

Strand-specific silencing of a picornavirus by RNA interference: Evidence for the superiority of plus-strand specific siRNAs

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Abstract

RNA interference triggered by small interfering RNAs (siRNAs) can be used to effectively contain viral spread. Here, we report on the mechanism of action of siRNAs targeting the medically important coxsackievirus B3 (CVB-3) as a typical representative of viruses with a non-segmented RNA genome in positive-strand orientation. Antiviral siRNAs can be designed to target the genomic (+)-strand, the (–)-strand that occurs as a replication intermediate, or both. In the present study, two complementary and systematic approaches are presented providing direct evidence that silencing of the viral (+)-strand is the key to inhibit CVB-3: first, we used rational siRNA design to direct silencing activity specifically against either of the two viral strands. As a second approach, we employed siRNA containing modified nucleotides to render them specific for one of the virus RNAs. Experiments with infectious coxsackievirus revealed that the inhibitory efficiency correlates exclusively with the activity of the siRNAs directed against the viral (+)-strand. Our finding that only (+)-strand specific siRNAs exert significant antiviral potency may hold true for other RNA viruses with (+)-stranded genomes as well and may therefore be helpful in the development of efficient strategies to inhibit virus propagation.

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1. Introduction

CVB-3 infection is one of the most frequent causes of myocarditis in humans. The infection becomes chronic in a significant number of patients, leading to dilated cardiomyopathy, a life-threatening disease that frequently requires heart transplantation (Kim et al., 2001). CVB-3 is a member of the genus Enterovirus within the family Picornaviridae, which comprises a number of clinically relevant pathogens including human rhinoviruses, polioviruses, echoviruses, and foot-and-mouth disease virus (summarized in Racaniello, 2001;

Zeichhardt and Grunert, 2003). After entering the host cell via the coxsackievirus-adenovirus receptor, the replication cycle of CVB-3 proceeds exclusively in the cytoplasm. The genome of picornaviruses consists of a single RNA strand of ~7200–8500 bases that serves as mRNA immediately after entering the host cell. A single open reading frame codes for a viral polyprotein which is cleaved cotranslationally. The genomic RNA strand, referred to as the (+)-strand, is then transcribed into a complementary (–)-RNA strand by the viral enzyme RNA-dependent RNA polymerase (RdRP) (Hung et al., 2002; Cornell et al., 2004). Resulting (–)-strands are used as templates for the generation of new viral mRNA and genomic RNA. In cells productively infected with poliovirus, considered to be the paradigm for enterovirus biology, viral (+)-strands were found at a 25–70-fold excess over (–)-strands (Giachetti and Semler, 1991; Novak and Kirkegaard, 1991). Similar ratios of (+)- to (–)-strand abundance were also found for other enteroviruses including CVB-3. Acutely infected cells contained approximately 100-

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times more CVB-3 (+)-strand than (–)-strand¹ (Hohenadl et al., 1991; Klingel et al., 1992).

Despite intensive efforts to develop specific therapeutic agents against coxsackieviruses and other picornaviruses (De Clercq, 2004; Barnard, 2006), current options for pharmacological treatment remain unsatisfactory. There is thus an obvious need to develop new antiviral strategies. RNA interference approaches have been shown to be a powerful means of repressing picornavirus reproduction in infected cells (e.g. Gitlin et al., 2002, 2005; Phipps et al., 2004). We and others have recently described the inhibition of coxsackievirus B3 (CVB-3) by means of RNA interference in cell culture (Ahn et al., 2005; Schubert et al., 2005; Werk et al., 2005; Yuan et al., 2005). Shortly afterwards, siRNA-induced RNAi was shown to slow down coxsackievirus propagation also in highly susceptible mice (Merl et al., 2005).

When designing a strategy to degrade virus RNA, the question arises whether to target the viral (+)-strand, the replication intermediate (–)-strand or both RNA species simultaneously. siRNAs, the triggering agents of RNA interference, are 21 mer double stranded RNA molecules. In principle, both strands are able to pair with their respective complementary sequences on one of the viral RNA species. Thus, both strands may induce degradation of the respective target RNA by recruiting the proteinaceous “RNA induced silencing complex” (RISC) to the virus RNA (for current reviews on RNA interference, see Eckstein, 2005; Leung and Whittaker, 2005). Most efficient siRNAs, however, are functionally asymmetric—only one of the two strands triggers target RNA degradation, while the other strand is degraded quickly. Thermodynamic parameters of the siRNA duplex determine which of the strands will be used as guide strand inducing target cleavage and which is the passenger strand that does not enter the RNAi pathway (Khvorova et al., 2003; Schwarz et al., 2003). For (+)-strand RNA viruses, using siRNA design algorithms on the genomic viral sequence will lead to the generation of (+)-strand specific siRNAs. In fact, recent studies have shown that (+)-strand specific siRNAs are efficient antiviral agents against picornaviruses (Gitlin et al., 2005; Yuan et al., 2005). Whether directing siRNAs against the viral (–)-strand enhances the antiviral potency of these agents remains unanswered.

