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# Small interfering ribonucleic acid design strategies for effective targeting and gene silencing

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***Introduction:*** Gene silencing mediated by siRNAs is becoming a promising therapeutic approach. Although many strategies and technologies have been applied to siRNA design, a key issue lies in the selection of efficient design predictors. Furthermore, the development of systemic siRNA delivery strategies, which would enhance the therapeutic effect, remains a central issue.

***Areas covered:*** The review discusses the basic principles of the sequence-specific design criteria of functional siRNAs and possible chemical modifications. Some of the most recent advances in the development of siRNA design algorithms and delivery strategies are also presented. Emphasis is given to the important design rule sets and predictors which determine the functionality of an efficient siRNA.

***Expert opinion:*** The potential and limitations of efficient design predictors obtained from computational algorithms play a crucial role in the development of target-specific siRNAs. Furthermore, the future success of RNA interference therapeutics will depend on their ability to efficiently cross the physiological barriers, selectively target cells-of-interest and finally silence the gene-of-interest without any side effects.

**Keywords:** efficient delivery, gene silencing, off-target effect, RNA interference, siRNA chemical modifications siRNA delivery, siRNA design algorithms, siRNA design rules

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## 1. Introduction

Over the past 40 years, researchers have been working on strategies to selectively silence genes that are responsible for the diseased state of cells/tissues. Following the initial discovery of RNA interference (RNAi)-mediated gene silencing [1], the therapeutic use of siRNA is gaining in popularity. The pioneering work of Fire *et al.* (1998) led to the identification of double-stranded RNAs (dsRNAs) with the potential to selectively and efficiently turn off genes in *Caenorhabditis elegans* [2]. In vertebrates, however, the dsRNAs were shown as causing cell death by the induction of the IFN response and the activation of dsRNA-dependent protein kinase R (PKR). Later, Elbashir *et al.* (2001) pioneered gene silencing in mammals by proving that diced dsRNAs can sidestep the IFN pathway and effectively switch off a targeted gene [3]. This mechanism opened a plethora of opportunities; one among them was the use of gene silencing against a variety of human diseases through an approach termed 'RNAi therapeutics'.

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**Article highlights.**

- RNA interference (RNAi) is a highly efficient process owing to its catalytic effects, in which a diligently designed high quality siRNA may be used over and over again to guide the cleavage of more and more targeted mRNAs.
- Application of siRNAs as potent sequence-specific inhibitors of mRNA transcripts is rapidly developing towards therapeutics. However, the key to successful siRNA delivery is dependent on the design of specific and effective siRNAs and the suitable delivery strategies.
- Successful silencing greatly depends on the selection of siRNA sequence based on different empirical rule sets because not all siRNAs designed based on a specific rule set for a target mRNA are efficient.
- Different design principles and chemical modifications may enhance the silencing potency, thermal and nuclease stability; reduce unintended off-target effects; and avoid the induction of pro-inflammatory cytokines and a type I IFN response.
- Integrating all design rules at a time may render inflexible selection as incorporation of all design rules into a computer-aided siRNA design algorithm may create complications in assigning proper scores to these published empirical rules. Hence, ranking these rules needs to be logically analyzed based on the experimental backgrounds.
- Despite the recent advancements in developing efficient siRNA design algorithms, prediction of specific and potent siRNAs is still far from accurate though different design predictors are taken into consideration.
- Algorithms developed by machine learning processes on artificial intelligence platform that are trained with recent RNAi experimental data sets may provide flexible integration of desired characteristics.
- Suitable biodegradable cationic nanoparticle carriers, functionalized with peptides and ligands, may enhance the internalization, stability, bioavailability, endosomal escape, intracellular trafficking and finally targeting of specific cells.

This box summarizes key points contained in the article.

Because siRNAs have the potential to be used as therapeutic drugs for human diseases, this review acknowledges the importance of effective design and delivery strategies being used for targeted gene silencing. Moreover, successful gene silencing greatly depends on the selection of the siRNA sequence. Thus, this review elaborates the key features of specific design rule sets, algorithms used for selecting an efficient siRNA and chemical modifications used for enhancing siRNA stability and target ability. At the end, a brief note on siRNA delivery strategies using synthetic carriers is detailed. Overall, the scope of the review explores the broad area of RNAi-based therapeutics involving a deep understanding of design predictors, computational algorithms, molecular cell biology, chemistry of the delivery vehicles and therapeutic molecules, and their interdependency for siRNA targeted delivery leading to gene silencing.

## 2. RNAi mechanism

RNAi-mediated gene silencing refers to the interruption or suppression of gene expression at the transcriptional or translational level. The RNAi mechanism is initiated by dsRNA that helps in endogenous gene regulation and controls the expression of cellular DNA. The two major players in RNAi mechanism are Dicer and Argonaute containing multiprotein RNA-induced silencing complex (RISC). **Figure 1** illustrates the mechanism of RNAi on introduction of dsRNA into the cytoplasm of a cell wherein Dicer along with its associated cofactors, consisting of an N-terminal RNA helicase domain, an RNA-binding Piwi/Argonaute/Zwille domain, two RNase III domains and a double-stranded RNA-binding domain (DRBD), trigger the generation of a pool of siRNAs. The formed siRNAs are ~ 21 base pairs (bp) in length with 2 nucleotide overhangs at both 3′ ends. The processed siRNAs are then delivered to an Argonaute-containing RISC. With a perfect sequence complementarity of the RNA duplex loaded onto the RISC, the Argonaute cleaves the passenger strand through RNA helicase activity. This produces activated RISC, retaining the guide strand with lower stability at the 5′ end, to act as an RISC-targeting cofactor. RISC is a stable protein–RNA complex guided by the bound strand of siRNA to the target mRNA [4]. The siRNA bound strand confers sequence-based specificity to its associated Argonaute containing-RISC complex, allowing recognition and base-pairing with the target mRNA. This reaction is carried out by the Piwi domain in RISC that folds into an RNaseH-like structure. The Argonaute in the RISC complex contains an endonuclease activity which causes a single-site cleavage of the target mRNA roughly in the middle of the siRNA-binding region [5,6]. The resulting cleaved fragments of the target mRNA have unprotected ends and are, hence, subsequently degraded by the cellular nucleases.

## 3. siRNA design profile

siRNAs are specifically designed to target and silence the gene expression at the mRNA level. The most effective siRNAs are found to be synthetic RNA duplexes with a length of 25 – 30 nucleotides. These are found as being 100-fold more potent than the 21-mer siRNAs [7]. The 27-mers did not induce IFN or activate PKR immune reactions [7]. Design rules to develop an efficient siRNA for gene silencing have proven to be crucial to improve siRNA activity and efficacy along with the site-specific characteristics of the target sequence. **Figure 2** illustrates a siRNA duplex with labeled specific sites that determine its efficiency. A variety of computational tools are available from academic and commercial sources to assist with high potency siRNA design [8]. **Table 1** summarizes some of the siRNA design tools currently being used. The following sections detail specific properties and design rule sets to determine siRNA target sites based on current literature and empirical observations by researchers through various experiments.

