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Optimization of shRNA inhibitors by variation of the terminal loop sequence

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This manuscript is dedicated to the memory of Rob Goldbach.

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ABSTRACT

Gene silencing by RNA interference (RNAi) can be achieved by intracellular expression of a short hairpin RNA (shRNA) that is processed into the effective small interfering RNA (siRNA) inhibitor by the RNAi machinery. Previous studies indicate that shRNA molecules do not always reflect the activity of corresponding synthetic siRNAs that attack the same target sequence. One obvious difference between these two effector molecules is the hairpin loop of the shRNA. Most studies use the original shRNA design of the pSuper system, but no extensive study regarding optimization of the shRNA loop sequence has been performed. We tested the impact of different hairpin loop sequences, varying in size and structure, on the activity of a set of shRNAs targeting HIV-1. We were able to transform weak inhibitors into intermediate or even strong shRNA inhibitors by replacing the loop sequence. We demonstrate that the efficacy of these optimized shRNA inhibitors is improved significantly in different cell types due to increased siRNA production. These results indicate that the loop sequence is an essential part of the shRNA design. The optimized shRNA loop sequence is generally applicable for RNAi knockdown studies, and will allow us to develop a more potent gene therapy against HIV-1.

1. Introduction

RNA interference (RNAi) is an evolutionary conserved pathway in eukaryotes that is triggered by double-stranded RNA (dsRNA), which is processed in the cytoplasm by the Dicer enzyme into small interfering RNA (siRNA) of 21–23 base-pairs with 2-nt 3′ overhanging ends. The siRNA duplex is incorporated into the RNA-induced silencing complex (RISC). The passenger strand is cleaved and removed such that the guide strand activated RISC complex can target a complementary mRNA for cleavage and degradation, resulting in silencing of the target gene (Elbashir et al., 2001; Hammond et al., 2001; Meister and Tuschl, 2004; Zamore et al., 2000). This endogenous pathway can be triggered by synthetic siRNAs that are transfected into the cell or by intracellularly expressed short hairpin RNAs (shRNAs) that are processed by the cell into siRNAs. Very effective and sequence-specific knockdown of genes has been reported. RNAi has also been induced against a wide range of viruses including hepatitis B and C virus (HBV and HCV), herpes virus, henipavirus, influenza A virus and human immunodeficiency virus type 1 (HIV-1) (Ely et al., 2008; Haasnoot and Berkhout, 2006; Liu et al., 2008b; McCaffrey et al., 2003; Mungall et al., 2008; Naito et al., 2007; ter Brake et al., 2006). A constant supply of siRNAs is required to combat chronic infections like HIV-1, thus much of the anti-HIV RNAi research is focused on the development of shRNA gene therapy strategies (Asparuhova et al., 2008; McIntyre et al., 2009; ter Brake et al., 2008, 2009).

We have successfully used shRNAs against HIV-1 that are based on the pSuper design as presented by Brummelkamp et al. (2002). However, in a pilot experiment in which we compared the activities of a set of siRNAs/shRNAs pairs that target the same sequence, we observed discrepancies in their activity. For instance, some siRNAs are active but the corresponding shRNAs are not, and vice versa. We speculated that the loop of the shRNA, which connects the sense and antisense strand of the siRNA, may be an important determinant of the shRNA activity. The effect of shRNA loop structure on knockdown performance is an issue of great practical importance. On-target knockdown efficacy is an important factor for RNAi-based experiments. ShRNA are used intensively for cell-based studies, increasingly for in vivo mouse studies, and are also considered a potential therapeutic RNAi delivery strategy. Much attention has been paid to the impact of hairpin loops on the effectivity of shRNA molecules thus far (Brummelkamp et al., 2002; Hinton et al., 2008; Kawasaki and Taira, 2003; Li et al., 2007; Miyagishi et al., 2004; Vlassov et al., 2007; Wei et al., 2009). In addition, alternative loop conformations in miRNA expression constructs have been tested (Boudreau et al., 2009; Li et al., 2007; Zeng and Cullen, 2004). Unfortunately, this string of reports has not left much clarity on loop effects, the results and conclusions have been contradictory. It is occasionally observed that the loop sequence matters, but with disagreement about whether certain loop characteristics or even specific loops are generally better or worse. This suggests that there have been confounding effects that interact with loop

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preference such that generalizations in this area should be made very cautiously.

We reasoned that the original loop design of the Brummelkamp shRNA molecule may be improved by novel loop sequences. We therefore tested the impact of different loop sequences, varying in size and structure, on shRNA-induced gene silencing. We demonstrate that the standard loop that is frequently used is sub-optimal (Asparuhova et al., 2008; Bernards et al., 2006; Huang et al., 2008; ter Brake et al., 2008), and we present new loops that yield a 7-fold increase in RNAi activity. The novel shRNA loop design provides an opportunity for significant improvement of shRNA-based gene therapy protocols in which potent knockdown is required.