We have now approached this problem in a systematic way and obtained direct evidence that targeting the viral (+)-strand is the superior strategy, even though the (+)-strand exists in 25–100-fold higher amounts within the cell (Giachetti and Semler, 1991; Novak and Kirkegaard, 1991; Hohenadl et al., 1991; Klingel et al., 1992). Using rational siRNA design (Amarzguioui and Prydz, 2004; Reynolds et al., 2004; Ui-Tei et al., 2004), we selected siRNA duplexes with different strand specificities. Only siRNAs with substantial activity against the (+)-RNA strand of CVB-3 were effective in inhibiting the virus.

Alternatively, siRNA specificity can be influenced by the incorporation of modified nucleotides like locked nucleic acids (LNA). These monomers are characterized by a methylene bridge connecting the 2'-oxygen and the 4'-carbon of the ribose (Fig. 1A) thereby restricting the nucleotide in its C3'-endo conformation. LNAs combine a number of desirable properties like low toxicity, nuclease resistance and high target affinity that make them valuable tools for antisense strategies and other types of biotechnological applications (for a review, see Kauppinen et al., 2006). Wahlestedt and co-workers recently reported that introducing LNA monomers at specific sites of an siRNA helix inhibits silencing by the modified strand of the siRNA without exerting negative effects on the unmodified strand (Elmén et al., 2005). The strand specificity of an siRNA double helix can be modified in this way without altering the sequence of the molecule. By selectively inactivating either strand of a functionally symmetric siRNA capable of silencing both viral strands, we were able to demonstrate that the antiviral potential relies almost exclusively on the siRNA strand complementary to the viral genomic RNA. Both methods show that inactivating the (+)-strand is the key to inhibiting picornaviruses. Targeting the (–)-strand has virtually no effect.

2. Materials and methods

2.1. siRNAs and oligonucleotides

Sequences of siRNAs used in this study are given in Table 1. LNA modifications were included as shown in the table and described in the text. siRNAs were designed using Dharmacon criteria (Dharmacon, Lafayette, CO) and scored according to the design criteria published by Reynolds et al. (2004): one point was given for a GC content between 30 and 52%; one point was added for each A or U at positions 15–19; another point was given for the lack of internal repeats; A at position 3 or at position 19, as well as U at position 10 resulted in addition of another point; a C or G at position 19, as well as a G at position 13 resulted in a one-point decrease. siRNA duplexes with or without LNA modifications containing symmetrical 3'-dTdT overhangs were purchased from MWG Biotech, Ebersberg, Germany, and Eurogentec, Liège, Belgium. Negative control siRNA without matches in human or viral genome was purchased from Qiagen, Hilden, Germany. Oligonucleotides used in PCR and cloning procedures were obtained from IBA, Göttingen, Germany and TIB MOLBIOL, Berlin, Germany.

2.2. Cloning procedures

cDNA of the RdRP of CVB-3 was obtained by reverse transcription and PCR-amplification of viral RNA as described elsewhere (Schubert et al., 2005). A translational UGA stop codon was added upstream of the viral subsequence during amplification. The resulting cDNA corresponding to bases 5911–7297 of CVB-3 (GenBank accession no. M33854) was then cloned into a pcDNA3.1/NT-GFP-TOPO vector (Invitrogen, Karlsruhe, Germany) to yield GFP-RdRP fwd. To obtain a plasmid producing the corresponding (–)-strand RNA, the gene of interest was cut

¹ Throughout the text, the terms (+)- and (–)-strand will be used for viral RNA only. In contrast, the terms sense and antisense strand will refer exclusively to siRNAs, with the sense strand being complementary to the viral (–)-strand and the antisense strand binding to viral (+)-strand in all cases.

