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siRNAs: their potential as therapeutic agents – Part I. Designing of siRNAs

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RNA interference (RNAi) is a novel and essential biological process, as well as a powerful experimental tool with the potential to be used in therapeutic development. RNAi-based strategies have the capability of being able to be driven from bench to bedside. It is very important to develop the precise tools for designing the siRNAs to get the most efficient knockdown of the target genes and to reduce any off-target effects. In this review we have discussed the strategies and parameters required for effective siRNA designing and synthesis, based on already published literature.

Introduction

RNA interference (RNAi) is a process that has been evolutionarily conserved across species and acts as an innate immune response against the deleterious effects of invasive nucleic acids of transposable elements, viruses and other pathogens such as bacteria [1,2]. RNAi plays an important and indispensable role in host defense and regulation of gene expression. The functionality of the RNA silencing pathway is maintained by small RNAs, along with many other protein complexes. Many naturally occurring small RNAs, such as microRNA (miRNA), Piwi-interacting RNA (piRNA), *trans*-acting small interfering RNA (tasiRNA), repeat-associated small interfering RNA (rasiRNA), natural small interfering RNA (nat-siRNA) and small interfering RNA (siRNAs) have been reported for their diverse regulatory roles in biological systems. In one of the most important discoveries of the past decade, Fire *et al*. reported that in *C. elegans* genes could be silenced by introducing dsRNAs directed against their particular coding regions [3]. This discovery revolutionized the field of genomics, which has now developed into a powerful tool to silence genes in a sequence-specific manner. Growing understanding of the mechanisms of biogenesis and the functional aspects of small RNAs have enabled us to use such RNAi tools more effectively to knock down genes in a temporally and spatially regulated manner. This is also known as post-transcriptional gene silencing (PTGS) or cosuppression in plants, RNAi in animals and quelling in fungi.

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miRNAs have an indispensable role in the regulation and tuning of the expression of genes. miRNAs originate from endogenous hairpin shaped transcripts of genes (Fig. 1). These miRNA genes are located in the intra- or intergenic regions. They are transcribed by their own promoters or by the promoters of genes in which they reside, either by RNA polymerase II or by RNA polymerase III. The transcribed precursor miRNAs (also called primary-miRNAs (pri-miRNA)) are processed by a microprocessor complex (Drosha–DGCR8 complex in humans, Drosha–Pasha complex in *Drosophila melanogaster* and *C. elegans*), followed by maturation of miRNA in the cytosol by a specific Dicer (DCR). Microprocessor complex binds to double stranded RNA (dsRNAs) at the dsRNA and single stranded RNA (ssRNA) junction close to the bottom of the 'stem loop structured pri-miRNA' and helps 'drosha' (RNase III type protein), to cleave it and produce precursor miRNA (pre-miRNA) with 2 nt 3′-overhang. The pre-miRNA hairpins are exported to the cytoplasm from the nucleus by Exportin-5 (Exp5) in the presence of Ran-GTP as a cofactor [4]. In the cytoplasm, Dicer interacts with its double stranded RNA-binding protein partner (TAR RNA binding protein (TRBP) in humans, RDE4 in *C. elegans* and Loquacious (Loqs) in *Drosophila*) and other partners to cleave the pre-miRNA to produce a small 21–23 nt RNA duplex with 2 nt 3′ overhangs [5]. Dicer and R2D2 (considered to be a homolog of the RDE-4 protein of *C. elegans*) and its partners bind to this small RNA duplex to form a RISC loading complex (RLC), which helps in the loading of the small RNA duplex into another multiprotein complex, with Argonaute protein as its core component, called RNA-induced silencing complex (RISC) or miRNA protein complex (miRNP). Only one

