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Therapeutic potential of chemically modified siRNA: Recent trends

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Abstract

Small Interfering RNAs (siRNAs) are one of the valuable tools to investigate the functions of genes, and are also used for gene silencing. It has a wide scope in drug discovery through *in vivo* target validation. siRNA therapeutics are not optimal drug-like molecules due to poor bioavailability, immunogenic and off-target effects. In order to overcome the challenges associated with siRNA therapeutics, identification of appropriate chemical modifications that improves the stability, specificity and potency of siRNA is essential. This review focuses on the various chemical modifications and their implications in siRNA therapy.

Graphical abstract

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Introduction

RNA interference (RNAi), one of the natural gene regulatory mechanisms in eukaryotes has become a prominent tool among the research community. It finds tremendous applications in fields such as functional genomics, and therapeutics. siRNA (small interfering RNA), a 21–

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Conflicts of interest

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25 nucleotide, double stranded RNA molecule plays a major role in gene silencing (1–3). The RNAi machinery involves various components such as sense strand (passenger strand), anti-sense strand (guide strand), enzymes like Dicer, Argonaute and the core part RISC (RNA induced silencing complex). RNAi mechanism was first discovered in the transgenic plant petunia in 1990 (4). Later, Fire and Mello, through their discovery of regulated protein production by mRNA degradation in *Caenorhabditis elegans* added light to the scientific community and they won the Nobel Prize for the year 2006 in Physiology and Medicine (1–5).

siRNA is gaining importance as a therapeutic tool for various diseases and disorders like cancers caused by viruses, hepatitis, metabolic and genetic disorders because of malfunctioning of genes and unregulated expression. siRNA selectively targets the gene and silences it by inhibiting the protein expression involved in the cell abnormalities. siRNA based therapeutic molecules for various diseases are under clinical evaluation. Recently, Dar et al developed specialized databank for chemically modified siRNAs which contains a total of 4894 chemically modified-siRNA sequences (6). Although, siRNA offers surplus significance as a therapeutic tool, it has few detriments which need to be eliminated before its usage as a drug. siRNA has the property of stimulating the innate immunity and may also exhibit non-specific binding. In addition to this, the stability of the duplex is also affected inside the serum and it is difficult for a negatively charged RNA to cross the cell membrane and hence the pharmacokinetic profile is affected. The thermodynamic stability and resistance to the nuclease activity need to be improved for effective usage of siRNA as a therapeutic tool. In order to eliminate all these detriments, the siRNA must be chemically modified to make it as an impressive therapeutic agent (7–11). This review focuses on the various modifications of siRNA carried out in the past seven years and their roles in improving the aforesaid discrepancies.

Ribose modifications

Several reports had given a proof that 2'-O modification in siRNAs acted as an effective tool for antisense technology (12–15). The most widely used sugar modifications at the 2' position of the sugar ring included 2'-OMe, 2'-F, 2'-O-methoxyethyl (2'-MOE). Such modifications allowed the oligonucleotide to adopt the energetically favourable conformation i.e. RNA-like C3'-endo sugar pucker. Thus, formation of thermally stable A-type duplexes was expected upon the introduction of these modifications into siRNAs (16–18). The L-isonucleoside (Figure 1) modification (**1** and **2**) had decreased the thermal stability of siRNA and the D-isoNA (Isonucleosides) modification had improved serum stability and the modification in the 3' end of the sense strand increased the silencing activity (Table 1). siRNAs (targeting MEK1) formed by the D-isoNA modified sense strand at position 15 (siMek1 S15D) and siRNAs (targeting MEK1) formed by the L-isoNA modified sense strand at position 15 (siMek1 S15L) had been reported to exhibit a high silencing effect and improved target selectivity compared to the native siMeK1 (19). Marimani et al were keen on the utilization of RNAi for inhibiting hepatitis B viral replication for which they incorporated guanidinopropyl (**3**) in the 2' position (2'-O-GP) of the ribose moieties of nucleotides from positions 2 to 21 of the antisense strand. The test was carried out in Liver-derived Human hepatoma 7 (Huh7) cells in which the modified siRNA

had high efficiency in inhibiting the viral gene expression. There is no report on toxicity and proof for inducing the interferon response in cultured cells (20). siRNAs were modified by the addition of 2'-O-GP nucleotide. The modified siRNA was used for testing against Hepatitis B virus and luciferase gene. The GP modifications improved the silencing activity of the HBSAg gene and the luciferase gene (21).