2. Materials and methods

2.1. Plasmids and siRNAs

ShRNA-expression plasmids targeting HIV-1 sequences pol47, pol9 and R/T5 and the furin mRNA were constructed as previously described (Brummelkamp et al., 2002; ter Brake et al., 2006). The oligonucleotides used to construct these shRNA are listed in supplementary Table S1. The target sites in furin mRNA (Accession No. BC012181) were selected with the Qiagen and Dharmacon algorithms https://www1.qiagen.com/GeneGlobe/Default.aspx and https://www.dharmacon.com/sidesign/default.aspx, respectively. SiRNAs were purchased from Dharmacon. The RNA secondary structure of the shRNA transcripts was predicted by the Mfold program (Zuker, 2003).

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 full-length HIV-1 molecular clone pLAI (Peden et al., 1991) was used to produce wildtype virus in transfected 293T cells and to study shRNA-mediated inhibition of virus production.

2.2. Cell culture

Human embryonic kidney 293T, Vero, HeLa and C33A cells were all grown 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% CO_2.

2.3. Transfection experiments

Co-transfection of pLAI or pGL-3 (Firefly luciferase reporter) with the shRNA vector was performed in the 96-well format. Per well, 2×10^4 cells (293T, Vero, HeLa or C33A) were seeded in 100 μl DMEM with 10% FCS without antibiotics. The next day, 100 ng pLAI or 25 ng pGL-3, 1 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). In case of furin inhibition experiments the indicated amounts of siRNA and shRNA-expression constructs were transfected in the titration experiments.

The co-transfections with pLAI were analyzed after 48 h. Cell culture supernatant was analyzed by CA-p24 ELISA, and cells were lysed for Renilla luciferase activity measurement with the Renilla Luciferase Assay System (Promega). Relative CA-p24 values were determined by dividing the CA-p24 values by the Renilla values. We set the restriction that the Renilla values should not vary by more than a factor 2 within an experiment.

The co-transfections with pGL-3 were analyzed after 48 h. Cells were lysed 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 variation as described previously (Ruijter et al., 2006).

2.4. siRNA detection by Northern blotting

Northern blot experiments were performed as previously described (Liu et al., 2008b). Briefly, human embryonic kidney 293T cells (5×10^5 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, 10 μg total RNA was electrophoresed in a 15% denaturing polyacrylamide gel (precast Novex TBU gel, Invitrogen, Carlsbad, CA). The RNA in the gel was electro-transferred to a positively charged nylon membrane (Boehringer Mannheim, GmbH, Mannheim, Germany). 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 ml [γ-32P] ATP (0.37 MBq/ml Amersham Biosciences). 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 (LNA positions underlined): 5′-ATGGCAGGAAGAAGCGGAG-3′ (R/T5), 5′-GTGAAGGGGCAGTAGTAAT-3′ (pol47) and 5′-TAGCAGGAAGATGGCCAGT-3′ (pol9). The signal was detected by autoradiography and quantified using a phosphorimager (Amersham Biosciences).

3. Results

3.1. Differential siRNA and shRNA activity

We wondered whether an active siRNA can be translated into an active shRNA, as this would enable shRNA design according to the existing siRNA algorithms. We compared the activity of seven siRNAs that target different sites in the furin mRNA, and the matching shRNA-expression plasmids. The furin protease is a co-factor for processing of the viral Envelope protein and a potential cellular target for RNAi gene therapy against HIV-1. The synthetic siRNAs were standard double-stranded RNAs (dsRNAs) with 2-nt 3′ overhangs characteristic for a Dicer product (Bernstein et al., 2001) (Fig. 1). For the shRNA-expression constructs, we cloned the same sequence as inverted repeats connected with a loop sequence under the transcriptional control of the RNA polymerase III H1 promoter with a T_5 termination signal (Brummelkamp et al., 2002) (Fig. 1). We determined the inhibition profile of the seven siRNAs and seven matching shRNAs on luciferase reporters with the furin target sequences inserted in the 3′UTR (Fig. 1 c1–c7). We titrated the siRNA/shRNA inhibitors and determined the relative luciferase expression by controlling for the transfection efficiency as determined by renilla co-transfection. The luciferase expression level obtained without inhibitor was set at 1 for each luciferase construct.

A similar inhibition profile was measured for three siRNA/shRNA pairs. Targeting of c1 and c4 resulted in strong inhibition with both the siRNA and shRNA, and targeting of c2 was not effective for both types of inhibitors. In contrast, differential siRNA/shRNA activity was scored on the other four targets. Only the siRNA was active

