring that they do not induce unwanted off-target effects.[18–20] siRNA therapeutics must also be cost effective in order to be viable replacements for small-molecule drugs. Appropriate chemical modifications are therefore required to decrease toxicity and to impart better pharmacokinetic and pharmacodynamic properties to siRNAs.[14,21–23]

Recently reported crystal structures[24–26] and biochemical studies[27] of the RISC have proven to be very important in the context of the aforementioned therapeutic challenges. The insight provided by these studies is helpful for determining the critical properties of the siRNA–Argonaute2 (Ago2) complex, and will be useful for determining appropriate modifications for siRNAs. Herein we discuss the currently available chemical modifications used in siRNAs from a structural perspective. We also outline the advantages and disadvantages associated with each modification. Additionally, we summarize the toxicity emerging from off-target effects and innate immune responses, and the ways in which these problems can be addressed by judicious use of chemical modification. Advances in siRNA delivery are also discussed, along with an overview of the best available delivery strategies. Thus, the overall scope of this review is to provide a set of comprehensive guidelines for the design and evaluation of therapeutically appealing siRNAs.

[a] *S. Shukla, C. S. Sumaria, Dr. P. I. Pradeepkumar*
Department of Chemistry
Indian Institute of Technology Bombay
Powai, Mumbai, 400076 (India)
Fax: (+91) 22-25767152
E-mail: pradeep@chem.iitb.ac.in

Table 1. Some siRNA drug candidates at various clinical trial phases.[14,226]

Compound	Condition	siRNA	Administration	Phase
ALN-RSV01 (Alnylam)	RSV	Unmodified	Nasal inhalation	II
Sirna-027 (Allergan)	CNV	–	Intravitreal injection	II[a]
PF-4523655 (Quark)	AMD/DME	Alternate 2′-OMe	Intravitreal injection	II
QPI-1002 (Quark)	Acute kidney injury	Alternate 2′-OMe	Intravenous saline injection	II
Bevasiranib (OPKO Health)	AMD	Unmodified	Intravitreal injection	III[a]
PRO-040201 (Tekmira)	Hypercholesterolemia	–	Intravenous SNALP-based injection	I
CALAA-01 (Calando)	Solid tumor	Unmodified	Intravenous polyplex injection	I

[a] Terminated due to safety concerns.

Siddharth Shukla completed his pre-college education at Christ The King College, Jhansi (India) with distinction in his 10th and 12th board exams. He was awarded by The Uttar Pradesh Government Technical Council for his class 10 performance with distinction in mathematics. He secured state rank 44 in the National Science Olympiad in 2004 and national rank 281 in a science talent exam conducted by the Unified Council in 2005. He entered IIT Bombay for his undergraduate studies in 2006 after securing an all-India rank of 2967 out of $>300\,000$ applicants in the joint entrance examination (JEE), and is currently in a five-year integrated masters program in chemistry. He completed a summer internship in 2009 at the University of Arizona (USA) with Professor Victor J. Hruby on hetero-multivalent ligands for cancer therapy. He is an active participant in various cultural activities such as dramatics, music, and debating. He has also served as a journalist for the IIT Bombay student newspaper *Aawaaz*. He likes to play football and cricket, and is a fitness enthusiast.

Chintan S. Sumaria completed his pre-college education at Ramnarian Ruia Junior College, Mumbai (India) with distinction in his 10th and 12th board exams. In 2006, he secured an all-India rank of 3037 out of $>300\,000$ applicants in the joint entrance examination (JEE), and is currently in a five-year integrated masters program in chemistry at IIT Bombay. He is the recipient of a prestigious KVPY scholarship awarded by the Indian government's Department of Science and Technology. He is active in various organizational activities at IIT Bombay; he has been a part of the organizational teams of the institute cultural festival, the technical festival, the entrepreneurship promoting body, and the hostel council. In 2008 he was promoted to sole manager of infrastructure, hospitality, and accounts for E-Cell, the largest entrepreneurship-promoting student body in India. He is an avid jogger and enjoys trekking.

2. Molecular Mechanism of RNA Interference

RNAi is induced by small (21–23-nt) dsRNAs, also known as small interfering RNAs (siRNAs), which are produced intracellularly by the action of Dicer on long dsRNAs (Figure 1 a).[28] These siRNAs can also be produced intracellularly from short hairpin RNAs (shRNAs), which in turn can be generated by the transcription of externally introduced viral vectors.[29] Among the two strands of siRNAs, the one that is complementary to the target is called the *guide* or *antisense* strand, and the other is known as the *non-guide*, *sense*, or *passenger* strand (Figure 1 b). The siRNA duplex binds a set of proteins such as Ago2, Dicer, and transactivating response RNA binding protein (TRBP) to form the RISC loading complex (RLC).[30,31] Upon activation by ATP, the siRNA duplex is unwound; the passenger strand is subsequently cleaved and separated, and the RISC is formed.[32] The part of the RLC responsible for cleavage of the

P. I. Pradeepkumar was born and raised in Trivandrum, Kerala, India. He received his BSc in chemistry from University College Trivandrum in 1995 and his MSc in chemistry (first rank) from Mahatma Gandhi University, Kottayam, Kerala in 1997. During his MSc studentship he carried out summer internships as a JNCASR Summer Research Fellow at the University of Hyderabad under the guidance of Professor Goverdhan Mehta. In 2004, he received his PhD in bioorganic chemistry from Uppsala University (Sweden), where he studied chemically modified oligonucleotides as antisense therapeutic agents under the mentorship of Professor Jyoti Chattopadhyaya. From 2004 to 2007, he carried out postdoctoral research with Professor Scott Silverman at the University of Illinois at Urbana-Champaign (USA), where he studied the in vitro selection and characterization of novel DNA enzymes capable of carrying out bioorganic reactions. In 2007 he joined IIT Bombay as an assistant professor of chemistry. He is the recipient of a Max Planck India Fellowship (MPI hosts: Professor Christian Griesinger and Dr. Claudia Höbartner). His research group studies various aspects of therapeutically promising chemically modified siRNAs, DNA enzymes, and telomeric DNA stabilizing agents.

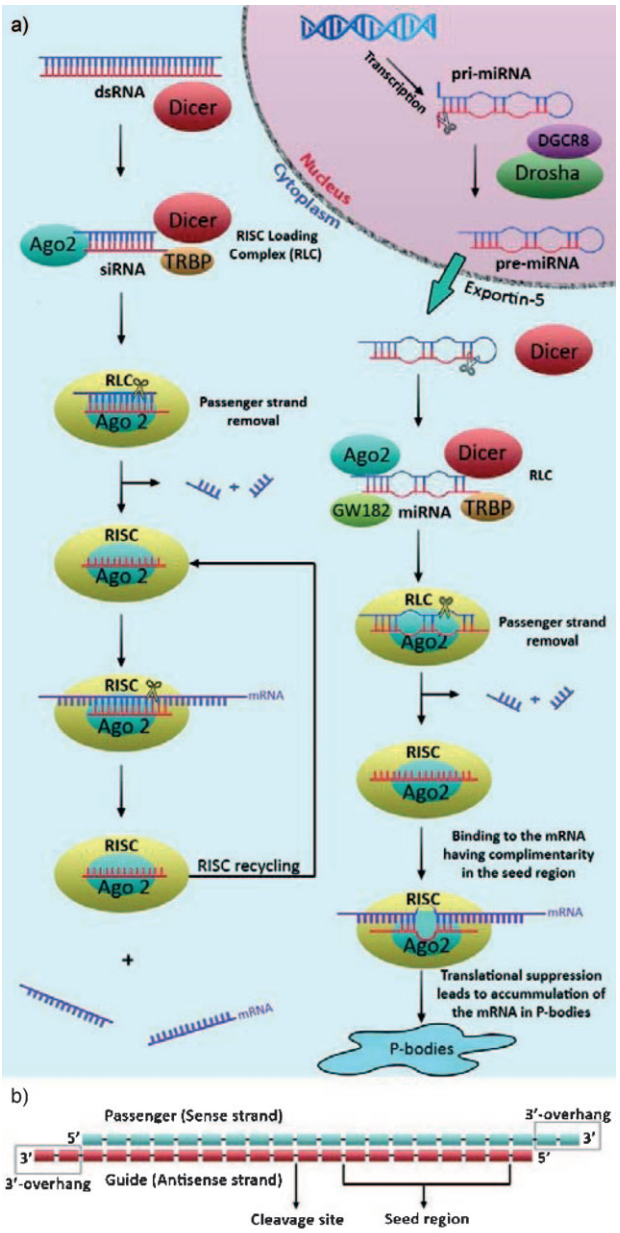


Figure 1. a) Detailed mechanism of RNAi in the siRNA and miRNA pathways. Left: Long dsRNAs are cleaved by Dicer into siRNAs; these associate with other proteins like Ago2, Dicer, and TRBP to form the RLC. Ago2 cleaves the passenger strand of the siRNA duplex to form the RISC, which contains the guide strand and Ago2 at its core. The target strand is incorporated into the RISC followed by its endonucleic cleavage by Ago2 at the position opposite from nucleotides 10–11 of the guide strand. Right: Endogenously expressed pri-miRNAs are processed by Drosha and DGCR8 to form pre-miRNAs. Exportin-5 transports these to the cytoplasm, where they are cleaved by Dicer into mature miRNAs. These miRNAs associate with various cellular proteins such as Ago2, GW182, TRBP, and Dicer to form the RLC. The RISC is formed in a manner similar to its formation in the siRNA-mediated RNAi pathway; it subsequently binds to the target sequence within the 3′ untranslated region (UTR) complementary to the seed region of the active strand, with up to two mismatches. This stops further mRNA translation, as it is removed from the translational machinery and confined into P-bodies followed by degradation. b) siRNA structure: A typical siRNA consists of two 21-nt guide and passenger strands, with two 2-nt overhangs at the 3′ ends. Nucleotides 2–8 on the guide strand constitute the seed region of the siRNA. The sequence of the seed region is involved in the initial binding of the guide strand to the target mRNA.

passenger strand is the endonuclease, Argonaute2 (Ago2).[33,34] Thermodynamic profiling of siRNAs that target several mRNAs have shown that the siRNA strand with lower thermodynamic stability at the 5′ end is incorporated as the guide strand during the formation of RISC.[12,13] Ago2 facilitates binding of the RISC to the accessible regions of the target mRNA, which is specifically complementary to the siRNA.[35] Ago2 also cleaves the mRNA at the position opposite to nucleotide 10 from the 5′ end of the guide strand and thus inhibits gene expression.[6]

RNAi is also induced by miRNAs in target cells (Figure 1 a).[20] Primary miRNAs (pri-mRNAs), which are formed in the nucleus by transcription of miRNA genes, are cleaved by Drosha/DGCR8 to form ~70-nt-long precursor miRNAs (pre-miRNAs).[36] These pre-miRNAs are carried to the cytoplasm by Exportin-5 (XPO5) and are then cleaved by Dicer, resulting in mature miRNAs.[37] The exact mechanism by which miRNAs induce gene silencing is not yet established, but two of the proposed mechanisms proceed as follows: In one pathway the miRNAs are unwound, and the strand with less thermodynamic stability at the 5′ end is picked up by Ago2 along with other proteins such as GW182 and TRBP to form the micro-RNA-induced silencing complex (miRISC), which results in site-specific cleavage if there is complete complementarity between the miRNA and the target strand.[37,38] In the second pathway, if the base pairing is incomplete, but predominant pairing is present in the seed region (nucleotides 2–8) of the miRNA with target mRNA, miRNAs decrease gene expression levels by a mechanism known as translational suppression followed by non-sequence-specific degradation of mRNA in P-bodies.[36] As a consequence, target genes with partial complementarity are silenced, and moreover, miRNAs can down-regulate the expression of several genes at a time, in contrast to siRNAs.[39] This property can be used to effectively target cancer cells that are prone to mutation and hence not amenable for targeting by siRNA drugs.[39] Several miRNA targets in cancer have been identified at this point, and this opens up opportunities for exploring their potential as cancer therapeutics.[40–42]

3. Mechanistic Interpretation of RNAi from Structural and Biochemical Studies

It is evident from the RNAi mechanism that the Ago2 protein forms the core of siRNA-mediated gene silencing. Studies on the structure of Ago2 revealed that it consists of four distinct structural components: N-terminal domain, PAZ domain, MID domain, and PIWI domain.[43–47] Out of these components, the MID, PAZ, and PIWI domains play an important role in the formation of the RISC owing to their distinct properties. The PIWI domain has structural similarities to the RNaseH enzyme due to the presence of a catalytic triad of aspartic acid residues, which impart endonuclease activity to this domain.[48–53] The PAZ domain specifically recognizes the 3′ end of ssRNA.[54–57] The MID domain has basic residues which are ideal for forming electrostatic interactions with positively charged species.[58–60] Cleavage is mediated by Mg^{2+} cations to generate two fragments: the 5′ portion terminates in a 3′-OH and the 3′ part is

terminated by a 5′-phosphate group.[6,59] Separate studies in 2005 by Patel and co-workers[58] and Barford and co-workers[61] on the crystal structure of the PIWI protein from *Archaeoglobus fulgidus* confirmed the anchoring of the 5′ end of the guide strand in a basic binding pocket located in the MID and PIWI domains.[58,61] Previous studies have also revealed the importance of the A-type helical structure in the siRNA–mRNA duplex for functional RNAi, which was shown to be the case in structural studies.[62–65] The bases at positions 1 and 2 of the guide strand are splayed apart, and this was seen as an indicator of the absence of pairing of the first base of the guide strand at the 5′ end with the first base of the target strand.[58,61] It was shown that the mRNA strand nucleates to the 5′ end of the guide strand, and the whole duplex assumes an A-type helical symmetry.[48,58,66] Structural features around the 10–11 nt of the guide strand, which is located opposite to the cleavage site of the target RNA, were also highlighted in these studies.[48,58,67] Because the PIWI protein of *A. fulgidus* is homologous to human Ago2, most of these results were expected to be verified in the Ago2 crystal structure. However, in these studies the information on critical steps involved in recognition and cleavage of the mRNAs was not available. A major breakthrough was reported by Patel and co-workers in 2008, when they successfully obtained a crystal structure of *T. thermophilus* Ago bound to a 5′-phosphorylated 21-base DNA guide strand at 3.0 Å resolution.[24] Although the guide strand was not an RNA due to experimental difficulties in obtaining a stable crystallizable complex, the X-ray structure provided ample information on how the mechanism exploits the structural features of the Argonaute regions to effect efficient and favorable loading of the guide strand.

