Design of Synthetic shRNAs for Targeting Hepatitis C: A New Approach to Antiviral Therapeutics

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Contents

Abstract Small hairpin RNAs (shRNAs) are widely used as gene silencing tools and typically consist of a duplex stem of 19–29 bp, a loop, and often a dinucleotide overhang at the 3′ end. Like siRNAs, shRNAs show promise as potential therapeutic agents due to their high level of specificity and potency, although effective delivery to target tissues remains a challenge. Algorithms used to predict siRNA performance are frequently used to design shRNAs as well. However, the differences between these two kinds of RNAi mediators indicate that the factors affecting target gene silencing will not be the same for siRNAs and shRNAs. Stem and loop lengths, structures of the termini, the identity of nucleotides

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adjacent to and near the loop, and the position of the guide (antisense) strand all affect the efficacy of shRNAs. In addition, shRNAs with 19-bp or shorter stem lengths are processed and function differently than those with longer stems. In this review, we describe studies of targeting the hepatitis C virus that have provided guidelines for an optimal design for short (19 bp) shRNAs (sshRNAs) that are highly potent, stable in biological fluids, and have minimal immunostimulatory properties.

Keywords Antivirals • Hepatitis C • RNAi • shRNAs • sshRNAs

1 Introduction

While antibiotics have greatly reduced the mortality and morbidity associated with bacterial infections, the development of effective antivirals has proceeded much more slowly. Hepatitis C virus (HCV) infections remain a worldwide health problem, and no vaccine is currently available. While a number of small molecule inhibitors of virally encoded enzymes are showing promise in clinical trials and some have now been approved by the US FDA, cures of HCV generally require multiple agents with different mechanisms of action due to the development of viral resistance. Such viral escape results from the high error rate of the viral RNA-dependent RNA polymerase, which allows rapid exploration of sequence space, creating myriad quasispecies that may include mutations conferring resistance to any given drug. Small interfering RNAs (siRNAs) and their cousins small hairpin RNAs (shRNAs) have two advantages over small molecule drugs that suggest a potential solution to the viral resistance problem: first, they can potentially target conserved but "undruggable" sequences anywhere in the viral genome, including noncoding sequences, and second, multiple small siRNAs or shRNAs can be combined to foil the ability of the virus to escape through single-site mutations. Chronic HCV infection, which represents the most common form of this disease, is also attractive as a disease target for RNAi because the course of the infection is slow. Thus, there is plenty of time for the biology of RNA interference to take place, in contrast to some very rapid viral infections in which viral replication may outrun treatments that require some hours to take full effect.

In this review, we describe the development of a new class of RNA interference (RNAi) effectors, short shRNAs, or sshRNAs, as HCV drugs. sshRNAs have been designed to target the conserved internal ribosome entry site (IRES) element of the HCV genome, one of the most highly conserved regions of the viral genome. Their short length (around 40 nt) distinguishes them from more traditional shRNAs in both potency and mechanism of action.

2 Hepatitis C Virus

HCV, a single-stranded, positive-sense RNA virus, is the most common blood-borne RNA virus, with an estimated 4.1 million persons currently infected in the United States and 180 million worldwide. A total of 3–4 million people are newly infected each year (Armstrong et al. 2000; Alter et al. 1999; Shepard et al. 2005; Ray Kim 2002). Approximately 250,000 human immunodeficiency virus (HIV)-infected persons in the United States are coinfected with HCV (http://www.cdc.gov/ncidod/diseases/hepatitis/c/). HCV is recognized as a major cause of end-stage liver disease, such as liver cancer (with 1–5% of HCV cases leading to this outcome) and cirrhosis (10–20% of cases), and is the leading indication for liver transplantation in the Western world (Shepard et al. 2005). Chronic liver disease ranks as the tenth leading cause of death in the United States, and HCV is estimated to account for 40–60% of these cases. Mortality related to HCV infection (death from liver failure or hepatocellular carcinoma) is expected to increase over the next two decades (Deuffic-Burban et al. 2007).

There are six major genotypes of HCV. The standard treatment is a combination of pegylated interferon (IFN)-α and ribavirin, which is effective in 40–80% of patients, depending on the genotype. Efficacy has been around 50% in patients infected with HCV genotype 1, which comprises nearly 70% of cases in the western hemisphere, although the success rate is improving with the addition of newly approved protease inhibitors to the standard treatment. The response rate for HIV–HCV coinfected patients is lower, estimated at 30–40% (Torriani et al. 2004). Because interferon is associated with severe adverse effects, including flu-like symptoms, hematologic abnormalities, and depression, patient compliance has been poor with an estimated 30% of patients refusing treatment. Considering that the incidence of HCV is increasing worldwide and that within 10 years more deaths from HCV than HIV are predicted by the Centers for Disease Control, safer and more efficacious HCV drugs are urgently needed (Deuffic-Burban et al. 2004, 2006; Law et al. 2003; Salomon et al. 2002).

More than 40 HCV drug candidates are under development. They can be divided into those that target the virus directly [direct-acting antiviral (DAA) agents] and those that affect host targets (Ronn and Sandstrom 2008; Liu-Young and Kozal 2008; Beaulieu 2007). DAAs include small molecules as well as ribozymes, antisense oligonucleotides (ASOs), decoy RNAs, RNA aptamers, siRNAs, and shRNAs. Host-targeting agents include small molecules such as cyclophilin inhibitors, antifibrotic agents, antibodies, modified interferons with improved pharmacokinetics, and oligonucleotides complementary to the required host factor microRNA-122 (miR-122) (Lanford et al. 2010). As mentioned above, the high genetic diversity and rapid mutation and turnover rates of HCV (10^{10}–10^{12} new particles produced per day with an error rate of 10^{-3}–10^{-5} mutations per nucleotide per genomic replication) result in the rapid emergence of viral resistance with many single DAAs (Okamoto et al. 1992; Neumann et al. 1998; Ogata et al. 1991; Sarrazin and Zeuzem 2010). For instance, although clinical studies showed that the use of protease inhibitors in

combination with pegylated interferon-α (peg-IFN) and ribavirin increased the rate of sustained viral response (SVR) by at least 20% compared with peg-IFN and ribavirin alone in patients infected with HCV genotype 1, the existence of viral variants with reduced susceptibility has been observed (Deuffic-Burban et al. 2004, 2009, 2012; Sarrazin and Zeuzem 2010; Robinson et al. 2011).