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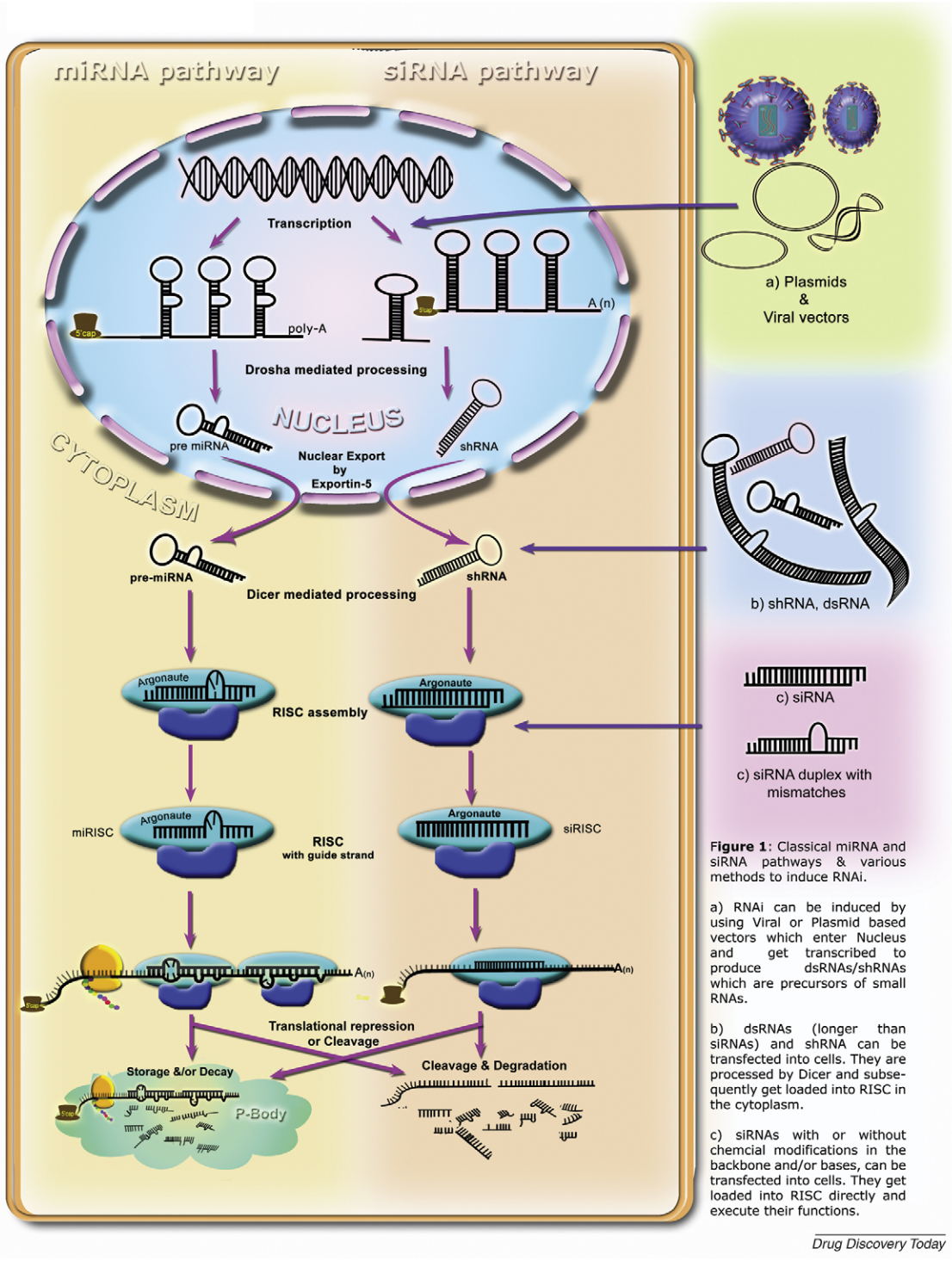

FIGURE 1

Classical miRNA and siRNA pathways and various methods to induce RNAi. **(a)** RNAi can be induced by using viral or plasmid-based vectors which enter nucleus and get transcribed to produce dsRNAs/shRNAs which are the precursors of small RNAi. **(b)** dsRNAs (longer than siRNAs) and shRNA can be transfected into cells. They are processed by Dicer and subsequently get loaded into RISC in the cytoplasm. **(c)** siRNAs with or without chemical modifications in the backbone and/or bases, can be transfected into cells. They get loaded into RISC directly and execute their functions.