The major insight provided by the Ago–DNA crystal structure is the strong anchoring of the 5′ and 3′ ends of the guide strand in the respective MID and PAZ domains of the Ago enzyme (Figure 2). It was observed that the phosphate group of the 5′ end is coordinated with the Mg^{2+} ion of the MID domain and the two lysine residues in the basic binding pocket (Figure 2a). Furthermore, the base of the 5′-terminal nucleotide stacks on the aromatic side chains of the Tyr residues of Ago. There is hydrogen bonding between the oxygen atoms of the 5′-phosphate and the hydrogen atoms on the side chains of highly conserved residues of Ago.[24] The bases at positions 1 and 2 are also splayed apart, as was observed in previously reported crystal structures;[58,61] this results from stacking of the base at position 1 over the side chain of Arg418 in the MID domain. The 3′ end is also anchored in the binding pocket in the PAZ domain, with base stacking interactions and hydrogen bonding serving as stabilizing forces, as observed in earlier biochemical and structural studies (Figure 2b).[24,55] The binding pocket is primarily hydrophobic, and the 3′-OH is hydrogen bonded to the neighboring amino acid residues. The crystal structure gives a fair idea of the factors that come into play when the target strand is incorporated into this binary complex, and how the cleavage site is situated 10 nt downstream from the 5′ end. The guide strand in the binary complex has a disruption around the cleavage site, which leads to a kink-like structure. The kink is formed by

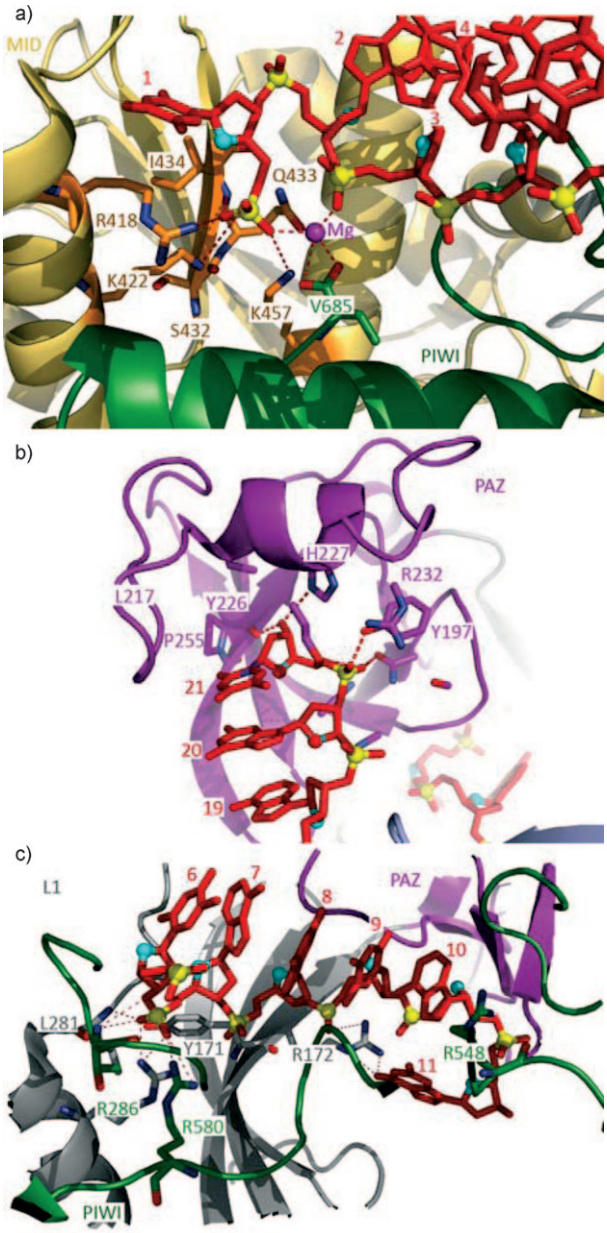


Figure 2. Crystal structure of the binary *T. thermophilus* Ago–DNA complex (PDB ID: 3DLH).[24] a) The 5′ end of the DNA strand is anchored in the basic binding pocket of the MID domain. The interaction between the 5′-phosphate and the neighboring residues of Ago2 coupled with the Mg^{2+} cation is electrostatic in nature. (DNA guide strand: red, phosphorus atoms: yellow, ribose oxygen atoms: light-blue, nitrogen atoms of the surrounding residues: dark-blue). b) The PAZ domain residues in the immediate surroundings of the 3′ end of the DNA strand. Hydrogen bonds between the 3′-OH and the neighboring Ago residues along with base stacking interactions anchor the 3′ end of the DNA strand in the PAZ domain. c) Disruption of the helical geometry of the DNA strand in the binary complex is observed after position 10. A kink is formed between nucleotides 10 and 11 due to the guanidinium group of Arg548 stacking on the base at position 10, making it orthogonal to base 11. Images were generated from PDB files using PyMol (http://www.pymol.org).

stacking of the guanidinium group of Arg548 on the base at position 10, making it orthogonal to the base at position 11 (Figure 2c). Another residue, Arg172, acts as a bridge between residues at positions 9 and 11.[24] This disruption in the align-

ment of the guide strand in Ago makes the binary complex thermodynamically unstable. As a result, when the target strand is incorporated into the binary complex, the kink is released, thus lowering the energy to form the stable ternary complex.

The insight provided by these studies allows speculation on the structural changes that may occur during the incorporation of the target RNA strand into the Ago–DNA complex. Along these lines, Patel and co-workers reported a 3.0 Å crystal structure of the ternary complex containing a 20-nt RNA strand bound to the guide DNA strand, which was phosphorylated at the 5′ end (Figure 3).[25] To enable the study of the entire complex, the RNA strand was incorporated with cleavage-preventing mismatches at the crucial base positions 10 and 11 nt from the 5′ end, though this led to incomplete mapping of the entire target strand beyond nt 11 in the crystal structure.[25] It was observed that both ends of the guide DNA strand were firmly bound to their respective anchors in the MID and PAZ domains even with a target strand bound to it. This was not expected after taking into account the formation of a double helix in the ternary complex, but can be attributed to the absence of base pairing beyond the cleavage site (Figure 3a). To validate the importance of base pairing in the seed region of the guide strand, the researchers studied the effect of mismatches in the DNA guide strand on gene silencing activity.[25] Mismatches were well tolerated near the 3′ end of the guide strand with little or no loss of silencing activity; however, alterations in the seed region or the cleavage site proved detrimental for silencing activity.[25] When the 2′-H of the deoxyribose ring was substituted with 2′-OMe, the modified nucleotides were shown to decrease silencing when placed in the seed region and adjacent to the cleavage site on the DNA strand. The reason for the tolerance of mismatches near the 3′ end could be the weak sugar–phosphate backbone interactions with Ago residues near the 3′ end relative to the 5′ end. Bulges introduced in the target RNA opposite to the seed region did not show a considerable effect on gene silencing because they have no interaction with the Ago2 domains (Figure 3b).[25]

Patel and co-workers recently reported the crystal structures of various ternary complexes of *T. thermophilus* Ago containing a 21-nt 5′-phosphorylated DNA guide strand and RNA strands of three different lengths: 12, 15, and 19 nt.[26] A key difference in this study from the previous ternary complex study is that the cleavage-preventing base mismatches were replaced by single mutations in the catalytic triad (Asp residues 478, 546, and 660) of the PIWI domain of Ago. As a result, these crystal structures could map the target RNA strand even beyond the cleavage site and hence provide detailed information on the cleavage mechanism of the RISC. A 2.6 Å crystal structure of a 21-nt DNA strand bound to a 12-nt RNA strand is quite similar to the previous structure of the incompletely paired ternary complex.[25] This complex contained an Asp546→Asn546 mutation in the catalytic triad.[26] It was seen that the first nucleotide of the guide strand is buried in the basic binding pocket in the MID domain, with base stacking interactions and, more importantly, N3 and O4 of the first nucleotide hydrogen bonded to the neighboring Ago residues Met413 and Asn436 (Figure 3c).[26] Due to these base-specific contacts, the nucleotides at positions 1 and 2 of the guide strand are splayed apart, which was also observed in the binary complex crystal structure.[24] As a result, the base pairing between the guide and target strands starts from the second nucleotide, and the first nucleotide of the target strand is untraceable.[26] The DNA–RNA base pairing is composed of an 11-bp duplex that adopts a perfect A-type helical geometry, resolving the kink at positions 10–11 of the DNA strand (Figure 3d). A 3.05 Å crystal structure containing a 21-nt DNA strand bound to a 15-nt RNA strand shows different structural features from those of the previous ternary structures (Figure 3e).[26] The most striking difference is that upon formation of the 14-bp duplex, the 3′ end of the guide strand is released from the PAZ domain. Similar features were observed in a 2.78 Å crystal structure containing a 21-nt DNA strand bound to a 19-nt RNA strand. Surprisingly, in this crystal structure only one extra base pair was observed after position 15 from the 5′ end of guide strand, with bases 17–19 of the target strand splayed apart from the guide strand.[26] It was observed that the N-terminal domain of Ago plays a key role in disrupting this base pairing beyond a 15-bp duplex.[26] The base at position 16 of the guide strand is stacked on the aromatic side chain of Tyr43, whereas the base at the opposite position on the target strand stacks with Pro44 (Figure 3f). Hence, propagation of the A-form helix stops beyond this point, and the guide and target are splayed apart. Thus, base pairing is prevented by the time the target strand reaches the PAZ domain of Ago; this supports the idea that the PAZ domain plays a very limited role in the formation of a competent guide–target duplex.[25,26] Moreover, replacement of the key residues of the N-terminal domain with mutations caused abrogation of cleavage, showing that the N-terminal domain is necessary for stabilizing the conformation of the ternary complex.[26]

In all the ternary complexes, the DNA–RNA duplex is accommodated in the nucleic acid binding channel through rotations of the N-terminal and PAZ domains with respect to the MID and PIWI domains, leading to a wider binding channel.[25,26] It was also observed that when the duplex length varies from 11 to 14 bp, the conformations of loops in Ago change in a manner to accommodate the duplex turn with the formation of extra hydrogen bonds within the loops of Ago residues.[68] In the previous ternary complex containing a 21-nt DNA–20-nt RNA duplex,[25] strong base pairing was seen in the seed region of the guide strand which is crucial in binding the guide strand to the target strand.[69,70] Moreover, mismatches in the seed region or near the cleavage site abolished silencing activity, but were well tolerated near the 3′ end of the guide strand.[25] In the three mutant Ago crystal structures, the seed region was found to be hydrogen bonded with the neighboring PIWI domain residues through its sugar–phosphate backbone.[26,71] This is in agreement with the previous study by Patel and co-workers, in which 2′-OMe modifications in the seed region or near the cleavage site abrogated silencing, which is due to disruption of guide strand interactions with the neighboring Ago residues.[25] In contrast, the sugar–phos-

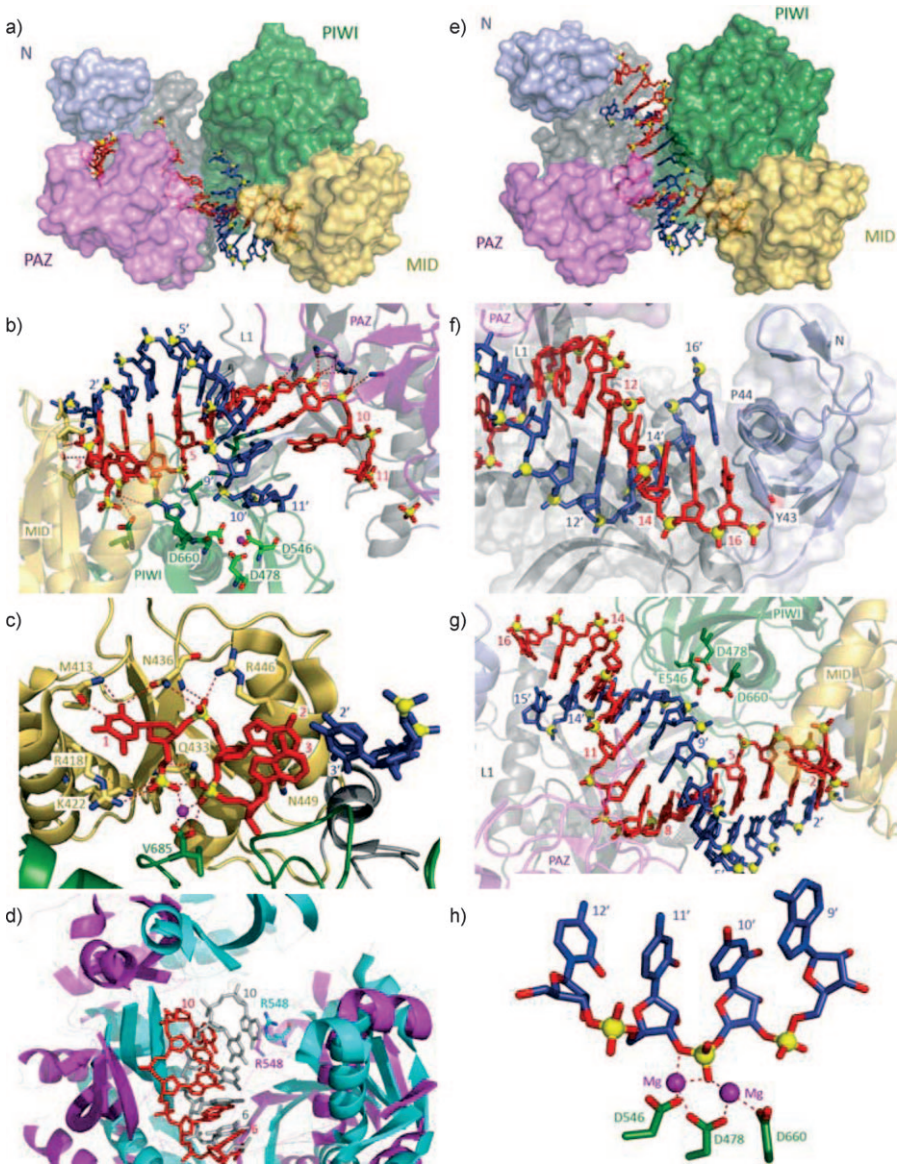


Figure 3. Crystal structures of ternary *T. thermophilus* Ago–DNA–RNA complexes.[25,26] a) Ternary complex of Ago with a 21-nt guide DNA (in red) and a 20-nt target RNA (in blue); the four major Ago domains are shown in color surface representation. Phosphorus atoms are shown as yellow spheres. The guide–target duplex has cleavage-preventing mismatches at positions 10–11 of the guide strand. The 3′ end of the guide strand is anchored in the PAZ domain. (PDB ID: 3F73). b) A closer look at the seed region base pairing between the DNA guide strand and the target RNA strand with cleavage-preventing mismatches at positions 10–11. The guide strand residues interact strongly with the neighboring PIWI domain residues through the sugar phosphate backbone; however, such interactions are absent for the target strand (PDB ID: 3F73). c) The first nucleotide of the guide strand has base-specific interactions with Ago, with the base O and N atoms forming H bonds with residues M413 and N436 of the MID domain. As a result of this strong interaction with Ago, the first nucleotide is buried in the basic binding pocket of the MID domain and is splayed apart from the second nucleotide; the base pairing starts from the second nucleotide of the guide strand. This image corresponds to the crystal structure of the ternary complex of Ago (Asn 546, catalytically inactive mutant form) with a 21-nt guide DNA and a 12-nt target RNA (PDB ID: 3HO1). d) The geometry of the DNA strands in the binary and ternary complexes are shown side by side in silver and red, respectively; the respective distances between Arg 548 and nucleotide 10 of the DNA strand differ significantly between the two cases, with the kink being resolved for the DNA strand in the ternary complex. This favors formation of the A-type helical duplex necessary for cleavage by the RNaseH fold of the PIWI domain (cyan ribbon: binary, magenta: ternary; PDB IDs: 3DLH, 3F73). e) Ternary complex of Ago (Glu 546 catalytically inactive mutant form) with a 21-nt guide DNA (in red) and a 15-nt target RNA (in blue) with the four major domains shown in color surface representation. The Asp 546→Glu 546 mutation results in abrogation of cleavage. It can be seen that the 3′ end of the guide strand is no longer anchored in the PAZ domain in this complex (PDB ID: 3HJF). f) View of the extra observable base pair beyond position 15 of the guide strand in the ternary complex of Ago (Asn 478 catalytically inactive mutant form) with a 21-nt guide DNA and a 19-nt target RNA. Only a single extra base pair is formed, resulting in a 15-bp duplex, as the target strand length is varied from 15 to 19 nt, as residues Tyr 43 and Pro 44 block further formation of the duplex beyond position 16 (PDB ID: 3HK2). g) A closer look at the 14-bp duplex between the DNA guide strand and the target RNA strand in the Ago (Glu 546) catalytically inactive mutant form. Cleavage is initiated from the nucleotide closest to the catalytic triad consisting of Asp 478, Asp 546, and Asp 660. This corresponds to the 10–11 step of the target strand. Oxygen atoms of the Asp residues are also shown in red (PDB ID: 3HJF). h) A closer view of the cleavage site in the Ago–DNA–RNA ternary complex of wild-type Ago with a 19-nt target RNA from crystals grown in a solution containing 80 mM Mg^{2+}. Oxygen atoms are shown in red, while nitrogen atoms are shown in light blue. Asp 660, Asp 478, and Asp 546 of the PIWI domain along with the Mg^{2+} ions (purple) are responsible for cleavage of the RNA target strand at the 10–11 position (PDB ID: 3HVR). Images were generated from PDB files using PyMol (http://www.pymol.org).

phate backbone of the target strand, and not the guide strand, spanning positions 10′–13′ was seen in hydrogen bonding contact with the neighboring Ago scaffold.[26] The catalytic residues of the PIWI domain are therefore positioned in a competent geometry along the cleavage site at the target strand (Figure 3g). Cleavage is also mediated by a pair of Mg^{2+} cations in the PIWI domain, with one cation facilitating nucleophilic attack at the phosphodiester backbone cleavage site, and the other stabilizing the transition state and leaving group (Figure 3h).[26]

In the previous ternary complex study, a 9-nt DNA guide strand showed effective cleavage of a 20-nt target RNA at 55 °C.[25] An explanation for this result may be that the DNA–RNA duplex adopts a stable A-type helical symmetry,[25,61,65] which is crucial for the RNaseH-like PIWI domain of the Ago2 protein to initiate cleavage of the target RNA.[50,51] Earlier studies by Rana and Chu in 2008 demonstrated that a 16-nt siRNA duplex can lead to more efficient silencing of a target mRNA than can the wild-type 19-nt siRNA duplexes.[72] It was observed that a 16-nt siRNA duplex can lead to the formation of a greater amount of RISC than can other siRNA duplexes, suggesting that the process has more to do with the better programming of the RISC by these siRNAs rather than the higher activity of the RISC itself.[72] This can be interpreted in terms of better clearance and removal of the passenger strand during RISC formation for these short RNAi triggers. Moreover, the authors suggested that a 42 Å A-form helical geometry, which amounts to ~1.5 turns, is sufficient for dsRNAs to trigger RNAi.[72] Patel and co-workers tried to underpin a structural basis for the minimum target strand length required to mediate efficient silencing. They found that a DNA target 16 nt in length is efficiently cleaved by a 21-nt guide DNA strand, while shorter targets lead to a decrease in cleavage capacity.[26] This result supports the structural observation that the maximum length of the duplex formed between the guide strand and the target strand is 15 bp, and guide strand residues beyond that do not play a major role in RISC cleavage activity.[26] Another key observation is that pairing of nucleotides 4′–16′ of the target strand to the guide strand is sufficient for target cleavage.[26]