Of the oligonucleotide-based DAAs, siRNAs and shRNA generally have the best efficacy and potency in vitro as well as in animals (Ronn and Sandstrom 2008; McHutchison et al. 2006). In addition, the ability of RNAi to efficiently limit viral replication, and to target multiple genes and/or sequences simultaneously, makes this an attractive therapeutic approach for limiting the emergence of resistant mutants. Both siRNAs and vector-expressed shRNAs have been shown to significantly decrease HCV RNA replication and protein expression in cell culture as well as in animal systems (Watanabe et al. 2007; Randall and Rice 2004; Kapadia et al. 2003; Wilson et al. 2003; Yokota et al. 2003; Seo et al. 2003; Takigawa et al. 2004; Prabhu et al. 2005; Sen et al. 2003; Kanda et al. 2007). More recently, synthetic shRNAs have gained attention, as discussed below.

3 RNA Interference

RNA interference (RNAi) plays a central role in the regulation of eukaryotic gene expressions associated with various biological processes ranging from development to cell homeostasis. Diseases, particularly cancers, are often associated with the dysregulation of particular miRNAs (Shenouda and Alahari 2009).

The mechanism of RNAi is complex and can involve alternative pathways (Filipowicz 2005; Siomi and Siomi 2009). Double-stranded RNA (dsRNA) molecules are recognized and processed by one or more RNase III-family enzymes (Hammond et al. 2000; Zamore et al. 2000; Bernstein et al. 2001). Primary transcripts encoding microRNAs (pri-miRs) are processed in the nucleus by a "microprocessor" complex to individual hairpins before export to the cytoplasm. In the cytoplasm, dsRNAs longer than ~23 bp, including hairpins, are processed by Dicer into double-stranded siRNAs whose strands are each 21–23 nucleotides (nt) in length. Dicer contains a PAZ domain that binds specifically to the 3′ end of single-stranded RNA and two RNase III domains that possess the catalytic cleavage activity. The distance between the 3′-overhang-binding PAZ domain and the active site of the RNase III domains provides a molecular ruler corresponding to the length of an siRNA duplex (Jinek and Doudna 2009). The cleavage products of Dicer have characteristic termini, a monophosphate group at the 5′ end, and a two-nucleotide overhang with a 3′-hydroxyl at the 3′ end (MacRae and Doudna 2007). sRNAs, whether generated by dicing of a precursor RNA or introduced from outside the cell, are loaded into an RNA-induced silencing complex (RISC) containing a protein of the Argonaute (Ago) family (Elbashir et al. 2001; Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004a, b; Jinek and Doudna 2009). Ago proteins contain PAZ, middle (MID), and PIWI domains. The binding of an siRNA to an

Ago is aided by the presence of a 5′ phosphate group and a 3′ dinucleotide overhang at the termini. As with Dicer, the 3′ overhang binds to the PAZ domain of Ago. The 5′ phosphate group binds in a pocket at the interface between the MID domain and the PIWI domain (Jinek and Doudna 2009).

Of the four human Agos, Ago2 is thought to be the only one able to mediate cleavage of a target mRNA (Hammond et al. 2001; Okamura et al. 2004; Meister et al. 2004; Rand et al. 2004; Liu et al. 2004; Song et al. 2004; Rivas et al. 2005). To render the "guide" (antisense) strand of an siRNA available to pair with its target, the passenger strand must be removed. The selection of which strand is to be removed and which retained is thought to be largely governed by asymmetry in the thermodynamic profile of the siRNA duplex termini (Schwarz et al. 2003; Khvorova et al. 2003). After the RISC-loading complex (which contains the dsRNA binding protein TRBP along with Dicer and Ago2) loads an siRNA duplex into Ago2, Ago2 cleaves (or "slices") the "passenger" (sense) strand at a position opposite 10 nt from the 5′-phosphate of the guide strand, facilitating the dissociation and/or degradation by C3PO (Ye et al. 2011) of the resulting passenger-strand fragments. The loss of the passenger strand from the complex produces the active RISC. The anchoring of the 5′ phosphate group into the binding pocket of Ago is essential, and the distance from it determines the position of the cleavage site in the passenger strand (this Ago2-mediated passenger-strand cleavage was also found in the processing of some microRNAs [miRNAs] (Diederichs and Haber 2007).

When passenger-strand slicing is blocked by chemical modification or by mismatches between the two strands of the siRNA, a slower, alternative pathway dissociates and destroys the passenger strand, possibly via an ATP-dependent helicase (RNA helicase A), yielding the active RISC (Matranga et al. 2005; Leuschner et al. 2006; Miyoshi et al. 2005; Kraynack and Baker 2006; Robb and Rana 2007; Rand et al. 2005). RISC uses the bound single-stranded RNA molecule as a guide to "search" the resident population of messenger RNAs (mRNAs) for complementary sequences, eventually cleaving these transcripts and thereby downregulating the expression of the targeted gene (Hammond et al. 2000; Dorsett and Tuschl 2004; Kim and Rossi 2008; Elbashir et al. 2001; Nykanen et al. 2001; Martinez et al. 2002). The currently favored model for target recognition and cleavage by Ago2 is as follows: The target binds to the seed region of the 5′ half of the guide sequence and then base pairing proceeds toward its 3′ end. This results in the 3′ end of the guide strand dissociating from the PAZ domain, leading to a conformational change that positions the active site of Ago2 at the cleavage site on the target (Filipowicz 2005; Tomari and Zamore 2005). The position of the scissile phosphate group of the target mRNA is similar to that in the passenger strand, i.e., 10 nt from the 5′-phosphate group of the guide strand. Mismatches at the 10th and 11th nucleotides prevent the slicing activity (Jinek and Doudna 2009).