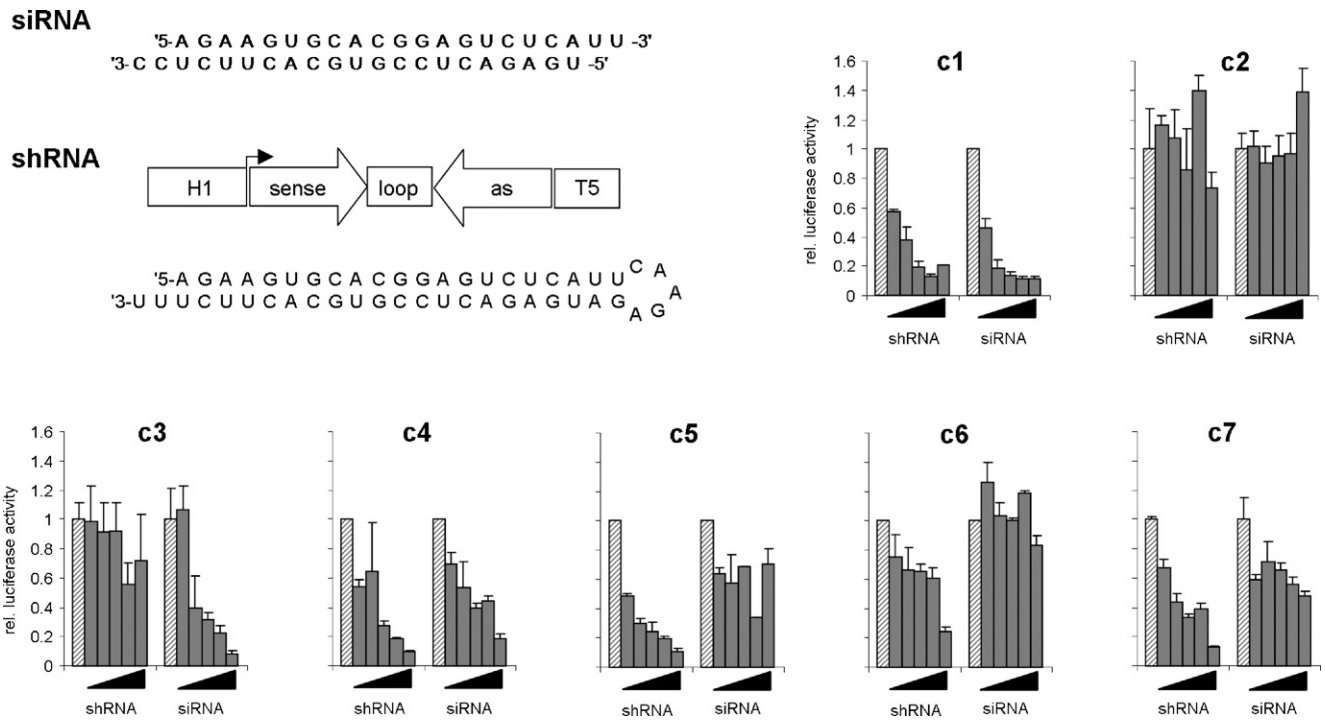

Fig. 1. Differential siRNA and shRNA activity. A schematic of the siRNA and shRNA constructs that were used. ShRNA sequences were cloned downstream of an H1 RNA polymerase III promoter and transcription was terminated by a T5 termination signal. The c1–c7 graphs show inhibition of a Luc reporter with all furin target sequences in the 3′UTR by siRNAs and shRNAs. SiRNAs and shRNAs (10 ng) against the HIV-1 nef gene were used as a negative control for non-specific effects on the Luc reporter. Luc reporters (100 ng) were co-transfected in 293T cells with increasing amounts of siRNA or shRNA-expression constructs (2, 5, 10, 20 and 50 ng) and 2.5 ng pRL as an internal control. Bars represent the mean ± standard deviation of three independent experiments.

on the c3 target, whereas the c5, c6 and c7 targets could only be attacked efficiently by the respective shRNAs. Thus, effective knockdown by a siRNA does not guarantee effective knockdown by the corresponding shRNA and vice versa. The obvious difference between these two entities is the loop sequence in the shRNA transcript that connects the sense to the antisense strand. Therefore, we wondered about the contribution of the loop sequence to the shRNA activity, and we became interested in the possibility to improve the shRNA activity by novel loop design.

3.2. RNAi activity is improved by novel shRNA loop design

To investigate the effect of the loop sequence on shRNA activity, novel loops were designed that vary in size and structure (Fig. 2A). All variants were tested in the context of the pol47 shRNA, a potent anti-HIV molecule with the original 9-nt hairpin loop sequence of Brummelkamp et al. (2002). In fact, the Mfold program predicts that the Brummelkamp loop consists of 5-nt only because 2 additional base-pairs (bp) are formed, including a U-G bp (Fig. 2A, wildtype (wt)). To examine the function of these extra 2 bp we made specific mutations in this area. To test whether the presence of a top U-G bp is important we interchanged the 2 bp, but maintained the 5-nt loop (mut1). The 2 extra bp were opened by a mutation on the 5′ side (mut2), the 3′ side (mut5) or both sides (mut3). All these variants have a 9-nt loop (see Table 1 for an overview). In addition, a mutant was designed lacking the 2-bp stem area of the original loop, leaving a 5-nt loop (mut4). We also introduced alternative loops: two microRNA derived loops on top of the 2 closing bp (mir-17 and mir-25) to explore whether naturally occurring loop structures are perhaps more active than artificial loop sequences. We also tested the particularly stable GUGA loop of the family of GRNA tetraloops (Prathiba and Malathi, 2008). Furthermore, two size variants were included; a 3-nt loop (min) and a 13-nt loop (max) to examine whether the loop size influences shRNA activity.