The tolerance for chemical modifications in the target DNA strand along the critical 10′–13′ position was also studied by Patel and co-workers.[26] The 2′-OH modification at position 11′ of the target strand showed decreased silencing, while the 2′-OMe modification at the same position completely abrogated silencing. However, a 2′-fluoro modification at the cleavage site showed a pronounced increase in silencing activity, which is due to its ability to stabilize the 3′-OH leaving group when cleavage takes place.[26] 2′-OH and 2′-OMe modifications also showed decreased cleavage at position 9′ of the target strand. The absence of residues in *T. thermophilus* Ago that can form hydrogen bonds with the target strand at position 11′ means that it cannot distinguish whether the target is a DNA or an RNA strand.[26] However, this may not be the case with human Ago2. Moreover, the effect of phosphorothioate modifications in the target strand was most prominent between positions 10′–11′.[26] Out of the two diastereomers of phosphorothioate, the S_p form was more sensitive to the cleavage mechanism, thereby implying that a pro-S_p oxygen atom is paired to both Mg^{2+} cations and plays a key role in stabilizing the transition state during cleavage. On the other hand, a pro-R_p oxygen is only paired to one Mg^{2+} cation, and its activity upon substitution with a sulfur atom is easily restored by a Mn^{2+} cation.[26]

These ternary complex crystal structures provide detailed insight into the cleavage mechanism of the target strand by a two-step model, which involves anchoring of the guide strand into Ago followed by release of the 3′ end of the guide strand from the PAZ domain during nucleation of the target RNA.[73] Moreover, base pairing can only take place up to position 16 of the guide strand, meaning that chemical modifications at the 3′ end of the guide strand are better tolerated than at the 5′ end, and that the PAZ domain does not play a significant role in the cleavage activity.[26] This can also explain why a 36-nt DNA guide strand showed efficient silencing of a target RNA strand in the previous study.[25] Although the guide strand in these cases was DNA, the structural features of the complexes are expected to follow a similar pattern for an siRNA guide strand. It is not completely certain that the mechanism of target RNA cleavage emerging from the structural studies is also valid for the passenger strand cleavage during RISC formation. This is because the passenger strand in the RLC is part of the preformed siRNA duplex, unlike the target RNA of the duplex in the RISC, which forms as a result of the nucleation of the guide strand, supported by the two-step model.[26]

A recent detailed biochemical study by Lima and co-workers on the cleavage and binding specificities of human Ago2 provides more insight into the structural and functional requirements of RNAi machinery.[27] The research group employed two different human Ago2 proteins: one was immunoprecipitated from HeLa cells using an HA antibody (HA-Ago2), and the other was a recombinant GST–Ago2 protein; the authors studied their binding properties toward a combination of siRNAs in order to gather information on how the guide strand directs cleavage of a target strand.[27] One of the key outcomes of this study is that the Ago2 showed very weak binding affinity for pre-formed guide RNA–target RNA duplexes relative to a single-stranded guide RNA, which was 19 nt in length. The two pre-formed duplexes studied consisted of a 19-nt guide strand–19-nt target strand duplex and a 19-nt guide strand–40-nt target strand duplex. The pre-formed duplexes showed very weak binding to Ago2 due to very slow association and rapid dissociation relative to the single guide strand studied.[27] Ago2 was subsequently unable to cleave the target strand from the preformed duplex, while the single guide strand mediated efficient cleavage of the target strand. This result is consistent with previous studies on the binding affinities of Ago2 toward various motifs, showing that the binding affinity decreases on moving from ssRNA to an RNA duplex.[43] A valid question here is whether Ago2 can cleave the target strand in the preformed duplexes with the help of additional proteins. To answer this, the group studied the effect of the TRBP, which forms a part of the RISC in humans.[5,55,74,75] It was observed that although TRBP plays a crucial role in binding to and stabilizing the PIWI domain of Ago2, it is itself unable to load the

siRNA duplex into the HA-Ago2, thereby preventing the target cleavage.[27] Therefore, the authors suggested that additional factors in the cellular medium are responsible for loading the siRNA duplex into the RISC.

To confirm that Ago2 interacts with the 5′ terminus of the guide strand, which was apparent in the crystal structures,[19,24–26,58] the group studied the effect of 5′-end modifications on the binding affinity of the guide strand to Ago2. Replacing 5′-phosphate with a hydroxy group decreased binding affinity by two orders of magnitude and showed poor cleavage of the target strand.[27] This has been attributed to the lack of coordination of the phosphate by divalent metal ions and the basic amino acid residues in the binding pocket of Ago2. Because the heterocyclic bases at the 5′ end stack on the aromatic side chain of Tyr residues in the basic binding pocket of Ago2,[58] modifying these bases with abasic modifications leads to decreased binding. The base stacking is one of the key factors in stabilizing the electrostatic interaction between the terminal phosphate and the divalent cation, which has been validated by experimental results using abasic modifications. A guide strand modified with abasic nucleotides showed weak association with Ago2.[27] Subsequently, such abasic guide strands showed decreased cleavage of the target strands, the effect being most pronounced for modification at the second nucleotide from the 5′ end.

Structural studies have shown that disruptions in the interaction between Ago2 residues and the seed region can lead to inefficient cleavage of target RNA.[25] To verify this, Lima and co-workers studied the effect of 2′-methoxyethyl (MOE) modification at position 2 from the 5′ end of the guide strand. As anticipated, this resulted in decreased target RNA cleavage.[27] Moreover, cleavage was also reduced when the modification was placed at nucleotide 13 or 14 from the 5′ end. This has been attributed to a disruption in binding of the catalytic triad of the PIWI domain with the guide strand, which could affect the formation of the kink-like structure at the nt 10–11 region of the guide strand.[24,34,58] The nature of the cation present also influences the binding and cleavage activities of the guide siRNA. Replacement of Mg^{2+} with Mn^{2+} led to improved binding and cleavage activity of the Ago2–guide siRNA complex.[27] This could be due to the difference in coordinating properties between Mg^{2+} and Mn^{2+}, with Mn^{2+} forming a smaller coordination sphere with the 5′ end residues.[76]

The structural and biochemical studies reported so far provide crucial information on the mechanism of, as well as various requirements for, RNAi machinery. The structural features of the seed region and the region surrounding the cleavage site have been determined to be important for cleavage activity of siRNAs.[25,26] The sites in the guide strand that may not be able to tolerate modifications have also been identified.[25–27] More insight may be gathered as soon as the crystal structure of the siRNA duplex with human Ago2 is obtained. However, with the existing structural and functional information, chemical modifications can be designed and used in a manner to enhance the binding of siRNAs to Ago2, leading to stable RISC formation and potent RNAi in vivo.

4. Challenges Involved in siRNA Therapeutics and Opportunities for Using Chemical Modifications

Although the application of siRNAs sounds useful in therapeutic practice, unmodified siRNAs have a variety of limitations.[15] In addition to their vulnerability toward nuclease activity in serum,[16] one of the biggest problems unmodified siRNAs face is that they act as potent triggers of the innate immune response,[17] particularly when associated with delivery vehicles that facilitate intracellular uptake. Unmodified siRNAs can also induce off-target effects, as they interfere with natural miRNA pathways.[14,20] Delivery of siRNAs is another substantial challenge, as these molecules cannot easily pass through cell membranes owing to their size and negative charge.[21] Currently, the most mature therapeutic approach using RNAi is the use of chemically modified siRNAs, which can address some of the problems discussed above.[14]

4.1. Imparting nuclease resistance without loss of RNAi activity

Unmodified siRNAs have a half-life of less than ~15 min in serum, which makes them impractical for use in RNAi-based therapeutics.[77] Recently, high-resolution mass spectrometry studies in rat and human serum have shown that the guide strand is more susceptible to degradation by exonucleases and the passenger strand is preferentially degraded by endonucleases.[78] The passenger strand has also been shown to be degraded by exonucleases in liver microsomes.[78] The most viable method to address the nuclease vulnerability of siRNAs is the use of various chemical modifications; however, the challenge here is to do so while retaining RNAi activity.

Broadly speaking, chemical modifications used in siRNAs fall into four classes: backbone, sugar, base, and terminal modifications (Figure 4). The backbone modifications involve alterations to the phosphate ester linkages in the nucleic acid (Figure 4a). One of the more common modifications of this type is the phosphorothioate (PS) modification, in which one of the nonbridging phosphate oxygen atoms is replaced by sulfur to give a PS group.[79] This modification is widely used in antisense oligonucleotide therapeutics and is now being employed in siRNAs, as it provides improved nuclease resistance and favorable pharmacokinetic properties.[22,63,80,81] However, the PS modification has a tendency to bind nonspecifically to cellular membrane proteins, which can lead to toxicity.[63,80] These modifications are also known to lower the melting temperature (T_m) values for siRNA duplexes, with a decrease of ~0.5 °C per modification.[64,80] Tuschl and co-workers reported the cytotoxicity of siRNAs in which every second nucleotide was modified with PS (50% PS content).[80] The toxicity can be decreased by restricting the use of this modification to the two ends of the siRNA and by reducing the overall PS content to the minimum.[80] It has been reported that incorporation of PS modifications at positions 3, 5, and 17 from the 5′ end of the passenger strand enhances the silencing activity of the modified siRNA by improving the RISC loading of the guide strand.[82]

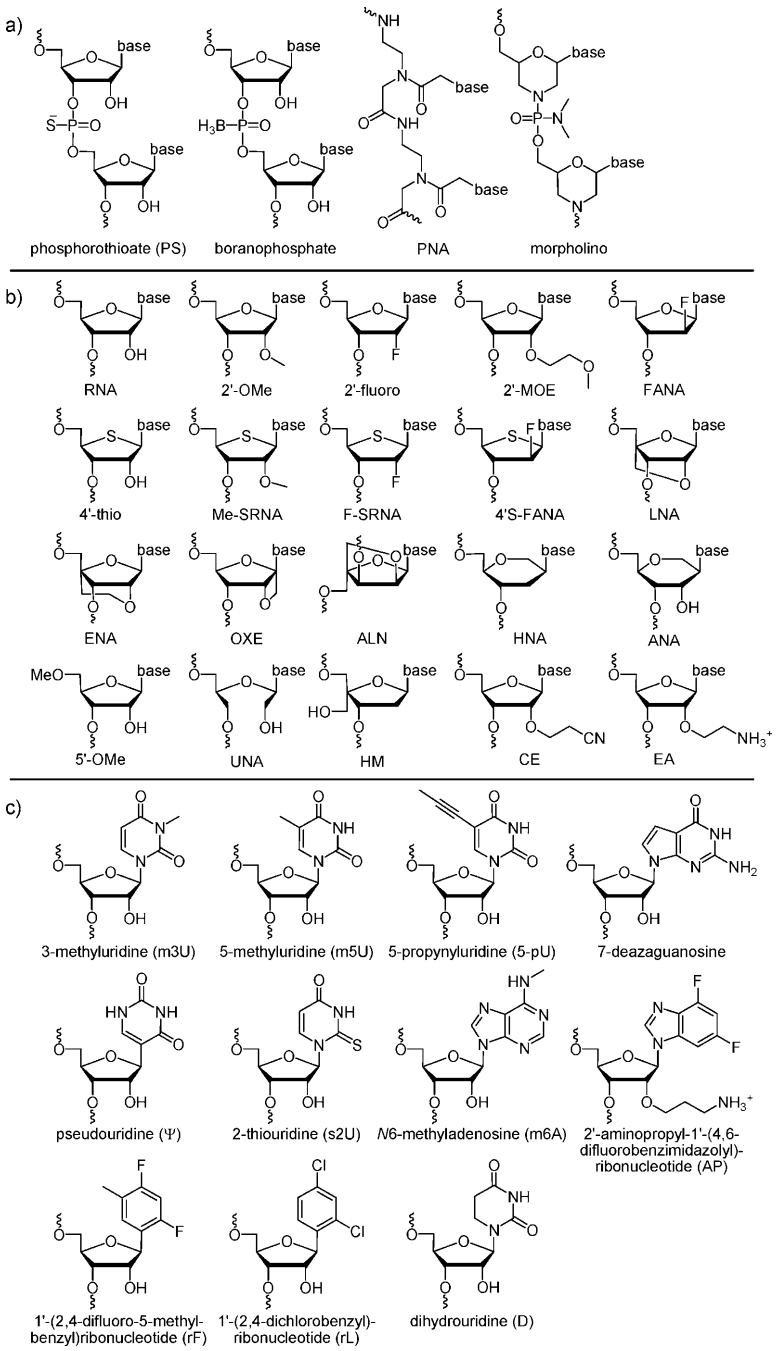


Figure 4. Selected chemical modifications used in siRNAs: a) Backbone modifications, b) sugar modifications, and c) base modifications.

The PS modification is not well tolerated if used heavily in the guide strand, and therefore a useful strategy is to use one or two PS modifications in the passenger strand in combination with sugar modifications in the guide strand.[64,80]

Another backbone modification used in siRNA is the boranophosphate modification, in which one of the non-bridging oxygen atoms of the phosphate moiety is replaced with a borane (BH_3) group (Figure 4a).[83] This modification is known to improve nuclease resistance by >300-fold relative to native siRNAs.[83,84] Boranophosphate-modified siRNAs have also been shown to be more potent than those modified with PS.[83] Studies have also shown that boranophosphate modifications slightly increase the T_m value of the siRNA duplex by 0.5–0.8 °C per modification.[83] The boranophosphate modification is hydrophobic in nature which can lead to a change in the RNA–Ago2 interactions around the modified site.[85] As with PS, it is important that the boranophosphate modification is not placed in the cleavage region of the guide strand; its presence in the guide strand should also be minimized, as it might interfere with the residues Ago2.[83]

A rather unconventional backbone modification used in siRNA is the peptide nucleic acid (PNA; Figure 4a).[86] PNAs are oligonucleotide mimics that have a peptide backbone rather than the sugar–phosphate backbone of RNA and DNA. Messere and co-workers have shown that a siRNA–PNA chimera can be used for efficient silencing along with improved serum stability and persistent activity.[87] The PNAs were conjugated to thymine dimers in the 3′ overhang of one or both strands of the siRNA. The siRNAs having PNA monomers at the passenger strand overhangs showed efficient gene silencing and also showed persistent silencing activity relative to other siRNAs tested. Chimeras consisting of siRNA–PNA can therefore be used as potent RNAi tools with the passenger strand preferably modified as outlined in this study, although the synthesis and purification of these duplexes can be a tedious process.[15] Morpholino-modified siRNAs with the modification present at the 3′ overhangs of the passenger strand have also given similar results, with the siRNAs showing efficient cleavage of the target strand at low concentrations.[88] These siRNAs also exhibited rather high resistance to nuclease degradation, showing that modifications in the overhangs can avoid exonuclease degradation of the siRNA duplexes.[88]

The most widely used sugar modifications in siRNAs are the 2′ modifications to the sugar ring, namely 2′-OMe,[89,90] 2′-fluoro,[90] and 2′-*O*-methoxyethyl (2′-MOE)[90] (Figure 4b). Due to their ability to favor the 3′-endo sugar conformation, all these modifications are expected to form thermally stable A-type siRNA duplexes once they are introduced into siRNAs. 2′-OMe-modified siRNA duplexes exhibited enhancements of 0.5–0.7 °C in T_m per modification relative to unmodified duplexes.[91] The 2′-OMe modification has been shown to improve nuclease resistance to a certain extent and to enhance silencing activity.[89,90] An siRNA in which either the guide or both strands are fully modified with 2′-OMe abolished silencing, which can be attributed to inefficient loading of the guide strand into the RISC.[91] Notably, passenger strands fully modified with 2′-OMe units were compatible with the RNAi machinery.[91] Although 2′-MOE modification improves the T_m values and nuclease resistance of siRNAs over 2′-OMe-modified siRNAs, this modification is generally not well tolerated in the guide strand relative to the passenger strand

of siRNAs.[90] This may be due to their bulky structure, which can cause steric clashes with Ago2 residues, thereby preventing guide strand loading into the RISC.[27,90] The 2′-fluoro modification is very well tolerated in both the guide and passenger strands of siRNAs.[90] Even modifications at the cleavage site of the passenger strand were very well tolerated.[26,90,92] Incorporation of the 2′-fluoro modification results in an increase in T_m by ~1 °C per modification.[93] The drawbacks of using individual 2′ modifications in isolation lay in their inability to render sufficient nuclease stability and pharmacokinetic properties, which are crucial for in vivo applications. When used along with the PS modification, the 2′-OMe modification shows a considerable synergistic enhancement in binding affinity of the guide strand with the target mRNA along with increased nuclease resistance without compromising the silencing activity.[91] It has also been demonstrated that an siRNA duplex containing nucleotides alternately modified with 2′-OMe and 2′-fluoro exhibited up to a 500-fold increase in silencing potency over the unmodified siRNA.[93] The use of 2′-fluoropyrimidines with 2′-methoxypurines can also result in siRNA duplexes with extreme resistance to nuclease activity in human serum (guide strand half-life up to three days).[94,95]

