Interplay between the RNA interference machinery and HIV-1
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DICER INDEPENDENT PROCESSING OF SHORT HAIRPIN RNAs

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ABSTRACT

Short hairpin RNAs (shRNAs) are widely used to induce RNA interference (RNAi). As the original shRNA design can potently induce RNAi, alternative shRNA designs have not been intensively explored. In this study, we tested a variety of shRNAs that differed in stem size and terminal loop (sequence and size) and revealed strikingly different RNAi activities and shRNA processing patterns. Interestingly, we identified a specific shRNA design that uses an alternative Dicer-independent processing pathway. Detailed analyses indicated that the stem length of the shRNA is critical for avoiding the Dicer route and activation of this alternative processing route. Importantly, alternatively processed shRNAs yield only a single RNA strand that effectively induces RNAi, whereas conventional shRNA processing results in an siRNA duplex of which both strands can trigger RNAi. The novel shRNA processing characteristics are reminiscent of AGO2-mediated cleavage, which was confirmed by sequence analysis of AGO2-associated small RNAs. One of the cleavage products remains associated with the RISC complex to induce RNAi activity. These results have important implications for the future design of more specific RNAi therapeutics.

Keywords: short hairpin RNAs, Argonaute, Dicer, RISC, RNA interference

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INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved post-transcriptional gene silencing mechanism in eukaryotes that is triggered by double stranded RNA (dsRNA) (Fire et al., 1998; Napoli et al., 1990). The key players in the RNAi mechanism are small noncoding dsRNAs of 20 to 30 basepairs (bp) in length with a 3’ dinucleotide overhang, a 5’ monophosphate and 3’ hydroxyl group (Bernstein et al., 2001; Nykanen et al., 2001). Several classes of small noncoding RNAs exist, including microRNAs (miRNAs) and small interfering RNAs (siRNAs). MiRNAs are transcribed in the nucleus as primary miRNAs (pri-miRNAs) by RNA polymerase (Pol II, occasionally Pol III) and are subsequently processed by the Microprocessor complex, comprising ribonuclease (RNase) III Drosha and subunit DGR8, into ~70 nucleotide (nt) premiRNAs (Denli et al., 2004; Gregory et al., 2004). The pre-miRNA associates with Exportin 5, which recognizes the 2-nt 3’ overhang and facilitates the export to the cytoplasm (Yi et al., 2003). The cytoplasmic complex that contains RNase III Dicer, TAR RNA-binding protein (TRBP) and PACT processes pre-miRNAs into the mature miRNA duplexes of 20-24 bp. The miRNA duplex associates with a specific member of the Argonaute protein family (AGO) within the precursor RNAi-induced silencing complex (pre-RISC) (Matranga et al., 2005; Rand et al., 2005). Either miRNA strand can mediate gene silencing, but many miRNAs show asymmetry, preferentially loading one of the strands (guide) into RISC. Thermodynamic properties largely determine which strand of the duplex will be incorporated as guide into RISC (Khvorova et al., 2003; Schwarz et al., 2003). The remaining strand of the duplex (passenger) is cleaved and degraded. The mature RISC contains the single-stranded guide RNA that finds a complementary target mRNA for post-transcriptional gene silencing (PTGS). The RNAi pathway can also be induced by artificial substrates, transiently by siRNAs that directly feed into RISC or stably by expressed shRNA precursors that are processed by Dicer into siRNAs and subsequently program RISC (Brummelkamp et al., 2002; Elbashir et al., 2001; Hammond et al., 2000).

A key component of RNAi related pathways is the AGO protein. Human cells have four AGO genes (AGO 1-4) and the siRNAs and miRNAs are assorted among these AGO proteins that exhibit different affinities for the different classes of RNA molecules. This sorting is influenced by Dicer processing, the structure of the small RNA duplex (thermodynamic properties) and its terminal nucleotides (Frank et al., 2010; Ma et al., 2005; Wang et al., 2008). Only the AGO2 protein is known to have “slicer” activity, executed by a catalytically active RNaseH-like domain that can cleave the target mRNA (Ender and Meister, 2010). Endonucleolytic cleavage of the target mRNA occurs opposite nucleotide position 10 and 11 of the annealed siRNA guide strand. The mRNA cleavage products are released from RISC and subsequently degraded by the endonuclease complex (Liu et al., 2009a).