The endogenous RNAi machinery has been exploited to advance a wide range of studies involving gene function analysis, pathway mapping, drug target validation, and host–pathogen interactions (Natt 2007; Dorsett and Tuschl 2004; Iorns et al. 2007). New understanding of how RNAi regulates gene expression is also leading to the rapid development of RNAi-based therapeutics, especially in the area of viral

disease. For example, RNAi approaches targeting viral genes, including those of the HIV, influenza, and hepatitis A, B, and C viruses (Table 1), respiratory syncytial virus, polio virus, the SARS coronavirus, alphaviruses, and the Marburg and dengue fever viruses, have been shown to limit viral replication in cell culture and, in some cases, in animals (Rossi et al. 2007; Barik and Bitko 2006; Watanabe et al. 2007; Arbuthnot et al. 2007; Seyhan et al. 2007; Gitlin et al. 2002; Fowler et al. 2005; Haasnoot et al. 2003). A number of siRNA drug candidates are in clinical trials (Castanotto and Rossi 2009). Moreover, mechanistic studies of dsRNA/siRNA/miRNA processing by Dicer and Ago2 have also led to the development of algorithms for efficient target selection; chemical modification for improved specificity, functionality, and longevity; and new designs for RNAi triggers such as Dicer substrate, ssiRNA, etc., that benefit both basic and applied research (Bolcato-Bellemin et al. 2007; Collingwood et al. 2008).

4 Structure–Activity Relationships of shRNAs

RNAi effectors can be generated by chemical synthesis of siRNAs and sshRNAs, Dicer cleavage of longer synthetic dsRNAs and shRNAs, or processing of shRNAs and long dsRNAs transcribed from DNA or viral vectors (Dorsett and Tuschl 2004; Chang et al. 2006; Bernards et al. 2006; Fewell and Schmitt 2006; Vlassov et al. 2006; Amarzguioui et al. 2006; Ge et al. 2010a, b). The transcription of shRNAs or dsRNAs can be driven by Pol II promoters or alternatively by Pol III promoters such as the H1 promoter of RNase P or the U6 snRNA promoter. shRNA has received considerable attention due to its widespread use in DNA vector-based shRNA libraries for various loss-of-function screens, generation of cell lines or transgenic animals that express silencing triggers against targets of interest, and therapeutic approaches (Grimm et al. 2006; Li et al. 2005a, b).

4.1 Parameters Involved

An shRNA consists of largely paired antisense and sense sequences connected by a loop of unpaired nucleotides. A duplex stem of typically 24–29 bp, either fully paired or with miRNA-style internal mismatches or loops, is commonly used in vector-expressed shRNAs (Silva et al. 2005; Stegmeier et al. 2005; Boudreau et al. 2008). Although target site selection is critical to silencing activity, the structural design of the shRNA also plays a significant role. With appropriate design, shRNAs can be at least as active as siRNAs targeting the same site. Although the structure–activity relationship of siRNAs has been extensively examined and a 19-bp RNA duplex with 2-nucleotide overhangs at the 3′ ends of each strand is widely used, there have been only a handful of studies on the effect of hairpin structures on efficacy of target silencing. The factors involved in shRNA design are loop size and

Table 1 Hepatitis C-specific siRNAs and shRNAs

Target region in HCV	Model	Biological effect	References
NS5B	Hydrodynamic injection of si/shRNA and the reporter plasmid of NS5B-luciferase fusion	Reduced luciferase expression in mouse liver by 75% (siRNA) to 98% (expressed shRNA)	McCaffrey et al. (2002)
NS3, NS5B	Selectable subgenomic HCV replicon cell culture	HCV RNA replication and protein expression were inhibited more than 30-fold	Kapadia et al. (2003)
Core	HCV replicon cell culture	80-fold decrease in HCV RNA	Randall et al. (2003)
NS3-1, NS5B	HCV replicon cell culture	Effectively suppressed replication of the HCV replicon without suppressing host gene expression	Takigawa et al. (2004)
NS3, NS5B	HCV subgenomic replicon cell culture	HCV RNA synthesis reduced by 90%	Wilson et al. (2003), Wilson and Richardson (2005)
E2, NS3, NS5B	Transient HCV1a replication model	Effectively suppressed HCV RNA replication and protein expression	Prabhu et al. (2005)
Core, E2	EGFP reporter in cell culture	Suppressed EGFP expression	Liu et al. (2006)
Core, NS3, NS4A, NS4B	Genomic HCV replicon cell culture and hydrodynamic injection mouse model	Effectively suppressed viral replication in a dose-dependent manner	Kim et al. (2006), Shin et al. (2009)
NS5A	Cell culture-grown HCV genotype 1a	Effectively inhibited NS5A and core protein expression	Sen et al. (2003)
Stem–loop II of 5′ UTR	HCV subgenomic and full-length infectious replicon cell culture	Suppressed GFP expression and IRES mRNA in the case of six different HCV genotypes	Prabhu t al. (2006)
5′ UTR	HCV IRES-reporter and HCV subgenomic replicon cell culture	~80% suppression of HCV replication with concentrations of siRNA as low as 2.5 nM	Yokota et al. (2003)
5′ UTR	HCV subgenomic replicon with the luciferase gene	Suppressed the luciferase reporter expression	Seo et al. (2003)
5′ UTR	Cell culture-grown HCV genotype 2a, 1a, and 1b replicon system	Inhibited viral genome replication and infectivity titers	Kanda et al. (2007)
5′ UTR	HCV serum infected Huh-7 cells that supports genotype-4 replication	Suppressed HCV RNA by ~25-fold	Zekri et al. (2009)