strand, called guide strand (miRNA), is retained in the RISC and the other passenger strand (miRNA*) is degraded. Although both miRNA–RISCs and miRNA*–RISCs are formed, they differ in their abundance. This biased selection of guide strand depends on the thermodynamic and structural properties of the miRNA::miRNA* duplex. The mature miRISC binds to its complementary target and executes its function by cleaving it or by repressing translation, followed by degradation [6,7]. Some miRNAs are known to regulate

DNA methylation and histone modifications without repressing translation or by cleaving their targets [8]. siRNAs also follow a closely related pathway sharing many of the components of miRNA pathway during their biogenesis and action. Discrimination between different types of small RNAs within the cell might be achieved at the level of their biogenesis, where their incorporation into respective effector complexes is decided based on their origin, sequence, structure and localization. Therefore, it is very important to have a clear understanding of the features contributing to this discrimination, which will form the basis for designing target-specific siRNAs for silencing. The RNAi pathway is well exploited in basic research to knock down genes of interest specifically. Several attempts have been made to utilize the potential of siRNA for therapeutic purposes against many viral diseases and other disorders. Even miRNAs are implicated to have roles in many diseases caused either by their overexpression or by their underexpression. RNAi can be used to reverse these under or overexpressed miRNAs, either by introducing functional precursor miRNAs, or by knocking them down, respectively.

Biochemical studies of the mechanism of biogenesis, as well as the function of small RNAs, have contributed to the better design of siRNAs and their utility *in vitro* and *in vivo*.

Important aspects of gene knockdown by siRNAs

The design of siRNAs includes the selection of siRNA sequences that are capable of knocking down the expression of their target genes. Many computational methods have been developed, based on the secondary structures of siRNAs/targets, or the nucleotide sequences of siRNAs and their targets [9–17]. The specificity of siRNA sequences can be improved by altering their sequences and/or by introducing some chemical modifications. The ways to reduce the off-target effects and development of suitable delivery methods are very important factors for consideration.

Major criteria for siRNA design

Naturally occurring small RNAs have undergone the process of evolution because of selection pressure and they exist in perfect balance with their precursors and targets, as well as associated machinery involved in this process. Gene silencing is performed by introducing artificially synthesized small RNAs into the cell or by expressing siRNAs/shRNAs within the cell, using specific expression cassettes, either in plasmids or in viral vectors. These siRNAs/shRNAs enter the endogenous RNAi pathway at different levels after their delivery into the cells. If the design parameters of siRNAs are not optimal they might cause imbalance in the endogenous small RNA mediated pathways, resulting in various deleterious/unwanted effects in the cells. Studies on various types of RNA-mediated silencing pathways have helped us better to understand the parameters required for efficient and specific siRNA-mediated gene silencing. There are several web-based tools available for the design of effective siRNAs, based on various studies (Table 1).

The following criteria are generally considered important for the conventional design of siRNAs: GC content of the sequence and other sequence parameters like inclusion or exclusion of specific nucleotides at particular positions; thermodynamic parameters of siRNA duplexes and their targets; structural requirements for their optimal action and consideration of identical/similar sequences in their targets to avoid nonspecific activity.

Previous studies have shown that 21 nt sequences, with 19 nt duplex and 2 nt overhang toward their 3′ termini, with AA bases at the 5′-end of their targets and GC content between 30 to 70%, provide the necessary thermodynamic stability for the siRNAs. The target sequence should preferably not be very close to the initiation codon (~75–100 bases), because those regions are usually occupied by different protein factors [18]. The following online tools (http://www.ambion.com/techlib/misc/siRNA_finder.html and http://www.jura.wi.mit.edu/bioc/siRNA) have been developed, based on the previously mentioned criteria [9,19]. Additional sets of tested and validated parameters have already been reported in the published literature elsewhere [20–23]. While using the 21 nt siRNAs with 2 nt overhang on the 3′ termini, the strand with low 5′ thermo-stability gets preferably incorporated into RISC. The use of the small RNAs of 25–27 bps [24] or shRNAs with 29 bp stem and 2 nt 3′ overhang [25] has been reported to be more effective than their 19 nt long counterparts.