Another important 2′ modification employed in siRNAs is the 2′-fluoro-β-D-arabinonucleotide (FANA; Figure 4b).[77] Incorporation of FANA increases the T_m of the siRNA duplex by ~0.5 °C per modification.[96] An siRNA duplex with a 3′ overhang in the guide strand and the entire passenger strand modified with FANA showed a 24-fold increase in serum half-life over unmodified siRNAs.[77] FANA differs from the other 2′ modifications because it is based on an arabinose sugar; the stereochemistry is opposite to that of a ribose 2′-fluoro group. Because FANA is structurally similar to DNA (2′-*endo* conformation), the placement of FANA modifications in an RNA duplex is bound to result in structural distortions.[77] Therefore, this modification is less tolerated in the guide strand.[77] However, the modification shows a considerable enhancement in RNAi potency when placed throughout the passenger strand and at the 3′ end of the guide strand.[77] Lingel and co-workers have shown that the PAZ domain forces the nucleotides at the 3′ overhangs into a DNA-like sugar pucker and an *anti* base conformation.[97] This implies better recognition of the FANA 3′ overhang by the Ago PAZ domain, and could hence be preferred over C3′-*endo* RNA as an overhang.[77,97]

The locked nucleic acid (LNA) modification is a specialized modification in which the 2′ and 4′ positions of the ribose ring are linked through a methylene bridge (Figure 4b).[98,99] The furanose ring of this modification is locked in a 3′-*endo* conformation, giving it the structural resemblance of a standard RNA monomer. The rigidity of the conformation of this modification also leads to better organization of the phosphodiester backbone, which enhances base stacking interactions as a result of stronger hybridization of the guide strand with the target strand.[100] A single LNA modification can bring about an increase of 5–10 °C in the T_m of the RNA–RNA duplex.[101] The modifications are position sensitive, and extensive modification with LNA in either strand is not tolerated by the RNAi machinery.[101,102] This arises due to the large gain in thermal stability, which probably interferes with duplex unwinding and/or release of the target RNA after its cleavage.[103] Moreover, RNAi activity is abrogated if the LNA is introduced at positions 10, 12, and 14 of the guide strand.[103] This must be due to the steric and conformational changes brought about by LNA modification near the cleavage site. LNA modifications at the 3′ overhangs of siRNA protect the duplex from 3′ exonucleases, which are particularly active in serum.[101,103] On the other hand, a related modification, 2′-*O*,4′-*C*-ethylene thymidine (eT), which belongs to the ethylene bridge nucleic acids (ENAs), completely abrogates RNAi when introduced at the 3′ end of either passenger or guide strand.[104] Mook and co-workers showed the efficacy of LNA-modified siRNAs to induce silencing activity in a murine xenograft cancer model.[102] However, extensive use of LNA-modified siRNAs in vivo warrants caution, as LNA-containing antisense oligonucleotides show profound hepatotoxicity.[105]

The 4′-thio modification involves substituting the oxygen atom of the ribose ring with a sulfur atom (Figure 4b).[106] Studies have shown that 4′-thio-modified siRNAs have considerable stability against nuclease activity and improved binding to the target mRNA.[106] These modifications are much better tolerated in the passenger strand than in the guide strand. The cleavage efficiency of this modification is highly position dependent when placed in the guide strand.[106] The C–S bond is longer than a C–O bond which may result in a different spatial conformation of the ribose ring.[107] This feature of 4′-thio-modified ribose may possibly confer nuclease stability to these modified siRNAs. Prakash and co-workers have shown that in order to take full advantage of the favorable properties of the 4′-thio modifications such as nuclease stability and biodistribution while improving silencing activity, it is advisable to use the 4′-thio modification along with 2′-sugar modifications like 2′-OMe or 2′-MOE.[108] In their studies of the nuclease stability of 2′-modified-4′-thio RNAs (Me-SRNA and F-SRNA) Matsuda and co-workers reported considerable synergistic effects of both modifications in the modified oligonucleotides.[109] These modifications assist the hybrid siRNAs to bind the target RNA strand with greater affinity than the guide strand made of only DNA moieties, due to the formation of a stable A-type helical structure.[109] F-SRNA modifications result in the formation of duplexes with moderately high T_m values (~1 °C per modification), but this modification does not render the duplexes nuclease resistant in human plasma. Duplexes modified with F-SRNA show less stability in human plasma than duplexes modified with 2′-OMe nucleosides. On the other hand, Me-SRNA modifications do not give noticeable increase in T_m values, but render the duplexes with approximately fourfold greater nuclease resistance than duplexes modified with 2′-OMe nucleosides.[109] Damha and co-workers explored the use of 4′-thio modifications along with FANA modifications (4′S-FANA) in RNAi.[96] The introduction of 4′-thio in FANA results in a conformational switch to the North-type, and hence this modification was expected to be compatible with the RNAi machinery. Incorporation of 4′S-FANA modifications in siRNA duplexes results in a decrease in T_m by ~1–1.4 °C per modification.[96] The authors demonstrated that the use of one or two 4′S-FANA

modifications in either the guide or passenger strand imparts duplexes with RNAi potency similar to that of unmodified duplexes. A passenger strand completely modified with FANA, leaving five unmodified RNA units at its 3′ end, was shown to be the most potent combination for a guide strand with one or two 4′S-FANA modifications.[96]

Manoharan and co-workers examined the effects of non-canonical base pairing in siRNA duplexes on RNAi potency.[110] A 1′-(2,4-difluoro-5-methylbenzyl)ribonucleotide (rF) was used as a substitute for a U or C moiety in the guide strand of the siRNA at various positions to determine its effect on silencing ability and serum stability (Figure 4c).[110] In the case of rF substitution for U, the silencing activity was the same as that of the unmodified duplex when placed at the 5′ end as well as positions 7 and 10 from the 5′ end of the guide strand. The rF modification is destabilizing (~5.5 °C decrease in T_m per modification) relative to unmodified duplexes, but was shown to protect the siRNA duplex from endonuclease cleavage in human serum when modified at position 16 from the 5′ end, although silencing activity was decreased to half.[110] Compared with the rF:A base pair, an rF:G pair showed complete loss of silencing, which is due to its structural similarity to a U:G wobble pair.[111] The crystal structure of an rF:G mismatch duplex shows a widening of the duplex, and the resulting structural distortion near the cleavage site may explain the poor interaction with the neighboring Ago2 residues and decreased endonuclease cleavage of the passenger strand in RISC formation.[111] It has been observed that if more than one U is substituted by rF, silencing is completely abolished.[111,112] Kool and co-workers have also shown that modifications in the major groove at position 7 from the 5′ end of the guide strand, which differ in Watson–Crick base pairing properties from natural nucleobases but resemble them sterically, exhibit silencing activity similar to that of wild-type siRNAs.[113]

In a recent study, Kool and co-workers modified the major groove of an siRNA using 5-methyluridine (m5U) and 5-propynyluridine (5-pU) in both the guide and passenger strands (Figure 4c).[114] The 5-pU modifications can lead to an increase in T_m for the siRNA strand by as much as 1.3–1.8 °C per modification.[114] Due to their bulky size, 5-pU modifications are not tolerated in the 5′ region of the guide strand, but have diminished effect on silencing activity when placed at the 3′ end.[114] This confirms that the major groove of the guide strand–target RNA duplex is highly sensitive to modifications in the 5′ region of the guide strand, which can interfere with the stabilizing interaction of the crucial seed region with the PIWI domain of Ago2.[26,115] In contrast to the 5-pU modification, modifying the 5′ and 3′ regions of the guide strand with m5U led to an increase in RNAi activity, which can be explained by the smaller size of m5U.[114] In a related study reported earlier, Rana and Chiu demonstrated the deleterious effects of the 3-methyluridine (m3U) modification, which affects the hydrogen bonding near the cleavage site in the guide strand on RNAi activity.[63] This contradicts the tolerance of the rF modification reported in the guide strand near the cleavage site, and an explanation for this anomalous observation is difficult to discern from the available structural and biochemical studies. Both the 5-pU and m5U modifications lead to an increase in serum stability of the siRNA duplex when placed in the guide strand, and the m5U modification should be further evaluated to explore its potential application in therapeutics.

Other base modifications that have been tested for their RNAi-inducing potencies are 2-thiouridine (s2U), pseudouridine (Ψ), and dihydrouridine (D) (Figure 4c).[116] The s2U and Ψ modifications do not result in noticeable changes in T_m, whereas the D modification shows a 3–5 °C decrease in T_m per modification. This must be because dihydrouridine preferably adopts the C2′-*endo* conformation, while s2U and Ψ support the formation of an A-type helical geometry. Use of s2U and Ψ modifications at the 3′ end or a D modification at the 5′ end of the guide strand results in a 25–50% increase in RNAi potency relative to unmodified siRNA.[116] However, placing the s2U and Ψ modifications at the 5′ end of the siRNA duplex leads to a 50% decrease in potency relative to the unmodified version. The positional effects on silencing activity can be attributed partially to the thermodynamic asymmetry imparted by these base modifications. The Ψ modification at position 10 or D modifications at positions 8, or 10 of the guide strand also result in decreased activity. Another important observation is that the effect of a wobble pair on siRNA activity can be nullified by placing an s2U modification at the position 3′ adjacent to the wobble pair. This effect has been attributed to conformational changes brought about by an s2U modification at the site adjacent to a wobble pair, which eventually favors the A-type helical structure in the resulting duplex.[116] The limited ability of an s2U modification to form a wobble pair may assist in the design of siRNAs with high target specificity. Notably, the nuclease stability of base modifications such as s2U can be enhanced by their use in conjunction with other 2′-sugar modifications like 2′-F or 2′-OMe.

In a recent study reported by a collaboration between various European research groups, several pairs of guide and passenger siRNA strands modified with 21 different modifications were used to generate a total of 2160 siRNA duplexes, which were studied for silencing activity and cell viability.[117] It was found that the thermodynamic asymmetry of the duplex and the 3′ overhangs are the major factors that affect silencing activity.[117] Stabilizing the 3′ end of the guide strand or the 5′ end of the passenger strand (by the use of 2′-OMe, 2′-fluoro, HNA, ANA, ALN, LNA, etc.), and destabilizing the 5′ end of the guide strand or the 3′ end of the passenger strand (by the use of DNA, OXE, AP, EA, CE, UNA, etc.) resulted in improved RNAi activity due to improved strand selection.[12,117] However, if the seed region is strongly destabilized, the corresponding weakening of the interaction between the guide strand and the target mRNA results in decreased silencing activity.[117] The highly stabilized duplexes were also shown to have decreased silencing activity.[117] It was thus suggested that destabilizing the 3′ end of the passenger strand is an optimal way of designing the desired efficient siRNAs. Certain favored 3′ overhangs (5′-LNA-LNA-RNA-3′) in the guide strand and disfavored 3′ overhangs (5′-RNA-UNA-RNA-3′ and 5′-RNA-HM-3′) in the passenger strand had been identified, which affect strand selection irrespective of the thermodynamic asymmetry of the

duplex.[117] The passenger strand was found to be more tolerant to modifications than the guide strand. However, it has been found that most of the highly active siRNAs are toxic, whereas most relatively less active siRNAs are nontoxic. The authors speculated that the toxicity emerges from either competition with the endogenous RNAi machinery or from participation in the endogenous miRNA pathways.[117] The distinctive exceptions are duplexes that contain single modifications such as unlocked nucleic acid (UNA) and hydroxymethyl (HM) in the seed region. Therefore, the authors concluded that fine tuning the thermodynamic properties of a duplex, the use of modified overhangs, and the exclusive use of UNA and HM in modified siRNAs are strategies to be considered for designing highly efficient siRNAs.[117]

Rossi and co-workers recently published their reports on a new class of dsRNAs called Dicer-substrate siRNAs (DsiRNAs).[118] These 27-bp RNAs have been shown to yield the 21-bp siRNA duplex upon Dicer cleavage, and have been found to be more effective in silencing the target gene than the 21-bp siRNAs.[118] It was also reported that 27-nt dsRNAs are more stable than 21-nt siRNAs in serum. An important outcome of this study is that the DsiRNAs show efficient cleavage at subnanomolar concentrations; this was not observed in the case of 21-bp siRNA duplexes.[118] This is highly advantageous in considering the toxicity associated with high dosages of siRNA drugs. In another study based on longer RNAs being processed into 21-bp duplexes by Dicer, Hannon and co-workers reported that 29-bp short hairpin RNAs (shRNAs) can yield efficient RNAi activity at lower concentrations than the 21-bp siRNA duplexes.[119] These are promising results, which should lead to more research into the role of chemical modifications in improving the activity of these RNAs even further.

4.2. Minimizing off-target effects of siRNAs

siRNAs are known to suppress the expression levels of unrelated genes in two ways. They can either participate in unwanted miRNA-like pathways,[120–122] or they can lead to the involvement of the passenger strand in gene silencing.[123]

Involvement in miRNA pathways results in the translational suppression of genes having up to two mismatches in the seed region corresponding to the matches in the 3′-untranslated region (UTR) of the mRNAs.[18,19,124–129] The mechanism of off-target down-regulation via miRNA pathways is not completely understood. Sharp and co-workers reported that most of these off-target effects might arise from Ago2-independent mRNA degradation processes.[130] Recently, Vickers et al. showed that the off-target mRNA silencing is Ago2-cleavage independent; nevertheless, interaction with either Ago1, Ago2, or the RLC is essential for off-target gene silencing.[131] Also, depletion of P-body-associated proteins was found to result in decreased off-target effects. This is because off-target mRNA degradation is a result of decapping and exonucleolytic processing, rather than endonucleolytic cleavage, and is hence P-body associated.[131] To address the involvement of siRNAs in miRNA pathways, a thorough seed region homology screening in the 3′-UTR of mRNAs can be done. However, this method might not be sufficiently sensitive to pick up all the sequences with relevant partial homology.[132] The chances of finding a six-base match corresponding to the seed region are very high, and thus more diverse methods must be applied to tackle off-target effects. Placing a single 2′-OMe modification at position 2 from the 5′ end of the guide strand can significantly decrease off-target effects.[133–135] While this modification improves siRNA specificity, it does not alter the silencing of perfectly matched targets.[133] Such sharp position dependence of the modification implies a particular role for position 2 of the seed region in the mechanism of translational suppression by miRNAs.[133] Considering that perfectly matched targets are silenced efficiently, the modification does not affect siRNA incorporation into the RISC and consequent separation of the passenger strand.[133] However, it has been speculated that the 2′-OMe modification results in conformational changes in neighboring PIWI residues of Ago2 that makes the guide strand inefficient at cleaving target strands with one or two mismatches.[133]

Ui-Tei and co-workers recently pointed out that the off-target effects of siRNAs can be correlated to the T_m of the duplex in the seed region, the Watson–Crick base pairing between the guide and the target strand, and the siRNA concentration.[70] The off-target effects were minimal for duplexes with low seed T_m values and negligible at lower siRNA concentrations.[70] It was also noted that the introduction of a G:U wobble pair in the seed region completely offset off-target effects, indicating that such structural features as Watson–Crick base pairing form an integral part of the molecular machinery associated with miRNA pathways.[70] Along these lines, it was reported that the incorporation of rF or 1′-(2,4-dichlorobenzyl)-ribonucleotide (rL) modifications (Figure 4c), which lack Watson–Crick paring, instead of U at position 7 from the 5′ end of the guide strand resulted in improved sequence selectivity relative to the unmodified siRNAs.[113] Ui-Tei and co-workers had earlier demonstrated that the replacement of the seed region of siRNAs with corresponding DNA nucleotides resulted in complete loss of off-target effects, while the on-target gene silencing was not substantially affected.[136] This supports the above conclusion, as the T_m of the seed region of the DNA-, rF-, and rL-modified siRNAs are considerably lower than those of unmodified siRNAs. Furthermore, a base mismatch at siRNA nt 16 was found to be the key to designing siRNAs that can distinguish between genes differing by a single nucleotide.[137] This is consistent with the results of structural and biochemical studies, which demonstrate that siRNA activity is decreased for siRNAs of lengths less than 16 nucleotides.[26,72]