(continued)

Table 1 (continued)

Target region in HCV	Model	Biological effect	References
5′ UTR	Cell culture-grown HCV and replicon system	Suppressed HCV replications	Ray and Kanda (2009)
5′ UTR	HCV replicon cell culture	Additive HCV inhibitory effects for combinations of ribozymes and siRNAs	Jarczak et al. (2005)
5′ UTR	HCV subgenomic replicon, HCV-luciferase reporter in cell culture	Effectively suppressed RNA replication in replicon; up to 98% knockdown of HCV-luciferase reporter	Ge et al. (2010a, b), Ilves et al. (2006), Vlassov et al. (2007)
5′ UTR	Hydrodynamic injection of HCV-luciferase reporter with shRNA in mice	Up to 99% knockdown of HCV-luciferase reporter in mouse liver	Wang et al. (2005)
5′ UTR	HCV-luciferase reporter expressed in mouse liver with lipid nanoparticle formulation	90% suppression of IRES-luciferase expression in mouse liver	Dallas, Ma et al. (submitted)
5′ UTR	HCV-infected chimeric uPA/SCID mice, lipid nanoparticle formulation	2.5 $\log_{10}$ viral load reduction	Dallas, Ma et al. (submitted)
5′ UTR NS3, NS4A, NS4B, NS5B	HCV subgenomic replicon cell culture	Suppressed HCV RNA and NS5B protein levels up to 75% with single siRNA and 90% with siRNA combinations	Korf et al. (2007)

sequence, stem length, presence of internal mismatches or single-stranded overhangs, and whether it is expressed or synthetic and directly delivered. As with siRNAs, stem length determines whether the molecule is a substrate for Dicer. In the context of vector expression, fully matched shRNAs have been compared with shRNAs containing internal mismatches from sequence alterations in the passenger arm, and the fully matched shRNAs were found to be more potent (Li et al. 2007; Boudreau et al. 2008). The loop can be almost any size, from 2 to 10 or more nucleotides. Brummelkamp et al. found that an expressed 19-bp shRNA with a 9-nt loop provided better target knockdown than similar shRNAs with 5- or 7-nt loops (Brummelkamp et al. 2002). The efficacy seen with expressed shRNAs depends on expression level as well as design parameters (Hinton et al. 2008; Kawasaki et al. 2003; Zhou et al. 2009). To investigate design parameters alone, several groups have examined the impact of structural changes to *synthetic* hairpins and found that the lengths of both stems and loops can affect efficacy. Li et al. reported that in the context of 4-nt loops, shRNAs with 29-bp stems silenced target gene expression more efficiently than those with 19-bp stems, but 19-bp shRNAs

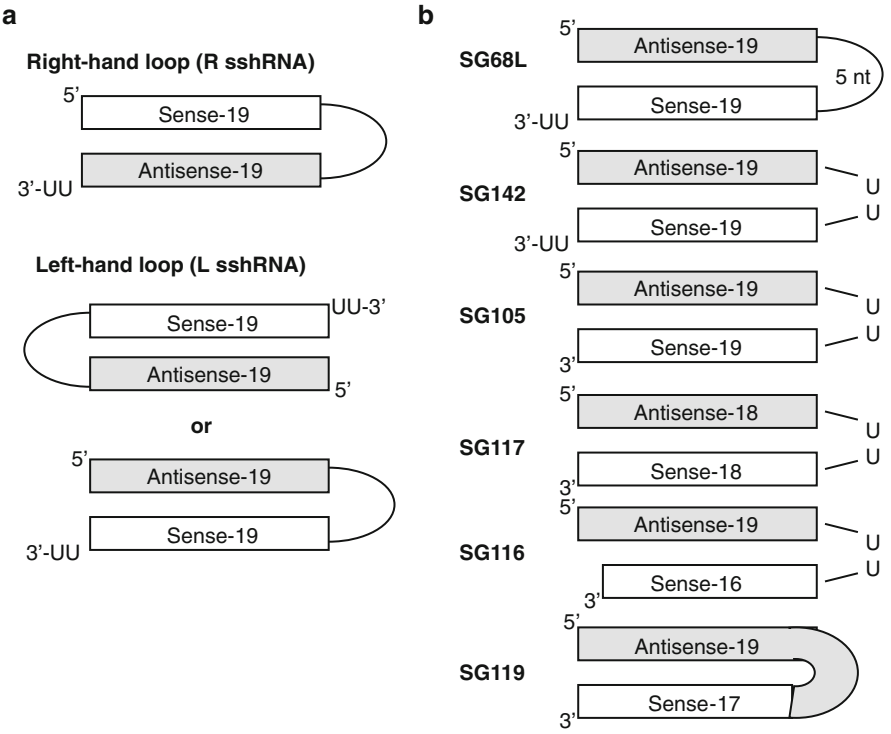

Fig. 1 Structure and activity of R- and L-sshRNAs. (**a**) General structures of R- and L-sshRNAs. Antisense and sense are relative to target sequence. The antisense strand normally becomes the guide strand in RISC. (**b**) Representative structures of L-sshRNAs, with sense and antisense strands of equal or unequal length. Adapted from Ge et al. (2010b)

with 9-nt loops outperformed shRNAs with longer stems, including 29-bp shRNAs with 4-nt loops (Li et al. 2007). Our group found that, in the context of 10-nt loops, 19-bp shRNAs were somewhat more potent than similar 19-bp and 25-bp siRNAs and 25-bp shRNAs were less potent than any of the 19-bp shRNAs or siRNAs tested (Vlassov et al. 2007).