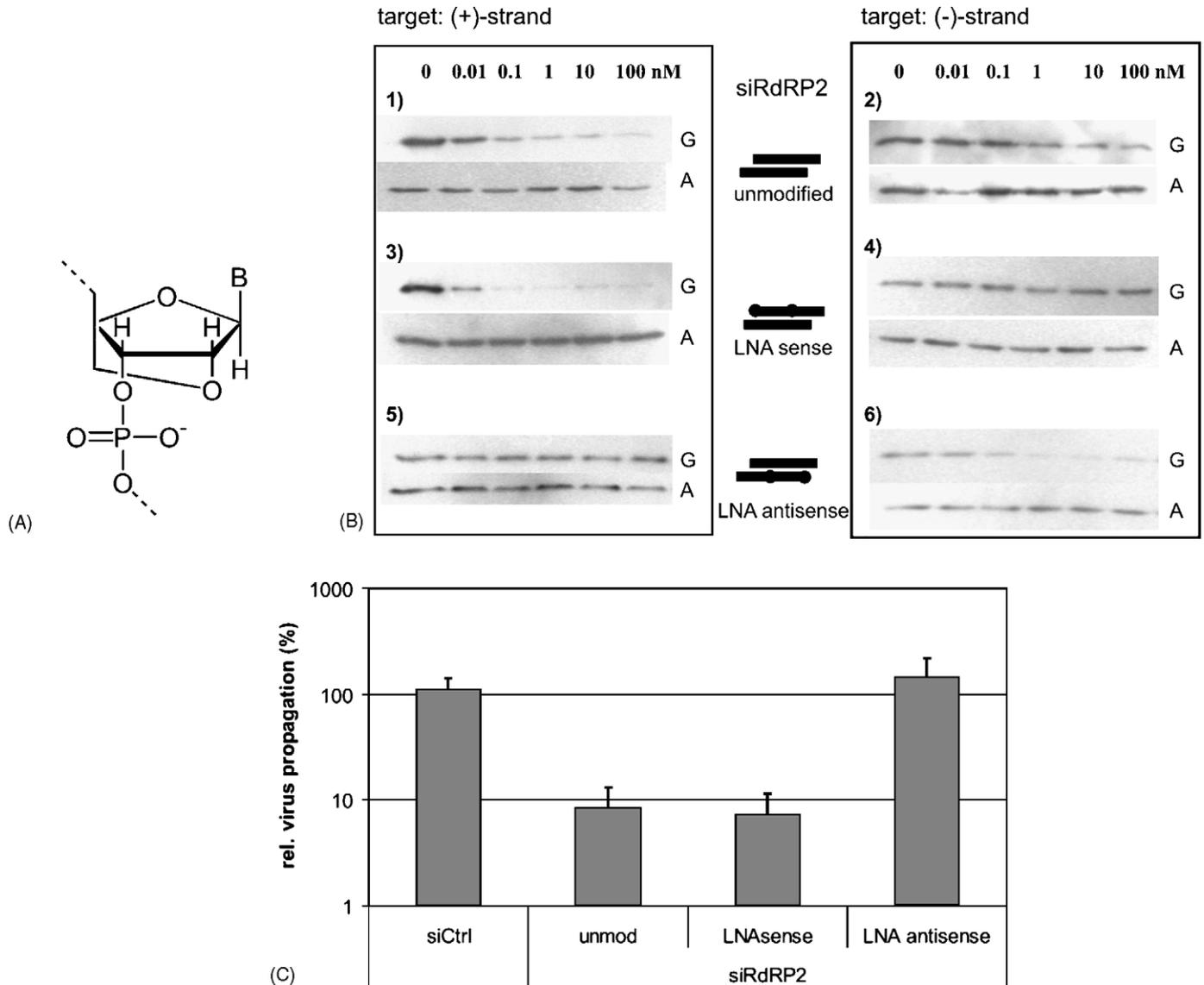


Fig. 1. Strand selectivity and antiviral activity of siRdRP2. (A) Molecular structure of locked nucleic acid (LNA). (B) Western blot of GFP-reporter assay. The target construct contains the siRdRP2 target site in (+)-strand (left) or (-)-strand orientation (right). Inactivation of the sense or antisense strand of the siRNA by the introduction of LNA monomers is indicated (LNA sense, LNA antisense). GFP-reporter expression 'G' and actin bands 'A' as a loading control are shown. (C) Plaque reduction assay. Relative reduction of plaque forming units by 100 nM of the siRNAs as indicated.

out of GFP-RdRP fwd using Bst XI and reinserted in reverse orientation yielding GFP-RdRP rev. In this construct, a translational stop-codon (UAG) appears at bases 25–27 of the inserted reverse RdRP sequence.

2.3. Cell culture, transfection and immunoblotting

Cos-7 cells were grown as described in Schubert et al. (2005). At 24 h prior to transfection, cells were plated in 24-well plates at a density of 0.7×10^5 cells/well in a volume of 500 μ l. Then, 0.8 μ g target RNA expressing vector and the appropriate amount of siRNA were transfected by using 2.5 μ l Lipofectamin 2000 (Invitrogen) per well, according to the manufacturer's instructions.

Cells were lysed 24 h after transfection. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF

membranes. Details of the immunostaining procedure are described elsewhere (Schubert et al., 2005). Equal loading of the lanes was confirmed by stripping the membrane and reprobing with actin-specific antibodies. The same results were obtained in at least two independent experiments.