The various shRNA constructs target the same HIV-1 sequence that was cloned in the 3′ UTR of the luciferase reporter gene, and we measured their RNAi activity upon co-transfection in 293T cells and luciferase measurement (Fig. 2B). The relative luciferase expression was determined by the ratio of the firefly and renilla luciferase activity. In fact, no significant variation in the expression of the renilla control was observed, indicating no acute cell toxicity effects. The luciferase expression measured with an irrelevant shRNA (shnef) was set at 100%. The wt version of the shpol47 inhibitor showed 70% knockdown of luciferase expression. Seven out of the ten new shRNA designs proved to be more effective than wt. The two notable exceptions mut4 and min showed a decrease in knockdown efficiency. When looking closely to the 'Brummelkamp' loop, a modest improvement (~10% increase in knockdown activity) was observed when the extra 2 bp stem segment of this loop was opened, as in mut2, 3 and 5. If the stem area remained base paired as in mut1, a similar activity as wt was observed. Thus, it is likely that the 2 extra bp are not important for the activity of the shRNA, and it even seems that the shRNA activity improves when these bp are opened. Deleting the extra 2 bp stem segment

Table 1
shRNA characteristics.

shRNA variant	Loop (nt)	Extra bp in stem	RNAi activity
wt	5	2	+
mut1	5	2	+
mut2	9	0	+
mut3	9	0	+
mut4	5	0	–
mut5	9	0	++
mir-17	7	2	+++
mir-25	10	2	+++
GRNA	4	2	++
min	3	0	–
max	13	3	+

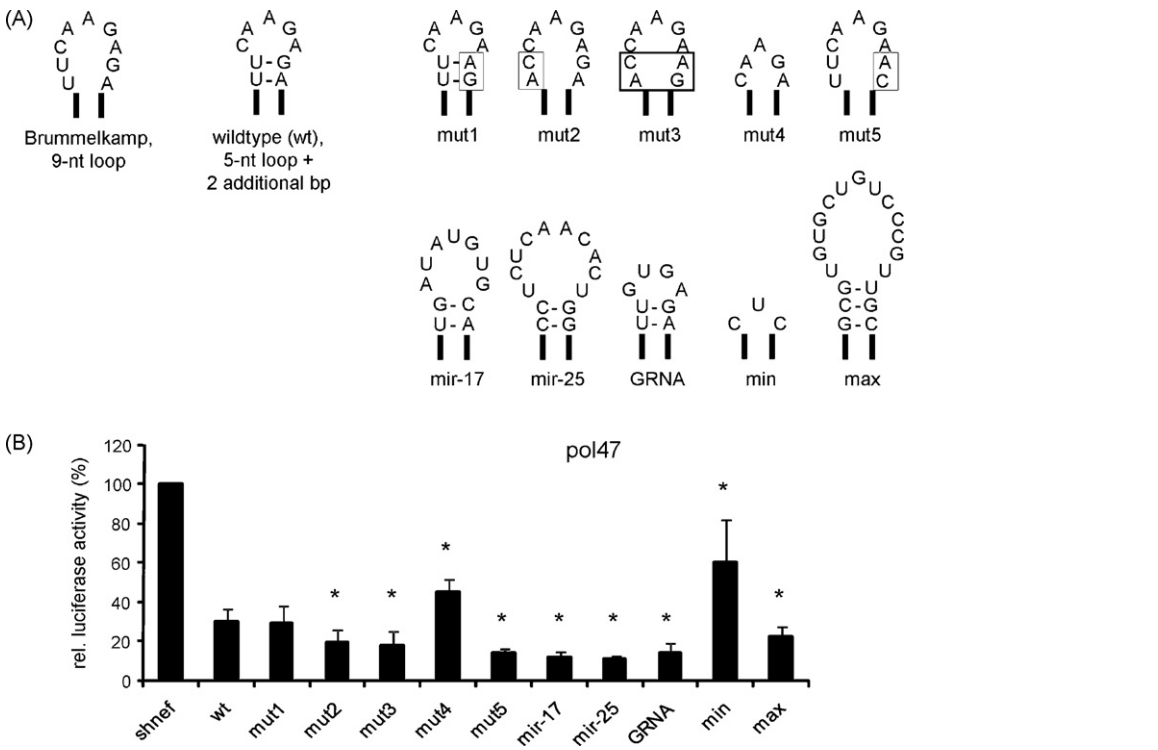

Fig. 2. RNAi activity is improved by novel shRNA loop design. (A) Structures of the shRNA loops are depicted. ShRNA stem sequences are depicted as black bars. The boxed area indicates the mutations made within the Brummelkamp loop (wt loop) sequence. Structures were predicted with the Mfold program. (B) The effect of novel loops on shpol47-induced silencing in a luciferase reporter assay. 293T cells were co-transfected with 25 ng firefly luciferase reporter plasmid (pol47 luc), 0.5 ng of renilla luciferase plasmid, and 1 ng shRNA constructs. Relative luciferase activities were calculated from the firefly and the control renilla luciferase expression levels. An irrelevant shRNA (shnef) served as negative control, which was set at 100% luciferase expression. Averages and standard deviations represent at least three independent transfections performed in quadruple. $^{*}p < 0.05$ compared to wt.

of the wt loop, leaving only a 5-nt loop as in mut4, has a detrimental effect on knockdown efficiency. We also tested a dramatic loss of activity for the min variant with a loop of only 3-nt. In contrast, the max variant with a loop size of 13-nt shows improved activity compared to wt. The GRNA tetraloop showed improved activity despite the short loop of 4-nt, suggesting that the stabilizing effect of this particular loop sequence overcomes the size restriction. Thus, the size of the loop is not the sole determinant of shRNA activity since the GRNA variant with a loop of only 4-nt is much more active than mut4 with a 5-nt loop. ShRNA constructs with loops derived from miRNAs (mir-17 and mir-25) were significantly more effective than the wt loop (90% versus 70% knockdown). These combined results indicate that it is better to open the 2 extra bp of the original pSuper shRNA design and that the loop size, sequence and structure has an impact on the RNAi knockdown efficiency.