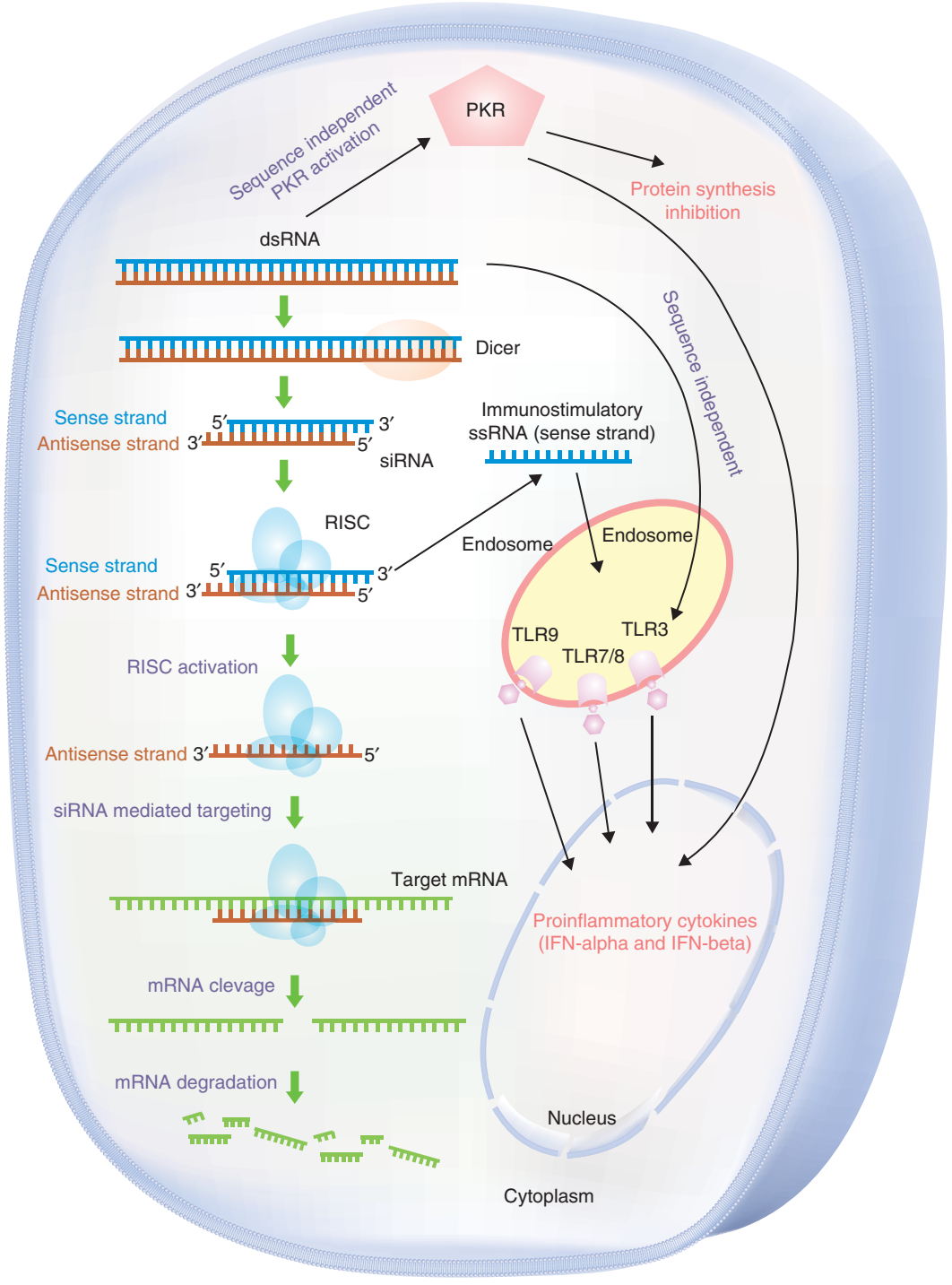


**Figure 1. siRNA-mediated gene silencing and off-target effects.** Long dsRNA entering into the cell is processed into siRNAs by Dicer. These siRNAs assemble into RISCs that unwind the sense strand. The antisense strand along with the RISC is guided to the complimentary mRNA strand. After the complimentary binding, RISC cleaves the target mRNA that is further degraded by cellular nucleases. dsRNA activates the dsRNA-dependent PKR leading to a global inhibition of protein synthesis. Toll-like receptors present in the endosome recognize double-stranded and single-stranded siRNAs in a sequence-dependent manner and induce pro-inflammatory cytokines.

dsRNA: Double-stranded RNA; PKR: Protein kinase R; RISC: RNA-induced silencing complex.

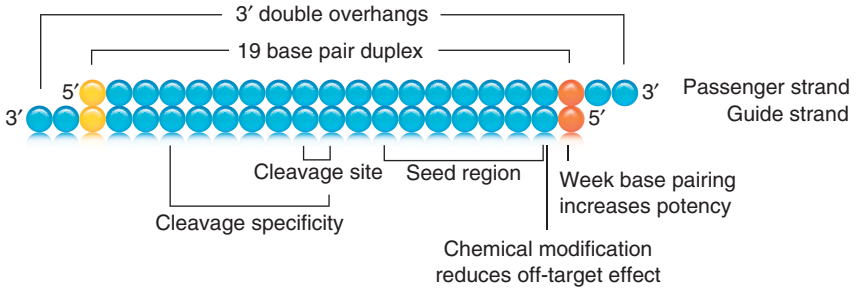


**Figure 2. Schematic representation of a siRNA duplex.** siRNAs have a well-defined structure, usually 21 nucleotide-long followed by 2 nucleotide 3′ overhangs. Each strand has a 5′ phosphate group and a 3′ hydroxyl group. This structure is the result of processing by Dicer, an enzyme that converts either long dsRNAs or hairpin RNAs into siRNAs. Important sequence-specific features such as seed-region, cleavage specificity region, mRNA cleavage site and key sites for chemical modifications and potency enhancement are shown.
dsRNA: Double-stranded RNA.

**Table 1. List of recent web-based siRNA design tools based on different empirical rule sets and algorithms.**

| Design tool | Rule set/algorithm | Features | Ref. |
|---|---|---|---|
| AsiDesigner | Sequence-based efficiency score | Selection of siRNAs for multiple target mRNAs considering alternative splicing | [122] |
| Block – iT RNAi Designer | Proprietary; Tuschl | Seed matches in 3′ UTR, BLAST option, integrated scrambled negative control design option | [15] |
| DSIR | siRNA and target sequence features | Linear regression-based model | [123] |
| E-RNAi | Reynolds; weighted scoring system | Flexible multi-species design of RNAi reagents | [124] |
| OligoWalk | Sequence and thermodynamic characteristics | Selection through SVM based on receiver operator characteristic curves and curves of positive predictive value | [125] |
| SiDesign Center | Reynolds | Updated scoring algorithm, BLAST option, seed matches in 3′ UTR | [10,18,27,28] |
| siDirect | Ui-Tei | Sensitive homology search algorithm, filtering low seed-target duplex stabilities | [122,126] |
| siDRM | DRM | Filtering immunostimulatory and cell toxic motifs, off-target checks | [127] |
| Specificity server | Mismatch tolerance scoring scheme | Specificity scoring based on various off-target effects filtering criteria | [128] |
| SVM siRNA Design (β*) | Sequence features | Features optimized based on hyper functional siRNAs through machine learning framework and SVM | [129] |

Every tool has some limitations as it is difficult to incorporate all possible criteria in the design algorithm. However, most of the tools are based on various sequence-specific features including off-target motifs, thermodynamic and structural properties of target as well as siRNA. Experimentally validated functional siRNA sequence databases and trained data sets are incorporated in most of the tools to generate ideal machine learning criteria to rationalize the siRNA design computationally.
*The β version of this tool is not available currently for public use.
DRM: Disjunctive rule merging; RNAi: RNA interference; SVM: Support vector machine.

### 3.1 Preferential siRNA loading to RISC based on GC content and thermodynamic profiles

The GC content of the siRNA sequence has been used as a vital condition for selecting target sequences. Ideally, the GC content should be < 50%, although successful gene silencing has also been reported with GC content between 50 and 60%. Reynolds *et al.* (2004), Ui-Tei *et al.* (2004), Amarzguioui and Pyrdz (2004) and Tuschl *et al.* (2006) reported guidelines for rational siRNA design based on position-dependent characteristics associated with siRNA functionality [9-12], as summarized in Table 2. Though it has been reported that low GC content in siRNAs correlates with increased silencing efficiency [8-11,13], it might not always be predictive of the efficacy because sequences with an extremely low GC content fail to form stable siRNA duplexes. On the other hand, siRNAs with a high GC content near the 3′ end of the antisense strand and a lower GC content near the 5′ end of the antisense strand offered significantly higher success rates [14]. Researchers at Cenix Biosciences recognized the strong impact of differential end stabilities on silencing

**Table 2. siRNA design rule sets proposed by lead researchers based on their empirical findings of the sequence-specific positional characteristics of functional siRNAs.**

| Ui-Tei (2004) | Reynolds (2004) | Amarzguioui (2004) | Tuschl (2006) |
|---|---|---|---|
| GC content: 30 – 60% | GC content: 30 – 52% | GC content: 32 – 53% | GC content 30 – 70% |
| A/U at the 5′ end of the antisense strand | At least 3 A/U bases at position 5 – 19 of the sense strand | High differential between A/U content in 3′ and 5′ ends | A/U at position 1 in antisense strand |
| C/G at the 5′ end of the sense strand | Absence of internal repeats (Tm of secondary structure < 20°) | Presence of G or C at position 1 | C/G (C is more common) at position 19 |
| A/U rich 5′ end of the antisense strand | An A base at position 19 of the sense strand | Presence of A at position 6 | A/U at position 10 in sense strand |
| No more than 9 GC pairs in a straight line | An A base at position 3 of the sense strand | Presence of A/U at position 19 | A/U richness at the 5′ end of antisense strand |
| | A U base at position 10 of the sense strand | Absence of U at position 1 | |
| | A base other than G or C at position 19 of the sense strand | Absence of G at position 19 | |
| | A base other than G at position 13 of the sense strand | | |

Gene-silencing efficacy depends on the target sequence positions selected from the target gene. Although these rule sets and guidelines are suitable for designing effective siRNAs for some specific genes, they are not always effective for many mammalian genes.
Tm: Melting temperature.



efficacy, as conferred by the choice of residues at or near the ends of the target sequence on silencing efficacy.

Schwarz *et al.* (2003) demonstrated the preferential loading of either the sense (passenger) or antisense (guide) strand of a siRNA to RISC through an *in vitro* study. Because the strand bound to RISC guides the target mRNA degradation [15], Schwarz *et al.* (2003) demonstrated that the siRNA strand with a lower GC content and higher A/U content at the 5′ end gets preferentially loaded to the RISC, as this condition makes the strand less thermostable (Tm) [15,16]. The high A/U content at positions 1 – 7 imparts lower stability due to a decreased count of hydrogen bonds between A and U compared to between C and G [15,17,18]. Based on this result, it was hypothesized that RNA helicase, an enzyme responsible for siRNA unwinding, selects a strand for incorporation into the RISC depending on the thermodynamic properties of the strand. This finding explains the sequence bias uptake by RISC between effective and ineffective siRNAs. **Figure 3** illustrates the two classes of siRNAs: (A) effective: Class I and (B) ineffective: Class III based on the GC and AU content, resulting in their preferential loading to RISC. It is noted that Class III siRNAs have opposing features to Class I and cause little or no mammalian gene silencing. The remaining siRNAs belong to Class II and are a mixture of highly or intermediately functional siRNAs and nonfunctional siRNAs. However, this classification is not widely accepted as it is difficult to categorize the siRNAs within the three classes.