Most research on the description of the RNAi machinery and improvement of the RNAi knockdown efficiency is focused on synthetic siRNAs or cellular miRNAs, but much less on shRNAs (Bartel, 2009; Boudreau et al., 2009; Li et al., 2007a; Siolas et al., 2005; Sun et al., 2008; Zeng and Cullen, 2004). The sequence and structural configuration are important features of shRNAs and have been shown to influence their RNAi activity (Brummelkamp et al., 2002; Hinton et al., 2008; Kawasaki and Taira, 2003; Li et al., 2007b; McManus et al., 2002; Miyagishi et al., 2004; Schopman et al., 2010; Vlassov et al., 2007; Wei et al., 2009), however recognition elements or sequences within the shRNAs that determine the processing efficiency
remain poorly defined. In this study we generated shRNA variants with different duplex lengths and loop sequences to test the effect on RNAi activity. Reporter constructs that score guide and passenger strand activity revealed a nearly complete switch from the regular guide to the passenger strand for a certain shRNAs design. Further analyses identified an alternative shRNA processing mechanism that is independent of Dicer, yet carries all the hallmarks of AGO2-mediated processing. AGO2 co-immunoprecipitation studies revealed that these alternatively processed shRNAs are indeed incorporated into RISC. Our findings indicate that the shRNA stem length is a critical factor for Dicer-independent processing. Importantly, the alternatively processed shRNAs induce a more specific and very potent RNAi-mediated gene knockdown compared to conventionally processed shRNAs.

MATERIAL AND METHODS

Plasmids
ShRNA expression plasmids targeting HIV-1 sequences pol47, pol9 and R/T5 were constructed as previously described (Brummelkamp et al., 2002; Schopman et al., 2010; Ter Brake et al., 2006). The RNA secondary structure of the shRNA transcripts was predicted by the Mfold program (Zuker, 2003). The plasmid expressing a Flag-tagged human AGO2 was purchased from Addgene (plasmid 10822). The firefly luciferase reporter plasmids were constructed by insertion in the 3’ untranslated region (3’UTR) of a 50- to 70-basepair fragment with the actual 19-nucleotide target in the centre. For this construction we used the EcoRI and PstI sites of the pGL-3 plasmid (Westerhout et al., 2005). The luciferase reporters containing the sense target sequences were described previously (Ter Brake et al., 2006), whereas the reporters harbouring the antisense target sequences were newly constructed as described above.

Cell culture
Human embryonic kidney 293T adherent cells and HCT-116 cells were grown as monolayer in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified chamber at 37°C and 5% CO₂.

Transfection experiments
Co-transfection of pGL-3 (Firefly luciferase reporter) with the shRNA vector was performed in the 96-well format. Per well, 2x10⁴ 293T cells were seeded in 100 μl DMEM with 10% FCS without antibiotics. The next day, 25 ng pGL-3, 10 ng of shRNA vector, and 0.5 ng pRL (Renilla luciferase reporter) were transfected using 0.5 μl Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Cells were lysed 48 hours post transfection to measure firefly and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. The ratio between firefly and renilla luciferase activity was used for normalization of experimental variations such as differences in transfection efficiencies. Transfection experiments were corrected for between session variations as described previously (Ruijter et al., 2006).
**siRNA detection by Northern blotting**