3.3. General shRNA loop improvements

It is important to determine whether the observed loop-trends are generally applicable. To test if these findings also apply to other shRNAs we put a selection of loops on two other anti-HIV shRNAs, R/T5 and pol9. We selected mut5, mir-17, mir-25 and GRNA as improved loops and mut4 as a less active loop variant. Silencing of luciferase reporters carrying the R/T5 and pol9 targets with the set of loop-substituted shRNAs revealed a trend similar to that obtained for pol47 (Fig. 3, compare to Fig. 2). The efficacy of both wt shRNAs improved significantly when the loop was replaced by a mir-17 or mir-25 loop, and the mut4 loop profoundly decreased the efficacy. The activity of the intermediate R/T5 inhibitor was improved from 50% to 90% knockdown efficiency and the weak pol9 inhibitor was improved from 40% to 60%. In general, these

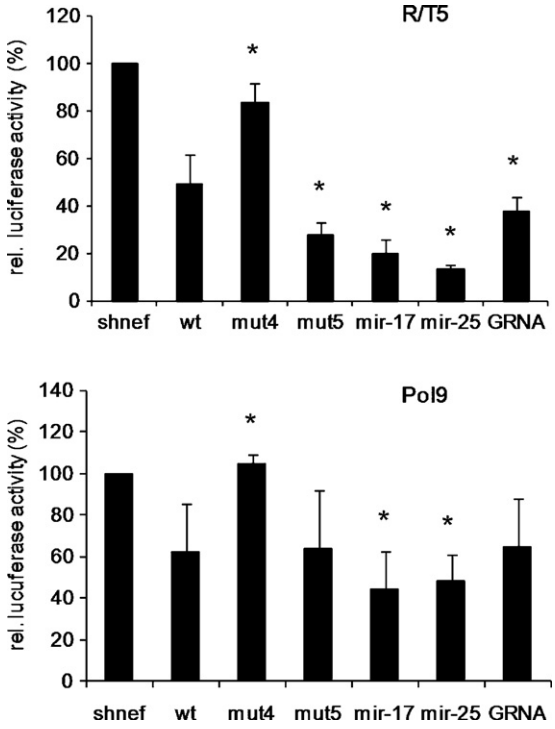

Fig. 3. The activity of any shRNA can be improved by novel loop design. 293T cells were co-transfected with 25 ng firefly luciferase reporter plasmid (R/T5 or pol9), 0.5 ng of renilla luciferase plasmid, and 1 ng of the corresponding shRNA constructs. Relative luciferase activities were determined from the firefly and the control renilla luciferase expression ratios. The shnef values were arbitrarily set at 100%. Averages and standard deviations represent at least three independent transfections performed in quadruple. $^{*}p < 0.05$ compared to wt.