4.2 Loop Position

The position of the antisense sequence within the hairpin also affects shRNA efficacy. shRNAs have often been designed with the sense sequence at the 5′ end of the hairpin (right-hand loop, R-type shRNAs) (Vlassov et al. 2007; Li et al. 2007) (Fig. 1). Harborth et al. reported that an shRNA with its antisense sequence at the 5′ end of the hairpin (left-hand loop, L-type shRNA) showed comparable silencing efficacy to an R shRNA if the stem length was 21–29 nt (Harborth et al. 2003). However, when the stem length was shortened to 19 bp (with a 4-nt loop), much less

potency was found with R shRNA, whereas L shRNA retained a potency comparable to that of shRNAs with 21–29-bp stems. Similar results were obtained by another group with a CD8-specific shRNA (McManus et al. 2002). Together, these results suggested that 19-bp shRNAs may be processed differently from shRNAs of 24 bp or longer. A major difference is that, of the longer shRNAs, both R and L types are processed by Dicer to generate the same ~19-bp siRNAs. In contrast, 19-bp shRNAs were found not to be Dicer substrates (Siolas et al. 2005; McManus et al. 2002). To distinguish shRNAs with 19 or fewer base pairs from longer, Dicer substrate shRNAs, we have designated the former as short shRNAs or sshRNAs.

4.3 Duplex Length Effects on Activity of sshRNAs

Shortening the sense sequence of a synthetic 19-bp L-sshRNA from its 3′ end to 17 or 16 nt while maintaining the length of the antisense arm at 19 nt significantly reduced gene silencing activity, suggesting that having duplex structure at the 5′ end of the antisense sequence is important(Fig. 2) (Ge et al. 2010b). However, the overall length of the duplex can be shorter than 19 bp. sshRNAs having 17- or 18-bp stems can be virtually as potent as similar 19-bp versions (Fig. 2). Further shortening of the stem to 16 nt in each strand (connected by UU, which might form a 15-bp stem with a GUUC loop) resulted in somewhat lower activity, and an sshRNA with 15 nt in each arm of the duplex had very little activity. This indicates that potent silencing activity requires a hairpin with a duplex length of at least 16 bp. Interestingly, an sshRNA with a stem of 16 bp consisting of a 19-nt antisense sequence connected directly to a 17-nt sense sequence showed similar potency compared to its parent molecule with 19-bp stem and UU linker.

4.4 Mechanistic Differences of L- and R-sshRNAs

To further examine the structure–function relationships of sshRNAs and how they may differ from Dicer-substrate shRNAs, our group undertook an extensive structure–activity and mechanistic study of sshRNAs targeting three partially overlapping sequences within the internal ribosome entry site (IRES) of HCV (Ge et al. 2010a, b; Dallas et al. 2012). For two of these target sites, L-type sshRNAs showed significantly higher potency than R-type; for the third, there was little difference between the two hairpin types. Unlike R-sshRNAs, where loop sizes of 5 nt or greater were optimal (Vlassov et al. 2007; Li et al. 2007), the L-sshRNAs were more potent when the loop size was very small (1 or 2 nt) than when it was larger (5 or 10 nt) (Fig. 3). The IC_{50} of an L-sshRNA of 19 bp and a UU loop was slightly more potent than a corresponding siRNA targeting the same region. The loop sequence appeared not to affect the sshRNA activity since three