Northern blot experiments were performed as previously described (Liu et al., 2008). Briefly, 1.5x10⁶ HCT-116 cells were transfected with equimolar quantities (5 μg) of shRNA constructs using Lipofectamine 2000. Total cellular RNA was extracted 2 days post-transfection with the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The RNA concentration was measured using the Nanodrop 1000 (Thermo Fisher Scientific). For Northern blot analysis, 15 μg total RNA was electrophoresed in a 15% denaturing polyacrylamide gel (precast Novex TBU gel, Invitrogen, Carlsbad, CA). RNA molecular weight marker (Ambion) was prepared according to the manufacturer’s protocol and run alongside the cellular RNA. To check for equal sample loading, ribosomal RNA was stained with 2 μg/ml ethidium bromide and visualised under UV light. The RNA in the gel was electro-transferred to a positively charged nylon membrane (Boehringer Mannheim, GmbH, Mannheim, Germany) and crosslinked to the membrane using UV light at a wavelength of 254 nm (1200 μJ x 100). Hybridizations were performed at 42°C with radiolabeled locked nucleic acid (LNA) oligonucleotides in 10 ml ULTRAhyb hybridization buffer (Ambion, Austin, TX) according to the manufacturer’s instructions. LNA oligonucleotide probes were 5’-end labeled with the kinaseMax kit (Ambion) in the presence of 1 μl [γ-32P] ATP (0.37 MBq/μl, Perkin Elmer). To remove unincorporated nucleotides, the probes were purified on Sephadex G-25 spin columns (Amersham Biosciences) according to the manufacturer’s protocol. We used the following oligonucleotides to detect the antisense strand of the siRNA (LNA positions underlined): 5’-ATGGCAGGAAGAAGCGGAG-3’ (R/T5), 5’-GTGAAGGGGCAGTAGTAAT-3’ (pol47) and 5’-TAGCAGGAAGATGGCCAGT-3’ (pol9). To detect the sense strand of the siRNA the following oligonucleotides were used (LNA positions underlined): 5’-CTCCGCTTCTTCCTGCCAT-3’ (R/T5), 5’-ATTACTACTGGCCCTTTGAC-3’ (pol47) and 5’-ACTGGCCATCTTCTCGCTA-3’ (pol9). The signal was detected by autoradiography and quantified using a phosphorimager (Amersham Biosciences).

**Argo2 Co-Immunoprecipitations of small RNAs**

HCT-116 cells (5x10⁶ cells) were co-transfected with 5 μg Flag-tagged AGO2 plasmid and 20 μg of the various shRNAs. At 36 hours post transfection, cytoplasmic cell extracts were prepared by the treatment of cells on ice for 20 min with IsoB-NP-40 (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1.5 mM MgCl2, 1% NP-40) followed by a centrifugation at 12,000g for 10 min at 4°C. The supernatant was incubated in 75 μl of anti-FLAG M2 agarose beads (Sigma) with constant rotation overnight at 4°C. The beads were washed three times in NET-1 buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5% Tween 20). Small RNAs associated with AGO2 were isolated by phenol chloroform extraction followed by DNase treatment.

**Cloning of small RNAs**

DNase treatment using the TURBO DNA-free kit (Ambion) was used to remove residual DNA from the small RNAs obtained from Ago2 co-immunoprecipitations. The purification and cloning strategy of small RNAs was performed as previously described (Sun et al., 2007). Briefly, small RNAs were polyadenylated with an A-Plus™ Poly(A) PolymeraseTailing Kit (EPICENTRE Biotechnologies) according to manufacturer’s protocol and a 5’adapter was ligated to the polyadenylated RNAs. Next, the 5’adapter-small RNA-poly(A)n was reverse transcribed into cDNA followed by amplification of the first strand cDNA by PCR. PCR products were gel-purified.
and subsequently used for TOPO-TA cloning (TOPO-TA cloning kit, Invitrogen). Positive clones were sequenced with the T7 or M13R primers.

RESULTS

To study whether we could improve the shRNA design, we tested several variants of shPol47 (Fig. 1) that generates an siRNA with the anti-HIV guide sequence encoded on the 3’ side of the hairpin duplex that targets a conserved sequence in the HIV-1 Pol gene. These shPol47 variants differ in length of the basepaired stem and terminal loop sequence. Several shRNA variants have been described in a previous study where we studied the impact of the terminal loop, varying in size and structure, on shRNA induced gene silencing (Schopman et al., 2010). We renamed the shRNAs according to the stem length and loop size, e.g. shPol47 with a stem length of 21 bp and a loop sequence of 5 nt was named 21/5. The 21/5A design that was originally proposed by Brummelkamp and co-workers and is commonly used in many laboratories. (Asparuhova et al., 2008;Bernards et al., 2006;Brummelkamp et al., 2002;Huang et al., 2008;Ter Brake et al.,