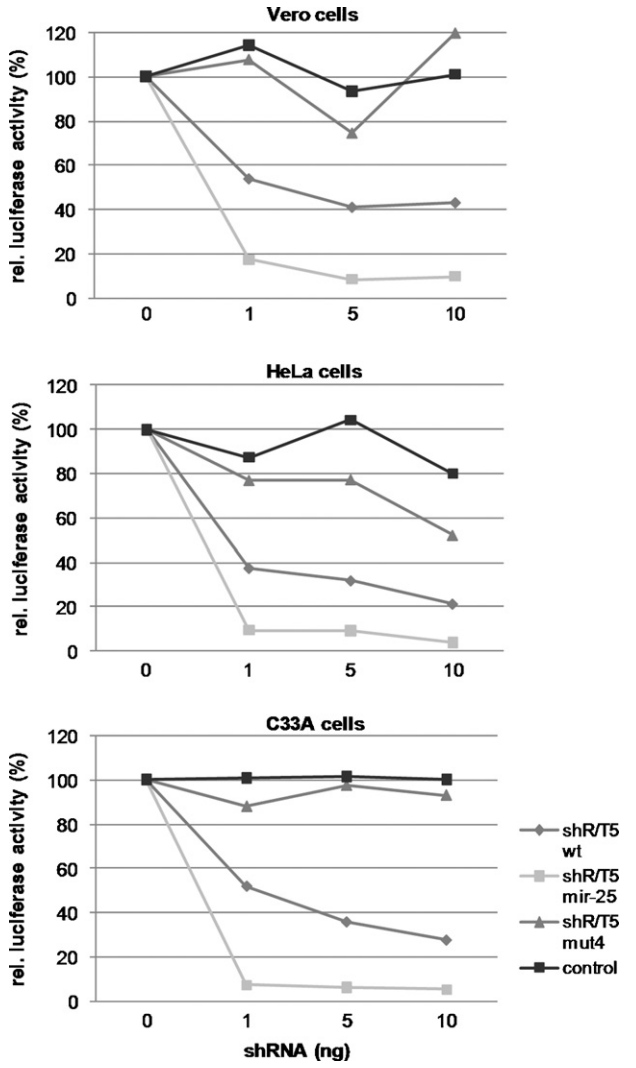

Fig. 4. Improved shRNA activity by novel loop design is cell line-independent. Vero cells, HeLa cells and C33A cells were co-transfected with 25 ng firefly luciferase reporter plasmid (R/T5), 0.5 ng of renilla luciferase plasmid, and with increasing amounts (0, 1, 5 and 10 ng) shR/T5 constructs with novel loops (wt, mut4 and mir-25). An irrelevant shRNA (shnef) was used as negative control. Relative luciferase activities were determined from the firefly and the control renilla luciferase expression ratios. The mean values are based on three independent transfections performed in quadruple.

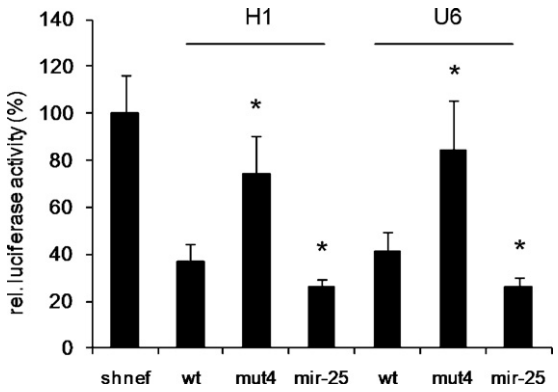

Fig. 5. Improvement of RNAi activity by novel loop design is independent of the shRNA-expression system. 293T cells were co-transfected with 25 ng firefly luciferase reporter plasmid (pol47), 0.5 ng of renilla luciferase plasmid, and 1 ng of the shpol47 constructs. The shnef was used as negative control. Relative luciferase activities were determined from the firefly and the control renilla luciferase expression ratios. The shnef values were arbitrarily set at 100%. Averages and standard deviations represent at least three independent transfections performed in quadruple. $^{*}p < 0.05$ compared to wt.

results indicate that the activity of any shRNA can be significantly improved by the novel loop design.

To investigate whether the loop optimization functions in different cell types, experiments with the set of R/T5 inhibitors were repeated in Vero, HeLa and C33A cells. These cells were co-transfected with the appropriate reporter plasmid and the shRNA constructs were titrated (Fig. 4). The highest knockdown activity was observed for the shR/T5 with the miRNA loop in all cell lines. The knockdown activity of shR/T5 mir-25 is on average 3–4 times greater as compared to shRT5 wt. The impaired shRNA mut4 showed a similar decrease in activity in all cell types.

To determine if the improvement of shRNA loop design can be used in different expression cassettes, we cloned shRNAs under the transcriptional control of the RNA polymerase III promoter U6. Three shRNA loop variants targeting pol47 were selected (wt, mut4 and mir-25) and their activity was compared to the original H1 constructs in transfected 293T cells. In both expression systems, we observed reduced knockdown efficiency for mut4 versus wt and improved activity for mir-25 versus wt (Fig. 5). These combined results suggest that the improvement of the shRNA design is a general phenomenon, which can be applied to any shRNA inhibitor, different cell lines and diverse shRNA-expression systems.

3.4. Improved shRNA loop design results in higher siRNA levels

To investigate why specific shRNA constructs are more effective in silencing their target gene, Northern blot analysis was performed to determine the siRNA levels in transfected 293T cells (Fig. 6). We tested the same set of loop variants (wt, mut4, mir-17, mir-25, GRNA) for the potent (pol47), intermediate (R/T5) and weak inhibitor (pol9). The mir-17 and in particular the mir-25 variants produced higher levels of siRNAs than the original wt shRNA in transfected 293T cells. Quantification of the bands showed a 1.4 (pol9) to 2.1-fold (R/T5) increase in siRNA concentration for the mir-25 loop. There may be subtle differences between the shRNAs with regard to the loop effect. For instance, mir-25 clearly seems to produce more siRNAs than mir-17 in the R/T5 context, but these two loops behave more similarly in the pol47 and pol9 context. Most importantly, the observed differences in siRNA concentration do correlate with the observed differences in RNAi knockdown activity. Thus, the mir variants are most active because they trigger the production of the highest concentration of siRNAs. In fact, the correlation also seems to hold on the negative side, as the poorly active mut4 variant produces hardly any siRNAs.