Ui-Tei *et al.* [23] have suggested four major criteria for the effective design of siRNAs:

TABLE 1

Popular siRNA design tools

Name	Address	Refs
RFRCDB-siRNA	http://www.bioinf.seu.edu.cn/siRNA/index.htm	[11]
sIR	http://www.biotools.swmed.edu/siRNA/	[17]
BIOPREDsi	http://www.biopredsi.org/	[12]
RNAi central	http://www.katahdin.cshl.org:9331/RNAi_web/	GJ Hannon lab
siDirect	http://www.design.RNAi.jp/	[13]
TROD	http://www.cellbio.unige.ch/RNAi.html	[14]
siVirus	http://www.siVirus.RNAi.jp	[15]
Oligo walk	http://www.rna.urmc.rochester.edu/	[16]
siRNA	http://www.jura.wi.mit.edu/bioc/siRNA	[9,10,19]
siRNA Selection Server	http://www.jura.wi.mit.edu/bioc/siRNA	[18]
siRNA Finder	http://www.ambion.com/techlib/misc/siRNA_finder.html	Ambion
DSIR	http://www.biodev.extra.cea.fr/DSIR/DSIR.html	[86]

1. The 10th and 19th bases of the sense strand should be A or U.
2. The 5′-end (~7 bases) of antisense strand should be AU rich.
3. The 1st base of the sense strand should be G/C.
4. The sense strand should have more than 3 A/U bases in the region between the 13th and 19th bases.

Reynolds *et al.* [26] analyzed 180 siRNAs with known efficiency and studied some salient features in those siRNAs showing maximum efficacy. They suggested the following parameters for the optimal design of siRNAs:

1. The GC content of the siRNA sense strand should be 30–50%.
2. The presence of more than 3 A or U bases in the region of 15–19th bases.
3. Stable hairpin-like secondary structure should be avoided.
4. The Tm should be less than 20°C.
5. The 3rd and 19th base of the sense strand should be A.
6. The 10th base of the sense strand should be U.
7. The 13th base of the sense strand should not be G.
8. The 19th base of the sense strand should not be either G or C.

Jagla *et al.* [27] analyzed a larger data set of 601 siRNAs targeting an exogenous gene (*lac z*) and three endogenous genes (*Rab6A*, *Tnfrsf1a* and *p65/RelA*) and suggested the following three major criteria:

1. The 10th and 19th bases of the sense strand should be A or U.
2. The 1st base of the sense strand should be G/C.
3. There should be more than 3 A/U bases within the 13–19th bases of the siRNA sequence.

Shabalina *et al.* [28] identified 18 parameters for siRNA design and derived a nucleotide position-dependent consensus for siRNA design. They reported the dinucleotide content to be a better predictor of siRNA efficiency, because the efficiency of those siRNAs and miRNAs with A/U dinucleotides alternating with G/C dinucleotides was found to be more effective than others lacking this periodicity. The nucleotide content and base stacking are important for efficient silencing. They suggested some preferred positions for particular bases in siRNA sequences as follows:

1. Nucleotides at positions 1–3 should preferably be A/U and U at the 1st position of antisense strand is particularly important.
2. Avoid having G at positions 1–3 and C at the 1st position.
3. At the 13–14th positions, U is preferable and G should be avoided.
4. The 17–19th positions should not contain A.
5. C and U are preferred in the 17th and 18th positions.
6. C and G are preferred in the 19th position.