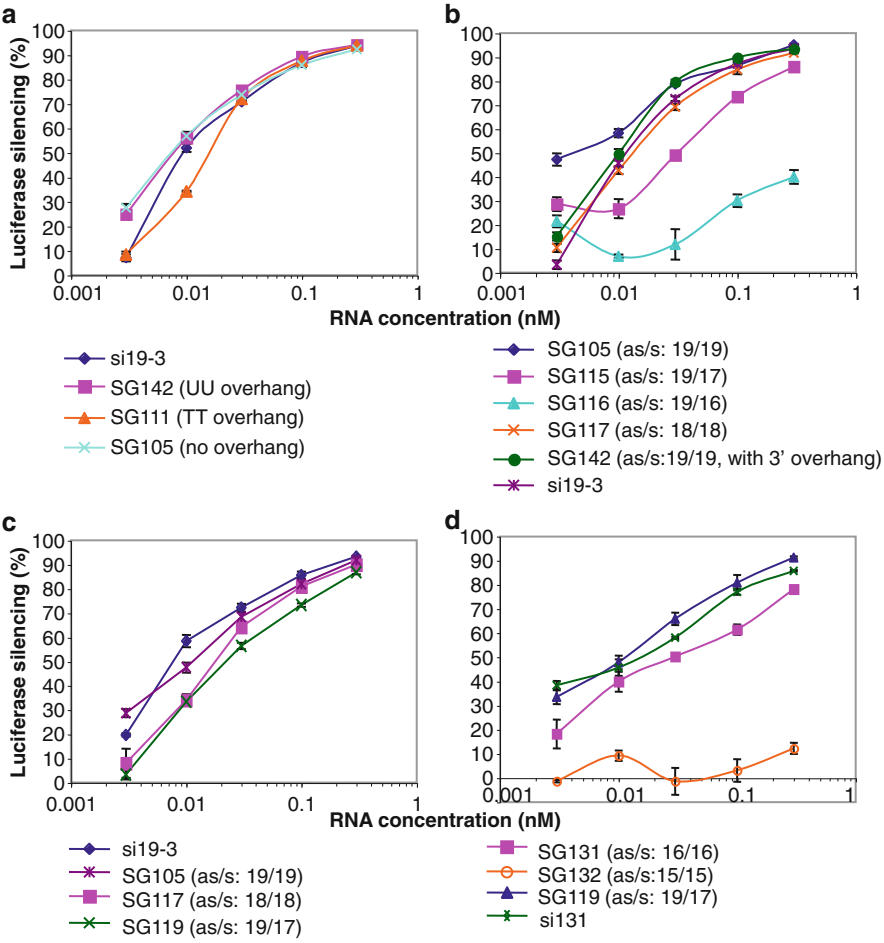

Fig. 2 Comparison of the activities of L-sshRNAs differing in 3′ overhang and stem length. (**a**) Overhang comparison. L-sshRNAs with and without a 3′ overhang as well as an siRNA, si19-3, all targeting the same sequence were chemically synthesized, and their ability to inhibit HCV IRES-dependent luciferase expression was compared in 293FT cells. (**b**) Stem length comparison. L-sshRNAs (UU loop) against the same target sequence but with different stem lengths were compared for their inhibitory activity in 293FT cells. SG105 differs from SG142 in lacking a 3′ UU overhang. (**c**) Effect of using part of the antisense sequence as the loop. SG119 has a 19-nt antisense sequence directly linked with a 17-nt sense sequence, probably forming a 16-bp duplex and 4-nt loop (UGCA). (**d**) Stem length comparison with SG119 derivatives. SG131 and SG132 have UU to connect the 3′ end of the antisense and 5′ end of the sense strands. They were compared with SG119 for target knockdown in 293FT cells. si131 has two complementary strands 16 nt in length with UU overhangs at their 3′ ends. as/s values represent the nucleotide lengths of antisense and sense strands. Adapted from Ge et al. (2010b)

different loop sequences, including one derived from the microRNA miR-23, gave the same results. However, nucleotides adjacent to the loop may affect the activity as they can affect the actual size of the loop. For example, a CG base pair next to a

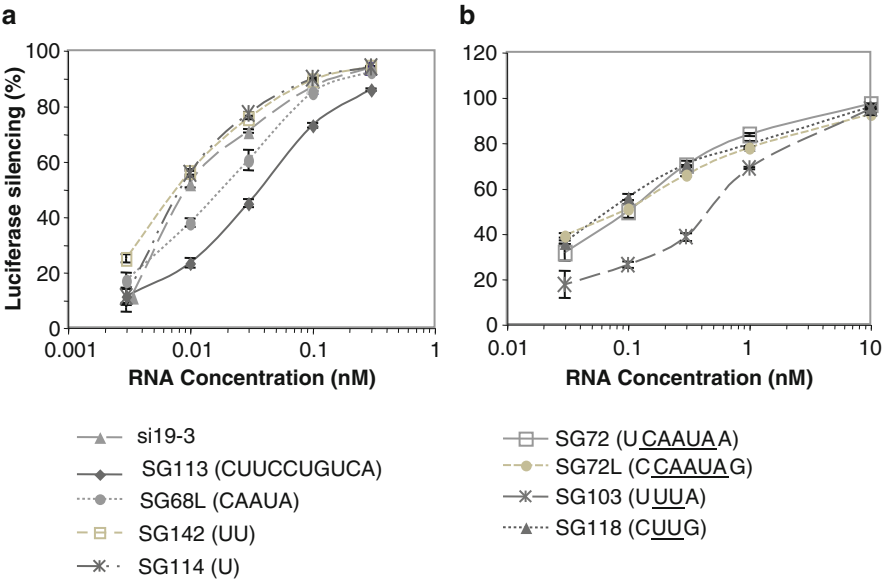

Fig. 3 Comparison of L-sshRNA activity with various loop structures and base pairs adjacent to the loop. (**a**) Loop length comparison, using L-sshRNAs against the same target region as sh68 and si19-3 but with various lengths and sequences of loops. sshRNAs were chemically synthesized, and their abilities to inhibit HCV IRES-dependent luciferase expression were compared in 293FT cells. (**b**) Loop-adjacent base pair comparison. sshRNAs against the same target region as si72 but with loops of 5 nt (SG72 and SG72L) and 2 nt (SG118 and SG103) and left- (SG72L and SG118) and right-loop (SG72 and SG103) orientation were compared for their inhibitory activity in 293FT cells. Loop sequences are *underlined*. Adapted from Ge et al. (2010b)

UU loop can be paired and result in an actual 2-nt loop (Jucker and Pardi 1995), whereas if that base pair is AU or UA, strain in a 2-nt loop may keep them unpaired, resulting in a 4-nt loop of AUUU or UUUA. Based on studies involving chemical modification, conditional dicer-knockout cells, and Ago immunoprecipitation, we concluded (Dallas et al. 2012) that L-sshRNAs having UU loops [which are naturally quite resistant to RNase cleavage (Ge et al. 2010a)] require passenger-arm cleavage to be maximally active, but the loops remain intact in the active RISC, with the guide strand breaking its base pairs with the truncated passenger strand to allow pairing with the target. Passenger-strand "slicing" is needed apparently to facilitate opening of the guide-passenger duplex. L-sshRNAs have their guide sequence at the 5′ side of the loop, so the 5′ end of the guide sequence is immediately available for phosphorylation and binding to the MID pocket of Ago2. R-sshRNAs, on the other hand, require a larger loop for full activity because the loop must be cleaved by some nuclease to create a terminal phosphate at the 5′ end of the guide strand (see Fig. 1). In this case, passenger-arm slicing is less important because the cleavage of the loop itself facilitates removal of the passenger strand in active RISC.