Figure 1. Design of a set of shPol47 variants. The encoded siRNA sequence targeting the Pol-region of HIV-1 boxed. The name of each shRNA indicates the stem length and loop size, e.g. a stem length of 19 bp and a loop sequence of 5 nt was named 19/5.
Figure 2. Knockdown activity of the 5' / 3' strands of the shPol47 variants. (A) The knockdown activity of the 5’ and the 3’ strands of the different shRNAs was determined by co-transfection of a luciferase reporter encoding either the sense- or antisense-target sequence, respectively. 293T cells were co-transfected with 25 ng of the respective firefly luciferase reporter plasmid, 0.5 ng of renilla luciferase plasmid, and 5 ng of the corresponding shRNA constructs. An irrelevant shRNA (shNef) served as negative control, which was set at 100% luciferase expression. (B) Processing of the 3’ strand (upper panel) and 5’ strand (lower panel) of shPol47 was analyzed by Northern blot analysis. HCT-116 cells were transfected with 5 μg of the shRNA constructs. An irrelevant shRNA (shGag) was used as negative control. Northern blot analysis was performed on total cellular RNA. The RNA size marker (nt) is shown on the left. The regular 21 nt siRNA products and the new ~30 nt product are indicated.
We tested both the knockdown activity mediated by the 5’ and 3’ side of the shRNA duplex by co-transfection with luciferase reporter constructs encoding the respective targets. An irrelevant shRNA (shNef) served as negative control that resulted in 100% luciferase expression. Most shPol47 variants efficiently inhibited gene expression of the luciferase reporter with the sense HIV-target and exercised very little activity on the antisense reporter, indicating that the 3’ strand of the shRNA is indeed selected as guide (Fig. 2A). However, the 19/5 design showed a significant reduction in sense-luciferase knockdown with a concomitant increase in antisense-luciferase knockdown activity. Moderate activity on the antisense-reporter was also observed for three other variants with a 19 bp duplex (19/9A, B and C), but not for shRNAs with a duplex of 21 bp. Thus, the 19 bp duplex seems to result in a shift in guide strand selection from the 3’ to the 5’ side of the shRNA.

To investigate this phenomenon in more detail Northern blot analyses were performed to analyze the processing of the different shPol47 variants. We used LNA oligonucleotides to detect the 3’ (upper panel) and 5’ side (lower panel) products of the shRNA (Fig. 2B). An irrelevant shRNA (shGag) was used as negative control. We observed 3’ side derived siRNAs (~21 nt) for most shRNA variants; except for the 19/5 variant and to a lesser extend for the 19/9 designs. This partial or nearly complete loss of the 3’ signal correlated with the reduced knockdown activity on the sense-luciferase reporter (Fig. 2A, 19/5 > 19/9A, B, C). A low level of ~21 nt fragments was detected for all constructs with the 5’ side probe, reflecting the passenger strand. Interestingly, a new and abundant RNA fragment of ~30 nt was observed for the 19/5 variant, which does not resemble a Dicer processed product.

To study whether this phenomenon also applies to unrelated shRNAs with a completely different stem sequence, we tested several stem/loop variants of shRT5 and shPol9 for knockdown activity on the two reporters, including the special 19/5 design (Fig. 3A). As expected, for most shRNAs we observed more potent inhibition of the sense reporter (normal guide or shRNA 3’ side activity) than the antisense reporter (passenger or shRNA 5’ side activity). However, the activity of the 19/5 shRNAs was shifted from 3’ towards the 5’ side, indicating a reversal of strand selection. Moreover, we again observed the loss of the 3’ side RNA fragment and appearance of a new ~30 nt 5’ side RNA fragment for the 19/5 shRNAs (Fig. 3B). These ~30 nt RNA fragments do not resemble regular Dicer processed RNA products. They seem to consist of 19 nt 5’ side, 5 nt loop, and approximately 6 nt from the upper 3’ side of the shRNA.