To investigate the role of sugar moieties in improving the efficacy of siRNA molecules, siRNAs were modified with 1-deoxy-D-ribofuranose (R^H), an abasic nucleoside (**4**). The modification was carried out in the 3' overhang region. The knockdown effect was tested against dual luciferase gene and the binding activity with PAZ domain was investigated. The silencing activity of the abasic nucleoside incorporated siRNA was reported to be high compared to the siRNAs possessing natural nucleosides. The modified siRNAs were more resistant to Snake venom phosphodiesterase (SVPD) than the unmodified one (22). The effect of destabilization of the siRNA duplex on RNAi activity was investigated by introducing mismatches in the central part of the 2'-O-methyl modified (**5**) siRNAs. Nucleotide substitutions and introduction of GU wobble base pairs in the central region decreased the thermodynamic stability. siRNA was targeted to MDR1 gene in human HEK293 cells. Mismatches in the 9–12 position from the 5' end increased the gene silencing (23).

The 2'-OH position of the siRNA was masked with acyloxymethyl groups, in particular pivaloyloxymethyl (PivOM) (**6**). Upon evaluation of the 2'-O-PivOM pro-RNA against the firefly luciferase gene, silencing effect was high compared to the conventional siRNA. The modified siRNA had increased cellular uptake since PivOM is lipophilic in nature. Hence, the modified siRNA can be a promising pro drug (24). Micura et al improved the structural and functional property of the siRNA by incorporating 2'-azido cytidine and 2'-azido guanosine (**7**). The silencing efficacy was tested in chicken fibroblast cells, in the genes coding for brain acid soluble protein (BASP1). 2'-azido modifications were tolerated in the guide strand and also exhibited increased nuclease resistance (25). Replacement of cytidine unit with 2',2'-difluoro-2'-deoxycytidine (gemcitabine, dFdC) (**8**) demonstrated a position-dependent tolerance. dFdC modifications introduced at the positions 2, 4 or 5 showed comparable silencing potential to that of the unmodified duplex (70% silencing). When these modifications were introduced in positions 1 or 8, approximately 15% silencing activity was observed. At position 10, dFdC unit almost switched off the silencing activity (below 10%) (26). When 2'-Deoxy-2',4'-difluorouridine (2',4'-diF-rU) modification (**9**) was incorporated in RNA:RNA duplex, thermal denaturation was not much affected. It gave a mild destabilizing effect when incorporated in the RNA strand of DNA:RNA duplex and high destabilization when incorporated in the DNA strand of DNA:RNA and DNA:DNA duplex. Modification in the central region of guide strand with 2',4'-diF-rU was more potent in silencing the gene expression (27).

siRNAs modified with 2'-O-Methyl guanosine and uridine residues profoundly decreased the immunostimulation and interferon response. The effect of modification by 2'-O-methyl-4'-thioRNA (**10**) on gene silencing was studied against HeLa-Luc gene. The modified siRNA had increased hybridization efficiency and improved resistance towards nuclease degradation in human plasma (28). Another potential modification that increased

the gene silencing activity of siRNAs is triantennary N-acetylgalactosamine (GalNAc). The modified siRNA was tested against asialoglycoprotein receptor (ASGPR); a hepatocytic receptor. It reduced the gene expression when monomeric GalNAc were introduced in the sense strand through ribose sugar or nucleobase (**11–13**). Thus modification of siRNA by GalNAc can be a better strategy to cure liver associated diseases (29).

Phosphate Backbone modifications

To make the siRNAs more stable, modifications with Phosphorodithioate (PS-2) (Figure 2) were performed (**14**). A series of PS-2 siRNAs were synthesized and evaluated for the gene silencing activity against BACE 1 gene expression and were also tested for its stability in serum. The substitution in the central part of the anti-sense strand and the sense strand provided a positive result where the BACE 1 gene expression was minimized and the serum stability was increased (Table 2) (30). Triazole (**15**) backbone dimers were incorporated in the siRNAs. The gene silencing effect was investigated in the firefly luciferase gene and Glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene transcript. Silencing of GADPH was found to be dose dependant. Modified siRNA was resistant to nuclease activity whereas the wild type siRNA was susceptible (31).