The above-mentioned conditions mainly suggest the need for the presence of higher A/U content at the 5′-end and higher G/C content at the 3′-end of the antisense strand. Studies on the specificity of miRNA targeting in mammals, conducted by Grimson *et al.* [29], throw some light on those parameters useful in the effective design of siRNAs. They suggested that AU-rich regions are the regions where miRNAs bind to their targets, therefore, the presence of AU-rich regions is preferred in targets. Good pairing of the 13–16th positions of miRNAs, along with their 2–8th bases (seed region) located in the 3′-UTR and at least 15 nts away from the stop codon is preferred. The miRNA binding sites should be positioned away from the center of UTRs. This strategy might be useful in avoiding the selection of regions often occupied by different protein factors. The above-mentioned considerations are quite helpful in designing siRNAs based on their target gene sequences. To increase the specificity of such selected sequences a few chemical modifications can be introduced into siRNAs, either at the sequence level or at the structural level.

As far as siRNAs are concerned, intramolecular secondary structures such as hairpins and intermolecular interactions, such as stable duplex formation should be avoided because duplexes with higher stability can hamper the dissociation and binding of siRNAs to their respective targets. In addition to the siRNAs, the structure of the target mRNA is also important, because this determines accessibility to the siRNAs. It is very important to avoid the presence of very rigid secondary structures, while designing siRNAs [30,31]. Many algorithms used to design siRNA use this feature with variable stringencies. Current tools to predict secondary structures from sequence are not sufficiently accurate for longer target sequences and require more computational resource.

Off-target effects of siRNAs

RNAi is sequence-specific but factors that determine the specificity depend on various parameters that might, in addition, exhibit some off-target activity. Off-target effects could be due to two factors: the binding of siRNAs to sequences other than the target, resulting in their suppression or the induction or enhancement of innate immune response by some motifs or patterns in the siRNAs.

Nonspecific effects dependent on sequences of siRNAs

In common with miRNAs, siRNAs are also capable of binding to sequences with partial complementarities. So, siRNAs with homology to sequences other than the desired target should be avoided. BLAST is the most commonly used tool to check homologous target sequences. In some cases, BLAST may not be sufficiently sensitive to pick up sequences with relevant partial homology, which can result in undesired silencing of off-target sequences. There are different schools of thought regarding the factors responsible for off-target silencing. Jackson *et al.* [32] have reported that siRNAs with as few as 11 nts homology can result in off-target silencing. Birmingham *et al.* [33] reported that off-target effects mostly take place as a result of 3′-UTR seed matches, rather than overall homology between the siRNAs and targets [34–36]. Off-target effects can be reduced by avoiding the incorporation of sense strand (siRNA*) and promoting the incorporation of antisense strand (siRNA) of siRNA duplex into the RISC complex. 5′-phosphate group is essential for the siRNA strand to act as a guide strand. So, modification of the 5′-phosphate group of the sense strand to a 5′-*O*-methyl, can effectively avoid sense strand RISC (siRNA*–RISC) formation [37].

Federov *et al.* described sequence-dependent, but target-independent toxic effects of siRNA. The toxicity in such cases was not as a result of activation of immune response. They found good correlation between the occurrence of specific sequence motifs like -UGGC- and other -AU- rich pentamers like -AUUUG, GUUUU, AUUUU, CUUUU, UUUUU, GUUUG- and toxicity, which results into loss of cell viability [38]. siRNAs with UGGC motif outside the seed region also exhibit toxic effects, indicating that regions other than 'seed sequences' might also contribute to nonspecific effects. It has been reported that miRNA-mediated silencing efficiency was greater when their binding sites were located next to rare codons. The efficiency of miRNA silencing machinery depends on the

speed of translation machinery of the mRNA [39]. Therefore, partial homology between siRNA sequences and the coding region of target sequences might not show very high off-target effects, though it cannot be ruled out. Some off-target effects can be reduced by using lower concentrations of siRNAs, but other non-specific effects cannot be avoided by lowering the concentration of siRNAs.