2.4. Luciferase assay

cDNA of viral RdRP was cloned in either forward or reverse orientation into the multiple cloning site of psiCheck-2 vectors (Promega, Mannheim, Germany) downstream of the renilla luciferase stop codon. Cos-7 cells were co-transfected with 0.8 μ g of vector and different amounts of siRNAs. At 24 h after transfection, cells were lysed with 100 μ l passive lysis buffer (Promega, Madison, WI). Expression of renilla luciferase and firefly luciferase from the same vector was measured in a Turner

Table 1
siRNA sequences used in this study and predicted strand preferences

	siRNA sequence	Target nucleotides	Predicted strand specificity (scores) (+):(-)
siRdRP2	CUAAGGACCUAACAAAGUdTdT dTdTGAUUCUGGAUUGUUCAA	6315–6333	8:4
siRdRP2 LNA sense	<u>C</u> UAAGGACCUAACAAAGUdTdT dTdTGAUUCUGGAUUGUUCAA	6315–6333	
siRdRP2 LNA antisense	CUAAGGACCUAACAAAGUdTdT dTdTGAUUCUGGA <u>A</u> UUGUUCAA <u>A</u>	6315–6333	
Sirev 1	UCUCAUAGCAUUGAUUACdTdT dTdTAGAGUAUCGUAACUAAUG	6594–6612	5:8
Sirev 2	UUACUCGUGAAGCUGGUAdTdT dTdTAAUGAGCGACUUCGACCAU	6937–6955	6:7
Sirev 3	AAUUAGGACACUAAUGCUAdTdT dTdTUUAAUCCUGUGAUUACGAU	6825–6843	6:8
Sirev 3 LNA sense	<u>A</u> AAUUAGGAC <u>A</u> CUAAUGCUAdTdT dTdTUUAAUCCUGUGAUUACGAU	6825–6843	
Sirev 3 LNA antisense	AAUUAGGACACUAAUGCUAdTdT dTdTUUAAUCCUG <u>U</u> GAUUACGA <u>U</u>	6825–6843	
siCtrl	UUCUCCGAACGUGUCACGUDdTdT dTdTAAAGAGGCUUGCACAGUGCA	None	

Underlined bases are LNA monomers. Numbering of target nucleotides using GenBank acc. no. M33854. Scores according to Reynolds et al. (2004), see text for details.

TD 20/20 luminometer using the Dual Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Inhibition of gene expression was calculated by comparing renilla/firefly ratios between cell lysates. Values given are means and standard deviation for three independent experiments.

2.5. Plaque reduction assay

Plaque reduction assays were performed as described in Werk et al. (2005). Briefly, HeLa (Wis) cells (courtesy of Dr. R.R. Rueckert, Madison, WI) were grown in confluent monolayers in 24-well plates, transfected with siRNAs and infected with CVB-3 (Nancy, ATCC no. VR-30) in serial dilutions for 30 min to achieve 10–50 pfu/well. Subsequently, cells were overlaid with agar. After 3 days of incubation at 37 °C, cells were stained with neutral red. Viral titers were determined by plaque counting. Values given are means and standard deviations of at least two independent experiments, each performed in duplicate.

3. Results

3.1. Strand preference of siRdRP2

We recently described the generation of an siRNA against CVB-3 that efficiently inhibited viral replication in cell cul-

ture experiments (Schubert et al., 2005). This siRNA, dubbed siRdRP2, was designed to target the gene for viral 3D RNA-dependent RNA polymerase (RdRP) within the CVB-3 RNA (+)-strand. However, the fact that siRNAs are double-stranded could also mean that the sense strand of the siRNA, which is complementary to the viral (–)-strand, mediates the siRNAs antiviral activity. We decided to investigate the ability of each individual strand of siRdRP2 to silence the expression of a matching sequence to clarify the question of which strand is the active agent. The subgenomic region of CVB-3 containing RdRP was placed in either (+)- or in (–)-strand orientation downstream of a GFP reporter gene. Silencing of gene expression by unmodified or modified siRNAs was analyzed by Western blotting with antibodies against GFP (labeled 'G' in Fig. 1B) and actin (A) as a loading control.

Fig. 1B shows downregulation of the GFP-reporter by siRdRP2. While the (+)-strand target was silenced at siRNA concentrations in the subnanomolar range (gel no. 1), interference with (–)-strand expression required 10–100-fold higher concentrations of the siRNA (gel no. 2); we conclude that siRdRP2 has a higher activity against the (+)-strand than against the (–)-strand. To verify that only one of the siRNA strands is involved in virus inhibition, we inactivated either of the two strands separately. Introduction of LNA monomers, depicted in Fig. 1A, at positions 1 and 10 of an siRNA strand has been

reported to prevent silencing by that strand without impeding activity of the other strand (Elmén et al., 2005). This method allowed us to render the siRNA (+)-strand specific by introducing LNA into the sense strand of the siRNA, or (–)-strand specific by introducing LNA into the antisense strand. As can be seen in Fig. 1B, incorporation of LNA into the sense strand of the siRNA drastically impeded silencing of the (–)-strand (gel no. 4), but even slightly increased silencing activity against the (+)-strand (gel no. 3). A similar observation has been reported by Elmén et al. (2005). The increase in activity is probably due to enhanced loading of the active strand into RISC, as competition by the complementary strand is lost. Incorporation of LNA into the antisense strand of the siRNA led to a loss of silencing activity against the (+)-strand (gel no. 5), while retaining its potency against the (–)-strand (gel no. 6).