One can address the problem of unwanted incorporation of the passenger strand into RISC by altering the thermodynamic asymmetry of the duplex.[117] This can be easily achieved by the judicious use of stabilizing and destabilizing chemical modifications, as outlined in the studies on the effects of various chemical modifications on siRNAs reported recently.[117] Stabilizing modifications such as LNA at the 5′ end of the passenger strand favor incorporation of the guide strand into the RISC.[102,103] On the other hand, even if the passenger strand is incorporated, LNA modifications prohibit phosphorylation of

the 5′ end, which results in inactivation of the RISC.[102] The 5′-OMe modification blocks phosphorylation or affects the 5′-phosphate recognition of the siRNAs by cellular kinases.[138] A 5′-OMe modification at the passenger strand blocks its incorporation into the RISC without hindering the incorporation of the guide strand, and this effect outweighs the strong strand preference for the passenger strand of an asymmetrically loading siRNA duplex.[138] However, whether RNAi is completely abolished by modification of one of the 5′-phosphate oxygen atoms of the guide strand is a matter of debate.[62,80,89,138] To explain the reported activity of 5′-phosphate-modified siRNAs, it has been suggested that the other unmodified oxygen atom of the 5′-phosphate continues to bind to the RISC depending on the bulk of the modification and its ability to avoid a steric clash with the RISC.[139]

To address the selective incorporation of the guide strand, a recent report outlines the use of novel siRNAs with an intact guide strand and two segmented passenger strands 9 and 13 nt in length, termed sisiRNAs ('small internally segmented RNAs').[140] These sisiRNAs are degraded in vivo, but when used along with LNA residues, they are imparted with sufficient serum stability.[140] This design can result in the incorporation of heavily modified guide strands that are otherwise not incorporated into the RISC if used as standard siRNAs.[140]

4.3. Combating the immunostimulatory effects of siRNAs

One of the major challenges in siRNA-based therapeutics is the undesired activation of the innate immune system in vivo.[21] The immune system has evolved to differentiate between viral or bacterial RNA and endogenously expressed RNAs.[141] The immunostimulatory effects of dsRNA are mediated primarily through three of the 13 Toll-like receptors (TLR3, TLR7, and TLR8),[142–144] and by proteins such as retinoic acid inducible protein (RIG-1), oligoadenylate synthetase (OAS), dsRNA-responsive kinase (PKR), and melanoma differentiation associated protein (MDA-5).[145–149] All these immunostimulatory receptors identify different forms of dsRNA and can potentially induce interferons such as IFNα/β, cytokines such as tumor necrosis factor α (TNFα), and interleukin-6 (IL-6) depending on the nature of the synthetic dsRNA.[144,150] Release of such inflammatory cytokines and interferons poses a significant toxicological hurdle in the development of RNAi therapeutics.[142] This demands the testing of various siRNAs against all the aforementioned receptors for assessing their immunostimulatory effects.

It is known from various studies that the activation of TLR7 and 8 is sequence dependent, whereas TLR3 activation depends primarily on the length of the siRNA.[151] TLR3, 7, and 8 are present within the endosomal compartments, and thus exposure to these receptors is maximized with the use of delivery vehicles that facilitate cellular uptake.[142] Upon endosomal acidification and release of siRNAs, the corresponding receptor pathways are activated.[152,153] Apart from the endosomal compartments, TLR3 is also found on the surface of certain cell types.[142] TLR3 is usually activated by dsRNAs longer than 21–23 nt; however, this length threshold varies among different cell types.[151] It has also been suggested that GU-rich motifs act as agonists for both TLR7 and 8, whereas AU-rich motifs activate only TLR8.[142] Although specific stimulatory motifs such as GUCCUUCAA and UGUGU have been identified, it is not yet possible to predict immunostimulatory potential from the siRNA sequence alone.[144,150]

The selective incorporation of as few as two 2′-OMe modifications at either uridine or guanosine residues in highly immunostimulatory siRNAs has been shown to significantly decrease the activation of TLR7 and 8.[154] Moreover, the effect of 2′-deoxyuridine was more significant than other 2′-deoxynucleotides in decreasing immunostimulation.[155] On the other hand, 5-methylcytidine and 7-deazaguanosine nucleotides do not affect IFNα secretion, indicating that recognition does not involve sites in the major groove of the siRNA.[155] The exact mechanism by which this recognition occurs is not known; however, it has been generally suggested that the 2′ position of the ribose sugar and uridine nucleotides play a crucial role in dsRNA recognition by TLR7 and 8.[156] The 2′-OMe modification renders protection such that only the passenger strand modification suffices to block immune activation, and the effect is independent of the position of the modification in the siRNA.[154] It has been demonstrated that 2′-OMe nucleotides act as competitive inhibitors by directly binding to TLR7 and posing as TLR7 antagonists.[157] A more robust approach would be to selectively modify both the guide and passenger strand with 2′-OMe nucleosides to completely block any potential minor activation of TLR7.[158] The LNA modification is also known to decrease TLR activation; however, unlike the 2′-OMe modification, the presence of this modification in both the guide and passenger strands is necessary.[142,157] Replacement of the uridine with 2′-fluoro-modified nucleotides also abrogates activation of TLR7 and 8.[159] However, Judge and coworkers pointed out that the use of 2′-fluoro and 2′-deoxy alone has an unpredictable effect on the immunostimulatory activity of siRNAs depending on the siRNA sequence as well as the position and extent of the modification.[142] Thus, the use of 2′-OMe is evidently the best way to deal with TLR7 and 8 activation, whereas the activation of TLR3 is not inhibited by this modification.[142,151]

The TLRs may have evolved to be preferentially deactivated by modifications that occur naturally in endogenously expressed human RNAs and that are absent in pathogenic RNAs.[141] Some of the naturally occurring modifications that have been tested for protection against TLR activation are *N*6-methyladenosine (m6A), m5U, s2U, and Ψ (Figure 4c).[141] These modifications provide protection against either TLR7 or 8, and the extent to which immunostimulatory activity is suppressed is directly proportional to the number of such modified nucleosides present.[141] The s2U and m6A modifications have been shown to protect against TLR3 activation.[141] Considering the fact that all these are base modifications, it is clear that factors other than site recognition in the minor groove and recognition of the ribose sugar ring may be responsible for the activation of TLR7 and 8.

The dsRNA binding domains in PKR can interact with dsRNAs as short as 11 bases, although PKR was previously considered to be activated only by dsRNAs >30 bases long.[147]

However, it has been demonstrated that 21-nt siRNAs with 3′ overhangs were able to induce some degree of PKR activation.[160–162] For long dsRNAs, the crystal structure of PKR in complex with dsRNA reveals that RNA–protein interactions are present largely in the minor groove, with water-bridged contacts to the 2′-OH group of the dsRNA, while the phosphates in the major groove are engaged by fewer water-bridged hydrogen bonds.[163] Thus the interaction between PKR and long dsRNAs is not sequence specific, and hence the absence of hydrogen bonding in the case of the 2′-deoxy modification hampers PKR activation.[163] In the case of 2′-fluoro-modified siRNAs, the 2′-fluoro group can serve as a hydrogen bond acceptor which results in PKR activation.[163] Thus, if PKR is likely to be activated by siRNAs, any modification that alters the hydrogen bonding interactions in the minor groove may decrease PKR activation, whereas major groove modifications may have little effect.[163] Also, modifications that disrupt the Watson–Crick base pairing and hence the A-type helical geometry were also shown to decrease PKR activation by long dsRNAs.[163]

Activation of cytoplasmic RIG-1 is independent of dsRNA sequence.[148,149] It increases as the dsRNA length increases from 21 to 27 nucleotides, but decreases in the presence of overhangs.[162] It has been shown that blunt-ended dsRNA can also bind to and activate RIG-1.[162] RIG-1 is also activated by dsRNAs containing uncapped 5′-triphosphate, which is characteristic of viral and bacterial RNAs.[148,149] However, RIG-1 is not activated by endogenously expressed RNAs with a monophosphate or a 7-methylguanosine (m7G) cap.[148,149] It has been reported that the 2′-OMe modification is capable of inhibiting interferon induction by RIG-1 resulting from stimulatory RNAs.[142,164,165] This may serve as a good strategy to decreases RIG-1-mediated activation of siRNAs.

Ambati and co-workers recently demonstrated that siRNAs inhibit choroidal neovascularization (CNV) by TLR3 activation in a sequence-independent fashion rather than RNAi activity.[151] Earlier clinical trials focusing on CNV inhibition using siRNAs targeting either the vascular endothelial growth factor-A (VEGFA) or its receptor (VEGFR1) were thought to be based on RNAi activity.[151] However, only siRNAs with a minimum length of 21 nucleotides could induce such TLR3-mediated CNV inhibition. In a more recent report, Ambati and colleagues have also shown that siRNAs suppress blood and lymphatic neovascularization in murine models via TLR3 activation.[166] This raises concerns of the undesirable effects of the presence of siRNAs on blood and lymphatic circulatory systems due to TLR3 activation.[151,166] Similarly, the anti-influenza properties of siRNAs in vivo, as demonstrated earlier, are now attributed to have arisen from immunostimulation.[167–169] The fault in the earlier conclusions arose from the use of a negative control siRNA targeting the green fluorescent protein (GFP), which has an unusually low immunostimulatory potential relative to most active siRNAs.[167] These unintended immunostimulatory properties of siRNAs warrant greater vigilance in the design of bona fide siRNAs and in the selection of appropriate control siRNAs. In an effort to address these serious issues associated with the therapeutic development of siRNAs, a recent report has shown the use of stable nucleic acid–lipid particle (SNALP)-formulated 2′-OMe modified siRNAs as antitumor therapeutics.[158] Thorough evaluation by various techniques has confirmed RNAi as the primary mechanism of action of these modified siRNAs. This is one of the first reports that has demonstrated the antitumor effects of siRNAs that are not attributable to their immunostimulation.[158]

4.4. Addressing siRNA delivery

Along with the rapid progress made in the design of efficient siRNAs, much effort is being directed at finding an appropriate delivery vehicle for siRNAs.[14,21] Naked siRNAs are much larger than small-molecule drugs, can be easily degraded in the bloodstream by endogenous enzymes, and are unable to cross the cellular membrane due to their negative charge.[170,171] However, naked siRNAs have been delivered in vivo using what is known as 'local delivery', in which the siRNAs are administered through an injection or passed through the respiratory tract directly to the affected tissues.[172–176] Clearly, this approach is only appropriate for organs such as lungs and eyes. Localized injection of unmodified siRNAs to the vitreal cavity in the eye has been shown to be beneficial in wet AMD-related complications; the drug has entered phase III clinical trials.[177–180] A similar approach is being taken in which the epithelial cells in the lung are targeted for the treatment of respiratory syncitial virus (RSV); the drug is currently in phase II clinical trials (ALN-RSV01, Alnylam Pharmaceuticals; Table 1).[181,182]

Not all cells of the human body, however, can be accessed through localized delivery, owing to the problem of the negative charge on siRNAs. In most cases, systemic delivery of siRNAs is essential. Systemic delivery involves intravenous injection of the siRNAs along with a delivery agent, which should be able to propagate in the bloodstream to the desired organ or tissue without being degraded or taken up by non-target tissues in the process.[183] A systemically injected siRNA has to undergo a series of processes before it can enter the cell and produce the desired result. The siRNA–delivery complex should be able to avoid kidney filtration, which will remove it from the bloodstream.[184,185] It should also be able to avoid uptake by phagocytes present in the body;[186] phagocytes are natural scavengers that are very potent at recognizing foreign particles in the blood and eliminating their harmful effects. After evading the phagocytes, siRNA complexes should be able to penetrate through the endothelial membrane in order to enter the targeted tissue. This is another hurdle, as it depends on the particle size. Diffusing through the endosome in order to reach the cytoplasm is difficult due to the viscosity of the vast network of polysaccharides and fibrous proteins.[187] This leads to problems with fluidity of the delivery package which may lead to elimination, and can also halt the delivery process. If they escape nuclease cleavage in the cell, siRNAs can finally be released to perform their task inside.

An appropriate delivery vehicle should be able to overcome all these hurdles and effectively release the siRNA in the cytoplasm of the target cell. To achieve this, it should possess the appropriate physical properties. As the cellular membrane is

negatively charged, an ideal delivery particle should be positively charged at the surface to ensure easier uptake.[188] This has been established through studies in murine graft models. However, the human serum itself is negatively charged, and will therefore form a complex with the siRNA–delivery complex and render it ineffective.[171] A way to address this is to increase the particle size by adding poly(ethylene glycol) (PEG) to the surface of the delivery vehicle, which can conjugate and prevent particle aggregation in serum.[189] PEG can also protect the delivery package from phagocytes and endogenous nucleases by surrounding it with a protective covering.[190,191] Increasing the particle size also prevents discharge of the naked siRNA through renal excretion and prevents its accumulation in certain organs such as the lung or spleen.[185] The most important factor, however, is the toxicity of the delivery vehicle. A toxic delivery particle will only compound problems. Viral vectors have been shown to harbor high levels of toxicity and are not considered ideal delivery vehicles for siRNAs.[192] These vectors have genes that express the siRNA sequences and can initiate a series of unwanted RNAi effects through prolonged gene expression in the targeted area; this can lead to a serious immune response from the body.[193]

Two known approaches for systemic delivery are 'complexation' and 'conjugation'.[171] The complexation method involves formation of complexes of siRNAs with motifs that bind to their negative charges. The techniques used for this include lipids and liposomes such as stable nucleic acid–lipid particles (SNALP), antibody complexes, polycationic peptides known as peptide transduction domains (PTDs), and polycationic polymers based on PEG and poly(ethylene imine) (PEI). The conjugation approach involves attaching the siRNA to the delivery vehicle and has shown success in targeting cell types that are specific to the siRNAs.[194–196] The conjugation approach is also useful, as the passenger strand can be conjugated to the delivery vehicle, leaving the guide strand to perform its desired function once the delivery vehicle is cleaved away.[197] Conjugates currently in use include lipophilic species such as cholesterol, as well as peptides and aptamers.[170]

siRNA delivery through complexation with liposomes is being widely explored these days.[168] A more recent development in this area is SNALP, which has been tested to target hepatitis B virus (HBV) RNA in mice, reported by Morrissey and co-workers.[95] Earlier studies on SNALP had proven its potency in efficiently delivering siRNAs to the ApoB target in mice and monkeys.[150,198] Alnylam Pharmaceuticals, in collaboration with Tekmira Pharmaceuticals, are using SNALP-based siRNA delivery for their liver cancer drug development program, and the drug candidate is currently in phase I trials (Table 1). The SNALP is composed of multiple layers of cationic lipids which complex with the bilayer membranes, PEG to increase circulation in the bloodstream, and cholesterol to provide lipophilicity to the delivery complex.[199] Anderson and co-workers have also synthesized a new lipid-like delivery molecule called Lipidoid for delivering siRNAs.[200] A library of ~1200 lipidoids was created, from which the best lipidoid–siRNA complexes were identified through in vitro delivery. In vivo tests have also been carried out in rats and Cynomolgus monkeys. Persistent silencing was observed in the primates with no treatment-related toxic effects.[200] Advanced reports on lipidoid-mediated delivery have suggested that in vivo efficacy depends on factors such as particle size, degree of PEG anchoring length, and siRNA loading.[201] The researchers used 98N12-5(1), which was identified as a superior lipidoid particle in the previous study along with PEGylation for bio-circulation and cholesterol for outer conjugation.[201] The final nanoparticle complex, called LNP01, was formulated and tested for efficacy in delivering siRNAs to the liver. The nanoparticle complex showed good stability in serum and efficient distribution of siRNAs in liver with no side effects on repeat delivery.[200] These results show that formulations of lipid-based particles are efficient in delivering siRNAs to the target tissues. Further studies should test the capacity for in vivo delivery in more complex target tissues, which may lead to potential therapeutic application of lipidoids.

