Chapter 15

Design and Chemical Modification of Synthetic Short shRNAs as Potent RNAi Triggers

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Abstract

Synthetic shRNAs that are too short to be Dicer substrates (short shRNAs or sshRNAs) can be highly potent RNAi effectors when properly designed, with activities similar to or more potent than the more commonly used siRNAs targeting the same sequences. sshRNAs can be designed in two possible orientations: left- or right-hand loop, designated L-sshRNAs and R-sshRNAs, respectively. Because L- and R-sshRNAs are processed by the RNAi machinery in different ways, optimal designs for the two formats diverge in several key aspects. Here, we describe the principles of design and chemical modification of highly effective L- and R-sshRNAs.

Key words: shRNA, sshRNA, Dicer, Ago proteins

1. Introduction

RNA interference (RNAi)-inducing triggers such as small interfering RNAs (siRNAs) and small hairpin RNAs (shRNAs) have been widely used for gene function analysis, pathway mapping, and drug target validation (1–9). Because of their high specificity and potency, siRNAs and shRNAs also show promise as potential therapeutic agents, although effective delivery to target tissues and organs remains a challenge.

While the most commonly used design for siRNA consists of 19 base pair duplexes with 2-nucleotide 3′-overhangs on each strand, the design of shRNAs involves more parameters. The basic structure of a typical shRNA comprises a Watson-Crick base paired duplex connected by a "loop" or connecting sequence. The length of the duplex usually varies from 19 to 29 base pairs, and the loop can be almost any length or sequence. We can further classify shRNAs into two subgroups depending on whether or not they are substrates for the RNase III-family endonuclease Dicer. shRNAs

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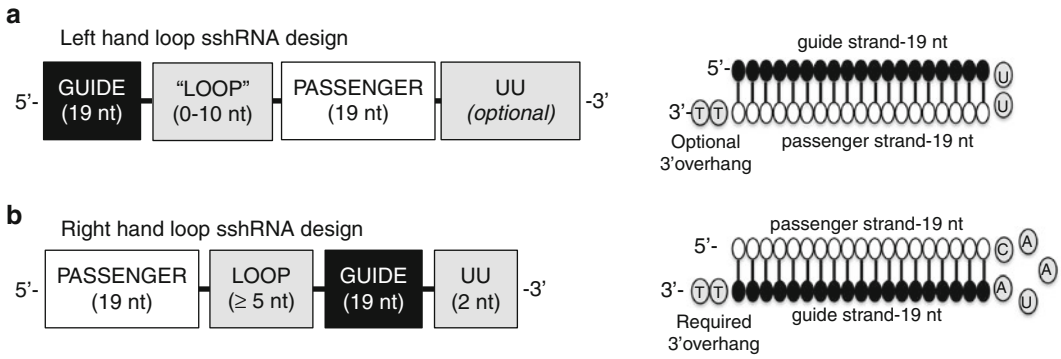

Fig. 1. Schematic diagram of sshRNA design. (**a**) Structural features and putative secondary structures of L-sshRNAs. (**b**) Structural features and putative secondary structures of R-sshRNAs.

having duplex lengths of ≥21 bp can be recognized and processed by Dicer before incorporation into the RNA-induced silencing complex (RISC) (10). In contrast, shRNAs with a duplex of 19 or fewer base pairs are not Dicer substrates (9–11). To distinguish shRNAs with 19 or fewer base pairs from longer, Dicer substrate shRNAs, we have designated the former as short shRNAs or sshRNAs (9). sshRNAs can be designed such that their efficacy may be as good as or, in some cases, better than siRNAs that target the same sequences (9, 10, 12–15). We have shown that sshRNAs can be extremely potent, with IC_{50}s in the low picomolar range, and that they are of interest for development as therapeutic agents (9).

sshRNAs have an intrinsic "handedness" to them because the guide sequence can be positioned either on the 5′ side of the loop (left-hand or L-type) or the 3′ side (right-hand or R-type) (Fig. 1) (16). Both L- and R-sshRNAs can be designed with high activity and specificity, but because their mechanisms differ, so do the structural aspects of their designs (17). L-sshRNAs can be loaded into RISC without any prior processing of their loops. Once loaded, RISC activation is completed by slicing of the passenger arm by Ago2 opposite nt 10–11 of the guide arm, measured from its 5′ phosphate (17). In contrast, potent R-sshRNAs need to have loops that can be cleaved prior to productive formation of an active RISC (17). Upon loop cleavage, a phosphate is produced at the 5′-end of the guide arm that can allow stable binding and accurate positioning of the guide arm in the RISC complex. The passenger arm may be sliced to facilitate its removal and leave the guide strand available for pairing with the target.