Most siRNA design rules exploit thermodynamic asymmetry by selecting naturally occurring sites within a gene sequence that conform to the desired pattern. However, it may occasionally be necessary to target a specific location even though the natural base sequence is thermodynamically unfavorable. It is possible to artificially manipulate thermodynamic asymmetry by introducing mismatches (to lower Tm) or by placing chemically modified bases (to increase Tm) in the siRNA duplex [8]. Furthermore, non-complementary bases can be introduced at the 3′ end of the sense (passenger) strand to lower the stability of the 5′ end of the antisense (guide) strand, without impairing the ability of the antisense (guide) strand to anneal to the native target [19-21].

## 4. siRNA design based on target mRNA patterns

Good accessibility with a low secondary structure in the target mRNA increases the chance of successful RNAi [22,23]. Several experimental analyses revealed that the most effective functional siRNAs comprised 2 nucleotide overhangs at the 3′ end. Elbashir *et al.* (2001) reported that siRNAs with UU or TT 3′ overhangs were more efficient and suggested that symmetric 3′ overhangs aid in the formation of RISC complexes with antisense (guide) and sense (passenger) strands in an equal ratio [8]. According to their study, an ideal target mRNA contains $AA(N_{19})TT$ motif, where (N) indicates a nucleotide and ($N_x$) indicates the length of the target sequence. But in the case of the absence of the $AA(N_{19})TT$ motif, the $NA(N_{19})TT$ or $NA(N_{21})$ motif were used as the target mRNA sequence, which would be similar to the sense (passenger) strand sequence of a siRNA, as summarized in **Table 3**. In the case of $NA(N_{19})TT$ or $NA(N_{21})$ motifs, a nucleotide at the 3′ end of the antisense strand was converted to a T nucleotide which, at position, was not complementary to the target mRNA site [8]. In the case of the $NA(N_{21})$ motif, a 3′ overhang of the sense strand was also converted to TT in order to generate symmetric siRNAs. The sense strand did not affect mRNA recognition and thus its 3′ overhang was not

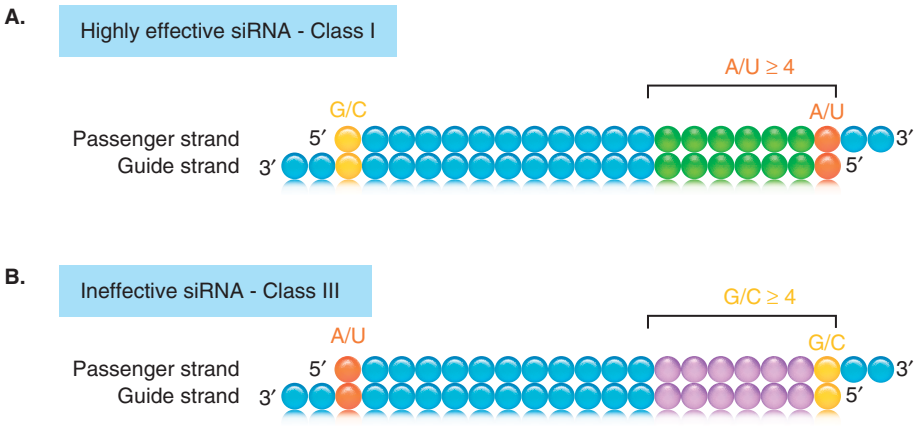


**Figure 3. Representation of class I and class II siRNAs. (A)** The highly functional class I siRNAs simultaneously satisfy the following four conditions. i) The 5′ antisense-strand end with A or U; ii) the 5′ sense-strand end with G or C; iii) more than four A/U nucleotides in the 5′-terminal belonging to a third of antisense strand and iv) lacking a long G/C stretch throughout the entire length. **(B)** Ineffective class III siRNAs that are nearly incapable of inducing effective mammalian RNAi possess features opposite to class I siRNAs with respect to the first three conditions. Rest all other siRNAs are considered to belong to class II (not shown in the illustration).

Adapted from [10] with the permission of Oxford University Press and Dr Ui-Tei.
RNAi: RNA interference.



required for to agree with the nucleotides of the mRNA target site [8,13].

Ui-Tei *et al.* (2004) represented the target mRNA pattern of $N_2(G/C)N_{17}(A/T)N_2$ for developing an effective siRNA sequence [10]. According to the design rules, the siRNAs that did not confer to the first three conditions, as mentioned in **Table 2**, have little or no effect on gene expression [10]. The design rule sets were confirmed by testing 57 potentially highly effective and 16 potentially ineffective siRNA constructs against the target mRNA pattern [10].

Reynolds *et al.* (2004) explored further sequence preferences. They proposed more effective siRNAs with reference to the target mRNA pattern of $N_4(A)N_6(T)N_2(A/T/C)N_5(A/U)N_2$ [9], as summarized in **Table 3**. They analyzed, experimentally, the silencing efficiency of 180 siRNAs targeting the mRNA of two genes. The silencing efficiency of each siRNA was then correlated with various sequence features of the individual siRNAs. Reynolds *et al.* (2004) (Dharmacon, Inc.) identified eight characteristics associated with siRNA functionality, as summarized in **Table 2**. These characteristics were used by rational siRNA design algorithms to evaluate the potential targeting sequences and were assigned scores based on hierarchy. Sequences which scored higher had a better chance of success for use in RNAi [9]. Reynolds *et al.* (2004) had projected several additional criteria based on their experimental analysis, including a statistical preference for certain residues at positions 1, 7, 10 and 17 in active siRNAs. While the biased preference at position 1 might be the requirement for a weak bonding at the 5′ end required for RISC inclusion, the reason for the preferences at the other positions was not clearly elucidated [9].

Pei and Tuschl (2006) proposed a target mRNA pattern of $N_2(G/C)N_8(A/T)N_8(A/T)N_2$ motif (**Table 3**) based on the empirical rules proposed by them for designing functional siRNA, formulated based on the experimental evidence obtained from the screening of sequence-specific siRNAs [12]. According to them, first the targeted region of 50 – 100 nucleotides downstream of the start codon should be selected from a given cDNA sequence. Following this, a 23-nucleotide sequence motif $AA(N_{19})$ should be searched for. In case no suitable sequence was found, a 23-nucleotide sequence motif $NA(N_{21})$ should be searched for, wherein the 3′ end of the sense siRNA should be converted to TT [12]. Otherwise, the $NAR(N_{17})YNN$ motif should be searched for (A = adenine; T = thymine; R = adenine or guanine (purines); Y = thymine or cytosine (pyrimidines); N = any nucleotide). Though the empirical rules provided a basis for designing siRNAs, their ability to predict knockdown efficiency still requires improvement [12].

Another guideline for effective siRNA design is based on the thermodynamic property that takes into account the energy difference between the sense and antisense strands as discussed in Section 3.1. Based on these factors, Yuan *et al.* (2004) proposed a siRNA design formulated on the thermodynamic scoring of a siRNA strand. The authors observed that if the 5′ end of an antisense strand could reflect the thermodynamic values of each end of the sequence, then a thermodynamic score could be determined**.** A negative score for a siRNA sequence led to its selection as a good candidate because it possesses the ability to effectively unwind and bind with RISC. Each model considered the first five nucleotides of the sense strand,

**Table 3. Target mRNA patterns and corresponding siRNAs.**

| Target Pattern | Sequence |
|---|---|
| *AA($N_{19}$)TT* [9] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ U U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... A C G G A A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |
| *NA($N_{19}$)TT* [9] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A G G C A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |
| *NA($N_{19}$)TT with converted nucleotide in 3′ end of antisense strand* [9] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ U U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A G G C A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |
| *NA($N_{21}$)* [9] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U A C A 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A U 5′ |
| mRNA | 5′... T A G G C A C G A G T G G A A T A A C C T C A T A C A G G T ...3′ |
| *NA($N_{21}$)with converted 3′ overhang of sense strand* | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A G G C A C G A G T G G A A T A A C C T C A T T C A G G T ...3′ |
| $N_2$(G/C)$N_{17}$(A/T)$N_2$ [12] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A A G C A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |
| $N_4$(A)$N_6$(T)$N_2$(A/T/C)$N_5$(A/T)$N_2$ [11] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A G G C A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |
| $N_2$(G/C)$N_8$(A/T)$N_8$(A/T)$N_2$ [13] | |
| siRNA – sense strand | 5′ C G A G U G G A A U A A C C U C A U U U U 3′ |
| siRNA – antisense strand | 3′ G U G C U C A C C U U A U U G G A G U A A 5′ |
| mRNA | 5′... T A A G C A C G A G T G G A A T A A C C T C A T T T T G G T ...3′ |

Target mRNA patterns - recommended nucleotides are green, arbitrary nucleotides are red and in corresponding siRNAs - recommended nucleotides are blue. Presented sequences are illustrative and do not agree with any mRNA and/or published siRNAs.

including a 3′ antisense overhang, and the first five nucleotides of the antisense strand, including a 3′ sense overhang [24].