Peptide complexes for siRNA delivery can offer several advantages. An example of such a complex involves peptide transduction domains (PTDs) or cell penetration peptides, which were shown to successfully deliver siRNAs to a large population of cells with high in vivo efficiency.[202,203] siRNA–PTDs can cross the cellular membrane, even though they are relatively large in size, and their cytotoxicity is low.[203] Recent studies have shown optimal siRNA release in primary cells when conjugated with a PTD–dsRNA binding domain fusion protein.[204] A shortfall with this strategy is that the endosomal release of the siRNA from the PTD complex is incomplete, leading to low activity.[205] Antibodies have also been shown to serve as efficient delivery vehicles for siRNAs.[206] A combination of protamine–antibody fusion protein has been used to target a series of cells via the HIV envelope protein gp160.[206] The siRNA is attached to the positively charged protamine and is delivered to the cells expressing the specific receptor. The siRNA–antibody complex is internalized, releasing the siRNA to down-regulate the targeted protein.[206]

Niidome and co-workers recently showed that an siRNA targeting the ApoB-related mRNA in mice complexed with a sixth-generation dendritic poly(L-Lys) effects efficient delivery of the ApoB-targeting siRNA in liver cells.[207] The intravenous injection of siRNA at 2.5 mg kg^{-1} body weight showed negligible levels of hepatotoxicity; however, the silencing activity was lower than that observed with lipid-based delivery systems. This has been attributed to the greater size of this dendritic complex, which makes cell membrane permeation difficult.[207] Another strategy is to use cationic polymers for siRNA delivery.[208–212] A lot of work on this approach is based on the fact that these polymers can efficiently bind to the siRNAs and condense them into stable nanoparticles; they can also facilitate endosomal movement, which overcomes a major hurdle.[213] However, significant toxicity can result from a high dose of polymer-complexed siRNAs or by increasing the molecular weight of the complex.[214]

Conjugation to lipophilic molecules such as cholesterol has been well documented in the delivery of siRNAs in murine models and has shown desirable results.[194] The 3′ end of a passenger strand was conjugated to cholesterol with the aim

to target the ApoB protein in mice.[194] The conjugated siRNA escaped kidney filtration, remained in circulation for a long period of time, and showed efficient silencing of the target gene. A disadvantage of this strategy is the high dosage required (50 mg kg^{-1}).[194] Gao and co-workers have also shown that cholesterol-conjugated siRNAs have improved pharmacodynamic properties and stay in circulation longer than unmodified siRNAs.[215] Other lipophilic species studied for siRNA conjugation are vitamins E and A.[216,217] A vitamin E–siRNA conjugate was systemically delivered to the liver and showed efficient delivery into the target tissues without any interferon response.[216] An aptamer-based approach works along similar lines.[195,218] The siRNA was conjugated to an aptamer that targets prostrate-specific membrane antigen (PSMA), which is abundant on prostrate cancer cells; the package efficiently delivered siRNAs into the tumor cells, leading to desirable silencing.[195,218] However, aptamer-based ligands are susceptible to nuclease degradation, and this limits their use in systemic delivery.[197] To overcome this problem, Giangrande and co-workers recently developed an optimal siRNA–aptamer chimera that targets PSMA-expressing cells. This conjugate showed potent silencing and good bio-distribution in the body through systemic delivery.[219] The aptamer length was shortened to 39 nt from the previous 71 nt, and various siRNA designs were tested, of which the presence of 3′ overhangs was shown to be critical for improved activity. In vivo tests in xenograft models involving a 2′-fluoro-modified siRNA–aptamer conjugate coupled with PEG showed potent RNAi activity in the tumor cells with no observable toxicity.[219] However, the dosage was very high, with 10 daily injections of the conjugate at 0.7 mg kg^{-1} body weight.[219] There are also a few reports of covalent siRNA–PTD conjugates that have shown desirable silencing in vitro, but which failed in vivo.[220]

The conjugation of peptides to siRNAs has been used to target the Japanese encephalitis virus.[221] The conjugated peptide belongs to the rabies virus glycoprotein, which binds to the neuronal cell acetylcholine receptor. An intravenous injection efficiently silenced the targeted gene in 80% of the treated mice. This study is important, as it shows that a peptide–siRNA conjugate can successfully deliver siRNAs across the blood–brain barrier.[221] Yu and co-workers recently published a study showing the efficacy of TLR9 agonist CpG oligonucleotides conjugated to siRNAs in silencing the tumor suppressor gene *Stat3* in mice.[222] Both localized and systemic delivery of conjugate siRNAs showed potent antitumor activity. Another recent in vitro study by Anderson and co-workers demonstrated the use of PEG-modified gold nanoparticles (AuNP) as conjugation agents for siRNA delivery.[223] The siRNAs were conjugated to the nanoparticles using disulfide linkages, and the particles were complexed with end-modified poly-β-amino esters (PBAEs). These AuNP–siRNA conjugates complexed with PBAEs showed efficient delivery without significant toxicity.[223] Further studies should be carried out to test the efficiency of these delivery systems in vivo, and also to detect any toxicity that may result from the premature decomposition of the complex. A report published very recently has shown that siRNAs can be efficiently delivered to mammalian cells using cationic spermines as conjugates.[224] A total of 30 spermine molecules were conjugated to the 5′ end of the passenger strand, leading to a net positive charge on the conjugated siRNA.[224] These modifications are promising considering the relative ease of delivering these conjugated siRNAs as naked entities versus the use of bulky complexes.

The field of siRNA delivery has undergone major improvements in recent years. New delivery methods are being tested, and some of the results are indeed very encouraging for addressing the issues associated with siRNA delivery. However, a perfect delivery vehicle that can overcome all associated hurdles has yet to be developed. Moreover, the viability of the existing delivery vehicles and strategies in clinical settings have yet to be evaluated. A comprehensive overview of siRNA delivery strategies can be found in recently published reviews.[170,171,225,226]

5. Guidelines for the Design and Use of Chemical Modifications in siRNAs

By scrutinizing the available literature on the use of chemical modifications in siRNAs as well as structural and biochemical studies of RNA–Ago complexes, a set of guidelines can be formulated which may be useful for the rational design and testing of siRNAs in in vitro and in vivo settings:

1. Bulky modifications that influence the minor groove environment (e.g., MOE, ENA) in the seed region (particularly at position 2 of the guide strand) as well as at the cleavage site affect the Ago2 interactions. Hence, such modifications should be avoided at these positions.[27,117]
2. Modifications at the 3′ overhangs of the guide strand are well tolerated due to the limited role of the PAZ domain interaction in the silencing mechanism, as the base pairing of the guide–target duplex effectively ends at position 16 from the 5′ end of the guide strand.[25–27] Therefore, most of the modifications can be incorporated at this position without risking loss of RNAi activity.
3. Modifications are generally well tolerated in the passenger strand. Modifying the 5′ end of the passenger strand with stabilizing modifications (e.g., LNA, 5-pU) can alter the thermodynamic asymmetry of the duplex leading to specific recognition of the 5′ end of the guide strand by Ago2.[101]
4. Bulky chemical modifications that are able to impart thermodynamic stability, high nuclease stability, and lipophilicity to siRNAs, but which cannot induce RNAi can be effectively used employing sisiRNA design strategies.[140]
5. Backbone modifications that do not affect siRNA structure (e.g., PS, boronophosphate) can afford protection against nuclease activity.[22,84] However, modifications that distort the siRNA structure (e.g., PNAs and morpholino) should be placed only at the 3′ overhangs of the passenger strand.[87,88]
6. Because position 7 of the guide strand from the 5′ end has been shown to tolerate non-hydrogen-bonding modifications (e.g., rF, rL, and m3U) without considerable loss

of silencing activity and also to improve target strand selection, this position can be altered with base modifications that affect the hydrogen bonding.[110,113]

7. The desired siRNA strand could be made to be selectively incorporated into the RISC by altering the thermodynamic asymmetry of the siRNA duplex. The most basic approach is to have C:G base pairs at the 3′ end and U:A pairs at the 5′ end in order to alter the thermodynamic stability during unwinding.[13] Chemical modifications that prevent phosphorylation, such as 5′-OMe, can be introduced at the 5′ end of the passenger strand of the siRNA duplex to enhance guide strand selection during formation of the RISC.[138] This may also be achieved by using the sisiRNA design.[140]
8. To offset the unwanted off-target effects arising from siRNA participation in miRNA-like pathways, the siRNA should have the minimum possible seed region homology with the 3′-UTRs of various other mRNAs. A 2′-OMe modification may be included at position 2 of the guide strand to prevent the unintended but probable partial homology of the seed region of the siRNA with other mRNAs.[133,134] In addition, guide strands that are likely to form a low-T_m seed duplex with the target strand should be favored.[70]
9. Immunostimulation by siRNAs depends on the structure and sequence of the siRNA as well as the delivery vehicle used. The most useful approach to avoid these undesired effects is to use 2′-OMe-modified uridine residues (at least three) in both the passenger and guide strand of siRNA duplex.[142,154,157]
10. Most chemical modifications can lead to improved utility when used as a combination in siRNAs (e.g., Me-SRNA, S-FANA).[96,109] This can help secure the maximal advantage of the synergistic effect of the two modifications. Even combinations such as backbone modifications at the overhangs and sugar modifications at the 2′ position can offset problems like nuclease resistance and off-target effects.
11. The structural and biochemical studies on the optimum length required for the formation of a potent RNAi trigger[26,72] call for testing 16-bp siRNA duplexes for their ability to silence a variety of targets in vivo. Such a design may also serve as a better alternative to avoid off-target effects and immunostimulation.
12. Target site accessibility has been shown to play a crucial role in effective RNAi; both experimental and computational approaches may be used in tandem in order to design the most efficient siRNA against a target.[227]
13. The issue of biodistribution should not be overlooked in the design of new modifications for siRNA therapeutics. One way to achieve this is by the incorporation of sulfur in the modification[108,109] or combining with the required number of PS moieties in the siRNA backbone.[22]
14. Care must be taken in in vitro studies such that any immune activation does not go unnoticed. Errors in studies arise from the use of non-responsive cell lines and from reaching a conclusion based on assay time points that miss the rapid induction of cytokines.[167] It is also necessary to test for different cytokine classes to evaluate the overall immunostimulation effects.[167]
15. The use of control siRNAs, such as the one that targets GFP with low immunostimulatory activity, should be avoided.[167] It is therefore necessary to characterize control as well as active siRNAs for their immunostimulatory potentials. It is important to ascertain that the desired effect results from RNAi and not from immunostimulation. This can be done by performing 5′ RACE PCR and DNA sequencing studies, which aid in detecting the mRNA cleavage products.[167,194]

6. Outlook

RNA interference has come a long way since its ground-breaking discovery in 1998. This is clearly evident from the fact that more and more siRNA-based drug candidates are entering clinical trials (Table 1). An important challenge is to establish that the silencing activity results from RNAi-mediated target cleavage rather than immunostimulation, which has been proven to be the case with a drug candidate in advanced clinical trials.[228] The improved design of siRNAs will be crucial, as the ideal drug should address such challenges as nuclease stability, immunostimulation, off-target effects, and delivery. More structural studies on human Ago–RNA complexes will certainly help in the design of better siRNA therapeutics. Notably, there is no clear understanding so far on the transition from the RLC to the RISC, as the passenger strand cleavage, its removal, and the geometry of the guide strand subsequent to these events cannot be deciphered from the available Ago–DNA crystal structures. The new designs being proposed to improve nuclease stability and potency such as DsiRNAs and 29-bp shRNAs should be evaluated with greater stress on their immunostimulatory potential.[165] Potential off-targeting may also result from the siRNAs produced by the cleavage of these long RNAs, as many different siRNA products are possible. Clearly these designs are in their nascent stage of development, and more in vivo studies are required to ascertain their full potential. Importantly, a majority of chemical modifications employed in siRNAs so far were originally devised for antisense technology. Although these modifications have proven useful to a certain extent, efforts to explore novel modifications for siRNAs are warranted, as their structural and functional requirements are different from those of antisense oligonucleotides. Improved delivery strategies can help in delivering siRNAs to a wide range of targets. Recent developments in PTDs and SNALP-based delivery methods are very promising, and much effort is being devoted to these aspects of delivery. Although it will take many more breakthroughs in structural studies and delivery, there is an overall excitement about the wider applications of siRNA therapeutics. Needless to say, with the amount of research being put into their development, the future of siRNA therapeutics looks very bright.

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[1] C. Napoli, C. Lemieux, R. Jorgensen, *Plant Cell* **1990**, *2*, 279 – 289.
[2] N. Romano, G. Macino, *Mol. Microbiol.* **1992**, *6*, 3343 – 3353.
[3] S. Guo, K. J. Kemphues, *Cell* **1995**, *81*, 611 – 620.
[4] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, *Nature* **1998**, *391*, 806 – 811.
[5] P. D. Zamore, T. Tuschl, P. A. Sharp, D. P. Bartel, *Cell* **2000**, *101*, 25 – 33.
[6] S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* **2001**, *15*, 188 – 200.
[7] M. T. McManus, P. A. Sharp, *Nat. Rev. Genet.* **2002**, *3*, 737 – 747.
[8] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* **2001**, *411*, 494 – 498.
[9] A. Grishok, A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun, C. C. Mello, *Cell* **2001**, *106*, 23 – 24.
[10] G. Hutvagner, J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl, P. D. Zamore, *Science* **2001**, *293*, 834 – 838.
[11] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, T. Tuschl, *Mol. Cell* **2004**, *15*, 185 – 197.
[12] D. S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, P. D. Zamore, *Cell* **2003**, *115*, 199 – 208.
[13] A. Khvorova, A. Reynolds, S. D. Jayasena, *Cell* **2003**, *115*, 209 – 216.
[14] M. A. Behlke, *Oligonucleotides* **2008**, *18*, 305 – 320.
[15] J. K. Watts, G. F. Deleavey, M. J. Damha, *Drug Discovery Today* **2008**, *13*, 842 – 855.
[16] J. J. Turner, S. W. Jones, S. A. Moschos, M. A. Lindsay, M. J. Gait, *Mol. Biosyst.* **2007**, *3*, 43 – 50.
[17] G. Hartmann, *J. Clin. Invest.* **2009**, *119*, 438 – 441.
[18] S. Saxena, Z. O. Jonsson, A. Dutta, *J. Biol. Chem.* **2003**, *278*, 44312 – 44319.
[19] X. Lin, X. Ruan, M. G. Anderson, J. A. McDowell, P. E. Kroeger, S. W. Fesik, Y. Shen, *Nucleic Acids Res.* **2005**, *33*, 4527 – 4535.
[20] J. G. Doench, C. P. Peterson, P. A. Sharp, *Genes Dev.* **2003**, *17*,438 – 442.
[21] D. M. Dykxhoorn, J. Lieberman, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 377 – 402.
[22] D. Bumcrot, M. Manoharan, V. Koteliansky, D. W. Y. Sah, *Nat. Chem. Biol.* **2006**, *2*, 711 – 719.
[23] M. Manoharan, *Curr. Opin. Chem. Biol.* **2004**, *8*, 570 – 579.
[24] Y. Wang, G. Sheng, S. Juranek, T. Tuschl, D. J. Patel, *Nature* **2008**, *456*, 209 – 213.
[25] Y. Wang, S. Juranek, H. Li, G. Sheng, T. Tuschl, D. J. Patel, *Nature* **2008**, *456*, 921 – 926.
[26] Y. Wang, S. Juranek, H. Li, G. Sheng, G. S. Wardle, T. Tuschl, D. J. Patel, *Nature* **2009**, *461*, 754 – 761.
[27] W. F. Lima, H. Wu, J. G. Nichols, H. Sun, H. M. Murray, S. T. Crooke, *J. Biol. Chem.* **2009**, *284*, 26017 – 26028.
[28] D. M. Dykxhoorn, C. D. Novina, P. A. Sharp, *Nat. Rev. Mol. Cell. Biol.* **2003**, *4*, 457 – 467.
[29] K. V. Morris, J. J. Rossi, *Gene Ther.* **2006**, *13*, 553 – 558.
[30] Q. Liu, T. A. Rand, S. Kalidas, F. Du, H. E. Kim, D. P. Smith, X. Wang, *Science* **2003**, *301*, 1921 – 1925.
[31] H. Wang, C. Noland, B. Siridechadilok, D. W. Taylor, E. Ma, K. Felderer, J. A. Doudna, E. Nogales, *Nat. Struct. Mol. Biol.* **2009**, *16*, 1148 – 1153.
[32] T. A. Rand, S. Petersen, F. Du, X. Wang, *Cell* **2005**, *123*, 621 – 629.
[33] T. A. Rand, K. Ginalski, N. V. Grishin, X. Wang, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14385 – 14389.
[34] C. Matranga, Y. Tomari, C. Shin, D. P. Bartel, P. D. Zamore, *Cell* **2005**, *123*, 607 – 620.
[35] J. Liu, M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, G. J. Hannon, *Science* **2004**, *305*, 1437 – 1441.
[36] C. Y. Chu, T. M. Rana, *J. Cell. Physiol.* **2007**, *213*, 412 – 419.
[37] X. C. Ding, H. Grosshans, *EMBO J.* **2009**, *28*, 213 – 222.
[38] G. Meister, M. Landthaler, L. Peters, P. Y. Chen, H. Urlaub, R. Luhrmann, T. Tuschl, *Curr. Biol.* **2005**, *15*, 2149 – 2155.
[39] R. W. Carthew, E. J. Sontheimer, *Cell* **2009**, *136*, 642 – 655.
[40] N. Lynam-Lennon, S. G. Maher, J. V. Reynolds, *Biol. Res.* **2009**, *84*, 55 – 71.
[41] F. Petrocca, J. Lieberman, *RNA Biol.* **2009**, *6*, 1 – 5.
[42] Y. S. Lee, A. Dutta, *Annu. Rev. Pathol. Mech. Dis.* **2009**, *4*, 199 – 227.
[43] F. V. Rivas, N. H. Tolia, J. J. Song, J. P. Aragon, J. Liu, G. J. Hannon, L. Joshua-Tor, *Nat. Struct. Mol. Biol.* **2005**, *12*, 340 – 349.
[44] Y. R. Yuan, Y. Pei, H. Y. Chen, T. Tuschl, D. J. Patel, *Structure* **2006**, *14*, 1557 – 1565.
[45] N. H. Tolia, L. Joshua-Tor, *Nat. Chem. Biol.* **2007**, *3*, 36 – 43.
[46] M. Nowotny, W. Yang, *Curr. Opin. Struct. Biol.* **2009**, *19*, 286 – 293.
[47] G. Hutvagner, M. Simard, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 22 – 32.
[48] J. S. Parker, S. M. Roe, D. Barford, *EMBO J.* **2004**, *23*, 4727 – 4737.
[49] J. J. Song, S. K. Smith, G. J. Hannon, L. Joshua-Tor, *Science* **2004**, *305*, 1434 – 1437.
[50] J. Martinez, T. Tuschl, *Genes Dev.* **2004**, *18*, 975 – 980.
[51] Y. R. Yuan, Y. Pei, J. B. Ma, V. Kuryavyi, M. Zhadina, G. Meister, H. Y. Chen, Z. Dauter, T. Tuschl, D. J. Patel, *Mol. Cell* **2005**, *19*, 405 – 419.
[52] M. Nowotny, S. A. Gaidamakov, R. J. Crouch, W. Yang, *Cell* **2005**, *121*, 1005 – 1016.
[53] M. Nowotny, *EMBO Rep.* **2009**, *10*, 144 – 151.
[54] J. J. Song, J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon, L. Joshua-Tor, *Nat. Struct. Biol.* **2003**, *10*, 1026 – 1032.
[55] J. B. Ma, K. Ye, D. J. Patel, *Nature* **2004**, *429*, 318 – 322.
[56] M. Jinek, J. A. Doudna, *Nature* **2009**, *457*, 405 – 412.
[57] S. L. Ameres, J. Martinez, R. Schroeder, *Cell* **2007**, *130*, 101 – 112.
[58] J. B. Ma, Y. R. Yuan, G. Meister, Y. Pei, T. Tuschl, D. J. Patel, *Nature* **2005**, *434*, 666 – 670.
[59] D. S. Schwarz, Y. Tomari, P. D. Zamore, *Curr. Biol.* **2004**, *14*, 787 – 791.
[60] J. S. Parker, D. Barford, *Trends Biochem. Sci.* **2006**, *31*, 622 – 630.
[61] J. S. Parker, S. M. Roe, D. Barford, *Nature* **2005**, *434*, 663 – 666.
[62] Y. L. Chiu, T. M. Rana, *Mol. Cell* **2002**, *10*, 549 – 561.
[63] Y. L. Chiu, T. M. Rana, *RNA* **2003**, *9*, 1034 – 1048.
[64] M. Amarzguioui, T. Holen, E. Babaie, H. Prydz, *Nucleic Acids Res.* **2003**, *31*, 589 – 595.
[65] T. Holen, M. Amarzguioui, E. Babaie, H. Prydz, *Nucleic Acids Res.* **2003**, *31*, 2401 – 2407.
[66] B. Haley, P. D. Zamore, *Nat. Struct. Biol.* **2004**, *11*, 599 – 606.
[67] S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *EMBO J.* **2001**, *20*, 6877 – 6888.
[68] T. M. Rana, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 23 – 36.
[69] J. S. Parker, S. M. Roe, D. Barford, *Cold Spring Harb. Symp. Quant. Biol.* **2006**, *71*, 45 – 50.
[70] K. Ui-Tei, Y. Naito, K. Nishi, A. Juni, K. Saigo, *Nucleic Acids Res.* **2008**, *36*, 7100 – 7109.
[71] J. S. Parker, E. A. Parizotto, M. Wang, S. M. Roe, D. Barford, *Mol. Cell* **2009**, *33*, 204 – 214.
[72] C. Y. Chu, T. M. Rana, *RNA* **2008**, *14*, 1714 – 1719.
[73] Y. Tomari, P. D. Zamore, *Genes Dev.* **2005**, *19*, 517 – 529.
[74] S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **2000**, *404*, 203 – 206.
[75] R. F. Ketting, S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon, R. H. Plasterk, *Genes Dev.* **2001**, *15*, 2654 – 2659.
[76] E. R. Goedken, S. Marqusee, *J. Biol. Chem.* **2001**, *276*, 7266 – 7271.
[77] T. Dowler, D. Bergeron, A. L. Tedeschi, L. Paquet, N. Ferrari, M. J. Damha, *Nucleic Acids Res.* **2006**, *34*, 1669 – 1675.
[78] Y. Zou, P. Tiller, I.-W. Chen, M. Beverly, J. Hochman, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1871 – 1881.
[79] F. Eckstein, *Biochimie* **2002**, *84*, 841 – 848.