Although target site selection is critical to silencing activity, details of the structural design of sshRNAs also play a significant role. Effective target sites can be identified and characterized by a number of methods, including rational design, by the use of one of a number of available algorithms, or by library-based screening

methods (18–23). We have studied the effects of design and various chemical modifications on the potency, stability, and immunostimulatory properties of sshRNAs. In this chapter, we describe the principles and guidelines for designing potent sshRNAs once an effective target site has been identified.

2. Materials

1. Commercial source of HPLC-purified synthetic sshRNA (e.g., IDT or ThermoFisher) or in-house equivalent.
2. Sterile, RNAse-free, pyrogen-free ddH_2O.
3. 1× sshRNA resuspension buffer: 20 mM KCl, 6 mM HEPES-KOH (pH 7.5), 0.2 mM $MgCl_2$.
4. An assay to measure the activity of sshRNAs in cultured cells expressing a target gene.
5. Microfuge tubes with low nucleic acid retention such as Eppendorf DNA LoBind tubes.
6. Disposable, sterile siliconized pipet tips.
7. Acrylamide solution (19:1 acryl:bis), TEMED, 10% ammonium persulfate.
8. Urea.
9. Formamide.
10. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0.
11. Apparatus for running polyacrylamide gels.
12. SYBR® Gold Nucleic Acid Gel Stain, 10,000× concentrate in DMSO (Life Technologies). Store at –20°C, protected from light. Dilute in 1× TBE for working solution.

3. Methods

3.1. Design of sshRNAs

Once a target sequence has been identified by the user's preferred method, highly potent non-Dicer substrate sshRNAs can be designed in either L or R orientation (Fig. 1). As noted above, the two orientations differ in the positions of the guide and passenger sequences with respect to the connecting loop sequence. The guide sequence of L-sshRNAs is located on the 5′-side of the loop, while the guide sequence of R-sshRNAs is placed to the 3′-side of the loop.

1. Design of L sshRNAs
   (a) Design L-sshRNAs to conform to the following general structure: 5′-19 nt guide sequence—"loop" connector sequence—19 nt passenger sequence—optional dinucleotide overhang-3′ (Fig. 1a).
   (b) L-sshRNAs should be designed such that they contain a 19 bp Watson-Crick base paired stem where the two strands (5′-guide arm and 3′-passenger arm) are joined by a connecting sequence that forms a hairpin loop. In addition, the guide sequence should be perfectly complementary to the target RNA to be silenced. While we have found that high silencing activity can occur with shorter stems (minimum of 16 bp), the reliability of such formats in many sequence contexts has not been explored in depth. By selecting a duplex length of 19 base pairs, the design of guide and passenger sequences is, in principle, similar to the standard design of siRNAs, and allows for molecules that are highly potent in their ability to silence their targets as well as maintaining a gene-specific effect.
   (c) While the connecting sequence that bridges the guide and passenger sequences can be varied from 0-to-10 nucleotides in length, we recommend a "loop" or connecting sequence length of 2 nucleotides with the sequence of UU (see Note 1).
   (d) Optionally include a 3′ overhang whose sequence may be either UU or TT. For some target sequences, we have found that L-sshRNAs have slightly higher activity if the molecules contain a 3′-overhang. It is recommended that both blunt and 3′-overhang-containing sshRNAs be synthesized and compared head to head in the gene knockdown assay of choice. One other point to consider is that blunt-ended hairpins induce expression of pro-inflammatory cytokines, which can lead to observation of nonspecific inhibition of gene expression. This interferon response can be abrogated by inclusion of 2′-*O*-methyl (2′-OMe) modifications at certain residues along the sshRNAs (see Subheading 3.2).
   (e) (Optional depending on application) We recommend that once a target site is selected, a sequence walk be performed around the target site. Several sshRNAs whose target sequences are positioned on either side of the target sequence should be synthesized and assayed in parallel to identify the sshRNAs with the highest activity. This step will be more important for development of sshRNAs to be used as therapeutic agents. Less optimization is required if sshRNAs are to be used as a research tool for inducing gene

knockdown. A scrambled-sequence sshRNA should also be included as a control for nonspecific gene knockdown.