Recent studies by Ui-Tei *et al.* [40] have shown that replacing the seed region of the guide strand by deoxynucleotides results in reduced off-target effects. This might be due to the strength of duplex formation between siRNA::target RNA. The duplex of siRNA::target RNA is stronger than duplexes between DNA-modified siRNAs::target RNA. siRNAs with modified nucleotides and the backbone can also be used to reduce the off-target effects. Recent reports suggest that locked nucleic acids (LNA)-modified siRNAs show higher stability, lower off-target effects and cellular toxicity compared to unmodified siRNAs both *in vitro* and *in vivo* [41–43]. siRNAs have been reported to function as miRNAs in a few cases. Recently, Jackson *et al.* reported that position-specific and sequence-independent chemical modifications, such as 2′-*O*-methyl ribosyl substitution in the seed region (2–8 nts) of antisense strands of the siRNA duplex can reduce off-target effects [36].

Nonspecific effects independent of siRNA sequences

In animals, the immune system is capable of discriminating between self- and nonself-nucleic acids, based on features that are not fully understood. Initial studies demonstrated that dsRNAs of greater than 30 bases can activate the interferon response, leading to apoptosis, and smaller RNAs (>30 base pairs) can induce RNAi without activating the interferon response. Many other reports have shown, however, that even smaller RNAs can also activate innate immune response by inducing cytokine and type 1 interferon expression [44]. In the cytoplasm, long dsRNAs are sensed by dsRNA-dependent protein kinase (PKR) that phosphorylates translation initiation factor eIF-2α causing inhibition of protein synthesis. PKR recognition of dsRNAs is sequence-independent and requires an optimum length of 30–80 bps to interact and become activated.

dsRNAs can also activate toll-like receptor 3 (TLR 3)-mediated pathways [45]. TLR 7/8 are, however, the major sensors of dsRNA compared to TLR 3 and PKR [46,47]. These TLRs are located in endosomes and produce proinflammatory cytokines upon activation. Sometimes TLR 7/8 induction is also sequence-dependent because they recognize certain siRNA sequence motifs like 5′-UGUGU-3′ [48] or a 9 nucleotide motif (5′-GUCCUUCAA-3′) [47]. Many other sequences, which do not have any GU bases and dsRNAs with high uridine content can, however, also activate immune response [49]. On the basis of the available information of self- and nonself-recognition and including other factors as mentioned above, we can improve siRNA design in many ways such as:

1. Avoiding sequence motifs and high uridine content, which are already known to stimulate immune pathways.
2. Using different siRNA delivery techniques, to avoid their retention in endosomes and subsequent induction of TLR 7/8. Avoiding the maturation/acidification of endosomes is essential and this can be done by using inhibitors like chloroquine and bafilomycin A [46]. These inhibitors have been reported to affect immunogenicity but not silencing efficiency.
3. siRNAs can be delivered by electroporation which has been reported not to induce immune response compared to other methods like liposome-mediated transfection. Because liposomes are taken up by endosomal pathways, they can potentially activate immune responses through TLRs.
4. siRNAs can be delivered to specific cells by antibody-mediated receptor specific delivery [50], avoiding the induction of immune response in cells that are not targeted.
5. Induction of immune response can be avoided by introducing modifications (like 2′-deoxy, 2′-*O*-methyl, 2′-fluoro and so on) in the nucleotides and/or their backbone to evade immune response without compromising efficiency [49,51].
6. Using naturally modified nucleotides, such as pseudo-uridine and 5′-methylcytidine, can reduce the potential of endogenous RNAs from being recognized as nonself.
7. It is known that 'RIG-1' a dsRNA sensor, can recognize dsRNAs with blunt ends but siRNAs with 2 nt 3′-overhangs can escape such recognition. These siRNAs might escape recognition because they resemble endogenous small RNAs processed by dicer, so siRNAs with 2 nt overhangs are preferred [52].
8. Often *in vitro* transcribed RNAs are also used to induce RNAi, but these RNAs with 5′-triphosphates are sensed by RIG1. Such RNAs also should be avoided [53,54].