[80] J. Harborth, S. M. Elbashir, K. Vandenburgh, H. Manninga, S. A. Scaringe, K. Weber, T. Tuschl, *Antisense Nucleic Acid Drug Dev.* **2003**, *13*, 83 – 105.
[81] R. S. Geary, R. Z. Yu, A. A. Levin, *Curr. Opin. Investig. Drugs* **2001**, *2*, 562 – 573.
[82] Z. Y. Li, H. Mao, D. A. Kallick, D. G. Gorenstein, *Biochem. Biophys. Res. Commun.* **2005**, *329*, 1026 – 1030.
[83] P. Li, Z. A. Sergueeva, M. Dobrikov, B. R. Shaw, *Chem. Rev.* **2007**, *107*, 4746 – 4796.
[84] A. H. Hall, J. Wan, E. E. Shaughnessy, B. R. Shaw, K. A. Alexander, *Nucleic Acids Res.* **2004**, *32*, 5991 – 6000.
[85] A. H. Hall, J. Wan, A. Spesock, Z. Sergueeva, B. R. Shaw, K. A. Alexander, *Nucleic Acids Res.* **2006**, *34*, 2773 – 2781.
[86] P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, *Science* **1991**, *254*, 1497 – 1500.
[87] N. Potenza, L. Moggio, G. Milano, V. Salvatore, B. DiBlasio, A. Russo, A. Messere, *Int. J. Mol. Sci.* **2008**, *9*, 299 – 315.
[88] N. Zhang, C. Tan, P. Cai, P. Zhang, Y. Zhao, Y. Jiang, *Bioorg. Med. Chem.* **2009**, *17*, 2441 – 2446.
[89] F. Czauderna, M. Fechtner, S. Dames, H. Aygun, A. Klippel, G. J. Pronk, K. Giese, J. Kaufmann, *Nucleic Acids Res.* **2003**, *31*, 2705 – 2716.
[90] T. P. Prakash, C. R. Allerson, P. Dande, T. A. Vickers, N. Sioufi, R. Jarres, B. F. Baker, E. E. Swayze, R. H. Griffey, B. Bhat, *J. Med. Chem.* **2005**, *48*, 4247 – 4253.
[91] B. A. Kraynack, B. F. Baker, *RNA* **2006**, *12*, 163 – 176.
[92] P. Muhonen, T. Tennilä, E. Azhayeva, R. N. Parthasarathy, A. J. Janckila, H. K. Väänänen, A. Azhayev, T. Laitala-Leinonen, *Chem. Biodiversity* **2007**, *4*, 858 – 873.
[93] C. R. Allerson, N. Sioufi, R. Jarres, T. P. Prakash, N. Naik, A. Berdeja, L. Wanders, R. H. Griffey, E. E. Swayze, B. Bhat, *J. Med. Chem.* **2005**, *48*, 901 – 904.
[94] D. V. Morrissey, K. Blanchard, L. Shaw, K. Jensen, J. A. Lockridge, B. Dickinson, J. A. McSwiggen, C. Vargeese, K. Bowman, C. S. Shaffer, B. A. Polisky, S. Zinnen, *Hepatology* **2005**, *41*, 1349 – 1356.
[95] D. V. Morrissey, J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer, L. B. Jeffs, A. Judge, I. Maclachlan, B. Polisky, *Nat. Biotechnol.* **2005**, *23*, 1002 – 1007.
[96] J. K. Watts, N. Choubdar, K. Sadalapure, F. Robert, A. S. Wahba, J. Pelletier, B. M. Pinto, M. J. Damha, *Nucleic Acids Res.* **2007**, *35*, 1441 – 1451.
[97] A. Lingel, B. Simon, E. Izaurralde, M. Sattler, *Nat. Struct. Mol. Biol.* **2004**, *11*, 576 – 577.
[98] A. A. Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldegaard, C. E. Olsen, J. Wengel, *Tetrahedron* **1998**, *54*, 3607 – 3630.
[99] S. Obika, T. Nanbu, Y. Hari, K. Morio, Y. In, T. Ishida, T. Imanishi, *Tetrahedron Lett.* **1997**, *38*, 8735 – 8738.
[100] K. Bondensgaard, M. Petersen, S. K. Singh, V. K. Rajwanshi, R. Kumar, J. Wengel, J. P. Jacobsen, *Chem. Eur. J.* **2000**, *6*, 2687 – 2695.
[101] D. A. Braasch, S. Jensen, Y. Liu, K. Kaur, K. Arar, M. A. White, D. R. Corey, *Biochemistry* **2003**, *42*, 7967 – 7975.
[102] O. R. Mook, F. Baas, M. B. de Wissel, K. Fluiter, *Mol. Cancer Ther.* **2007**, *6*, 833 – 843.
[103] J. Elmen, H. Thonberg, K. Ljungberg, M. Frrieden, M. Westergaard, Y. Xu, B. Wahren, Z. Liang, H. Orum, T. Koch, C. Wahlestedt, *Nucleic Acids Res.* **2005**, *33*, 439 – 447.
[104] M. Hamada, T. Ohtsuka, R. Kawaida, M. Koizumi, K. Morita, H. Furukawa, T. Imanishi, M. Miyagishi, K. Taira, *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 301 – 309.
[105] E. E. Swayze, A. M. Siwkowski, E. V. Wancewicz, M. T. Migawa, T. K. Wyrzykiewicz, G. Hung, B. P. Monia, C. F. Bennett, *Nucleic Acids Res.* **2006**, *35*, 687 – 700.
[106] S. Hoshika, N. Minakwa, H. Kamiya, H. Harashima, A. Matsuda, *FEBS Lett.* **2005**, *579*, 3115 – 3118.
[107] M. Yokoyama, *Synthesis* **2000**, 1637 – 1655.
[108] P. Dande, T. P. Prakash, N. Sioufi, H. Gaus, R. Jarres, A. Bardeja, E. E. Swayze, R. H. Griffey, B. Bhat, *J. Med. Chem.* **2006**, *49*, 1624 – 1634.
[109] M. Takahashi, N. Minakawa, A. Matsuda, *Nucleic Acids Res.* **2008**, *37*, 1353 – 1362.
[110] J. Xia, A. Noronha, I. Toudjarska, F. Lee, A. Akinc, R. Braich, M. Frank-Kamenetsky, K. G. Rajeev, M. Manoharan, *ACS Chem. Biol.* **2006**, *1*, 176 – 183.
[111] F. Li, P. S. Pallan, M. A. Maier, K. G. Rajeev, S. L. Mathieu, C. Kreutz, Y. Fan, J. Sanghvi, R. Micura, E. Rozners, M. Manoharan, M. Egli, *Nucleic Acids Res.* **2007**, *35*, 6424 – 6438.
[112] M. Zacharias, J. W. Engels, *Nucleic Acids Res.* **2004**, *32*, 6304 – 6311.
[113] A. Somoza, A. P. Silverman, R. M. Miller, J. Chelliserrykattil, E. T. Kool, *Chem. Eur. J.* **2008**, *14*, 7978 – 7987.
[114] M. Terrazas, E. T. Kool, *Nucleic Acids Res.* **2008**, *37*, 346 – 353.
[115] A. J. Pratt, I. J. MacRae, *J. Biol. Chem.* **2009**, *284*, 17897 – 17901.
[116] K. Sipa, E. Sochacka, J. Kazmierczak-Baranska, M. Maszewska, M. Janicka, G. Nowak, B. Nawrot, *RNA* **2007**, *13*, 1301 – 1316.
[117] J. B. Bramsen, M. B. Laursen, A. F. Nielsen, T. B. Hansen, C. Bus, N. Langkjaer, B. R. Babu, T. Højland, M. Abramov, A. Van Aerschot, D. Odadzic, R. Smicius, J. Haas, C. Andree, J. Barman, M. Wenska, P. Srivastava, C. Zhou, D. Honcharenko, S. Hess, E. Müller, G. V. Bobkov, S. N. Mikhailov, E. Fava, T. F. Meyer, J. Chattopadhyaya, M. Zerial, J. W. Engels, P. Herdewijn, J. Wengel, J. Kjems, *Nucleic Acids Res.* **2009**, *37*, 2867 – 2881.
[118] D. H. Kim, M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi, J. J. Rossi, *Nat. Biotechnol.* **2005**, *23*, 222 – 226.
[119] D. Siolas, C. Lerner, J. Burchard, W. Ge, P. S. Linsley, P. J. Paddison, G. J. Hannon, M. A. Cleary, *Nat. Biotechnol.* **2005**, *23*, 227 – 231.
[120] A. L. Jackson, S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, P. S. Linsley, *Nat. Biotechnol.* **2003**, *21*, 635 – 637.
[121] S. P. Persengiev, X. Zhu, M. R. Green, *RNA* **2004**, *10*, 12 – 18.
[122] L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, J. M. Johnson, *Nature* **2005**, *433*, 769 – 773.
[123] P. R. Clark, J. S. Pober, M. S. Kluger, *Nucleic Acids Res.* **2008**, *36*, 1081 – 1097.
[124] P. C. Scacheri, O. Rozenblatt-Rosen, N. J. Caplen, T. G. Wolfsberg, L. Umayam, J. C. Lee, C. M. Hughes, K. S. Shanmugam, A. Bhattacharjee, M. Meyerson, F. S. Collins, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 1892 – 1897.
[125] C. Dahlgren, H. Y. Zhang, Q. Du, M. Grahn, G. Norstedt, C. Wahlestedt, Z. Liang, *Nucleic Acids Res.* **2008**, *36*, e53.
[126] A. Birmingham, E. M. Anderson, A. Reynolds, D. Ilsleytyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K Robinson, J. Karpilow, W. S. Marshall, A. Khvorova, *Nat. Methods* **2006**, *3*, 199 – 204.
[127] Q. Du, H. Thonberg, J. Wang, C. Wahlestedt, Z. Liang, *Nucleic Acids Res.* **2005**, *33*, 1671 – 1677.
[128] J. G. Doench, P. A. Sharp, *Genes Dev.* **2004**, *18*, 504 – 511.
[129] A. L. Jackson, J. Burchard, J. Schelter, B. N. Chau, M. Cleary, L. Lim, P. S. Linsley, *RNA* **2006**, *12*, 1179 – 1187.
[130] L. M. Alemán, J. Doench, P. A. Sharp, *RNA* **2007**, *13*, 385 – 395.
[131] T. A. Vickers, W. F. Lima, H. Wu, J. G. Nichols, P. S. Linsley, S. T. Crooke, *Nucleic Acids Res.* **2009**, *37*, 6927 – 6941.
[132] E. M. Anderson, A. Birmingham, S. Baskerville, A. Reynolds, E. Maksimova, D. Leake, Y. Fedorov, J. Karpilow, A. Khvorova, *RNA* **2008**, *14*, 853 – 861.
[133] A. L. Jackson, J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J. M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova, P. S. Linsley, *RNA* **2006**, *12*, 1197 – 1205.
[134] Y. Fedorov, E. M. Anderson, A. Birmingham, A. Reynolds, J. Karpilow, K. Robinson, D. Leake, W. S. Marshall, A. Khvorova, *RNA* **2006**, *12*, 1188 – 1196.
[135] O. Snove, Jr., J. J. Rossi, *ACS Chem. Biol.* **2006**, *1*, 274 – 276.
[136] K. Ui-Tei, Y. Naito, S. Zenno, K. Nishi, K. Yamato, F. Takahashi, A. Juni, K. Saigo, *Nucleic Acids Res.* **2008**, *36*, 2136 – 2151.
[137] D. S. Schwarz, H. Ding, L. Kennington, J. T. Moore, J. Schelter, J. Burchard, P. S. Linsley, N. Aronin, Z. Xu, P. D. Zamore, *PLoS Genet.* **2006**, *2*, 1307 – 1318.
[138] P. Y. Chen, L. Weinmann, D. Gaidatzis, Y. Pei, M. Zavolan, T. Tuschl, G. Meister, *RNA* **2007**, *14*, 263 – 274.
[139] S. Shah, S. H. Friedman, *Oligonucleotides* **2007**, *17*, 35 – 43.
[140] J. B. Bramsen, M. B. Laursen, C. K. Damgaard, S. W. Lena, B. R. Babu, J. Wengel, J. Kjems, *Nucleic Acids Res.* **2007**, *35*, 5886 – 5897.
[141] K. Karikó, M. Buckstein. , H. Ni, D. Weissman, *Immunity* **2005**, *23*, 165 – 175.
[142] M. Robbins, A. Judge, I. MacLachlan, *Oligonucleotides* **2009**, *19*, 89 – 102.
[143] K. Karikó, P. Bhuyan, J. Capodici, D. Weissman, *J. Immunol.* **2004**, *172*, 6545 – 6549.