3.5. Stronger inhibition of HIV-1 production with novel loop design

The optimized shRNAs were tested for their ability to inhibit HIV-1 production. We used a relatively low amount of the wt and loop-mutated shRNA constructs in a co-transfection assay with the HIV-1 molecular clone pLAI. Virus production was measured by CA-p24 ELISA in the culture medium after 2 days. The amount of virus production with each inhibitor was related to that obtained in the control co-transfection with pBluescript (pBS), which was set at 100%. An irrelevant shRNA (shluc) served as negative control. The same loop effects were observed for the different shRNAs on HIV-1 production as in the luciferase assays (Fig. 7, compare to Figs. 2 and 3). Knockdown activity was lost for mut4 and the two miRNA loops yielded significant improvements. The most extreme example is R/T5 with the mir-25 loop, showing 90% knockdown compared to only 40% for the wt R/T5.

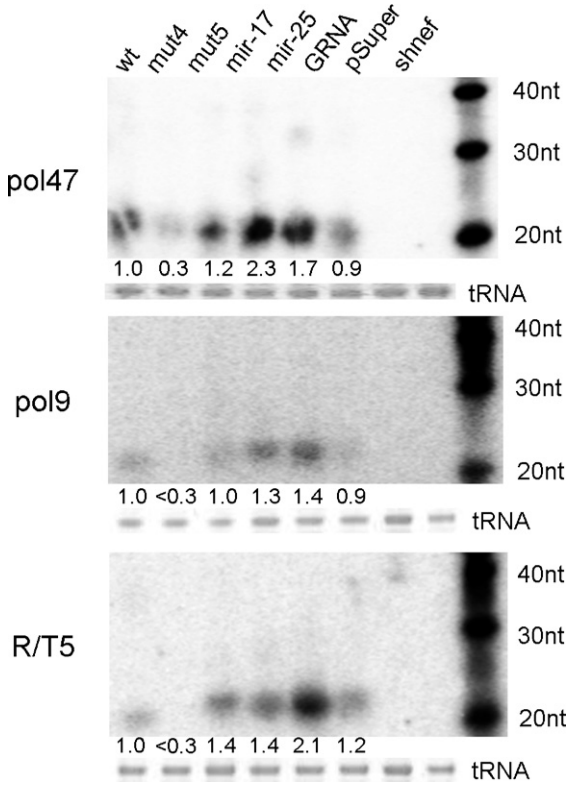

Fig. 6. Improved shRNA by novel loop design result in higher siRNA levels. 293T cells were transfected with shpol47, shpol9 or shR/T5 constructs with novel loops and the siRNAs were detected by Northern blot of total RNA using a 19-nt antisense complementary LNA probe. An irrelevant shRNA (shnef) and empty vector (pSuper) were used as negative controls. Ethidium bromide staining of tRNA served as sample loading control. Numbers below indicate the relative siRNA concentration compared to the wt sample.

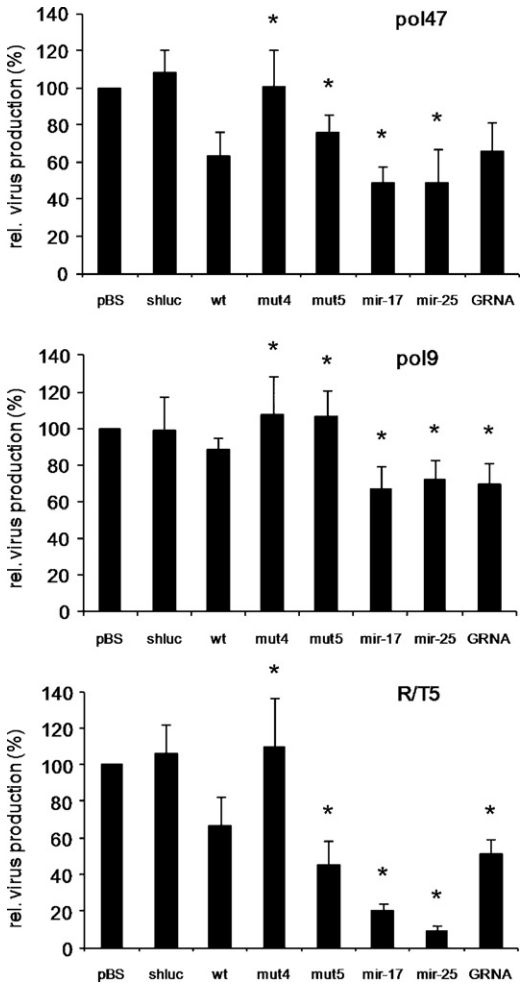

Fig. 7. Inhibition of HIV-1 production by the shRNA with novel loops. 293T cells were co-transfected with 100 ng pLAI, 0.5 ng of renilla luciferase plasmid and 1 ng of the corresponding shRNA constructs. CA-p24 levels in culture supernatant and renilla luciferase expression were measured. Values obtained for the irrelevant shRNA (shluc) served as a negative control and pBS values were set at 100%. Averages and standard deviations represent at least three independent transfections performed in quadruple. $^{*}p < 0.05$ compared to wt.

4. Discussion

RNAi is widely used as a research tool for gene knockdown studies and it is in development as a therapeutic approach, for instance against pathogenic viruses. The RNAi mechanism can be induced by transfected siRNAs or intracellularly expressed shRNAs. When comparing the efficacy of matching siRNA and shRNAs pairs, we frequently observed discrepancies in their activity, e.g. the siRNA was active and the shRNA was not. Thus, caution should be taken when translating an active siRNA into a shRNA species. The obvious difference between siRNA and shRNA molecules is the loop sequence in the latter that connects the sense and antisense sequence. Other differences include the intracellular synthesis (shRNA) versus delivery route (siRNA). ShRNAs are expressed in the nucleus and transported to the cytoplasm where they are processed by Dicer, in contrast to siRNAs that are transfected as active molecules in the cytoplasm (Leung and Whittaker, 2005).