The internucleotide phosphodiester linkages of unmodified siRNAs were negatively charged at physiological pH and can be cleaved by endo and exonucleases. Chemical modifications of phosphodiester linkages with amide modification (**16**) using U_{AM1}U and U_{AM1}A had been investigated by our group (selvam et al (32) & Mutisya et al (33)). 1–5 amide modifications were introduced in the sense and the antisense strand. It was found that amide modifications were well tolerated at internal positions of both the guide and passenger strands of siRNAs. It increased the silencing activity when placed near the 5'-end of the passenger strand. Most interestingly, the heavily modified (five amide modifications) sense strand was more active than the unmodified siRNA. Hence, amide modifications were proved to be an excellent replacement for internucleotide phosphodiester linkages.

The backbone of the siRNA was modified with amide bonds at both 3' overhangs and the internal regions by Desaulniers et al (34). The strategy used for the amide backbone construction is through Uracil based peptide nucleic acid (PNA)–RNA dimer unit incorporated via phosphoramidite chemistry. A large thermal destabilization effect was observed for the internally modified siRNA but no gene silencing effect was observed when tested in firefly luciferase gene (pGL2). Internal modifications near the 3' end showed high degree of compatibility with RNAi machinery although showed no potency for gene silencing. siRNA with 3' overhang modification exhibited some increase in potency. Evidence of decreased potency is observed when the 3' overhang modification is carried out in the anti-sense strand (34).

Tanui et al replaced the phosphodiester with non-ionic amide (**17**) linkages. RNA2 (three consecutive amide linkages, UaUaUaU), RNA3 and RN4 (two consecutive amide linkages, UaUaU were introduced at different positions) were synthesized. Only a slight modification in the silencing activity was reported when RNA2 was modified at the 5' end with three consecutive amide linkages (35).

Substitution of boranophosphate instead of the phosphodiester backbone of siRNA was reported to be an effective modification. The silencing efficiency of boranophosphate (BP) (**18**) modified siRNA was tested in HeLa cells that express enhanced green fluorescent protein (EGFP). Double stranded BP siRNA exhibited potent downregulation of EGFP in HeLa cells. The ss-siRNA with BP modified adenosine and cytidine residues potentially inhibited the GFP expression (99%) compared to its native ds-siRNA (70%). A significant increase in the stability of BP siRNAs compared to native siRNA was demonstrated. BP siRNAs were found to be resistant to RNase degradation, however there was no correlation found between the greater silencing activity and the stability. The boranophosphate modifications were hydrophobic in nature which can change RNA-Ago2 interactions (increases siRNA duplex stability) (36, 37). Modifications of phosphodiester linkages of siRNA by phosphorothioate (PS) offered good stability against nucleases. The PS group was chiral with either *Rp* (**19**) or *Sp* (**20**) configuration at phosphorous. Jahn et al reported that the PS siRNAs composed of oligoribonucleotides (ORNs) with a higher fraction of *Rp* centres showed greater resistance to nucleases in serum and were more effective inhibitors in cells than their *Sp* counterparts. Introduction of phosphorothioate (PS) modifications in the place of phosphodiester linkages of siRNA exhibited improved potency when tested in HeLa cells by Luciferase assay (38).

Nucleobase and sugar modifications

5-fluoro-2'-deoxyuridine (FdU) (Figure 3) (**21**) moieties were introduced in multiple positions of siRNA targeting thymidylate synthase (TS). The cytotoxicity level of the modified siRNA was improved up to 10–100 fold compared to the unmodified siRNA (Table 3) (39). siRNAs modified with 2'-*O*-methyl phosphorodithioate (2'-O-MePS2) (**22**) was reported to have effective therapeutic targeting. This was confirmed by targeting this modified siRNA against GRAMD 1B gene. It increased the loading of siRNA to RISC which increased the gene silencing and was position independent. It also increased the serum stability of siRNA (40).

The siRNA against mRNA of BACE 1 gene was modified with a lipophilic boron cluster, 3-*N*-[(1,12-dicarba-closo-dodecacarboran-1-yl)propan-3-yl]thymidine (C₂B₁₀H₁₁, CB) (**23**) at N3 position of the sense and anti-sense strand. The thermodynamic stability of these modified siRNAs was much higher than the unmodified siRNA duplex. The gene silencing activity remained the same as that of the parent siRNA, which may be because of the reduced affinity of siRNA to RISC complex due to the large size of the boron cluster. The modification protected the siRNA from nucleolytic degradation (41).