## 5. Overcoming off-target effects

siRNA silencing of unintended mRNA transcripts containing partial homology to the target poses an important barrier to the success of effective siRNA design. This undesirable effect is known as the 'off-target effect' [16,25]. Sequence specificity of the siRNA is very stringent, with even a single base pair mismatch between the siRNA and its target mRNA dramatically reducing the efficacy of silencing. The incidence of nonspecific targeting is dependent on the concentration of the siRNA, with a higher concentration leading to a greater off-target effect. Thus, sub-nanomolar concentrations of highly active siRNAs are used in order to minimize off-target effects [16,25]. Off-target effects, from high concentrations of siRNAs in the cells, are likely to result from the sense strand or from the dsRNA-binding proteins that stimulate the antiviral-response pathways, inducing the expression of antiviral-response genes. The use of sub-nanomolar concentrations of siRNA allows the transfection of multiple siRNAs simultaneously, an important advantage.

Seed-region analysis determines the number of exact matches of the siRNA seed-region with the mRNA's

3′ UTRs. Complementarity between the 3′ UTR of the mRNAs and the seed-region of the antisense (guide) strand, especially with the nucleotides at positions 2 – 7 or 2 – 8, has been associated with off-targeting [26]. It has been observed that siRNAs with low seed-region frequency are less likely to cause off-targeting when compared to siRNAs with higher seed-region frequencies [26,27]. Interestingly, it was reported that long dsRNA does not evoke off-targeting because a pool of siRNAs with different sequences arise following Dicer digestion [28], which subsequently reduces the concentration of a particular siRNA in relation to the total siRNA. This, in turn, reduces off-targeting. Therefore, the use of a pool of chemically synthesized siRNAs, with different sequences, plays a key role in reducing off-target effects [29]. The loading of the sense (passenger) strand to the RISC may be another source of off-target effects. Even if a siRNA sequence satisfies the conditions aforementioned, the possibility of a small percentage of sense (passenger) strand being incorporated into RISC and serving as a guide strand can change the expression of many genes. In such a case, chemical modifications of the sense strand would serve to solve this problem [16].

Longer and unmodified siRNAs are more likely to trigger off-target effects. In the cytoplasm, such effects are associated with the activation of PKR that is not sequence-dependent (**Figure 1**). The sequence-dependent immunostimulatory effects, that is, IFN and TNF-mediated innate immunity, are induced by specific siRNA motifs that activate toll-like receptors 7 and 8 (TLR7/8) [30,31] and the retinoic acid-inducible gene I pathway [32]. The systemic therapeutic delivery of siRNA *in vivo* is intrinsically related to a potential recruitment by the immune cells, as both TLR7 and TLR8 are located in the endosomal compartment and can sense endocytosed single-stranded RNA (ssRNA)/dsRNA (**Figure 1**) [33]. Such immunostimulatory activation caused by siRNAs can be mitigated by different chemical modifications [34,35]. Bifunctional siRNA approaches combining gene silencing and immunostimulation for the enhancement of antiviral and antitumoral synergy between innate immune recruitment and gene-specific targeting have recently been published [32,36]. A recent review describes the details on recognizing and avoiding different siRNA off-target effects [37].

## 6. Chemical modification

Optimal downregulation with siRNA could be achieved by overcoming the transient nature of siRNA that makes it less stable and less active. In addition to the rational design criteria mentioned in Section 4, the chemical modification of the siRNA has been shown to improve the siRNA function by enhancing its stability, reducing the off-target effects and avoiding the stimulation of the innate immune system [31,38]. Most researchers currently use chemically synthesized siRNAs, comprising single-stranded 21-mer RNA oligonucleotides (ssRNAs) annealed into duplexes [39]. These siRNAs mimic the natural siRNAs that result from Dicer processing of long dsRNAs. Several studies reveal that some chemical modifications can improve the properties of siRNAs but certain chemical modifications have none or little influence on the efficacy of the siRNA (**Table 4**) [40]. In general, these chemical modifications can greatly increase the specificity and stability of the siRNAs, resulting in a prolonged effect of gene-silencing activity.

Natural siRNAs have a phosphorylated 5′ end, essential for their function, but phosphorylation of the 5′ end of chemically synthesized siRNAs is not required as they get naturally phosphorylated by cellular kinases following their transfection. Moreover, blocking the 5′ end of the antisense strand leads to the loss of RNAi activity as described in Section 3. Thus, it is not encouraged to phosphorylate the antisense strand of the chemically synthesized siRNAs. Blocking of the 3′ end of either or both the antisense and the sense strand has no significant effect on RNAi activity [41,42].

Combinations of 2′-ribose siRNA modifications enable effective gene silencing and improve siRNA stability in human serum. Introduction of as few as two different or identical 2′-ribose modifications at a specific position of either strand was previously suggested to suppress unintended off-target effects, enhancing the thermodynamic stability of siRNA duplexes, without altering the overall A-form helical geometry required for silencing. Stable siRNAs downregulate gene expression for a more prolonged period of time. In many cases, chemical modification reduces the activity when applied to sensitive sites within the siRNA, for example, the 5′ phosphorylation of antisense strand of chemically synthesized siRNAs [39]. However, *in vivo* activities of such chemically modified siRNAs required testing and evaluation for any therapeutic use. A few of the chemical modifications of siRNA are described below.

### 6.1 Phosphorothioate backbone

A phosphorothioate (P-S) backbone increases the resistance to ribonucleases, thus, increasing the RNA stability. A P-S backbone at a given position on the RNA can be achieved by the oxidation of phosphite with Beaucage reagent during oligonucleotide synthesis. Chemically stabilized siRNAs with a partial P-S backbone and 2′-O-methyl sugar modifications on the sense and antisense strands showed a significant resistance towards degradation by exo- and endo-nucleases in serum and in tissue homogenates [43]. Experiments revealed that P-S linkage lowers both RNAi and toxicity in a dose-responsive manner. An alternative backbone modification that increases siRNA stability is the boranophosphonate linkage (**Figure 4**).

### 6.2 2′-Fluoro modifications

Fluorine has an interesting combination of properties. Electronegativity of a fluorine atom is similar to that of a hydroxyl group (**Figure 4**). siRNAs synthesized with 2′-F pyrimidines showed an increased stability in human plasma when compared to 2′-OH siRNA [15]. 2′-Fluoro-cytidine (2′-FC) and 2′-fluoro-uridine (2′-FU) could protect siRNAs from nuclease

**Table 4. Effect of few chemical modification and bioconjucation on siRNA.**

| Passenger strand | Guide strand | RNAi function | siRNA character |
|---|---|---|---|
| 2′-OMe, 2′-F and related substitutions | Unmodified | Increased | Endonuclease resistance, reduced off-target |
| Unmodified | 2′-OMe at position 2 | Not affected | Off-target reduction |
| 2′-O-MOE | Unmodified | Not affected | Endonuclease resistance |
| Unmodified | 2′-O-MOE | Moderate to severe based on position | Endonuclease resistance |
| 2′-OMe and 2′-O-MOE | Unmodified | Not affected | Endonuclease resistance, reduced off-target |
| Phosphorothioate backbone | Unmodified | Not affected | Exonuclease resistance |
| Unmodified | Phosphorothioate backbone | Moderately affected | Exonuclease resistance |
| Phosphorothioate backbone | Phosphorothioate backbone | Moderately affected | Exonuclease resistance |
| 3′ end modification by 2′-OMe and phosphorothioate substitutions | Unmodified | Increased | Higher nuclease stability |
| Unmodified | Blocking of the 5-hydroxyl terminus | Severely affected | No significance |
| Blocking of the 3′ terminus | Unmodified | Not affected | No significance |
| Bioconjugation with cholesterol or carrier molecule | Unmodified | Not affected | Increased cellular uptake, biodistribution and site specificity |

RNAi function (when compared with the gene-silencing effect of unmodified siRNA): not affected - > 50% gene silencing; moderately affected - 20 – 50% gene silencing; severely affected - < 20% gene silencing (based on the data in [40]). The increase in RNAi activity is not because of the direct influence of chemical modification on silencing activity of the guide strand. The enhanced potency of siRNA due to chemical modifications such as nuclease stability, off-target reduction and specificity results in optimal guide strand entry into RISC and facilitates multiple turnover.
RISC: RNA-induced silencing complex; RNAi: RNA interference.



degradation. Chiu and Rana (2003) identified that siRNAs with a 2′-FC- and 2′-FU-modified sense strand or modified sense and antisense strands were more stable when compared to unmodified siRNAs, without any compromise on the silencing activity [39]. Studies were performed to evaluate the capacity of 2′-F-modified siRNAs to knockdown target mRNA and their cellular toxicity was also monitored [44]. All 2′-F-modified siRNAs resulted in the target knockdown at nanomolar concentrations despite their high thermal stability. These experiments provide the first evidence that RISC activation not only allows 2′-F modifications at the sense-strand cleavage site but also increases the biological efficacy of modified siRNAs *in vitro*.