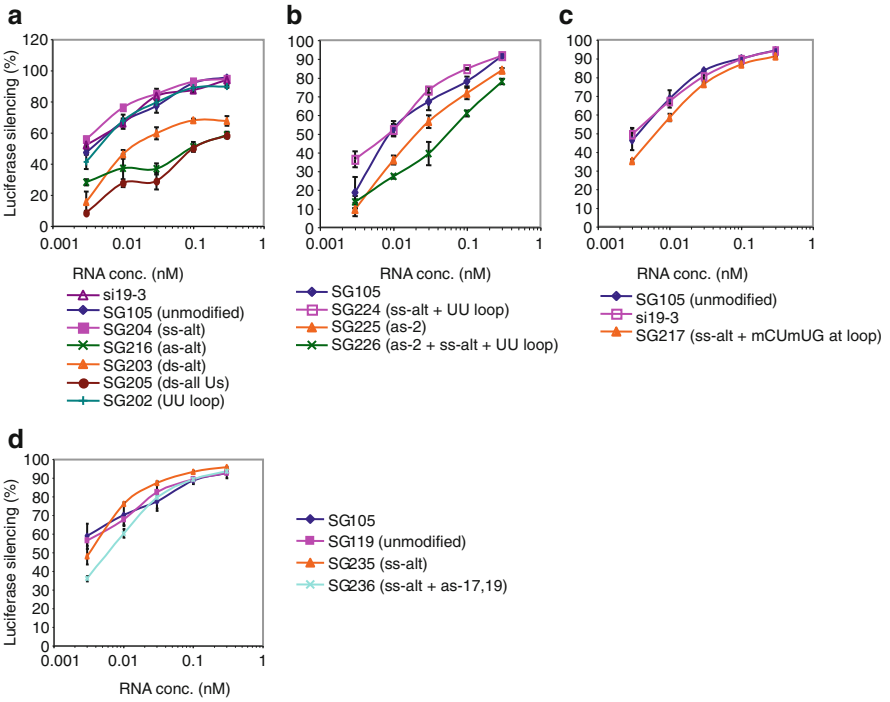

Fig. 4 Effect of 2′-*O*-Me modifications in the stem and/or loop regions on activities of L-sshRNAs. Activity was determined by potency in suppressing the expression of an HCV IRES-fLuc reporter in 293FT cells, by cotransfecting them in triplicate with the reporter DNA. An unmodified siRNA specific for the same target, si19-3, was used as a positive control. (**a**–**c**) Comparison of an unmodified sshRNA (SG105) and its derivatives containing 2′-*O*-Me modifications in the stem and/or loop. (**d**) Comparison of SG119 and its derivatives containing 2′-*O*-Me modifications in the stem and loop. Each construct is identified by sequence number and a shorthand notation for the region modified, e.g., as-2 means 2′-*O*-methylated at position 2 of the antisense strand, ss-alt means 2′-*O*-methylated at every other nucleotide of the sense strand except for the slicer site. Adapted from Ge et al. (2010a)

Whereas the presence of short flanking sequences such as a 3′-overhang generally enhance the efficiency of gene knockdown for R shRNAs (Siolas et al. 2005; Vlassov et al. 2007), the presence or absence of a 3′ overhang has relatively little effect on the silencing ability of L-sshRNAs (Fig. 2a). This appears to be because the loop is generally not cleaved in L-sshRNAs, and when the stem opens up, the loop is available to bind to the PAZ domain of Ago2 (Dallas et al. 2012).

5 Monomeric Versus Oligomeric shRNAs

Synthetic shRNAs are usually simply dissolved in H_2O or a buffer prior to in vitro or in vivo use. Whether these shRNAs form the expected hairpin structures in solution is frequently ignored. We have found that synthetic shRNAs, irrespective of stem

Table 2 Heating and snap cooling reduces immune stimulation by unmodified sshRNAs

	IFN-β		TNF-α	
Heating/snap cooling	–	+	–	+
SG105	47.5 ± 0.4	5.0 ± 0.6	6,192 ± 2,422	319 ± 81
SG117	23.1 ± 2.7	4.6 ± 0.7	2,552 ± 73	195 ± 37
SG119	7.3 ± 1.8	2.6 ± 0.6	5.9 ± 2.8	5.4 ± 2.1

Three different unmodified sshRNAs (100 nM), either untreated or subjected to 95°C heating (4 min) and snap cooling, were transfected into MRC5 cells in triplicate. RNA was extracted from cells 24 h post-transfection, and IFN-β and TNF-α mRNAs were quantified by RT-PCR. The mean values and standard errors of the relative RNA levels (fold differences) of cytokine genes were calculated and normalized to levels of GAPDH. Adapted from Ge et al. (2010b).

length, loop size, or L vs. R loop orientation, can form dimers or even oligomers (Ge et al. 2010b). Dimerization of hairpin RNAs has been also documented in retroviral RNAs, tRNAs, and some artificial RNA hairpins (Sun et al. 2007). The propensity of hairpin RNAs to dimerize depends on their loop size, sequence, and concentration as well as how they are handled (Bernacchi et al. 2005; Liu et al. 2005). When shRNAs are heated to 95°C and quickly cooled in an ice bath (snap cooling), dimerization can be eliminated (Ge et al. 2010a). Interestingly, sshRNAs showed efficient target knockdown both before and after the heating/snap cooling procedure, even for some sshRNAs that were predominantly dimers before the treatment, suggesting that the dimers of certain sshRNAs are functional molecules that can be processed and utilized by the RNAi machinery with similar efficiency as the monomers. However, the presence of long duplex regions in dimers can provoke immune stimulation. The heating/cooling procedure greatly reduces this immune stimulation (Table 2).

6 Chemical Modification to Improve Pharmacological Properties

Like all RNAs, shRNAs are nonideal as drugs due to their susceptibility to degradation by nucleases and the tendency of some sequences and structural features to cause unwanted immune stimulation. In a study of the effects of 2′-*O*-methyl, 2′-deoxy (DNA), and phosphorothioate modifications at various positions in the stem, loop, and overhangs, we found that placing a 2′-*O*-Me on each nucleotide of the loop and alternate nucleotides of the passenger arm, but leaving an unmodified window of 4 nt at the slicer site, provided significantly greater stability in 10% human serum (Fig. 5) while abrogating induction of the innate immune system (Ge et al. 2010a). It can be seen from Table 3 that two sshRNAs of the same structure but different sequences can have very different immunostimulatory properties. Blunt sshRNAs are particularly efficient at inducing RIG-I. In each case, however, 2′-*O*-methylation renders them non-stimulatory, by blocking recognition by pattern recognition sensors such as RIG-I. These modifications had essentially no effect on potency, but placing modifications in most positions of the guide arm or the slicer