A synthetic siRNA chemically modified with thymidine and 5-bis(aminoethyl)-aminoethyl-2'-deoxyuridine (**24**) in the 3' overhang region of the siRNA showed increased gene silencing against HNF 4 α (Hepatocyte nuclear factor 4 α). This was tested in human hepatoblastoma HepG2 cells that expressed high levels of endogenous HNF 4 α cells. The nuclease resistant property was increased which was confirmed using snake venom phosphodiesterase (42).

Modification to the termini and conjugate groups

Palmitic acid was conjugated at the 5' end of the siRNA (**25**) (Figure 4) and its efficacy of gene silencing was tested against Renilla luciferase gene by transfecting the HeLa cells. It proved to be a good gene silencer as well as had a greater stability against the nuclease enzyme. Another modification involving inverted thymidine (idT) was coupled to the 3' end of the siRNA with 5' end conjugated topalmitic acid. This modified siRNA was targeted against Vascular endothelial growth factor (VEGF) in HeLa cells. The greater inhibitory effect for long term and increased membrane permeability had been reported with the use of C16-siRNA-idT (Table 4) (43). An efficient *in vivo* delivery system for siRNA targeting the EphA2-positive tumors were developed by Xie et al using cell permeable peptide (CPPs) delivered through ultrasound sensitive Nano bubbles (NBs). The CPP-siRNA (**26**) was trapped in ephrin mimetic peptide modified Nano bubbles. The penetration of CPP-siRNA was temporarily blocked and the movement was triggered by ultrasound into human breast adenocarcinoma cells (MCF-7, EphA2 cells). The cellular uptake ability was high when CPP was conjugated with siRNA and triggered by ultrasound (44).

Conjugation of siRNAs with aromatic compounds like phenyl, hydroxyphenyl, naphthyl, and pyrenyl derivatives (**27**) protected from nuclease degradation. They were also proved to be highly stable thermodynamically. The silencing activity of the siRNAs conjugated with aromatic compounds was tested against *Renilla* luciferase in HeLa cells (45). The level of membrane permeability through HeLa cells was high for the modified siRNAs compared to the conventional siRNAs. The ability of siRNAs to inhibit tumour necrosis factor (TNF- α) and the anti-inflammatory properties were evaluated by covalently linking the amino lipid groups in the 3' end of the passenger strand (**28**). The anti-sense activity was studied in HeLa cells (46).

siRNAs and miRNAs were chemically modified with urea/thiourea bridged aromatic compounds to utilize them as a therapeutic tool against human bladder cancer T 24 cells. siRNAs containing urea and thiourea derivatives in their 3' overhang region (**29**) had the desired gene knock down effect. Thermal denaturation studies were carried out to assess the thermostability of the modified siRNAs and there were no major changes in the stability due to the incorporation of urea and thiourea. Renilla and firefly luciferase gene were used for evaluating the silencing efficiency, which was reported to be increased in dose-dependent manner. Nuclease resistance was tested using Snake venom phosphodiesterase in which the modified siRNAs had a good nuclease resistance (47).

Polyethylene glycol (PEG) modified siRNAs against breast cancer cells were studied by Gaziova et al. Attachment of PEG increased the bioavailability. The reaction of N-hydroxysuccinimide (NHS) activated PEG esters with primary amino groups produced active PEG conjugated siRNA attached at the 3' end of the sense and anti-sense strand (**30**). The target was chosen to be the mRNA of bcl-2 gene. The PEGylated siRNA produced 70% gene silencing and the circulation time was prolonged by reducing the renal filtration rate (48).

Automated synthesis of the triphosphate groups to be attached to the 5' end of the passenger strand of the oligonucleotide was performed which was considered to be a higher end technique in Anti-sense technology. The modified siRNA was reported to have gene silencing effect that down regulated the proteins involved in the tumour cell growth and survival, but the efficacy was the same as that of the unmodified siRNAs. The immunostimulatory profiles were found to be increased in triphosphate modified siRNA (31) (49).