The shRNA mutant data indicate that variation in the stem and/or loop can induce alternative shRNA processing. An additional set of shRNAs was designed to critically test this idea. The stem of shRT5 was shortened or extended at the bottom of the hairpin (Fig. 4, shRT5 variants 15/5-23/5). The hairpin loop was varied between 3-8 nt (Fig. 4, 19/3-19/8) and in addition we reversed the 5-nt loop sequence (19/5R). We tested the knockdown activity of the 5’ and 3’ strands of these shRNAs on the respective luciferase reporter targets. The irrelevant shNef served as negative control for which luciferase expression was set at 100%. The original 21/5A hairpin design was included as a positive control. The guide strand switch from 3’ to 5’ side, resulting in a switch from knockdown on the sense- to antisense-reporter was apparent for all variants with a 19 bp duplex (Fig. 5). A partial reversal of strand activity was seen for the shRNAs with a duplex of 17 or 18 bp, but all activity was lost for shorter shRNAs. The shRNAs of at least 20 bp in length do show the regular 3’ side activity on the sense-reporter. The loop size does clearly modulate the 5’ side activity on the antisense-reporter, with optimal activity for 19/3 and
Figure 3. Knockdown activity of the 5’/3’ strands of several shRT5 and shPol9 variants. (A) The knockdown activity of the 5’ and the 3’ strands of the indicated shRNAs was determined by co-transfection of a luciferase reporter encoding the sense or antisense target sequence, respectively, in 293T cells. See Figure 2A for details. (B) Processing of the 5’ / 3’ strands of the indicated shRNAs was analyzed by Northern blot analysis. We used LNA oligonucleotides to detect the 3’ (upper panel) and 5’ strand (lower panel) of the siRNA. An irrelevant shRNA (shGag) was used as negative control. The regular 21 nt products are marked and the * indicates the ~30 nt RNA products.
Figure 4. Design of additional shRT5 mutants varying in loop size and stem length. The shRT5 with a 19-bp stem and a 5-nt loop (19/5) was used as backbone for this design. The shRNA stem length was reduced/extended from the bottom of the hairpin, resulting in shRT5 variants 15/5-23/5. In addition, shRNA terminal loops ranging in size from 3-8 nt were designed (19/3-19/8) and the loop sequence was reversed (19/5R). The original shRT5 21/5A variant was also included.

...a gradual decrease towards larger loops. In fact, 19/7 and 19/8 seem to regain some regular 3' side activity at the expense of 5' side activity.

We next investigated the processing of these shRT5 variants by Northern blot analyses. Consistent with the luciferase knockdown data, we observed no 3' strand siRNA production for the 19/3-19/6 mutants, but a modest regain of regular siRNA production (~21 nt) was apparent for 19/7 and 19/8 (Fig. 6). All 19-bp shRNAs produced the new ~30-37 nt RNA fragment as detected with the 5' side probe. The increased loop size of the 19/3 to 19/8 variants corresponds with a gradual increase in size of this RNA fragment, proofing that this new RNA fragment contains the terminal loop sequence. We observed regular guide 3' strand siRNA production (~21 nt) for hairpins with a stem length from 20-23 bp, with the original 21/5A design being the most optimal. These shRNAs also showed some passenger 5' strand siRNA production (~21 nt). The 20/5 and 21/5A variants also showed weak expression of the ~30 nt RNA fragment. These shRNAs are therefore likely processed in different ways by the conventional and a novel processing route. The shRT5 variants of 15 and 16 bp did not show any 3' or 5' siRNA expression, consistent with the lack of knockdown activity. The 17/5 and 18/5 variants produced some 5' strand derived RNAs around 30 nt. These combined results indicate that a shRNA with minimal duplex length...
(19 > 18 > 17 bp) in combination with a small loop (3 > 4 > 5 > 6 > 7 nt) favors an alternative shRNA processing route. The alternative processing route produces an RNA fragment that does not resemble conventional Dicer processed shRNA products and most likely consist of 19 nt 5’ side, the terminal loop, and approximately 6-8 nt from the upper 3’ side of the shRNA.