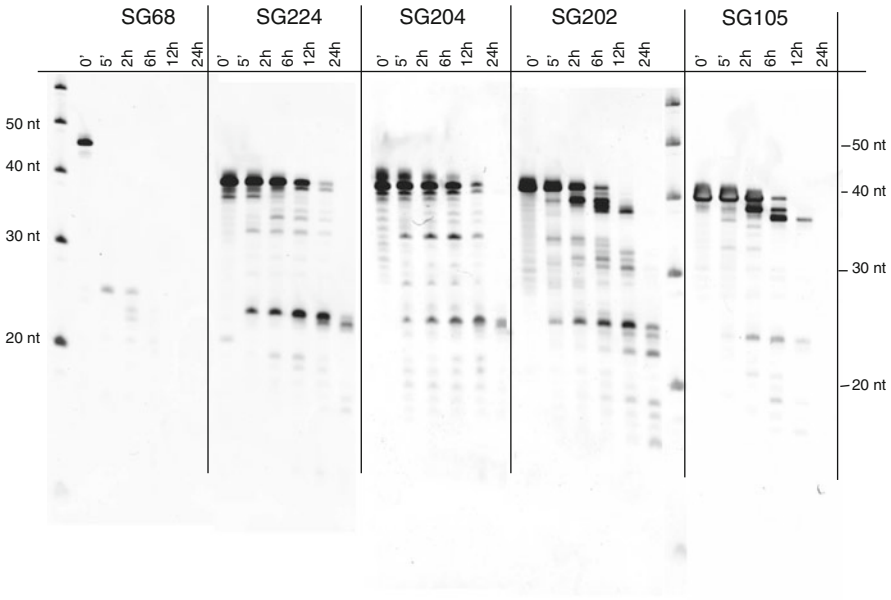

Fig. 5 Effect of 2′-*O*-Me modifications and loop size on serum stability of sshRNAs. sshRNAs were incubated with 10% human serum at 37°C for the times shown. Aliquots were analyzed by denaturing 12% PAGE. Adapted from Ge et al. (2010a)

Table 3 Effect of 2′-*O*-Me modification on immune stimulation by sshRNAs

2′-*O*-Me	SG142 (3′-overhang)		SG118 (3′-overhang)		T7 transcribed shRNA	Blunt-ended version of SG142	
	–	+	–	+	–	–	+
IFN-b	1.5 ± 0.2	1.3 ± 0.1	192 ± 7	2.2 ± 0.5	393 ± 35	7.3 ± 1.8	0.6 ± 0.1
IL-6	2.8 ± 0.1	1.3 ± 0.1	21.5 ± 1.7	1.5 ± 0.2	83 ± 27	4.6 ± 1.4	0.9 ± 0.1
TNF-a	7.7 ± 0.4	1.5 ± 0.4	548 ± 92	1.8 ± 0.6	319 ± 49	5.9 ± 2.8	1.3 ± 0.2
TLR3	4.3 ± 0.2	1.3 ± 0.1	67.6 ± 3.7	1.5 ± 0.1	95 ± 5	8.0 ± 0.9	1.1 ± 0.1
TLR7	1.5 ± 0.4	7.0 ± 3.0	6.5 ± 0.8	2.6 ± 1.2	7.8 ± 4.0	1.2 ± 0.4	0.3 ± 0.04
TLR8	1.4 ± 0.1	4.5 ± 1.7	5.3 ± 0.4	N.D.	4.1 ± 1.1	2.1 ± 0.4	0.5 ± 0.1
RIG-I	28.4 ± 10.2	2.8 ± 1.5	18.9 ± 8.5	3.9 ± 1.6	141 ± 35	289 ± 73	13.5 ± 6.1
PKR	3.6 ± 0.4	1.2 ± 0.1	12.1 ± 2.2	1.2 ± 0.1	6.2 ± 2.2	4.3 ± 0.4	1.1 ± 0.2

Shown are mean values and standard errors of the mRNA levels (relative to an untreated control and normalized to GAPDH) of genes of interest. 100-nM sshRNAs with (+) and without (–) 2′-*O*-Me modifications on the loop and alternate nucleotides of the passenger strand were transfected into human MRC-5 cells in triplicate (without heating and snap cooling). SG142 and SG118 are 19-bp sshRNAs with UU loops and 3′-UU overhangs targeting different sequences on the HCV viral RNA. RNA was extracted from cells 24 h post-transfection, and quantitative RT-PCR was performed. Cells that received the transfection reagent Lipofectamine 2000, alone showed no change in levels of the tested genes. A T7-transcribed shRNA, used as positive control, was transfected into cells in equivalent amounts (on a mononucleotide basis). N.D., not done. Adapted from Ge et al. (2010a).

site of the passenger arm reduced their efficacy. Phosphorothioate modifications were found to induce interferon-beta (IFN-β) and TNF-α in MRC-5 cells.

7 Summary: Design of Active sshRNAs

Synthetic sshRNAs can be highly potent RNAi effectors when properly designed. 19-bp R-sshRNAs require longer loops (at least 5–6 nt) and the presence of a 3′ dinucleotide overhang for maximal efficacy (Li et al. 2007; Vlassov et al. 2007; Siolas et al. 2005). In contrast, L-sshRNAs, at least the ones tested, can possess a loop as short as 1–2 nt or even a direct connection between the two strands. A UU loop is particularly effective. A 3′ overhang is not essential for many active L-sshRNAs, and both antisense and sense sequences can be either 18 or 19 nt. Modification of every other nucleotide on the passenger arm, except around the slicer site, is helpful for increasing stability against nucleases (e.g., those found in serum) and minimizing immune stimulation, particularly for blunt-ended sshRNAs. A heat–snap cooling step immediately prior to use is advisable to eliminate dimers.

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