2. Design of R sshRNAs
   (a) Design R-sshRNAs to conform to the following general structure: 5′-19 nt passenger sequence—loop sequence of at least 5 nt—19 nt guide sequence—dinucleotide overhang-3′ (Fig. 1b). The minimum length of R-sshRNAs of this design is 45 nucleotides.
   (b) The guide and passenger sequences should be fully complementary, and the guide sequence should have perfect complementarity to the target RNA to be silenced.
   (c) Many sequences are possible for the loop, but for optimal activity, the loop must have at least 5 nucleotides (15, 17) (see Note 2). Because we have found that R-sshRNAs are more potent if they can be cleaved by an endonuclease in the loop, we recommend including a 5′-Pyr-A-3′ sequence in the loop, which is the recognition motif for ribonuclease-A type endonucleases. In practice, we have found that the sequence 5′-CAAUA-3′ is a good choice for the loop as long as it is not complementary to either the guide or passenger sequence (9, 15). Whatever loop sequence is chosen, it is important to check that it does not have such complementarity, which could lead to misfolding of the hairpin.
   (d) It is essential to include a 3′-dinucleotide overhang for highly potent R-sshRNAs. The sequence can be either UU or dTdT.

3.2. Chemical Modification of sshRNAs

Chemical modification of shRNAs and siRNAs can be beneficial for several reasons: to enhance nuclease stability, to mitigate potential undesirable immune stimulatory effects, to reduce off-target effects, and to aid in conjugation to delivery agents (24–31). One of the factors that govern the overall pharmacokinetics of oligonucleotide-based drugs is their sensitivity to nucleases found in serum. Although dsRNAs are more stable than single-stranded RNAs (ssRNAs), sshRNAs without chemical modification are still relatively sensitive to nucleases. The inclusion of certain chemical modifications in sshRNAs can substantially increase their serum half-lives (16).

Numerous studies have demonstrated the capability of unmodified shRNAs and siRNAs to induce the undesired expression of proinflammatory cytokines such as type I interferon (IFN), IL-6, and TNF-α (28). Although sshRNAs have a duplex length of 19 bp or less, shorter than “ordinary” shRNAs, they may still be immune activators if they contain certain sequences (e.g., GU motifs) or structural features (e.g., blunt ends, which can stimulate RIG-I). Because L- and R-sshRNAs differ in their mechanisms of action and how they are processed in intracellular environments (16, 17), the rules governing chemical modification differ for each class of hairpin RNA.

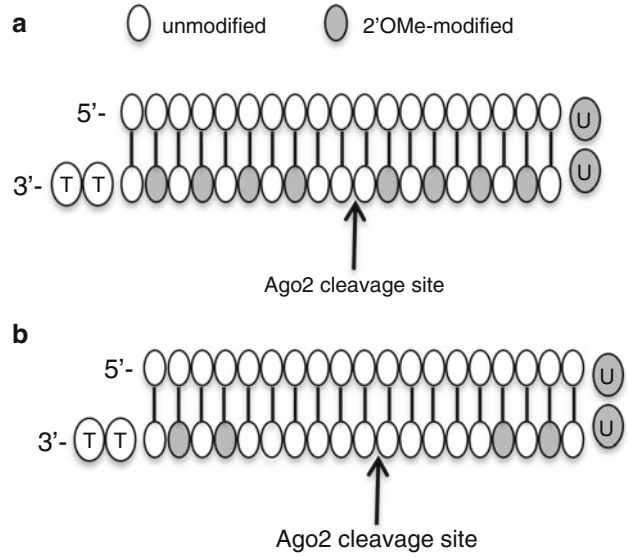

Fig. 2. Effective 2′-OMe-modification patterns for L-sshRNAs. *White ovals*: unmodified residues; *gray-ovals*: residues with 2′-OMe substitutions.