### 6.3 2′-O-methyl modifications

siRNA molecules with internal 2′-O-methyl modifications at specific positions confer nuclease resistance especially against serum nucleases, without any significant loss of RNAi activity (**Figure 4**) [42]. The RNAi activity is only reduced with an increase in nucleotide modification, that is, modification of a whole strand (either or both of the sense and antisense strands) severely affects silencing [45]. Jackson *et al.* (2006) hypothesized that siRNA modification could weaken or disrupt the RISC–mRNA interaction, due to imperfect complementarity between the mRNA and the siRNA's guide strand. They demonstrated that a 2′-O-methyl group at the second nucleotide from the 5′ end of the antisense strand reduced the off-target effect, without having any effect on the downregulation of the targeted mRNA [46]. 2′-O-methyl modifications judiciously placed on guanosine and uridine backbones along the siRNAs prevented type I IFN induction and cytoplasmic TLR activation, which is a pathogen pattern receptor that signals macrophages and other immune activating cells leading to an immune response [47].

### 6.4 ssiRNA

Sticky siRNA (ssiRNA) allows siRNA molecules to form more stable structures that can potentially enhance *in vivo* delivery. ssiRNAs adhere together due to short complementary runs of adenosine (A) and thymine (T) bases (A5–8/T5–8) added at the 3′ end of each molecule. These larger, more stable structures bind tightly to cationic polymeric delivery vectors by concatemerization [48]. Due to these interactions, ssiRNAs can easily avoid non-optimal condensation and leaching from cationic polymeric complexes, thus enhancing their cellular uptake and gene-silencing activity. Hence, the delivery of ssiRNA with an efficient and safe polymeric carrier may provide an alternative strategy for RNAi-based research.

### 6.5 SisiRNA

Small internally segmented interfering RNAs (sisiRNAs) are characterized by an intact antisense strand and two shorter sense strands or a nicked sense strand of ~ 9 to 13 nucleotides in length that can reduce nonspecific downregulation caused by the loading of the sense strand of siRNA to RISC. Such a structural design increases the specific gene silencing by the antisense strand and reduces the genome-wide off-target effects by the sense strand. Bramsen *et al.* (2007) observed that the two shorter nicked sense strands of sisiRNA of 10 and 12 nucleotides length did not incorporate into RISC, which eventually eliminated

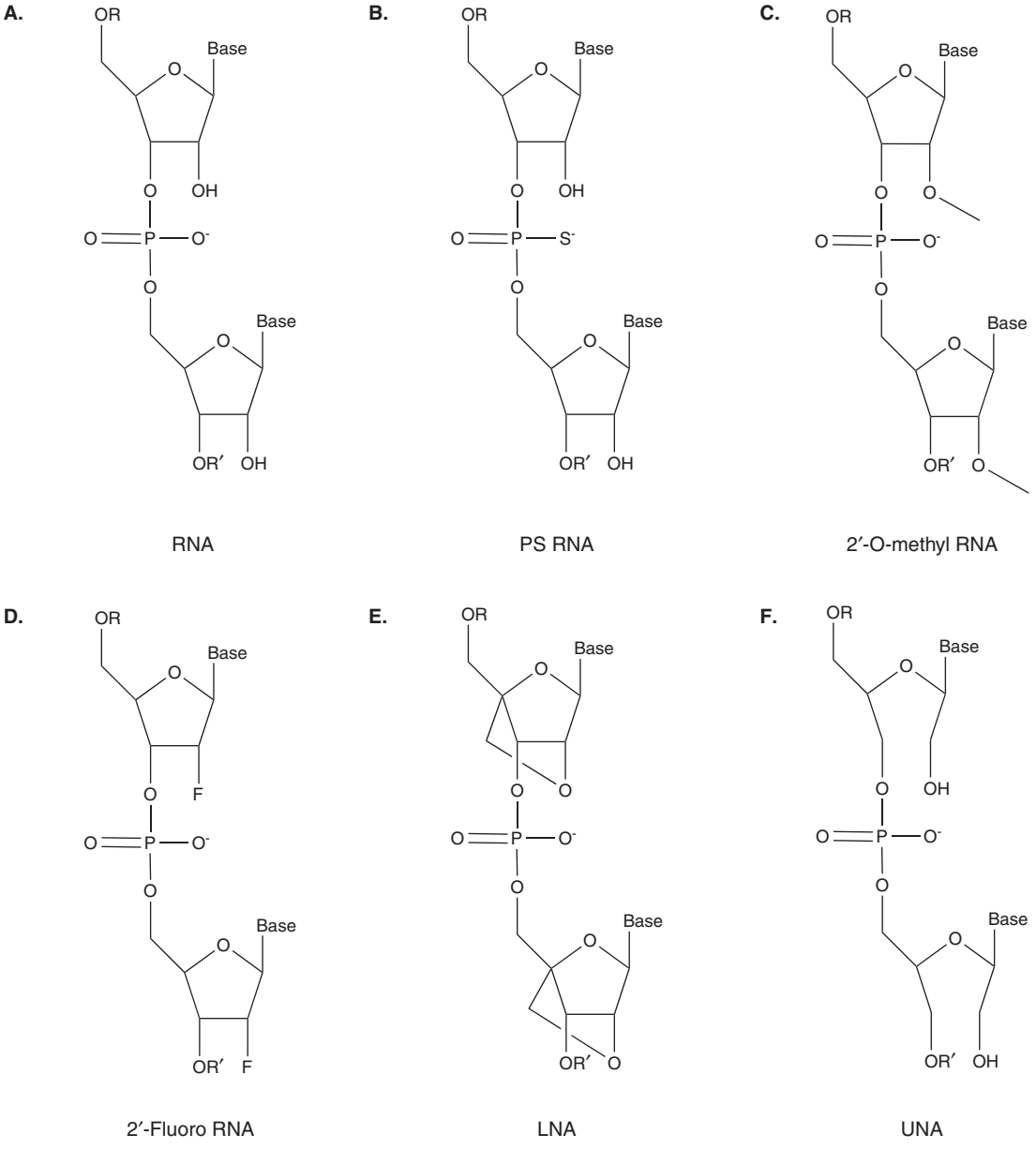


**Figure 4. Possible chemical modifications that enhances the potency of the siRNA. A.** The backbone structure of RNA is given for comparison. **B.** The phosphodiester linkage can be replaced by phosphorothioate that retains the negative charge of a phosphate. Introduction of a phosphorothioate (P=S) backbone linkage at the 3′ end protects against exonuclease degradation. **C and D.** 2′-Sugar modification such as 2′-O-methyl or 2′-Fluoro provides endonuclease resistance. 2′-O-methyl modification suppresses off-target effects and evades induction of IFN response. **E.** LNA is conformationally locked to (contains a methylene bridge connecting the 2′-oxygen with the 4′-carbon of the ribose ring) nucleotide analogs with high nuclease resistance. **F.** UNA is an acyclic analog of RNA in which the bond between the C2′ and C3′ atoms has been cleaved. The destabilizing properties of UNA are suited to enhance the potency of siRNAs that can be modified by other chemical modifications such as LNA or 2′-O-methyl-RNA.

LNA: Locked nucleic acid; UNA: Unlocked nucleic acid.

sense strand-mediated off-target activity [49]. Such nicked sense strands have no influence on the generation of the functional RISC [50]. As strand selection is primarily determined by the thermodynamic asymmetry of siRNA duplex ends, highly efficient siRNA may be difficult to design if the target sequence is restricted to a thermodynamically unfavorable region, for example, when the intention is to target a single nucleotide mutation or junctions between fused genes (such as Pax3-Fkhr). In such cases, the sisiRNA design will ensure that only the unsegmented antisense strand contributes to the gene silencing,

irrespective of the thermodynamic profile of the sisiRNA duplex, thus, eliminating the possibility of unwanted silencing [49]. Unmodified sisiRNAs most probably do not function *in vivo* due to their low stability. A locked nucleic acid (LNA) modification is used to stabilize the sisiRNAs. Although sisiRNA animal studies are still at a nascent stage, preliminary data suggest that these molecules may have benefits over other RNAi agents in a therapeutic setting.

### 6.6 LNA

LNAs make up a class of nucleic acid analogs possessing unprecedented binding affinity towards complementary DNA and RNA. For efficient gene silencing and serum stability *in vitro* and *in vivo*, fully modified or chimeric LNA oligonucleotides can be used. LNAs are conformationally locked nucleotide analogs that contain a methylene bridge connecting the 2′-oxygen and the 4′-carbon of the ribose ring (**Figure 4**). This connection locks the ribose ring in a 3′ end conformation, typical for an A-form helix. The use of LNA has been tried in siRNA design to develop siLNA that may improve serum stability, bioavailability and pharmacokinetic properties. LNA is compatible with the siRNA machinery and increases the biostability of the siRNAs without compromising their efficiency. Incorporation of LNA in siRNA can effectively reduce the off-target effects of siRNA, as a substitution on the 5′ sense-strand position of the siRNA encourages the loading of the antisense strand and provides a higher affinity for binding to complementary nucleotides while any other additional internal substitution in the sense strand might impair its ability to participate in target cleavage [51,52].