The inhibitory effect of siRdRP2 on the infectious virus is in line with these observations (Fig. 1C): inactivation of the sense strand made the siRNA (+)-strand specific. This modification had no detrimental impact on virus inhibition. In contrast, eliminating antisense strand activity resulted in an siRNA that was active against the viral (–)-strand. This modification completely abolished the antiviral activity of siRdRP2. These results demonstrated that the high efficacy of siRdRP2 resides in the strand directed against viral (+)-RNA.

3.2. (–)-Strand silencing by rationally designed siRNAs

siRdRP2 had been deliberately targeted against the viral (+)-strand. In a study of the parameters governing asymmetric incorporation of siRNA strands into the effector complex RISC, Reynolds et al. (2004) established a scoring system to quantify the propensity of an individual siRNA strand to enter RISC. In this system, a score greater than 6 denotes an siRNA strand that will be loaded into RISC efficiently and is thus likely to be a good silencing agent. The scores for the two siRdRP2 strands binding to viral (+)- and (–)-RNA were calculated to be 8 and 4, respectively, meaning this siRNA will probably be (+)-strand silencing. The fact that (+)-strand inhibition is the primary mode of action of siRdRP2 therefore came as no surprise, but the question remained: which approach is superior in preventing the spread of virus—silencing the abundant genomic (+)-strand or targeting the (–)-strand which occurs during replication?

First, it was necessary to find siRNAs with specificity for the respective viral strand in order to evaluate the applicability of (–)-strand silencing. Three siRNAs were selected whose thermodynamic parameters would favor (–)-strand silencing over (+)-strand inhibition (sirev 1–3). The freely available web-based tool by Dharmacon (www.dharmacon.com) was used, which employs the latest criteria for the design of active siRNAs. An experimental evaluation of strand specificity was conducted using a luciferase reporter assay. The RdRP sequence in either orientation was contained in the 3'-UTR of a renilla luciferase reporter. Fig. 2A and B shows inhibition of renilla luciferase by each of the siRNAs normalized to the constitutive expression of firefly luciferase from the same vector. Estimated values for the concentration at which half-maximal inhibition was observed (IC_{50}) and for maximum inhibition are summarized in Table 2.

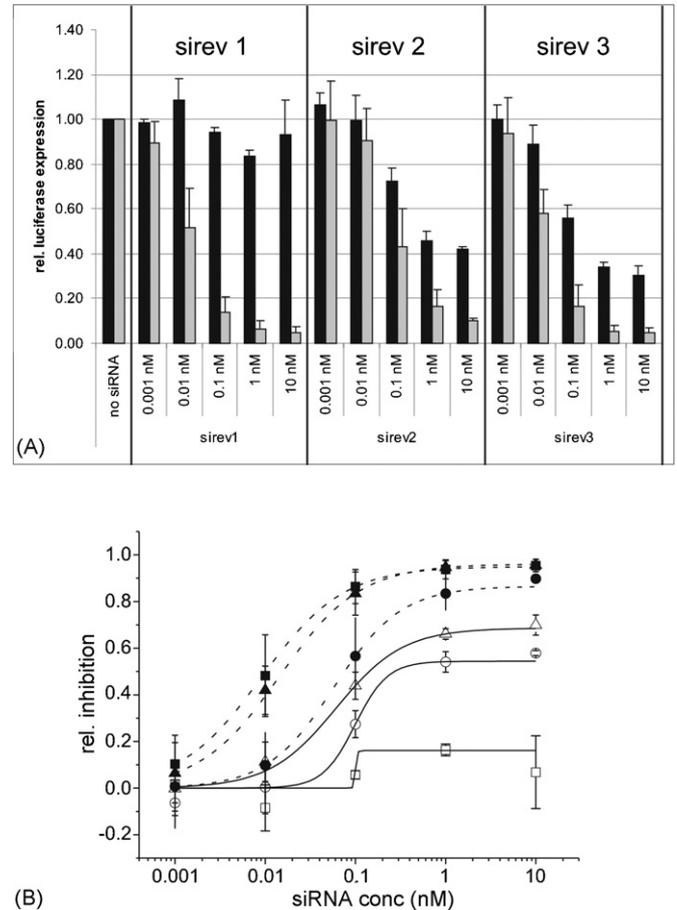


Fig. 2. Strand specificity of sirev 1–3. (A) Knock-down of luciferase reporter expression by sirev 1–3. Reporter constructs contained the target sequence in (+)-strand (black bars) or (–)-strand orientation (gray bars). Luminescence of renilla luciferase was normalized to firefly luciferase activity for each experiment individually. (B) Quantification of reporter gene inhibition with the target site in (+)-strand orientation (open symbols, solid line) or (–)-strand-orientation (closed symbols, dashed line). Squares, sirev 1; circles, sirev 2; triangles, sirev 3.

These results confirm the theoretically predicted activity of the siRNAs against the (–)-strand: all three siRNAs showed 80–90% knock-down of the reporter, even at a concentration as low as 1 nM, when acting against their respective target sites in (–)-orientation. Efficacies against viral (+)-RNA varied between ~10% and almost 70%: sirev 1 is strictly (–)-strand specific, sirev 2 has significant activity against the (+)-strand, and sirev 3 is a strong inhibitor of both (+)- and (–)-strand expression.