These results indicate that the new ~30 nt RNAs are very active in the RNAi mechanism causing efficient knockdown of reporter gene expression, which implies that they end up in the mature RISC complex. To proof this and to determine the exact nature of the processed RNA

Table 1. AGO2 associated small RNAs from HCT-116 cells transfected with shRT5 19/5 or 21/5A

<table>
<thead>
<tr>
<th>AGO2 product</th>
<th>shRTS 19/5 n=48</th>
<th>shRTS 21/5A n=48</th>
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<tbody>
<tr>
<td>5’ 21 nt siRNA</td>
<td>0x</td>
<td>0x</td>
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<tr>
<td>3’ 21 nt siRNA</td>
<td>0x</td>
<td>0x</td>
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<tr>
<td>~30 nt siRNA</td>
<td>7x (33 nt)</td>
<td>1x (37 nt)</td>
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<tr>
<td>miRNA</td>
<td>4x</td>
<td>16x</td>
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<tr>
<td>tRNA</td>
<td>1x</td>
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<tr>
<td>Other RNAs (rRNA, mitRNA)</td>
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*a a total of 48 clones was sequenced
fragments, we performed immunoprecipitation of the AGO2-associated small RNAs. HCT-116 cells were co-transfected with Flag-tagged AGO2 plasmid and shRT5 19/5 or 21/5A expression plasmids. At 36 hours post transfection cytoplasmic cell extracts were prepared and the AGO2 complexes were captured with an anti-FLAG M2 agarose resin. The RNA content of purified AGO2 complexes was extracted, cloned and subsequently sequenced to determine the exact identity. Many of the small RNA sequences found in AGO2 complexes were of miRNA origin that serve as a control. The identified small RNA sequences that originate from either shRT5 19/5 or 21/5A are listed in Table 1. Perhaps surprisingly, no regular 21 nt siRNAs were detected for these shRNAs. However, shRNA 19/5 yielded 7 identical sequences that represent the new siRNA of 33 nt including the complete 5’ strand, terminal loop and part of the 3’ strand of the shRNA. Thus the shRNA is cleaved half-way the 3’ side of the duplex. This finding implicates AGO2 as its slicer activity will cleave duplexes between nucleotide 10 and 11 from the 3’ end, which coincides exactly with the new cleavage site. A similar fragment was observed for shRT5 21/5A (1x), albeit 37 nt in length due to the 2 bp increase in duplex length. This shift is consistent with the proposed AGO2-action that will cleave between nt 10 and 11 from the 3’ end of the shRNA template. Both the 33 and 37 nt RNA fragments were observed on Northern blot (Fig. 6, lower panel).

**DISCUSSION**

The sequence and structure of miRNAs and shRNAs are important features that have been shown to influence their RNAi activity. Recognition elements or sequences within the shRNAs
that determine the processing efficiency have not been examined in detail. The shRNA loop sequence is likely to have an effect on Dicer-mediated recognition or processing (McManus et al., 2002; Schopman et al., 2010; Trabucchi et al., 2009; Vermeulen et al., 2005) and also the stem length is an important determinant for RNAi activity (Ge et al., 2010; Liu et al., 2009b; McIntyre et al., 2011). We tested a variety of shRNAs for RNAi activity and processing characteristics. We identified a specific shRNA design with a minimized stem length that used an alternative processing mechanism, which resulted in the production of a new characteristic RNA fragment of ~30 nt. The new cleavage event half way the 3’ side of the shRNA duplex suggests a role for AGO2, which cleaves dsRNA between nt 10 and 11 from the 3’ end. Sequencing of AGO2-associated small RNAs confirmed this exact cleavage event. In other words, the minimized shRNAs of 17-19 bp are not processed by Dicer and seem to end up in RISC for cleavage and subsequent RNAi-silencing of an appropriate mRNA (Fig. 7). We tested this specific design for other shRNAs that all were
alternatively processed, demonstrating the universal value of the new shRNA design. Notably, this shRNA design results in the production of only a single RNAi-active RNA fragment, whereas the conventional shRNA design yields two siRNA strands of ~21 nt.