1. Modification of L sshRNAs
   (a) The connecting sequence between the guide and passenger arms may be modified with 2′-OMe groups, deoxy substitutions of the 2′-OH, or PS groups, or it may be completely substituted with nonnucleotide linkers such as C_3C_3 without loss of activity.
   (b) To improve serum stability while abrogating any induction of the innate immune system (see Note 3) without loss of gene silencing activity, the following chemical modification pattern is highly effective (shown schematically in Fig. 2a): place a 2′-OMe on each nucleotide of the loop and alternate nucleotides of the passenger strand, leaving an unmodified window of 3 nt at the slicer site. If this pattern of modification reduces potency of the sshRNAs beyond an acceptable level for the application of interest, we found that reducing the number of modified residues to the pattern shown in Fig. 2b was also highly effective in improving serum stability and eliminating immune response stimulation. As few as two 2′-OMe substitutions can reduce the innate immune response to background levels (16).
   (c) Avoid modification of the guide arm with 2′-OMe moieties (except at the last 2 residues at the 3′-end), as these reduce activity of the L-sshRNA.

(d) Phosphorothioate (PS) modifications at the 5′ and 3′ termini of the stem of L-sshRNAs do not reduce activity but have been found to be immune activating (16) (see Note 4). If PS modifications are desired, they should be combined with 2′-OMe modifications to avoid an immunostimulatory response.

(e) 3′-end conjugation with relatively bulky groups such as groups containing a disulfide linkage (e.g., 3′-S-S-C_6) does not adversely affect the activity of L-sshRNAs, whereas conjugation at the 5′-end does impair activity (e.g., 5′-S-S-C_6). These types of modifications can be useful for conjugation of delivery agents such as peptides, lipid nanoparticles, or antibodies that could enhance cellular uptake of sshRNAs (see Note 5). Again, L-sshRNAs are more permissive of modifications to the passenger strand than the guide strand and require a free 5′-phosphate on the guide strand. Because 5′-conjugation presumably blocks the phosphorylation of 5′-OH ends of synthetic RNAs that normally occurs upon their transfection into the cell, the loss of RNAi activity by this modification is not surprising.

2. Modification of R sshRNAs

(a) Do not modify the loop residues of R-sshRNAs

(b) R-sshRNAs are more tolerant than L-sshRNAs of 2′-OMe in guide strand (e.g., at position 2)

(c) Bulky groups can be conjugated to the 5′-end but not the 3′-end of R-sshRNAs without loss of activity.

3.3. Avoiding Multimeric Forms of shRNAs

Lyophilized synthetic sshRNAs have been found upon hydrating to comprise at least three major species that resolve in native polyacrylamide gels, regardless of whether they are resuspended in ddH_2O or a mildly buffered solution (9). In contrast, under denaturing conditions (12% polyacrylamide gel containing 8 M urea and 20% formamide), only a single band is usually observed (9). The three major bands behave in a manner consistent with their being monomer, dimer, and trimer forms of sshRNAs. We have seen this mix of structures with all the sshRNAs we have procured and purified, irrespective of stem length, loop size, or L vs. R loop orientation. In some cases, even higher-order multimeric complexes were observed. Although it will usually contain an internal loop at the middle (unless the loop is self-complementary), a dimer sshRNA has a duplex length of well over 30 bp and is thus a good candidate for protein kinase R (PKR) recognition (32). Before characterization of the functional activities of these sshRNAs in

knockdown assays, the mixed population should be treated, so that it consists solely of monomeric hairpins, using a heating and quick-cooling procedure described as follows.