Table 2
Approximated IC_{50} values and maximum inhibition by sirev 1–3

	IC_{50} (nM)		Maximum inhibition (%)	
	(+)-Strand	(–)-Strand	(+)-Strand	(–)-Strand
Sirev 1	0.11 ± 0.01	0.012 ± 0.009	16 ± 2	96 ± 4
Sirev 2	0.10 ± 0.01	0.06 ± 0.03	57 ± 3	87 ± 10
Sirev 3	0.06 ± 0.02	0.02 ± 0.02	70 ± 2	95 ± 3

All values are means and standard deviations for at least three independent experiments.

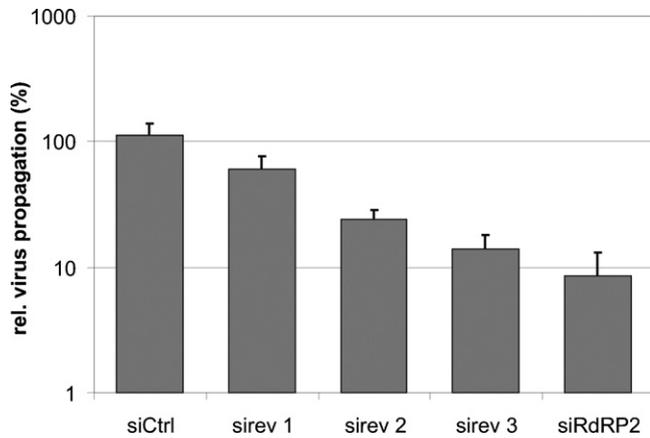


Fig. 3. Antiviral effect of siRNAs. Relative CVB-3 propagation in a plaque reduction assay with 100 nM of the indicated siRNAs normalized to virus-infected cells without siRNA.

We were now equipped with four siRNAs whose strand specificities varied from higher (+)-strand inhibition (siRdRP2) to virtually exclusive activity against the (–)-strand (sirev 1). These siRNAs were then employed in plaque reduction assays with infectious CVB-3 to determine which strategy would be superior: targeting the viral (–)-strand (by sirev 1) or both strands simultaneously (with sirev 3) or attacking solely the viral (+)-strand. The impact of the siRNAs on viral propagation normalized to virus replication in untransfected cells is shown in Fig. 3. Interestingly, efficacy of virus inhibition was found to correlate with the ability of the respective siRNA to target the viral (+)-strand, while mainly (–)-strand specific siRNAs were clearly inferior. We conclude from these findings that (+)-strand specific siRNAs are more promising antiviral agents than (–)-strand specific species.

3.3. Selective strand inactivation by introduction of LNAs

We then chose to approach the question in a different and complementary way by investigating how each strand of a functionally symmetric siRNA contributes to virus inhibition. As shown above (Fig. 2), sirev 3 works with almost equal efficiency against both strands of CVB-3. It may be argued that the sirev 3's high antiviral activity results from the fact that its strands are functional against both virus RNAs. We tested this hypothesis by modifying the sense and antisense strand of sirev 3 individually as described above, i.e. we introduced LNA monomers to stop silencing activity of the strands individually. We were thus able to turn sirev 3 into a strand-specific siRNA targeting either only viral (+)- or (–)-strand. Fig. 4A demonstrates that unmodified sirev 3 silences reporter gene expression in both orientations of the target RNA, whereas specific siRNA inactivation leads to orientation-specificity for subgenomic viral RNAs.

The question was: if this bispecific siRNA only targets one strand, how would that effect its antiviral efficacy? Fig. 4B shows the antiviral effect of the three sirev 3 species as quantified in a plaque reduction assay. sirev 3 in its (–)-strand specific form, that is LNA antisense, is completely inactive against CVB-3. Rendering the siRNA (+)-strand specific by

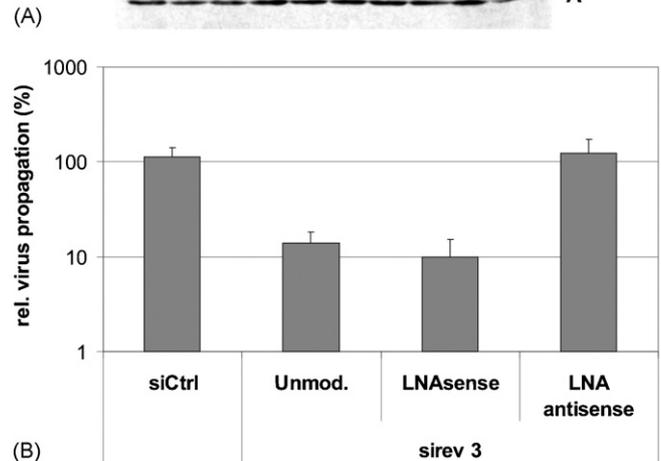
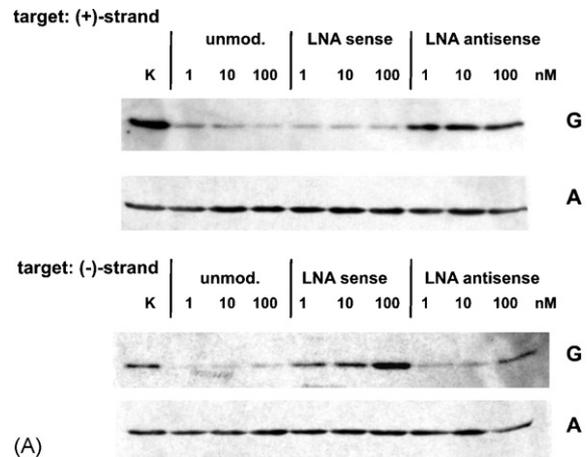