Mutagenesis studies indicated that a stem length of 17-19 bp is critical to trigger alternative processing, whereas the loop size had a modest effect. For shRNAs with a stem length smaller than 17 bp no target knockdown was observed and no processing was apparent on Northern blot. The larger shRNAs are good substrates for Dicer, which requires >19 bp stem in addition to a 2-nt 3’ overhang for efficient hairpin cleavage (Siolas et al., 2005). An intermediate stem length of 17-19 bp was needed to induce the alternative shRNA processing path that results in RNA fragments of ~30 nt, which do not resemble Dicer-mediated cleavage. Another group previously described minimal-size shRNAs that potently induced RNAi via mRNA cleavage, yet were not processed by Dicer in vitro and in cells (Ge et al., 2010; Siolas et al., 2005). The Siolas report suggested that the shRNA loop could be cleaved by a cellular endonuclease of unknown origin. It seems very likely that the new AGO2-processing route explains these results. The original shRNA 21/5A design resulted in a mixed processing profile according to the conventional Dicer and the alternative AGO2 mechanism. This may relate to the fact that the upper 2 bp of the shRNA are relatively weak (U-G and U-A). Thus, this structure may exist of a mixture of hairpins with 19, 20 or 21 bp. The conformers with 19 bp stem will be alternatively processed, whereas conformers with an extended stem of 20 or 21 bp will be conventionally processed.

The loop size of the shRNA has a modest influence on this new processing path as increasing the loop size to 8 nt caused a partial return to regular Dicer processing. We hypothesize that a too large loop (e.g. > 8 nt) sterically hinders uptake of the shRNA by RISC and subsequently AGO2-mediated processing. The 3’ segment of RISC-associated guide strands is accessible and thus not shielded by the PAZ domain. Extended segments are allowed to loop out, but extended guide strands of 36 nt may adopt alternate trajectories for the 3’ segment (Wang et al., 2008). When AGO2 processing is hindered by a too large loop (19/8 mutant), a partial return to regular Dicer processing was observed, suggesting that the two processing paths are in direct competition with each other. Dicer processing will obviously remove the loop region such that efficient siRNA uptake in RISC is allowed.

We identified a specific shRNA design that triggers an alternative processing pathway that bypasses the Dicer endonuclease and instead requires AGO2 for processing and subsequent mRNA cleavage. Such non-canonical processing has previously been reported for miR-451 and has been suggested for several other miRNAs (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). The slicer activity of AGO2 cleaves the passenger strand of these miRNA duplexes, after which the cleaved products dissociate from RISC. The RISC loading determinants for such a Dicer independent processing of miRNAs have not been examined in detail.

It seems that alternatively processed shRNAs are more active than the conventional shRNAs. More potent target knockdown was observed in the luciferase reporter assays with the 17-19 bp minimized shRNAs compared to the >19 bp regular shRNAs, although a direct comparison remains difficult as different reporters are targeted. The design of optimised RNA therapeutics may allow one to use a lower shRNA dosage, thus further reducing the chance of adverse effects, e.g. due to saturation of the RNAi machinery. Increased activity may be related to the extended length of the 30 nt siRNA product, although this does not extend the baseparing potential with the targeted mRNA. In addition, the AGO2 co-immunoprecipitation
experiments showed these AGO2-related small RNAs of 33-37 nt for shRNAs 19/5 and 21/5A, but no ‘regular’ 19-21 nt siRNAs (Table 1). Northern blot analysis indicates that such regular siRNAs are abundantly produced by Dicer processing of the 21/5A shRNA, suggesting either inefficient uptake by RISC or rapid release. One possibility is that the 33-37 nt AGO2-processing products are present much longer in active RISC than the conventional 19-21 nt siRNAs, thereby increasing the RNAi activity.

This alternative shRNA processing yields only one active guide strand, the other (passenger) strand is in fact destroyed by AGO2-mediated cleavage. This feature is an important property to restrict off-target effects induced by the passenger strand. It remains possible that a further increase of the basepairing complementarity of the ~30 nt RNA fragment with the mRNA target, e.g. by loop sequence adaptation, will further increase the RNAi efficiency. Experiments are ongoing to test whether conversion of the regular loop sequences into anti-HIV sequences will generate more potent and possibly more escape-proof antivirals.

REFERENCES