Melanoma siRNA (named siMB3), targeting the mRNA of the mutant BRAF protein was modified by conjugating with a peptide, KALLAL at the 3' end of the sense and anti-sense strands (32). The gene silencing efficiency of Bis-pep-siMB3 was better at 48h and 72 h after transfection with HEK293 cell. The results showed that the modified siRNA had prolonged inhibitory activity and were resistant to endogenous nucleases. The Bis-pep-siMB3 induced apoptosis and reduced the tumors efficiently (50). Cholesterol conjugated siRNAs were targeted to silence the muscle specific gene, Myostatin (Mstn) in mice. Myostatin deficiency leads to increased muscle size and mass. The modified siRNA produced a knockdown of 85–95% of the Mstn mRNA in skeletal muscles and greater than 65% reduction in the circulation. The cholesterol conjugated siRNAs helped in achieving target specific delivery and also had increased silencing duration (51).

siRNA binding

Analysis of the crystal structure of the PAZ domain (52) from human Argonaute eIF2c1 (Ago2) bound to both ends of 9-mer siRNA-like duplex (pdb entry 1SI2) was done and the results are shown in figures 5 and 6. The PAZ domain consists of a β barrel and $\alpha\beta$ components that together form a conserved pocket structure (Figure 5a). The figure 5a shows the interaction of PAZ domain with a siRNA-like end. The surface view of the co-crystal structure of PAZ domain and a 9-mer RNA (5'-CGUGACUCU-3') is shown in figure 5b. Figure 6 shows the interactions between the siRNA and the PAZ domain. The two nucleotides at the 3'-end of siRNA enter the pocket and the non-bridging oxygens of the phosphodiester backbone make hydrogen bonds with the residues Tyr309, Tyr314 and His269 (Figure 6a). Stacking interaction was observed between the portions of the sugar ring and base of the terminal nucleotide with an aromatic residue Phe292. Hydrogen bond and electrostatic interactions between five phosphodiester groups of the siRNA strand and the basic residues Arg275, Arg278, K333, K264 (Figure 6b, 6c) were seen. The first nucleotide was placed in the pocket composed of the hydrophobic residues Leu294, Phe292, Leu337, Pro293, and Phe310. Mutation studies suggested that the residues Arg278, Phe292, L337, Y309, Y314 were crucial for effective binding. Other residues Val306, Phe310, Pro338 and Val341 were not directly contacting RNA, but were also reported to be responsible for maintaining hydrophobic environment.

In order to strengthen the interaction between the 3'-end of the siRNA, Xu et al (53) designed and synthesized a series of chemically modified nucleotide monomers containing an aromatic hydrophobic structure by applying bioisosterism strategy. Pyrimidine ring was substituted with benzene to enhance the binding between modified motif and hydrophobic residues Phe292, Leu337, and Phe310 in the PAZ domain. The pentose ring was opened and

replaced by the straight chain to have strong hydrogen bond interactions between residues Tyr312 and Tyr309 and the original phosphate groups. Binding energies of the modified siRNAs for Ago2 were calculated and the predictions were confirmed experimentally by a Surface Plasmon Resonance (SPR) assay in vitro.

Immunostimulation

One of the major challenges faced by siRNA as a therapeutic molecule is their immune stimulation. Chemical modifications in siRNA may reduce its immunostimulatory properties. The receptors involved in the recognition of RNAs are Toll like receptors (TLR), Protein kinase R (PKR) and few helicases (54). Activation and release of cytokines and interferons are the major effects of immunostimulation by siRNA. It has been reported that, siRNA duplexes modified with 2' Fluoro and 2' methyl moieties showed reduced stimulation of interferons and cytokines. Immunostimulatory properties of siRNAs were investigated in human peripheral blood mono nuclear cells (PBMCs). Ribose backbone modified siRNAs showed no immune-stimulatory effects in PBMCs (55). In vivo study on these modified siRNAs demonstrated the reduced liver toxicity. Since toll like receptors are majorly involved in immunostimulation, the immunostimulatory sequence motifs can be modified such that it cannot be recognized by TLRs.

In vivo application

The most important factors of siRNA that must be considered for efficient in vivo action are nuclease stability, immunostimulatory effects, bioavailability and off target effects. Degradation of siRNA is prevented by modification in the 2' hydroxyl group. Backbone modifications also make the siRNA less susceptible to nuclease degradation. Methyl modifications in the 2' position had proved to have potent silencing efficiency without any inflammatory activity. Backbone modification by phosphorothioate had prolonged the life of the duplex and the boranophosphate modification increased the efficacy. Cholesterol conjugates to siRNA had prolonged the duration and also improved the tissue uptake and distribution (56). The methods used for local delivery of siRNA under in vivo conditions like lipid mediated transfection, electroporation and microinjection are toxic and harsh to the tissues and hence it is necessary to find a solution for effective delivery without any toxicity and damage. The sites used for local delivery include, intravaginal, intranasal, intracerebral, intramuscular and intratumoral. These routes proved to be good for local in vivo delivery. Non-viral nanoparticles are gaining importance as a delivery vehicle because of good patient compliance and low cost (57). It has been reported in a recent article that invivo delivery of siRNA through lipoplexes and polyplexes has mediated an undesired induction of type I interferon response (58). Pulmonary route of administration acts as an effective method to treat disorders related to the lungs and also has minimized systemic adverse effects (59). Hence, modifications that improve the potency of siRNA under in vivo conditions require more research.