Fig. 4. Strand specific antiviral activity of sirev 3. (A) Western blot of GFP-reporter assay. Indicated amounts of unmodified sirev 3 or sirev 3 containing LNA monomers in sense or antisense strand were employed. GFP 'G' and actin 'A' levels are shown. (B) Plaque reduction assay. Relative virus propagation in cells transfected with 100 nM of the indicated siRNA.

incorporating LNA into the sense strand has no adverse effects on virus inhibition over the bispecific species—it even slightly increases efficacy of sirev 3. These observations confirm our results that efficient siRNAs against CVB-3 function only by targeting viral (+)-strands. Using an siRNA specific for both virus strands does not increase antiviral efficacy.

4. Discussion

In recent years, RNA interference has been established as a suitable approach to inhibit virus propagation both in vitro and in vivo (reviewed by Haasnoot et al., 2003; Colbère-Garapin et al., 2005; Ryther et al., 2005; Haasnoot and Berkhout, 2006; Schubert and Kurreck, 2006). Several classes of medically relevant viruses consist of RNA genomes in (+)-strand orientation, among them Picornaviridae (e.g. polio-, coxsackie- and rhinoviruses), Flaviviridae (e.g. hepatitis-C-virus) and Coronaviridae. We consider these viruses to be particularly attractive targets for RNAi approaches because their genomic (+)-stranded RNA is directly employed as an mRNA by the cellular translational machinery and should therefore be accessible to siRNA-mediated degradation. We and others have successfully inhibited the important heart-pathogen coxsackievirus B3, a member of

the picornavirus family, using RNA interference (Ahn et al., 2005; Merl et al., 2005; Schubert et al., 2005; Werk et al., 2005; Yuan et al., 2005).

If the antiviral approach is to be further developed and optimized, we must identify the most critical virus-specific steps in the viral life cycle where the pathogen is most vulnerable to an attack by RNA interference. Possible targets for siRNAs could be incoming viral genomic RNA, mRNA or RNA serving as template in the replication process. It is still unclear whether the incoming (+)-strand can be targeted since this RNA may be protected in virions or by bound proteins. Circumstantial evidence has been accumulating that siRNAs directed against viral mRNAs are most efficient, but no direct and systematic approach has been developed which would clarify this pivotal question.

In the present study, we aimed to investigate virus inhibition by siRNAs in more detail so as to optimize the silencing strategy. During replication, (+)-strand RNA viruses produce a small number of antigenomic (–)-strands that serve as a template for the generation of further mRNA and genomic RNA molecules. In cells harboring actively replicating picornaviruses, (+)-strand RNA occurs in approximately 25–100-fold excess over the (–)-strand (Giachetti and Semler, 1991; Novak and Kirkegaard, 1991; Hohenadl et al., 1991; Klingel et al., 1992). Targeting the large pool of (+)-strand RNAs might therefore be much harder for the RNAi machinery than degrading the low abundant (–)-strands. Theoretically, each siRNA should target both strands, since the sense strand of the siRNA is complementary to the viral (–)-strand and the antisense strand should be capable of hybridizing to the viral (+)-strand. However, in practice a functional asymmetry of both siRNA strands due to thermodynamic features of the duplex has frequently been found to influence the potency of siRNA molecules (Khvorova et al., 2003; Schwarz et al., 2003). For this reason, if an siRNA is devised which contains an efficient antisense strand, the sense strand will often be a much weaker silencing agent. The important and as yet unanswered question is: which of the strands is the superior target for RNAi approaches? Or should siRNAs be designed to target both strands simultaneously?

Recently, Yuan et al. (2005) reported having inhibited CVB-3 replication by an efficient siRNA. The authors analyzed the mode of action of this particular siRNA. By introducing mismatches into the central region of either the sense or the antisense strand, they found that only a perfect match between viral (+)-strand and siRNA antisense strand supported efficient target cleavage. In this way, the authors proved that the siRNA used in their study was (+)-strand specific. This finding was not unexpected, since the design of siRNAs according to the currently used criteria automatically results in (+)-strand specificity. However, the results reported by Yuan et al. (2005) allow no general conclusions about the antiviral potential of (–)-strand specific siRNAs. It might be possible that such an approach may lead to the generation of superior antiviral tools.