The observed differential shRNA/siRNA activity may suggest that the loop sequence of the shRNA affects its activity. We tested the influence of different shRNA loops that were compared to the standard pSuper design, the Brummelkamp loop (Brummelkamp et al., 2002). We documented that RNAi activity can be improved significantly by novel loop design, indicating that the Brummelkamp loop is not the most optimal one for effective shRNA-induced gene silencing. The miRNA-derived loops resulted in the most efficient target knockdown in a variety of cell lines, even transforming moderate inhibitors into very strong inhibitors. Furthermore, the use of a different RNA polymerase III promoter (U6 instead of H1) did show the same improvement of RNAi activity by novel loop design. Table 1 gives an overview of all novel loops regarding size, structure and activity. All together, it seems that the size, structure and sequence of the shRNA loop affects knockdown efficiency of shRNAs. Establishing conclusively the generalizability of loop effects is very difficult, to some extent because of possible experimental confounders, but especially because of the large number of potential co-factors that contribute to knockdown (delivery method, stem length, stem sequence, plus multiple facets of loop structure itself such as length, structure, specific sequences).

The sequence and structural configuration are important features of shRNA molecules and have been shown to influence the silencing activity of the hairpin (McManus et al., 2002). The miRNA-derived loops significantly improved gene knockdown in reporter assays and inhibition of HIV-1 production. We showed by Northern blot analysis that more siRNA molecules are produced from these miRNA variants, which explains the improved RNAi activity. The loop may influence the intracellular shRNA stability, the nuclear export or Dicer processing. Future studies should address the possibility that loop sequences affect the actual Dicer cleavage site, which will lead to the production of different siRNA molecules with potentially different activity.

The loop sequence is likely to have an effect on the processing of the shRNA, e.g. Dicer recognition and/or cleavage may occur more efficiently (Hinton et al., 2008; McManus et al., 2002). Recog-

nition elements or sequences within the shRNA that determine the processing efficiency have not been examined in detail. The loop sequence of the hairpin may function as a binding site for a co-factor that determines Dicer specificity (Vermeulen et al., 2005). Our results do suggest that such a putative co-factor should be expressed in diverse cell types. Recently it was shown that a class of miRNAs is recognized by KSRP (KH-type splicing regulatory protein) through high affinity binding to sequences in the loop, which facilitates both nuclear Drosha- and cytoplamic Dicer-mediated processing (Trabucchi et al., 2009). The latter protein is able to adapt to a broad range of single stranded RNA sequences, thus explaining its activity with a wide variety of miRNA precursors. For pre-miRNAs it has been shown that the loop nucleotides play a critical role in controlling the distinct activities of these molecules, both the strength and specificity were controlled by the pre-miRNA loop sequence. Mutations within these loop sequences affected or even completely abrogated miRNA processing (G. Liu et al., 2008a; Michlewski et al., 2008). It was described that hnRNP A1, a protein implicated in many aspects of RNA biosynthesis and processing, binds to a miRNA cluster to specifically promote the production of the pri-miR18a unit (Guil and Cáceres, 2007). This ubiquitously expressed protein was shown to act as an auxiliary factor at the level of Drosha processing. Subsequent studies indicated that hnRNP A1 binds to the loop of pri-miR-18a and induces a relaxation of the stem, creating a more favorable cleavage site for Drosha (Michlewski et al., 2008). Interestingly, the same study mentioned that the high sequence conservation of certain miRNA loop sequences throughout evolution suggests the requirement for other auxiliary factors that bind to facilitate miRNA processing. This could be the explanation for the superiority of miRNA sequences among the set of loop-mutated shRNA variants in this study. Consistent with this idea is the observation that improved shRNA activity coincides with improved shRNA processing.

The finding that target inhibition by shRNA can be improved dramatically by novel loop design is very useful for the development of antiviral shRNA used in clinical applications. The shRNA optimized in this study are effective HIV-1 inhibitors, in which shpol47 is classified as a strong inhibitor, and R/T5 and pol9 as an intermediate and weak inhibitor, respectively (von Eije et al., 2009). It is interesting to document that weak shRNA inhibitors can be transformed into intermediate or even strong inhibitors by simple replacement of the loop sequence. This is important as most designed shRNA molecules turn out to be poorly active. On average, about one of four shRNAs are active, due to the absence of efficient shRNA design algorithms (Sen et al., 2004; Shirane et al., 2004; ter Brake et al., 2006). In contrast, there are multiple efficient siRNA design algorithms, but our results indicate that these cannot be applied to shRNA design. A previous study indicated that siRNA rules, while quite good at predicting effective siRNA, are bad at predicting effective shRNAs (Taxman et al., 2006). Altogether, this work shows that there is a lot to gain from novel loop design, which can trigger the development of much more effective shRNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.02.320.

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