Conclusion

Small interfering RNAs are evolving as one of the better therapeutic strategies for various ailments. Although there are many advantages in the use of RNA interference mechanism to treat various disorders, siRNAs have certain drawbacks inside the biological system and hence the efficacy of the treatment is reduced. The major discrepancy in the use of siRNA as a therapeutic tool is its decreased stability under *in vivo* conditions and susceptibility to nuclease as well as off-target immunostimulatory activity. In order to overcome all these disadvantages, several chemical modifications were done by changing the sugar moiety, backbone phosphodiester, nucleobase, termini and conjugate groups of siRNA. Such modifications resulted in improvement in the discrepancies mentioned above. Hence, modified siRNAs have a wide scope in therapeutic application when compared to unmodified siRNAs. Apart from conventional methods of chemical modifications, rational drug design approach has been successfully applied by utilizing the available experimental structural information of siRNA-PAZ domain. It was demonstrated that the modifications of phosphodiester linkages of siRNA by phosphorothioate (PS) or boranophosphate (BP) resulted in good nuclease stability. Reduced off-target activity without altering the silencing activity was achieved by the 2'-O-methyl modification in the seed region of siRNA. Cholesterol conjugated siRNAs were successfully used in achieving target specific delivery to silence the muscle specific gene, Myostatin (Mstn) in mice. Although many promising studies have been reported on modified siRNAs, more research on the suitable chemical modifications that provides the silencing effect without any toxicity, immunostimulation, and off target effects needs to be carried out. A detailed *in vivo* study is also essential to be performed in order to better understand the mechanism, specificity, gene silencing efficacy and pharmacokinetic properties of the siRNAs.

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Figure 1.
Chemically modified sugars used in siRNA for gene silencing

Figure 2.
Chemically modified Phosphate Backbone successfully applied in siRNA

Figure 3.
Nucleobase and sugar modifications

Figure 4.
Modification to the termini and conjugate groups

Figure 5.

The PAZ-siRNA-like duplex subcomplex (Crystal structure of the PAZ domain of human Ago2.) (PDB entry 1SI2). **a.** The entire complex of two PAZ domains bound to each end of an siRNA-like duplex. Protein and RNA are presented in ribbon and stick representations; respectively. **b.** Surface view of PAZ domain is shown with columbic surface colouring option (blue= positive potential, white= neutral, red= negative potential) and the RNA is presented in stick representation.

Figure 6.

The details of PAZ–siRNA-like duplex interaction **a**). The second 3′-overhang segment positioned within the conserved pocket, **b**). Interactions involving the RNA duplex segment. **c**). Interactions involving the RNA duplex segment (view 2) Potential hydrogen-bonding interactions are shown by dashed lines. Phosphorus atoms are coloured in orange, oxygens in red, nitrogens in blue, and protein side chains in grey.