1. Handle all reagents, pipets, tubes, and other consumables with gloved hands.
2. We recommend the use of low nucleic acid binding microcentrifuge tubes and pipet tips especially when working with low concentrations of sshRNAs.
3. Dissolve sshRNA in either sterile, RNAse-free, pyrogen-free ddH_2O (commercially available from numerous suppliers) or a low ionic strength buffer such as 1× sshRNA resuspension buffer to a final concentration of 100 μM. In practice, we initially dissolve the RNAs to a relatively high concentration, which is more stable for long-term storage, and then make dilutions to working concentration. If resuspending in a different buffer than the one suggested, avoid pH > 8 and the inclusion of divalent cations in millimolar concentrations as these conditions will promote degradation of the RNA in subsequent steps.
4. Dilute to a working concentration if necessary. We typically dilute to 5 μM, but we have confirmed that the following steps can be performed at up to 150 μM concentration.
5. Heat the RNA at 95°C for 4 min.
6. Transfer the RNA immediately to an ice-water bath and let sit for 10–20 min until ready for further use.
7. sshRNAs can be stored at −20°C and can be thawed at room temperature and frozen repeatedly.
8. Confirm that the sshRNAs have been converted to monomeric hairpins by analyzing by both non-denaturing (Fig. 3) (see Note 6) and denaturing PAGE (see Note 7) for monomer formation. Stain with SYBR Gold to visualize RNA bands (see Note 8). In both non-denaturing and denaturing gels, only a single band should be observed. For comparison, load an aliquot of non-heat-treated RNA in an adjacent lane.

4. Notes

1. Dinucleotide UU connecting sequences, even when they are unmodified, dramatically improve resistance to serum nucleases compared to longer sequences (16). In addition, these L-sshRNAs can be loaded efficiently into Ago2-containing RISC complexes directly as intact hairpins (i.e., they are not cleaved by Dicer or some other cellular endonuclease to remove the loop) (17). Instead, the full-length hairpins are sliced in

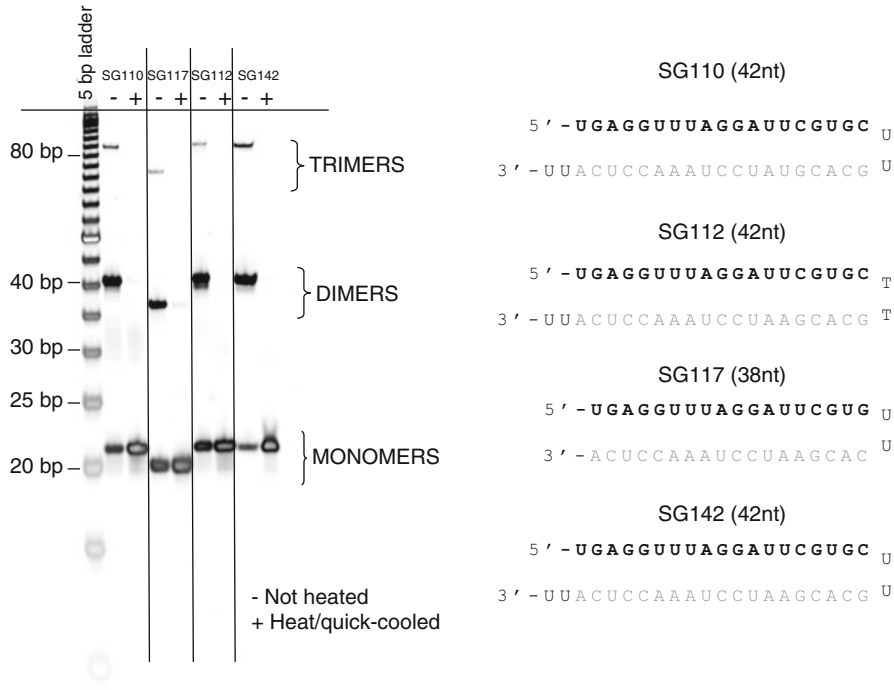

Fig. 3. Non-denaturing PAGE analysis of the conversion of mixed multimeric sshRNA species to monomeric hairpins. 10% non-denaturing PAGE of sshRNAs before (lanes labeled –) and after heating and quick-cooling treatment (lanes labeled +). The gel was stained with SYBR Gold. Sequences and putative secondary structures of sshRNAs analyzed are depicted to the *right* of the gel. Guide strand sequences are in *bold, black text*; passenger strand sequences are in *gray text.*

the passenger strand by Ago2 to generate the active molecule. sshRNAs with 2-nucleotide connectors will also be more cost-effective to synthesize since their overall length is shorter.