### 6.7 UNA modified siRNA

Unlocked nucleic acid (UNA), an acyclic analog of RNA, has an incomplete ribose ring open between the positions of the 20- and 30-carbon. Prominent properties of LNA and UNA monomers are their high and low affinity towards complementary nucleotides, respectively. Modification of siRNA with both UNA and LNA nucleotides showed efficient gene silencing, especially the UNA-modified siRNAs which exhibited a lower cellular toxicity [49]. These modifications destabilize the base pairing at the 5′ end of the antisense strand, while strengthening the base pairing at the 3′ end. Consequently, the polarities of the siRNAs are reversed due to the modulation of the relative stability of the termini with LNA and UNA. Though it was observed that the potency of the reversed siRNA against the full-length target decreased significantly, the introduction of a limited number of UNA and LNA monomers into a siRNA maintains the high level of silencing activity [53]. UNA modifications at various positions were found to be detrimental to the siRNA activity, especially, UNAs at positions 1 and 2 which prevented the phosphorylation of the siRNA, thereby abrogating the RISC loading [54]. Such a strategy would improve silencing efficiency by inhibiting the loading of the RISC by the passenger strand. UNA modification at position 7 was observed to reduce off-target effects, causing destabilization of the siRNA–target interactions but, importantly, did not reduce siRNA potency [55]. Thus, such position-specific designs can be utilized for the design of future safe and effective siRNA. Another study revealed that the incorporation of a UNA in the seed-region of the guide strand eliminated microRNA (miRNA)-like off-target activity without affecting the siRNA-like activity [56]. Thus, it was concluded that UNA substitutions at different positions can confer desirable properties to the siRNAs. In addition, a combined modification of siRNAs with LNA and UNA provides a promising approach to alter and improve the properties of the siRNAs.

## 7. Control siRNAs–scrambled siRNA

The importance of a control siRNA is to observe and demonstrate the specificity of the delivered siRNA's silencing activity on the gene of treatment with no off-target effects due to any transfection methods. Various experimental controls such as a negative control, positive control, IFN control, cell death control and reporter control are utilized depending on the objective of the research task. The best negative control (scrambled) siRNA can be selected based on a lack of homology between the candidate scrambled siRNA and the genome. Scrambled siRNAs should have the same length and nucleotide composition as the siRNA of interest but have at least 4 – 5 mismatched bases when compared to the test siRNA. Till date, there are no specific design rules available for making a proper and appropriate scrambled siRNA negative control.

Online tools such as Invitrogen's Block-iT RNAi designer and InvivoGen's siRNA wizard provide an option to design negative scrambles. However, it is not possible to assure the odds of using such scrambled siRNAs, as there is a possibility for some nonspecific side effects. Predesigned negative controls are offered by several vendors and are based on the different levels of GC content to match that present in the siRNA to be tested, with no homology to the test organism. They are also tested for any sequences that can potentially induce a stress response. However, even with prevalidated scrambled siRNAs, altered expression levels of a number of different proteins nonspecific to the target have been observed, suggesting that the optimal selection of a negative control is critical in order to get the specific interpretation of siRNA down-regulation [57]. Hence, it is important to ensure the non-reactiveness of such scrambled siRNAs through different validation experiments such as genome-wide microarray expression analysis and multiparametric cell-based assays such as cell proliferation, viability and morphology.

## 8. Combining the goodness of different rule sets to develop better algorithms in selecting efficient siRNA

Though various investigators, research institutions and companies have proposed different empirical rule sets and

design algorithms to predict potential siRNAs, an experimental verification would still be needed to determine its efficiency. The first generation of siRNA prediction algorithms were searched through the target mRNA to find possible siRNAs according to the decisive criteria, detailed in Section 4. The decisive criteria included the GC content, the thermodynamic stability, the base composition/preference and the specific occurrence of nucleotide bases in critical positions of the siRNA motif. A combination of these criteria determines a set of rules, assigning a score to each siRNA sequence that is being screened. The siRNA candidates scoring above a predefined threshold are thus selected as valid siRNA candidates. However, these tools provide a limited flexibility to users and make the selection of a particular rule set critical as per the user's preferences, based on the validated research data [58]. The use of these algorithms, with specific rule sets, yields at least a 50% of functional siRNAs that result in more than a 70% gene knockdown.

Based on various publications and validated empirical findings, an attempt to combine some of their rule sets into a single effective tool is illustrated in **Figure 5.** However, it is impossible to include all known rules as they may render stringent selection criteria through which only few siRNA candidates will pass, which in turn can result in the loss of some potentially efficient siRNA candidates. Apart from this, reliable prediction techniques should also be considered that take into account secondary structure predictions of siRNA and target mRNA to be silenced (**Figure 5**). Along with these applications, an effective filter for the minimization of off-targeting is also essential. This could be achieved by incorporating homology search engines that avoid partial-matching between the antisense strand and unintended targets. Studies have revealed that a good specificity is achieved when the selected siRNA has at least three mismatches in any of its off-target gene alignments. Most prediction tools (**Table 1**) involve a homology search by BLAST, which helps to find nonspecific or partially specific siRNAs that lead to unintentional targeting. However, an accurate mismatch tolerance is tedious to calculate because it demands an entire search of the sequence database in order to check if an individual siRNA is potentially able to cross-hybridize with irrelevant sequences. The Smith–Waterman algorithm can be used for more accurate results that involve an exhaustive search of local alignments but require an extensive search of the entire genome, including spliced exon overlaps and alternative splicing genetic targets thus making the process time consuming to execute. Although BLAST is much faster than the Smith–Waterman algorithm, it may overlook significant alignments. For example, in the case of short input sequences of 19 bases, BLAST, with its default parameter values, may fail to notice the best alignments with a minimum number of mismatches.

Many RNAi applications demand better functional efficiency than that offered by current algorithms. Recent siRNA design algorithms are based on various bioinformatic analyses of experimentally tested siRNA candidates by machine learning methods to better understand the link between a siRNA's sequence, its target location, its thermodynamic properties and its silencing efficiency. Several machine learning algorithms have been used in the computational modeling of efficient siRNA design. Among such algorithms, support vector machine methods, artificial neural network methods, decision tree and linear regression method are best suited for rational analysis. For example, artificial neural networks can identify complex patterns from experimentally validated siRNA data sets through rigorous analysis and make accurate predictions that aid in generating algorithms for designing highly potent target-specific siRNA sequences that are effective even at a low concentration.

In addition, these algorithms use position specific scoring matrices (PSSM) and knowledge of the most important siRNA seed-region to predict the highly effective and specific siRNA sequences for an intended target. These algorithms incorporate the common properties of highly effective siRNAs from databases and selectively search for similar properties among the siRNA candidates being screened for the intended target mRNA. However, not all the algorithms are trained with the recent feedbacks of experimental and empirical findings of gene silencing using different siRNAs, hence failing to update and ensure the *in silico* data rule sets of the algorithm. Initially, the structure of the target mRNA was not considered as an essential criterion in the siRNA design, but recent studies showed that siRNA efficacy could depend highly on the pattern of the target mRNA secondary structure [59,60]. A filtering algorithm developed by Yiu *et al.* (2005) based on the observations of secondary structure of mRNA and verified by artificial intelligence techniques such as decision trees and support vector machines was compared with the existing siRNA design tools. The results indicated that this filtering algorithm can effectively identify ineffective siRNAs [61].

Matveeva *et al.* (2007) assessed several algorithms with statistical analysis based on the published experimental data and derived a new efficient method to predict siRNA efficiency, which utilizes linear regression fitting with local duplex stability, nucleotide position-dependent preferences and total G/C content of siRNA duplexes as input parameters [62]. Though this method did not use time-consuming RNA secondary structure calculations, the method's ability to discriminate between the ability of efficient and inefficient siRNAs was comparable with that of the best methods identified, but its parameters were observed to be more related to the mechanisms of siRNA action. This permits insight into the underlying physical features and the relative importance of the parameters. The existing design rule sets and guidelines for selecting effective siRNAs for mammalian genes may not estimate the probability of a siRNA candidate to silence the target gene. However, the prediction methods such as radial basis function network and decision tree for selecting effective siRNA target sequences from many possible candidate sequences are really different from the previous PSSM-based siRNA design techniques [63].