Table 1

Effects of chemical modification of siRNA ribose sugar

Chemical Modification	Target	SS/AS/3' or 5' overhang	Modification nucleotide	Number of modification	Silencing activity (RNAi), Nuclease resistant and Tm	Ref
D- or L-Isonucleoside (isoNA) modified siRNAs	Firefly luciferase gene normalized by Renilla luciferase gene in HEK 293 cells	SS & AS 5' end, cleavage and seed regions	A & U	1 per each strand	(- to +) silencing activity tm (-4.9 to +2.0)	(19)
2'-O-GP modified siRNAs (GP-siRNAs)	Hepatitis B virus (HBV) gene in Huh7 cells and Renilla/firefly luciferase gene	AS	All nucleosides from position 2-21 of 5' end	1 per sequence	(+++) Silencing activity	(20)
2'-O-GP modified siRNAs (2'-O-GP-siRNA)	Firefly / Renilla luciferase gene in HEK 293 cells	AS	UGA	8 (seed region, diagonal)	(-/+ to -) Silencing activity tm (-0.3 to -2.4) (+) Stability	(21)
1-deoxy-D-ribofuranose (R ^H)	Firefly luciferase gene normalized by Renilla luciferase in HeLa cell lines GAPDH gene in HeLa cell lines	SS & AS 3' overhang	TT	2	(++)Silencing activity (+)Nuclease resistance	(22)
2'-O-methyl modified siRNAs with mismatches in the central region of the duplex	EGFP/MDR1 gene in HEK 293 cell	SS and AS Central region	GGG UUU AAA CCC	3	(-)silencing activity Tm (-3.3 to -4.0)	(23)
Fully modified 2'-O-PivOM pro-RNA Partially modified 2'-O-PivOM pro-RNA	firefly luciferase genes / FDA normalized in Human ECV304 cells	SS and AS AS; 5' -end	Full modified Partial modified	38 5	(-)Silencing activity and Cellular uptake Tm (+0.2 to 1.0)	(24)
2'-Azido RNA (2'-N ₃ Cytidine & 2'-N ₃ guanosine)	Brain acid soluble protein 1 (BASP1) gene/GAPDH normalized; in Chicken DF-1 cell line	SS & AS Central region	C and G	6	(+++)Silencing activity (+) Nuclease resistance	(25)
Locked 2'-deoxy-2',4'-difluororibonucleoside modified Nucleic Acids (2',4'-difluoro RNA)	Firefly luciferase gene in HeLa cells	AS Near cleavage site and seed region	U	3	(+ to -) silencing activity relative to 2'-F-RNA and LNAs tm(-15.6 to +3.5)	(27)
2'-OMe-4'-thionucleoside modified siRNA (2'-OMe-4'-thioriboside siRNAs)	Firefly luciferase gene in HeLa-luc cells	SS & AS 5' & 3' ends	ACG	0-8 modifications per strand	(+ to -) silencing activity	(28)
Trivalent N-Acetylgalactosamine Conjugated siRNAs (siRNA-GalNAc conjugate)	mRNA encoding mouse transthyretin (mTTR) relative to PBS	SS 3' end & near 3' end	AU & C	3	(+ to -) Silencing activity	(29)

SS, sense strand; AS, antisense strand; Tm, melting temperature; (+++), high activity; (++), medium activity; (+), slightly active; (-), low activity; (+/-) activity retained.

Table 2

Effects of chemical modification of siRNA phosphate backbone

Chemical Modification	Target	SS/AS/3' or 5' overhang	Modification nucleotides	Number of modification	Silencing activity (RNAi), Nuclease resistant and Tm	Ref
Phosphorodithioate modified siRNAs (PS2-siRNA)	BACE1-GFP gene /RFB	SS & AS 5', 3' ends & central region	GCAU—all 4 nucleosides	1-3 depending on the sequence	(++ to +) silencing activity (+) Serum stability	(30)
Triazole Linked siRNAs (TR-siRNAs)	Firefly luciferase/ GAPDH genes normalized by Renilla luciferase in HeLa cell lines	SS & AS 3', 5' overhangs, Near central, 3' & 5' ends	C _{TR} U U _{TR} U	1-3 TR mod per sequence	(+++ to ++) Silencing activity tm (+1.1 to -20.3)	(31)
Isolated Amide Linked siRNAs	Firefly luciferase gene normalized to Renilla luciferase in HeLa cells	SS & AS Near 5' & 3' ends, 3' & 5' overhangs and Central region	Uam1U Uam1A	1-5 mod/strand	(+++ to ++)Silencing activity	(32-33)
Consecutive Amide Linked siRNAs	Firefly luciferase gene normalized to Renilla luciferase in HeLa cells	SS Seed region	U	3	(+ to -) Silencing activity	(35)
Boranophosphate (BP)	EGFP mRNA expressed in HeLa cells	SS & AS 3' overhang	A, C and U	5-17 mod per seq	(+++)Silencing activity	(36-37)
Phosphorothioate siRNAs (PS-siRNAs)	Renilla / firefly normalized in HeLa cell lines	AS & SS Central region & 5', 3' ends	UsU, AsA, CsC&GsG	Majority of the phosphate groups	(++) silencing activity depending on base modified	(38)

SS, sense strand; AS, antisense strand; Tm, melting temperature; (+++), high activity; (++), medium activity; (+), slightly active; (-), low activity; (+/-) activity retained.