The aim of the present study was therefore to design siRNAs with clear preferences to silence either of the viral strands. First, we employed rational siRNA design to generate siRNAs with a high degree of strand specificity. The scores of the siRNAs according to the classification system established by Reynolds

et al. (2004) are given in Table 1. All three siRNAs have a value greater than 6 for (–)-strand specificity and can be expected to exhibit good silencing of the (–)-strand; (+)-strand silencing can be expected to be less effective. Reporter assays confirmed that our set includes siRNAs with higher (+)-strand activity, with comparable activity against both strands and with almost exclusive (–)-strand inhibition.

When we challenged the siRNA with the complete virus in replication assays we found that siRNAs with almost exclusive (sirev 3) or predominant (sirev 2) minus-strand specificity had only minor inhibitory activity against CVB-3 (Fig. 3). The siRNA which is active against the viral RNA in both orientations had an improved antiviral potency, while the siRNA with higher activity against the viral (+)-strand revealed the highest efficacy. As a result of our initial reporter assays, we can be confident that the weak protection of the two former siRNAs against CVB-3 challenge is due to their inability to target the viral (–)-strand and not caused by poor loading of the siRNA sense strand into RISC.

We independently confirmed this finding by introducing LNA monomers into the siRNA to inactivate either of the strands (Elmén et al., 2005). Using the siRNA with stronger activity against the viral (+)-strand (i.e. siRdRP2), we observed a complete loss of antiviral activity when the antisense strand was inactivated by introducing LNA, rendering the siRNA (–)-strand specific. In contrast, the efficacy of the siRNA was not compromised by modifying the sense strand with LNA (Fig. 1C). This does not answer the question, however, as to which of the viral strands is the superior target for an RNAi approach.

To solve this problem, we selectively inactivated either one of the strands of sirev 3, which displayed comparable activity against both viral RNA species and potent inhibition of viral spread. Virus replication assays (Fig. 4B) then clearly showed that inactivating the siRNA antisense strand that targets viral (+)-RNA stops virus silencing completely; introduction of LNA into the sense strand which is complementary to virus (–)-RNA does not compromise antiviral activity, but even slightly enhances its potency. Most importantly, these findings demonstrate that the antiviral action of sirev 3, which is active against the target RNA in both orientations, is mediated exclusively by its activity against viral (+)-RNA.

Three explanations can be given for the superiority of (+)-strand silencing: (1) RISC acts as a sequence-specific endonuclease with a K_M value in the low nanomolar range (Haley and Zamore, 2004). It might therefore be that the concentration of the low abundant (–)-strand RNA is below the K_M , rendering the silencing machinery inefficient. (2) Picornavirus replication has been observed to occur in complexes that are known to be associated with membranous vesicles within cells (Egger et al., 2000). It is therefore tempting to speculate that the (–)-strand is shielded by the membrane system, proteins, or nascent RNA (+)-strands in the replication intermediate and therefore might not be accessible to RISC. In contrast, the (+)-strand might leave the replication complex as it also serves as mRNA and will then become accessible to silencing by RNAi. (3) Certain structures of viral genomes have been shown to be resistant to silencing by siRNAs (Westerhout et al., 2005).

Possibly the (–)-strand could adopt a conformation that blocks RNAi.

A recent study by Gitlin et al. (2005) points in the same direction. Rather than supplying siRNAs, the authors made use of the endogenously expressed microRNA let-7, only one strand of which is biologically active. Poliovirus was modified to contain the target site of let-7 in sense or antisense orientation, so that the microRNA would interfere with (+)- or (–)-strand viral RNA. Only when the target sequence was situated in the viral (+)-strand was the endogenous silencing agent let-7 capable of interfering with viral RNA. Even though the modes of action of microRNAs and siRNAs are closely related, the exact molecular mechanisms of microRNAs may be dependent on factors different from those that govern siRNA action. In addition, using exogenously applied siRNAs allows us to give a detailed characterization of siRNA action at various concentrations. Furthermore, our approaches enabled us to use the wild type coxsackievirus, whereas Gitlin et al. (2005) employed an artificially modified virus that might produce unnatural features.

In our present study, we investigated the mode of RNAi-mediated silencing of CVB-3 as a typical representative of the picornavirus family. We employed two complementary approaches to design strand-specific siRNAs and confirmed in reporter assays that our set of siRNAs covers molecules that are active specifically against either of the strand or against both strands simultaneously. Subsequent virus inhibition assays clearly revealed that virus silencing exclusively correlates with siRNA activity against the viral (+)-strand. This bias is most probably due to virus biology. Other (+)-stranded RNA viruses might thus show similar resistance to (–)-strand silencing. Based on our findings, siRNA approaches for these viruses should focus on targeting the genomic RNA. We expect that our conclusions may be of value for antiviral siRNA strategies against a large number of clinically relevant pathogens.

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