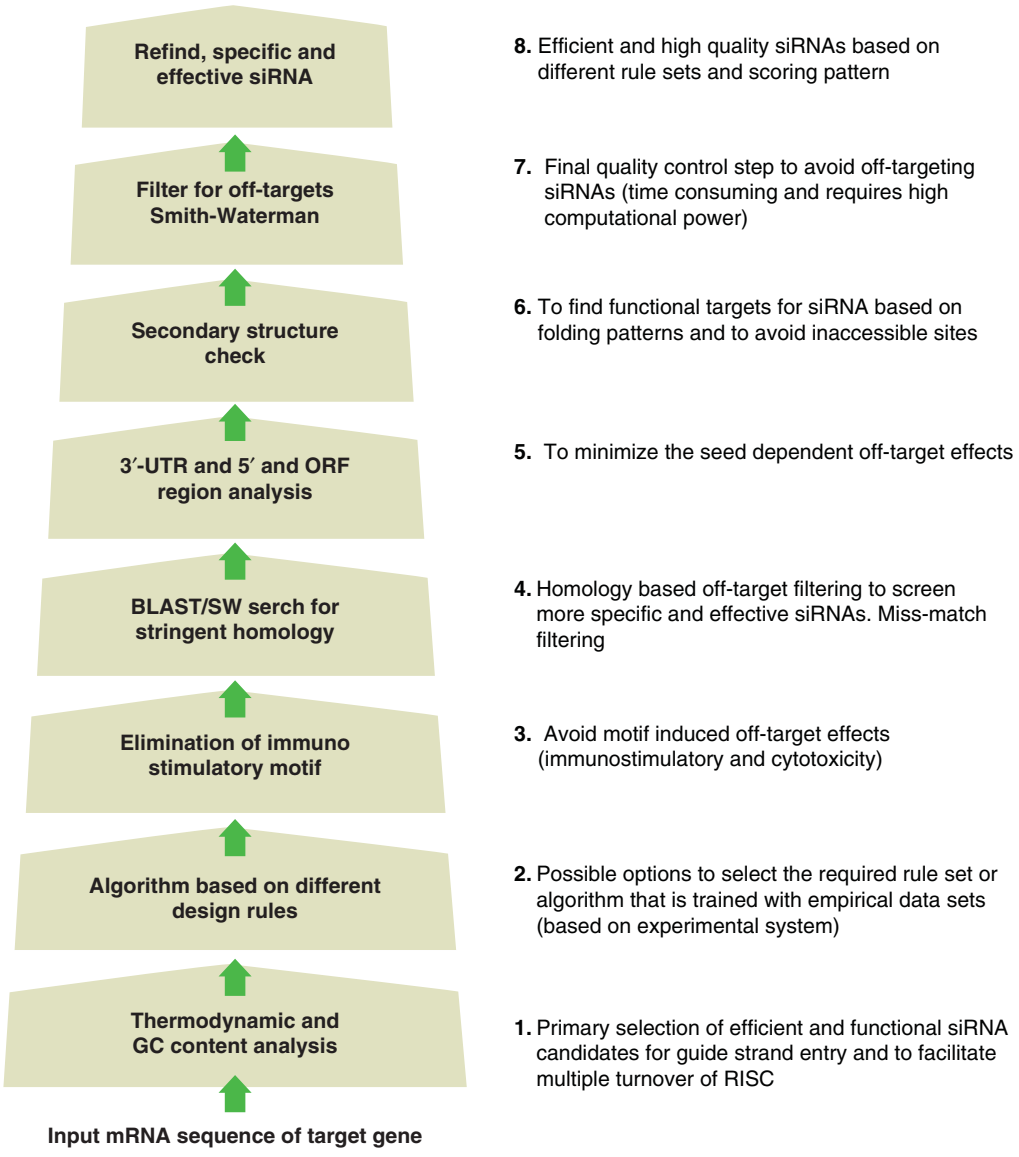


**Figure 5. A schematic model of an improved siRNA design algorithm based on: 1. Thermodynamic asymmetry, 2. Selected empirical rules and guidelines, 3. Filtering known stimulatory motifs, 4. Selection for more specific siRNA candidates through homology analysis, 5. Seed frequency analysis for siRNA design to minimize off-target effects by eliminating common seed-regions 6. siRNA target site secondary structure predictions to confirm functional mRNA structures from non-functional sites, capable of forming an A-helix for correct positioning of the scissile phosphate bond for cleavage in RNAi and 7. Stringent filtering through Smith–Waterman algorithm for off-targets as a final quality control step.**

RNAi: RNA interference.

Another algorithm using a stochastic logistic regression-based method with approximate Bayesian feature selection has been designed to explore a vast model space of 497 compositional, structural and thermodynamic features to identify a highly potent siRNA. Novel siRNA potency motifs 'UCU' and 'ACGA' are identified in such a large siRNA metadata set analysis [64]. Application of least absolute shrinkage and selection operator model has been used to measure the contribution of each siRNA sequence. This feature has suggested that only few positions, that is, 5′ and 3′ ends and the seed-region of siRNA sequences are influential to determine the siRNA efficacy [65]. These novel prediction algorithms imply high estimation accuracy for selecting and predicting effective siRNA candidates.

The recent incorporation in precise siRNA design is chemoinformatics descriptors involved in siRNA chemical modification that avoid off-target effects and enhance the potency. Along with chemoinformatics, quantitative structure–activity relationship modeling studies help to reveal the efficacy prediction of therapeutically active siRNAs. In

addition, accurate homology search through extensive Smith–Waterman algorithm to avoid splice variants, single nucleotide polymorphisms and allelic specificity is also used to design therapeutic siRNAs. Due to such improvements, most of the web-based online designing tools may not generate accurate hits in the real time designing. As more specific details of the RNAi mechanism have been defined, these recent methods have been incorporated into more complex siRNA selection algorithms, increasing the reliability of selecting active siRNAs against a single target [66].

## 9. Combinatorial targeting approaches in siRNA design

Several reports validate the new concept of combinatorial RNAi and illustrate its versatility which involves the design of dual-targeting siRNAs, wherein both strands are intentionally designed to separately target different mRNA transcripts with complete complementarity [67]. The advantage of such an approach, over the use of two separate duplexes, is that only two strands, as opposed to four, are competing for entry into the RISC. These dual-targeting siRNAs were designed as Dicer substrate 25-/27-mer siRNAs. They were as effective as their corresponding single-targeting siRNAs in target knockdown. Such structural flexibility enables multifunctional therapeutic siRNAs. The algorithm developed in this study should prove to be useful for predicting the dual-targeting siRNAs with a variety of different targets. Long synthetic siRNAs as long as 38 bp were also used to knockdown two targets without any nonspecific off-target responses [68]. Collectively, these efforts open up exciting new therapeutic avenues but could also augment the inherent risks of RNAi technology, including immune responses, off-targeting and over saturation of endogenous pathways [69].

Multivalent (MV) siRNA provides three-in-one siRNA capabilities for targeting multiple sites via RNAi. A single MV-siRNA can suppress one gene at several sites or suppress multiple genes all at once. This novel design delivers greater potency than a single siRNA, removes off-target elements and replaces the inaccurate 'pooling' methods of traditional siRNA. By combining the characteristics of siRNA's sequence-specific mRNA cleavage and miRNA's complementary pairing to the 3′ UTR regions that results in the suppression of translation (not discussed in this review), a novel rational design was developed to achieve bifunctional siRNAs that simultaneously direct the cleavage and the translational suppression of HIV RNAs [70]. These bifunctional siRNAs triggered the inhibition of HIV infection and replication in cell culture. Such design principles have wide applications throughout the genome of HIV, as nearly 90% of genes harbor sites that make the design of bifunctional siRNAs possible [70]. Due to the complexity of the combinatorial RNAi approach that involves expression of various methods such as shRNA and miRNA expression system along with siRNA and other protein therapeutics, it is difficult to comment on the therapeutic viability of this method at this point.

## 10. Effective siRNA delivery strategies

Once a siRNA has been successfully designed and synthesized, the next challenge is to effectively deliver siRNAs into the target cell/tissue. This requires the siRNAs to migrate and cross the physiological barriers to initiate successful gene silencing. Genetically-modified viral vectors have extensively been investigated as gene/siRNA delivery vehicles but they possess high immunogenicity and toxicity risks. Therefore, non-viral delivery systems, especially biodegradable cationic lipids and polymers, have attracted much attention because these systems do not have the risks associated with the viral systems. In the next step, cell-specific targeting becomes significant to further improve on the effectiveness of these siRNA delivery systems. Systemic delivery is currently a major obstacle in the development of siRNA and other nucleic acid-based therapeutics. Before siRNAs can be used therapeutically, researchers must overcome the problems of *in vivo* stability [71,72], tissue-specific targeting [73,74] and unwanted immune system activation [30,31]. Recent studies have demonstrated rapid advancements in overcoming the efficient delivery challenges in RNAi-based therapeutics.

Animal studies involve either naked or formulation-based siRNA delivery to the target tissue. The advantages of administering siRNA locally are the utilization of lower siRNA dose and reduced systemic side effects. However, this form of delivery is invasive in nature, whereas formulation-based siRNA delivery involves a higher dosage but remains less invasive. The success of local and formulation-based siRNA delivery has been demonstrated to be well tolerated and efficacious in animal models of ocular neovascularization and scarring targeting VEGF and is now being further tested in a clinical setting as a treatment for age-related macular degeneration [75-78]. Further success with RNAi therapeutics has been shown for pulmonary diseases targeting viral genes, respiratory syncytial virus and parainfluenza virus [79,80] and in CNS, locally administering naked siRNA via intracerebroventricular or intrathecal infusion [81-83] and with formulation-based siRNA requiring 10 times lesser dose with efficient silencing [84,85]. For an extensive review on siRNA-based therapies, delivery strategies and clinical studies, readers are encouraged to follow reviews published elsewhere [86-89].