2. It is important to keep in mind that although R-sshRNAs may be highly potent, they are rapidly degraded in serum when they are unmodified because of the longer loop lengths required for activity (16).
3. 2′-OMe modification has been found to be particularly effective in preventing the recognition of siRNAs by TLR7/8 and RIG-I (28, 33–35). The modified groups interact with TLR7 without triggering signaling cascades, antagonizing TLR7-mediated activation by both RNA and small-molecule TLR7 agonists (34, 36).
4. The presence of many PS bonds in oligonucleotides can result in cytotoxicity, and the ability of PS oligonucleotides to nonspecifically bind to proteins may explain some if not all of its toxicity (37, 38). The addition of as few as three PS bonds in sshRNAs has caused significant induction of cytokines, particularly TNF. For blunt-ended hairpins, one possibility is that PS linkages may enhance the interaction with RIG-I. However,

some single-stranded oligonucleotides with PS backbone have been shown to inhibit the signaling of TLR3 and RIG-I induced by poly I:C (39), suggesting that the immune activation by PS-modified dsRNAs may be partially due to RIG-I having different recognition mechanisms for single- and double-stranded oligonucleotides (39). The up-regulation of TLR3 and RIG-I may be an indirect effect of the release of inflammatory cytokines or their direct recognition of the modification.

2′-OMe substitution in the sense strand (at alternate nucleotides) of sshRNAs containing PS groups largely eliminated the innate immune responses. Inclusion of 2′-OMe residues allows the substitution of PS without triggering an innate immune response.

5. The introduction of a 3′ end conjugation induced high levels of IFN-β and TNF-α expression compared to the same sshRNAs lacking the conjugation. TLR3 and RIG-I were also up-regulated. Interestingly, the up-regulation of cytokines and RIG-I was not seen when the same group was conjugated to the 5′ end of L-sshRNAs. It is not clear how the activity of RIG-I was affected differently by a disulfide group conjugated to the 3′ or 5′ ends of a blunt-ended RNA hairpin.
6. For sshRNAs with a typical length of 38–45 nucleotides, we recommend 10% acrylamide (19:1, acryl:bis), 1× TBE for non-denaturing gel electrophoresis, which will resolve monomers from dimers and higher-order multimeric species. Any commercially available native gel loading buffer can be used to prepare the samples, but we suggest avoiding the use of loading buffer containing bromophenol blue tracking dye as the dye may comigrate with RNA species of interest and can interfere with visualization of bands if imaging with a phosphorimager. Do not heat samples prior to loading on the gel. For sufficient sensitivity without overloading the gel, load approximately 100 ng per lane.
7. For sshRNAs with a typical length of 38–45 nucleotides, we recommend the following PAGE conditions for denaturing gel electrophoresis: 12% acrylamide (19:1, acryl:bis), 20% formamide, 8 M urea, 1× TBE. Samples should be diluted 1:1 with 2× Loading buffer (95% formamide, 50 mM EDTA, 0.015% xylene cyanol, and 0.015% bromophenol blue) and heated to 95°C for 2 min prior to loading on the gel. Because of the high degree of secondary structure in hairpin RNAs, it is essential to include formamide in the gel to provide complete denaturation. Run the gel at 45 W.
8. Do not substitute ethidium bromide stain for SYBR gold for visualization of small, denatured RNAs. Ethidium bromide

binds preferentially to double-stranded structures and does not accurately reflect the relative population of single-stranded RNA oligonucleotides. Because of the highly structured nature of hairpin RNAs, also do not rely on the intensity of 5′-end-labeling with a radioisotope such as ^{32}P to assess relative amounts of sshRNAs. The efficiency of 5′-end-labeling is very sensitive to the availability of the 5′-terminal residue, which, in the case of sshRNAs, may be either recessed if a 3′-overhang is included in the design, or blunt-ended. Consequently, even minor degradation products with comparatively available termini will be labeled with much higher efficiency than full-length products and will not accurately reflect the relative population of RNA species present in the sample.

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