In an interesting study Kim *et al.* added -AA bases at the 3′-end, followed by treatment with RNase T1 and calf intestinal phosphatase to remove the terminal triphosphate. These RNA sequences were left with a blunt end, which, interestingly, did not induce interferon response [55]. This was in contrast to an earlier report where blunt ended siRNAs had been reported to induce interferon response. Further, Gondai *et al.* reported that the T7 phage polymerase-transcribed products do not induce interferon response by the addition of two to three bases of G with a terminal triphosphate group to the shRNAs [56,57]. To induce RNAi by T7 RNA polymerase-transcribed products, one has to consider the constraints and limitations of using T7 RNA polymerase along with the guidelines for effective designing of siRNAs.

9. shRNAs expressed using plasmid-based vectors did not induce interferon response at least in some cases compared to siRNAs of the same sequence [52,58] so plasmid-based expression of siRNAs or shRNAs can be used instead of using naked siRNAs or shRNAs in some cases to avoid immune response. The induction of immune response against RNA depends on its sequence [59]. The recognition of RNA danger signals via TLR 7 and TLR 8 seems to be dependent on the compartmentalization of the RNA in endosome and not based on self- versus nonself-signals [51,58,60]. Even dsRNAs can be used to elicit RNAi in mammals without inducing interferon response. Mouse oocytes [61], undifferentiated embryonic stem cells [62] and embryonal teratocarcinoma cells [63] were targeted by dsRNAs to demonstrate specific RNAi without inducing interferon response. So, whenever new siRNA sequences are used, it is advised to examine their ability to induce immune responses.

The exact nature of siRNAs, responsible for activating the immune response is not known, but the usage of known, specific, sequence motifs capable of inducing immune response should be avoided. Overexpression of siRNAs can lead to saturation of some factors in the RNAi pathway, like Exportin-5, and that could result

TABLE 2

siRNA databases		
HuSiDa	http://www.human-siRNA-database.net	[87]
siRNAdb	http://www.siRNA.cgb.ki.se	[88]
sIR	http://www.biotools.swmed.edu/siRNA/	[17]
siRecords	http://www.siRecords.umn.edu/siRecords/	[89]

in the deregulation of vital pathways dependent on endogenous RNAi [64]. So it is better not to use excessive dose of higher concentrations of siRNAs to avoid such effects [65]. There are various databases of siRNAs which have been validated experimentally (Table 2). Such databases can be checked to gather the information, whether the gene of interest has been targeted by siRNA and, if so, with what efficiency.

Ways to improve efficiency of RNAi

Sometimes it is difficult to design siRNAs using all the criteria mentioned above, because of various constraints such as nature of sequence and nucleotide content or secondary structures. In cases where options may be limited, one should think of using the maximum possible crucial parameters for improving the efficiency of siRNA. The efficiency can be improved by increasing specificity and reducing the off-target effects by using different modifications in the backbone of the siRNA and/or by using modified bases.

Loading of siRNAs into RISC assembly

siRNAs, which are produced by dicer, become loaded into the RISC complex. Once the native siRNA::siRNA* duplex enters RISC, one strand is preferentially retained as a guide strand. This preferential loading of a particular strand into RISC takes place in many ways. A few studies have shown that the thermodynamic stability of the strands plays a major role in strand selection. A recent study by Sano *et al.* [66] suggests that siRNA terminal structures determine strand selection, rather than its thermodynamic stability (the strand having lower free energy is considered to be thermodynamically stable). They reported the following guidelines for the design of siRNA:

- An siRNA duplex with a 3′ overhang on the antisense strand and a symmetric end toward the 5′-end of the antisense strand results in preferential selection of the antisense strand as a guide strand.
- Lower stability at the 5′-end of antisense strand relative to 3′-end results in increased preference for the antisense strand.
- Introducing mismatches at the 5′-end of the antisense strand further increases its preference to become selected as a guide strand.
- The preference of selection of one strand of the duplex to become selected as guide strand can be increased by deleting a few nucleotides from the other strand.