[144] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, *Nat. Med.* **2005**, *11*, 263 – 270.
[145] G. R. Stark, I. M. Kerr, B. R. Williams, R. H. Silverman, R. D. Schreiber, *Annu. Rev. Biochem.* **1998**, *67*, 227 – 264.
[146] M. Yoneyama, M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, T. Fujita, *Nat. Immunol.* **2004**, *5*, 730 – 737.
[147] L. Manche, S. R. Green, C. Schmedt, M. B. Mathews, *Mol. Cell. Biol.* **1992**, *12*, 5238 – 5248.
[148] A. Pichlmair, O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, C. Reis e Sousa, *Science* **2006**, *314*, 997 – 1001.
[149] V. Hornung, J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K. Conzelmann, M. Schlee, S. Endres, G. Hartmann, *Science* **2006**, *314*, 994 – 997.
[150] A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, I. MacLachlan, *Nat. Biotechnol.* **2005**, *23*, 457 – 462.
[151] M. E. Kleinman, J. R. Smith, E. W. Taylor, J. Ambati, *Nature* **2008**, *452*, 591 – 597.
[152] Z. Ma, J. Li, F. He, A. Wilson, B. Pitt, S. Li, *Biochem. Biophys. Res. Commun.* **2005**, *330*, 755 – 759.
[153] M. Sioud, D. R. Sorensen, *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1220 – 1225.
[154] A. D. Judge, G. Bola, A. C. Lee, I. MacLachlan, *Mol. Ther.* **2006**, *13*, 494 – 505.
[155] F. Eberle, K. Giessler, C. Deck, K. Heeg, M. Peter, C. Richert, A. H. Dalpke, *J. Immunol.* **2008**, *180*, 3229 – 3237.
[156] S. S. Diebold, C. Massacrier, S. Akira, C. Paturel, Y. Morel, C. Reis e Sousa, *Eur. J. Immunol.* **2006**, *36*, 3256 – 3267.
[157] M. Robbins, A. Judge, L. Liang, K. McClintock, E. Yaworski, I. MacLachlan, *Mol. Ther.* **2007**, *15*, 1663 – 1669.
[158] A. D. Judge, M. Robbins, I. Tavakoli, J. Levi, L. Hu, A. Fronda, E. Ambegia, K. McClintock, I. MacLachlan, *J. Clin. Invest.* **2009**, *119*, 661 – 673.
[159] L. Cekaite, G. Furset, E. Hovig, M. Sioud, *J. Mol. Biol.* **2007**, *365*, 90 – 108.
[160] Z. Zhang, T. Weinschenk, K. Guo, H. J. Schluesener, *J. Cell. Biochem.* **2006**, *97*, 1217 – 1229.
[161] S. Puthenveetil, L. Whitby, J. Ren, K. Kelnar, J. F. Krebs, P. A. Beak, *Nucleic Acids Res.* **2006**, *34*, 4900 – 4911.
[162] J. T. Marques, T. Devosse, D. Wang, M. Zamaniandaryoush, P. Serbinowski, R. Hartmann, T. Fujita, M. A. Behlke, B. R. Williams, *Nat. Biotechnol.* **2006**, *24*, 559 – 565.
[163] S. R. Nallagatla, P. C. Bevilacqua, *RNA* **2008**, *14*, 1201 – 1213.
[164] M. Zamanian-Daryoush, J. T. Marques, M. P. Gantier, M. A. Behlke, M. John, P. Rayman, J. Finke, B. R. Williams, *J. Interferon Cytokine Res.* **2008**, *28*, 221 – 233.
[165] M. A. Collingwood, S. D. Rose, L. Huang, C. Hillier, M. Amarzguioui, M. T. Wiiger, H. S. Soifer, J. J. Rossi, M. A. Behlke, *Oligonucleotides* **2008**, *18*, 187 – 200.
[166] W. G. Cho, R. J. C. Albuquerque, M. E. Kleinman, V. Tarallo, A. Greco, M. Nozaki, M. G. Green, J. Z. Baffi, B. K. Ambati, M. D. Falco, J. S. Alexander, A. Brunetti, S. D. Falco, J. Ambati, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 7137 – 7142.
[167] M. Robbins, A. Judge, E. Ambegia, C. Choi, E. Yaworski, L. Palmer, K. McClintock, I. MacLachlan, *Hum Gene Ther.* **2008**, *19*, 991 – 999.
[168] S. M. Tompkins, C. Y. Lo, T. M. Tumpey, S. L. Epstein, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8682 – 8686.
[169] Q. Ge, L. Filip, A. Bai, T. Nguyen, H. N. Eisen, J. Chen, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8676 – 8681.
[170] S. K. Singh, P. B. Hajeri, *Drug Discovery Today* **2009**, *14*, 859 – 865.
[171] K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discovery* **2009**, *8*, 129 – 138.
[172] D. R. Thakker, F. Natt, D. Husken, H. van der Putten, R. Maier, D. Hoyer, J. F. Cryan, *Mol. Psychiatry* **2005**, *10*, 782 – 789.
[173] G. Dorn, S. Patel, G. Wotherspoon, M. Hemmings-Mieszack, J. Barclay, F. J. Natt, P. Martin, S. Bevan, A. Fox, P. Ganju, W. Wishart, J. Hall, *Nucleic Acids Res.* **2004**, *32*, e49.
[174] X. Y. Niu, Z. L. Peng, W. Q. Duan, H. Wang, P. Wang, *Int. J. Gynecol. Cancer* **2006**, *16*, 743 – 751.
[175] J. Q. Hou, J. He, X. L. Wang, D. G. Wen, Z. X. Chen, *Chin. Med. J. (Engl.)* **2006**, *119*, 1734 – 1739.
[176] X. Zhang, P. Shan, D. Jiang, P. W. Noble, N. G. Abraham, A. Kappas, P. J. Lee, *J. Biol. Chem.* **2003**, *279*, 10677 – 10684.
[177] S. J. Reich, J. Fosnot, A. Kuroki, W. Tang, X. Yang, A. M. Maguire, J. Bennett, M. J. Tolentino, *Mol. Vision* **2003**, *9*, 210 – 216.
[178] M. J. Tolentino, A. J. Brucker, J. Fosnot, G. S. Ying, I. H. Wu, G. Malik, S. Wan, S. J. Reich, *Retina* **2004**, *24*, 132 – 138.
[179] M. J. Tolentino, A. J. Brucker, S. Wan, S. J. Reich, J. Gordon, Y. H. Du, *Retina* **2004**, *24*, 660 – 661.
[180] J. Shen, R. Samul, R. L. Silva, H. Akiyama, H. Liu, Y. Saishin, S. F. Hackett, S. Zinnen, K. Kossen, K. Fosnaugh, C. Vargeese, A. Gomez, K. Bouhana, R. Aitchison, P. Pavco, P. A. Campochiaro, *Gene Ther.* **2006**, *13*, 225 – 234.
[181] J. P. DeVincenzo, J. E. Cehelsky, R. Alvarez, S. Elbashir, J. Harborth, I. Toudjarska, L. Nechev, V. Murugaiah, A. van Vliet, A. K. Vaishnaw, R. Meyers, *Antiviral Res.* **2008**, *77*, 225 – 231.
[182] R. Alvarez, S. Elbashir, T. Borland, I. Toudjarska, P. Hadwiger, M. John, I. Röhl, S. S. Morskaya, R. Martinello, J. Kahn, M. Van Ranst, R. A. Tripp, J. P. DeVincenzo, R. Pandey, M. Maier, L. Nechev, M. Manoharan, V. Kotelianski, R. Meyers, *Antimicrob. Agents Chemother.* **2009**, *53*, 3952 – 3962.
[183] Y. Dorsett, T. Tuschl, *Nat. Rev. Drug Discovery* **2004**, *3*, 318 – 329.
[184] D. A. Braasch, Z. Paroo, A. Consantinescu, G. Ren, O. K. Oz, R. P. Mason, D. R. Corey, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1139 – 1143.
[185] D. W. Bartlett, M. E. Davis, *Nucleic Acids Res.* **2006**, *34*, 322 – 333.
[186] F. Alexis, E. Pridgen, L. K. Molnar, O. C. Farokhzad, *Mol. Pharm.* **2008**, *5*, 505 – 515.
[187] J. Zamecnik, L. Vargova, A. Homola, R. Kodet, E. Sykova, *Neuropathol. Appl. Neurobiol.* **2004**, *30*, 338 – 350.
[188] V. P. Torchilin, T. S. Levchenko, R. Rammohan, N. Volodina, B. Papahadjopoulos-Sternberg, G. G. D'Souza, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1972 – 1977.
[189] D. T. Auguste, K. Furman, A. Wong, J. Fuller, S. P. Armes, T. J. Deming, R. Langer, *J. Controlled Release* **2008**, *130*, 266 – 274.
[190] M. S. Martina, V. Nicholas, C. Wilhelm, C. Menager, G. Barratt, S. Lesieur, *Biomaterials* **2007**, *28*, 4143 – 4153.
[191] S. Mao, M. Neu, O. Germershaus, O. Merkel, J. Sitterberg, U. Bakowsky, T. Kissel, *Bioconjugate Chem.* **2006**, *17*, 1209 – 1218.
[192] D. Grimm, K. L. Streetz, C. L. Jopling, T. A. Storm, K. Pandey, C. R. Davis, P. Marion, F. Salazar, F. A. Kay, *Nature* **2006**, *441*, 537 – 541.
[193] J. Barquinero, H. Eixarch, M. Perez-Melgosa, *Gene Ther.* **2004**, *11*, S3-S9.
[194] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Röhl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H. P. Vornlocher, *Nature* **2004**, *432*, 173 – 178.
[195] J. O. McNamara II, E. R. Andrechek, Y. Wang, K. D. Viles, R. E. Rempel, E. Gilboa, B. A. Sullenger, P. H. Giangrande, *Nat. Biotechnol.* **2006**, *24*, 1005 – 1015.
[196] M. Thomas, S. A. Kularatne, L. Qi, P. Kleindl, C. P. Leamon, M. J. Hansen, P. S. Low, *Ann. N.Y. Acad. Sci.* **2009**, *1175*, 32 – 39.
[197] J. H. Jeong, H. Mok, Y. K. Oh, T. G. Park, *Bioconjugate Chem.* **2007**, *18*, 1483 – 1489.
[198] T. S. Zimmermann, A. C. H. Lee, A. D. Judge, M. Manoharan, I. MacLachlan, *Nature* **2006**, *441*, 111 – 114.
[199] L. M. Jarvis, *Chem. Eng. News* **2009**, *87*, 18 – 27.
[200] A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J. R. Dorkin, K. N. Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Sah, J. Soutschek, I. Toudjarska, H. P. Vornlocher, T. S. Zimmermann, R. Langer, D. G. Anderson, *Nat. Biotechnol.* **2008**, *26*, 561 – 569.
[201] A. Akinc, M. Goldberg, J. Qin, J. R. Dorkin, C. Gamba-Vitalo, M. Maier, K. N. Jayaprakash, M. Jayaraman, K. G. Rajeev, M. Manoharan, V. Koteliansky, I. Röhl, E. S. Leshchiner, R. Langer, D. G. Anderson, *Mol. Ther.* **2009**, *17*, 872 – 879.
[202] J. S. Wadia, S. F. Dowdy, *Adv. Drug Delivery Rev.* **2005**, *57*, 579 – 596.
[203] J. S. Wadia, R. V. Stan, S. F. Dowdy, *Nat. Med.* **2004**, *10*, 310 – 315.
[204] A. Eguchi, B. R. Meade, Y. C. Chang, C. T. Fredrickson, K. Willert, N. Puri, S. F. Dowdy, *Nat. Biotechnol.* **2009**, *27*, 567 – 571.

[205] P. Lundberg, S. El-Andaloussi, T. Sutlu, H. Johansson, U. Langel, *FASEB J.* **2007**, *21*, 2664 – 2671.
[206] E. Song, P. Zhu, S. K. Lee, D. Chowdhury, S. Kussman, D. M. Dykxhoorn, Y. Feng, D. Palliser, D. B. Weiner, P. Shankar, W. A. Marasco, J. Lieberman, *Nat. Biotechnol.* **2005**, *23*, 709 – 717.
[207] K. Watanabe, M. Harada-Shiba, A. Suzuki, R. Gokuden, R. Kurihara, Y. Sugao, T. Mori, Y. Katayama, T. Niidome, *Mol. Biosyst.* **2009**, *5*, 1306 – 1310.
[208] U. Lungwitz, M. Breunig, T. Blunk, A. Göpferich, *Eur. J. Pharm. Biopharm.* **2005**, *60*, 247 – 266.
[209] A. Zintchenko, A. Philipp, A. Dehshahri, E. Wagner, *Bioconjugate Chem.* **2008**, *19*, 1448 – 1455.
[210] S. Hu-Lieskovan, J. D. Heidel, D. W. Bartlett, M. E. Davis, T. J. Triche, *Cancer Res.* **2005**, *65*, 8984 – 8992.
[211] J. D. Heidel, Z. Yu, J. Y. Liu, S. M. Rele, Y. Liang, R. K. Zeidan, D. J. Kornbust, M. E. Davis, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 5715 – 5721.
[212] O. M. Merkel, A. Beyerle, D. Librizzi, A. Pfestroff, T. M. Behr, B. Sproat, P. J. Barth, T. Kissel, *Mol. Pharm.* **2009**, *6*, 1246 – 1260.
[213] D. Putnam, *Nat. Mater.* **2006**, *5*, 439 – 451.
[214] A. Kichler, *J. Gene Med.* **2004**, *6*, S3 – S10.
[215] S. Gao, F. Dagnaes-Hansen, E. J. B. Nielsen, J. Wengel, F. Besenbacher, K. A. Howard, J. Kjems, *Mol. Ther.* **2009**, *17*, 1225 – 1233.
[216] K. Nishina, T. Unno, K. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa, T. Yokota, *Mol. Ther.* **2008**, *16*, 734 – 740.
[217] Y. Sato, K. Murase, J. Kato, M. Kobune, T. Sato, Y. Kawano, R. Takimoto, K. Tawada, K. Miyanishi, T. Matsunaga, T. Takayama, Y. Niitsu, *Nat. Biotechnol.* **2008**, *26*, 431 – 442.
[218] T. C. Chu, K. Y. Twu, A. D. Ellington, M. Levy, *Nucleic Acids Res.* **2006**, *34*, e73.
[219] J. P. Dassie, X. Y. Liu, G. S. Thomas, R. M. Whitaker, K. W. Thiel, K. R. Stockdale, D. K. Mayerholz, A. P. McCaffrey, J. O. McNamara II, P. H. Giangrande, *Nat. Biotechnol.* **2009**, *27*, 839 – 849.
[220] J. J. Turner, S. Jones, M. M. Fabani, G. Ivanova, A. A. Arzumanov, M. J. Gait, *Blood Cells Mol. Dis.* **2007**, *38*, 1 – 7.
[221] P. Kumar, H. Wu, J. L. McBride, K. E. Jung, M. H. Kim, B. L. Davidson, S. K. Lee, P. Shankar, N. Manjunath, *Nature* **2007**, *448*, 39 – 43.
[222] M. Kortylewski, P. Swiderski, A. Herrmann, L. Wang, C. Kowolik, M. Kujawski, H. Lee, A. Scuto, Y. Liu, C. Yeng, J. Deng, H. S. Soifer, A. Raubitschek, S. Forman, J. J. Rossi, D. M. Pardoll, R. Jove, H. Yu, *Nat. Biotechnol.* **2009**, *27*, 925 – 932.
[223] J. S. Lee, J. J. Green, K. T. Love, J. Sunshine, R. Langer, D. G. Anderson, *Nano Lett.* **2009**, *9*, 2402 – 2406.
[224] M. Nothisen, M. Kotera, E. Voirin, J. S. Remy, J. P. Behr, *J. Am. Chem. Soc.* **2009**, DOI: 10.1021/ja908017e.
[225] A. Eguchi, S. F. Dowdy, *Trends Pharmacol. Sci.* **2009**, *30*, 341 – 345.
[226] D. Castanotto, J. J. Rossi, *Nature* **2009**, *457*, 426 – 433.
[227] H. Tafer, S. L. Ameres, G. Obernosterer, C. A. Gebeshuber, R. Schroeder, J. Martinez, I. L. Hofacker, *Nat. Biotechnol.* **2008**, *26*, 578 – 583.
[228] J. J. Rossi, *Gene Ther.* **2009**, *16*, 579 – 580.

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