Owing to the transient nature of siRNA, the most common siRNA-delivery methods used are liposomes [90,91], cationic lipids [92,93], lipid conjugant, and stable nucleic acid lipid conjugates [94,95], and polymers such as poly-L-lysine, polyamidoamine dendrimers [96,97] and polyethyleneimine have been widely used for nucleic acid/siRNA delivery [98,99] and cyclodextrins [100-103]. Other natural cationic polymers that have been used for siRNA delivery are chitosan [104,105] and atelocollagen [106,108]. Cheng *et al.* (2006) have demonstrated the enhanced cellular uptake of siRNAs

conjugated to cholesterol via systemic administration. The cholesterol conjugation helped in increasing the hydrophobicity of the siRNA and thus enhanced the stability and cell membrane penetration [109]. siRNA conjugated to RNA aptamers specific to prostate-specific membrane antigen has also shown successful siRNA delivery both *in vitro* and *in vivo* as a local delivery, targeting the survival genes (PLK1 and BCL2) [110,111].

Some transfection systems utilize a cell penetrating peptide (CPP) that non-covalently binds to siRNA to form a nanoparticle. These CPPs are protein transduction domains (PTDs) that consist of positively charged amino-acid sequences mainly arginine and lysine that interact with the negatively charged head groups present on the cell membrane, allowing for the diffusion across the cell membrane and the delivery of the siRNA directly into the cytoplasm. CPPs such as penetratin and transportan have been used to conjugate siRNA and enhance cellular uptake [112]. Other cationic peptides that have been used for gene delivery are MPG (27-mer peptide) [113] and cholesteryl oligo-D-arginine (Chol-R9) [114]. CPPs can also be attached on to polymer surfaces or cationic lipids in order to enhance vector-mediated delivery, for example, the RGD peptide [115]. The coupling between the polymer surface and the CPPs for vector-mediated delivery is facilitated either via covalent coupling of active functional groups naturally present on either of the molecules or by the introduction of a chemically modified, reactive functional moiety.

Cationic lipids tend to aggregate and release their cargo on their interaction with serum proteins, and thus are an unstable mode of delivery. To enhance their stability, hydrophilic polymers such as polyethylene glycol can be used, which reduce macrophage clearance by the reticulo-endothelial system. A bifunctional PEG molecule acts a spacer between the vector carrying the cargo and the CPP. This conjugation enables an improved stability and the target specific delivery of the therapeutic molecule. Cationic polymers, on the other hand, pose cytotoxic and inflammatory effects *in vivo* [74]. This problem can again be addressed through the use of PEG by surface coating the polymers to reduce their toxic effects. Another recent class of siRNA delivery system being used is the fusion protein complexes, termed PTD and DRBD. Dowdy and co-workers (2009) reported that direct conjugation of negatively charged siRNA to a positively charged PTD resulted in an inefficient/loss of cellular delivery due to the aggregation of complexes [116]. While the DRBD part of the fusion protein had high affinity to bind to siRNAs, by masking its negative charge and PTD, it facilitates the cellular delivery of the complex. This complex offered excellent transfection with no apparent cytotoxicity/immune responses in both primary cells as well as cancer cell lines [116].

## 11. Conclusion

RNAi mediated by the introduction of long dsRNA has been used to investigate gene function in a variety of organisms. Various factors contribute to the optimization of the siRNA used in RNAi studies. A major challenge, however, is to ensure the efficient delivery of siRNA drugs *in vitro* and *in vivo* in animals and eventually in humans. Design, quality and delivery represent three of the most important elements that enable the accurate downregulation of a target gene. So far, three published clinical studies *in vivo* have shown that the RNAi technique is safe and effective in the treatment of cancers and various other diseases, with considerable benefits such as a high specificity, an ability to be administered through various routes and the possibility of silencing multiple genes simultaneously [89]. Though clinical studies represent a promise of RNAi therapeutics, the availability of a therapeutic approval for human use with precision and efficiency is yet to be validated in areas such as long-term therapeutics/side effects. In addition, the amount of siRNA to be administered must be accurately optimized for the clinical standards, because a slight deviation from the desired amount may trigger an unexpected and undesired immune response. Moreover, the response may vary from siRNA to siRNA based on the sequence, activity, effectiveness, sequence length, stability and other factors related to recipient. It is important to investigate the relationship of the siRNA-mediated target-specific gene silencing induced by the stimulation of the IFN pathway. This would require rigorous testing of the backbone modifications, carrier system and *in vivo* delivery mechanisms in order to ensure the application of siRNA for targeted downregulation as the sole cause of observed phenotypes.

## 12. Expert opinion

Sequence characteristics for designing siRNAs are the most important factors determining effective siRNA sequences. The rules sets and guidelines proposed by various researchers may result in the generation of many siRNA candidates which can in turn make the screening process stringent for synthesis and therapeutic application. Despite numerous efforts, the design of potent siRNA remains inadequate and the design rules resulting from different studies frequently disagree with each other, and are often unsatisfactory. Several siRNA design algorithms have emerged over the last few years claiming to offer high success rates for silencing human genes but it is estimated that only one out of eight siRNAs are actually functional. Rational guidelines in analytical reviews, websites and commercial algorithms have facilitated the easy selection of functional siRNA candidates with high efficacy and specificity. Publicly available siRNA design programs have so far shown success rates of 50 – 60% in generating siRNAs that can yield > 70% downregulation of the targeted mRNA. In collaboration with Ambion, Cenix BioScience has chosen the more rigorous path of directly measuring the effectiveness of ~ 1100 of its own algorithm-designed siRNAs in silencing almost 400 endogenously expressed human transcripts. This empirical study, therefore, represents the most physiologically

relevant, direct and comprehensive performance analysis of any siRNA design algorithm available today [118].

Another important aspect of siRNAs is their stability for which chemical modifications have been used and are considered critical for optimizing and validating siRNAs for therapeutic use. By modulating siRNA thermodynamic asymmetry and engineering the 3′-overhangs, the activity of siRNA is primarily enhanced by favoring the incorporation of the intended antisense strand during RISC loading [49]. Some specific positional modifications such as 2′-O-methyl ribosyl substitution at position 2 in the guide strand can effectively reduce off-targeting, resulting in more specific and reliable siRNAs [46]. Although chemical modifications can alter the properties of siRNAs, only selective modifications can improve the desired properties of cellular uptake, biodistribution, thermal stability, efficacy, nuclease stability, reduced innate immune response, lowered incidence of off-target effects and improved pharmacodynamics. The incorporation of chemical modifications in siRNA strands is likely to give rise to another set of challenges. The complexity of interpreting the effects of different chemical modifications on siRNA activity exemplifies the importance of using multiple biochemical, cell-based and *in vivo* assays to validate and rationally design chemically modified siRNAs destined for therapeutic use. Though chemical modifications do enhance the desired characteristics of siRNAs, the selection of target-specific sequences of siRNA remains the most important criteria.

Several groups have compared and assessed the performance of published siRNA efficacy predictor algorithms [62,118,119]. Every prediction algorithm emphasizes a unique set of rules; hence, different tools select different siRNA candidates. However, updating the data sets with newly published data on siRNA efficiency and specificity enables a check as a quality control step of the program. Though it is not possible to say which tool is better in designing efficient siRNA, a comparison between various tools may help in the identification of efficient siRNA candidates. Usually, multiple siRNAs against a specific target are used to ensure similar phenotypic response resulting from on-target gene silencing. However, using such multiple siRNAs per target fails in potential therapeutic applications [120]. Hence, further refinement in the integration of different empirical rule sets and predictors of design algorithms is crucial for synthetic siRNAs for therapeutic applications. In the future, it is expected to merge miRNA target prediction algorithms with those developed for siRNA design to eliminate candidate siRNAs with potential off-target gene silencing through undesirable miRNA-related mechanisms [120]. The quality parameters for the selection of highly effective siRNAs are certainly going to change over the next years due to the tremendous growth in RNAi research that is generating vast empirical data and also the developments in computational integration methods.

As the large mass of siRNA may reduce the potency, in cell culture, the $IC_{50}$ for siRNA-mediated target knockdown can be as low as 1 – 10 pM. Hence, the experimental screening for potent siRNAs that gives sub-nanomolar $IC_{50}$ and > 80 – 90% knockdown is essential to enable lower doses, which, in turn, should reduce the toxicity and off-target effects. Ideally, a delivery vehicle should carry the highest possible siRNA dose for a required amount of delivery vehicle, as it helps to reduce both toxicity and cost. A reasonable approach is required to make siRNA drugs an attractive therapeutic option. Recent innovations in RNAi research aim at the successful delivery of siRNAs in order to facilitate the prolonged effect of gene silencing. Owing to such innovations, a multistage nanovector has been proposed, which comprises mesoporous silicon particles, loaded with neutral nano liposomes containing siRNA targeted against ovarian cancer [121]. Other techniques related to siRNA delivery involve the use of pH sensitive lipoplexes/polyplexes as delivery vehicles or incorporation of fusogenic proteins/aptamers/ligands/antibodies to siRNA, which aid in their delivery across the biological barriers and their release into the cellular cytoplasm. Thus, an adequate siRNA delivery method should be extensively studied because a properly designed siRNA packed in an incorrect carrier could be delivered to an unintended site and produce undesired effects. Researchers are continually exploring the areas to resolve technical challenges, unwanted cellular/immunological reactions, compound stability, dose optimization and delivery aspects for siRNA-based therapeutic applications.

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## Declaration of interest

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