Specificity becomes a very important issue in the case of allele-specific silencing, which differs either by only one or by very few bases [67–69]. Specificity can be increased by introducing mismatches in siRNA sequences. Ohnishi *et al.* [70] found that the introduction of mismatches in the seed region and in the central position (10–11th position) of siRNA increases its ability to discriminate between alleles. These features, along with those mentioned above, will be helpful in increasing the siRNA-target specificity. They further reported, when siRNA sequence is introduced in the form of shRNA, they exhibit higher specificity [70]. The specificity can be further improved by using forked siRNAs of the same sequence. Forked siRNAs have 2 nt mismatches on the 3′-end of sense strand [71]. Schwarz *et al.* showed that introduction of mismatches at the 3′-end of seed region also results in robust discrimination between alleles [72].

Modifications to increase stability of siRNAs

Different chemical modifications have been tried to improve the properties of siRNAs [73,74]. Some modifications can be introduced at most of the bases of both strands of the siRNA duplex. Other modifications cannot, however, be placed at certain positions [74]. Phosphorothioate linkages (PS) have been reported to be one of the potential modifications. It has been reported that PS linkages between and within DNA increases its resistance to serum nucleases. PS also increase the half-life of oligonucleotides in the circulation by increasing their binding to serum proteins [74]. Many studies have demonstrated that the silencing ability of siRNAs remains unaltered after the introduction of PS linkages into siRNA [75–77]. Preliminary studies on boron phosphorus (BO) linkages have similarly shown improved gene silencing activity at lower concentrations and enhanced resistance to degradation by nucleases [78]. 2′-*O*-Methyl, 2′-*O*-methoxyethyl and 2′-F modifications display high specificity to their targets and have been reported to be resistant to nuclease activity [36,76]. The siRNAs with modifications viz., 2′-deoxy, 2′-methoxy, 2′-hydroxyethylmethyl or 2′–5′-formacetal-linkage [79], sulphur-modified nucleotides combined with 2′-*O*-substitution and LNAs have been reported to be resistant to nucleases and, hence, more stable [79]. LNA modification increases the thermal stability of siRNAs and makes them nuclease resistant [80]. Antisense oligonucleotides (ASOs), containing LNA bases, are highly potent but have been reported to cause liver toxicity in a mouse model [81]. Another class of 2′ modification is 2′-deoxy-2′-fluoro-β-arabinonucleic acid (FANA). FANA nucleotides are based on the sugar, arabinose, rather than ribose [74]. Compared to unmodified RNAs, siRNAs having completely FANA-substituted sense strands are more potent and stable [74,82,83].

Murao *et al.* [84] used bases chemically modified with *N*-phenylcarbamoyl or *n*-naphthylcarbamoyl groups at the N4 position of cytosine and N6 position of adenine in the overhanging 3′-end of sense strand to increase their resistance to nucleases. Ittig *et al.* [85] developed siRNAs with Tricyclo-DNA (Tc-DNA) in their sense strand and found them more stable in serum. They reported enhanced silencing activity of Tc-DNA containing siRNAs than their natural unmodified siRNA counterparts. Apart from modifications, the efficiency can also be improved by adopting suitable ways of delivering siRNAs into cells.

Conclusion

The scientific data emerging from genome-wide siRNA screening, along with the many other technological advancements, such as bioinformatics, high-throughput screening assays and other technical approaches are quite helpful in the development of siRNA design tools. The perfect algorithm for siRNA design has not yet been developed because of the complexity of predicting the var-

ious parameters affecting siRNA function. It is worthwhile to follow the siRNA design guidelines defined in various pieces of software, but one should be very clear that the real efficiency of siRNA should be validated experimentally. In-depth studies are required to develop the tools for the prediction of cross silencing and immunostimulatory activities during the siRNA design. Such improvements would facilitate the development of siRNA-based drugs from bench to bedside.

Conflict of interest statement

There are no financial interests associated with this manuscript between the authors. Authors would like to mention that there are many popular siRNA design tools available either online or elsewhere but it was not possible to discuss all of them here due to the limitation of the space. Authors have included only handful websites of siRNA design tools. It does not mean that the websites, which are not mentioned here are not popular or precise. Authors would like to apologize for all the investigators, whose websites or references for siRNA designing could not be included in this article owing to space constraint.

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