Table 3

Effects of chemical modification of siRNA nucleobase and ribose sugar

Chemical Modification	Target	SS/AS/3' or 5' overhang	Modification nucleotide	Number of modification	Silencing activity (RNAi), Nuclease resistant and Tm	Ref
5-fluoro-2'-deoxyuridine modified siRNA	firefly luciferase/Renilla luciferase in pGL3 plasmid TS-mRNA in RKO cells	SS & AS 5' & 3' ends	Du	0-5 mod/strand	(+ to -)Silencing activity (+)Cytotoxicity	(39)
2'-OMe-phosphorothioate modified siRNAs (MePS2-siRNAs)	GRAM domain containing 1B (GRAMD1B) in taxane-resistant cell lines	SS & AS Near 3', 5' end & central region	A, U, & G	2 mod/strand	(+++silencing activity (+)serum stability tm (+0.7 to -1.24)	(40)
Boron Cluster modified siRNAs(CB-siRNAs)	BACE1 gene	SS & AS Near 3' & 5' ends	U/T	1 per each strand	(+++Silencing activity (++)Nuclease resistance	(41)
C-5 PLA-substituted pyrimidine nucleoside	Hepatocyte nuclear factor 4α (HNF4α) in HepG2 cells	SS and AS 3' overhang	TT modified to TZ & ZZ	2	(++) Silencing activity (++) Nuclease resistance	(42)

SS, sense strand; AS, antisense strand; Tm, melting temperature; (+++), high activity; (++), medium activity; (+), slightly active; (-), low activity; (+/-) activity retained.

Table 4

Effects of modification to the termini and conjugate groups

Chemical Modification	Target	SS/AS/3' or 5' overhang	Modification nucleotides	Number of modification	Silencing activity (RNAi), Nuclease resistant and Tm	Ref
Palmitic Acid-Conjugated siRNAs : C16-siRNAs & C16-siRNAs-idT	Exogenous Renilla/firefly luc gene; In HeLa cells/ Endogenous VEGF/ GAPDH gene; in HeLa cells	SS & AS 3, and 5' end	TT	2	(+++ to ++)/Silencing activity (+ to +)/cell permeability	(43)
Cell-Penetrating peptide-siRNA conjugate (CPP-siRNA)	Human c-MycmRNA gene in MCF-7 cells	SS & AS 5' end	A	1	(-) Silencing activity (+ to -)/cellular uptake	(44)
siRNAs conjugated with aromatic compounds via - (CH ₂) ₆ -Linker (R-(CH ₂) ₆ -siRNAs); R: phenyl, hydroxyphenyl, naphthyl, pyrenyl.	Exogenous Renilla luciferase gene normalized by firefly luciferase in HeLa cells	SS 5' end	G	4 Aryl derivatives	(+++ Silencing activity (++) Nuclease resistance tm(+1.0 to +5.3)	(45)
Amino-lipids Linked Oligos Oligos-R; R= C ₁₂ NH ₂ , C ₁₂ triazol CH ₂ NH ₂ And C ₁₂ triazol (CH ₂) ₄ NH ₂	Tumor necrosis factor-α (TNF-α) mRNA gene in HeLa cells	SS 3' end	T	1	(+/-) Silencing activity	(46)
Urea/Thiourea-bridged aromatic-siRNAs (U/TU-Ar-siRNAs)	Renilla luciferase normalized by firefly luciferase using psiCHECK-2 vector in HeLa cell lines	SS & AS 3' overhang	TT	2	(++ to +) Silencing activity (+ +) Nuclease resistance	(47)
Polyethylene glycol siRNA conjugates (PEG-siRNAs)	Renilla luciferase gene normalized with firefly luciferase genes in MCF-7 breast cancer cells	SS & AS 3' terminal	dT	1	(- to +++ silencing activity for AS and SS mods respectively	(48)
5'-triphosphate siRNAs (ppp-siRNAs)	firefly luciferase gene normalized by Renilla luc. gene in HEK 293T or HepG2.15 cells	SS	A and G	1	(++ to +++)/Silencing activity	(49)
3', 3'-Bis-Peptide-siRNA (Bis-Pep-siRNA)	Firefly luciferase gene / Renilla luc. gene normalized in HEK293 cells	SS & AS	TT	1	(++)inhibition of the target tumor	(50)
Cholesterol conjugated siRNAs (Chol-Conj-siRNAs)	myostatin (Msn) mRNA gene in Hepal-6 cells	SS & AS	T	1	(++) Silencing activity	(51)

SS, sense strand; AS, antisense strand; Tm, melting temperature; (+++), high activity; (++) medium activity; (+), slightly active; (-), low activity; (+/-) activity retained.