Design of LNA-modified siRNAs against the highly structured 5′ UTR of coxsackievirus B3

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Abstract This study describes a strategy to develop LNA-modified small interfering RNA (siRNAs) against the highly structured 5′ UTR of coxsackievirus B3 (CVB-3), which is an attractive target site due to its high degree of conservation. Accessible sites were identified based on structural models and RNase H assays with DNA oligonucleotides. Subsequently, LNA gapmers, siRNAs, siLNAs and small internally segmented interfering RNA (sisiLNAs) were designed against sites, which were found to be accessible in the in vitro assays, and tested in reporter assays and experiments with the infectious virus. The best siLNA improved viability of infected cells by 92% and exerted good antiviral activity in plaque reduction assays.
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1. Introduction

Coxsackievirus B3 (CVB-3) is a member of the picornavirus family, which contains a single stranded RNA genome in plus-strand orientation [1,2]. The 5′ UTR of CVB-3 plays an important role in key steps of the viral life cycle, replication and translation. To initiate the latter process, it contains an Internal Ribosome Entry Site (IRES), which allows protein synthesis in a cap-independent manner. CVB-3 can cause viral heart muscle infections, against which no specific drugs exist to date.

In the last years RNA interference (RNAi) has emerged as a technique that might develop into a new class of therapeutics [3]. It has particularly been used as a novel antiviral approach and the first clinical trials to use small interfering RNAs (siRNAs) or vector-expressed short hairpin RNAs have already been initiated [4]. Consequently, a number of groups have used RNAi-based approaches against CVB-3 [5–10]. Since the emer-

gence of escape mutants is a major challenge for all types of antiviral applications, the 5′ UTR of CVB-3 is a preferable target region for siRNAs: It is highly conserved and therefore unlikely to tolerate mutations and it is functional by structural features. While protein-coding regions will tolerate mutations in wobble positions, substitutions in the IRES will lead to structural alterations, thereby compromising its function. A previous study demonstrated that siRNAs against the conserved cis-acting replication element within the enterovirus coding region drastically reduces the risk to enrich escape mutants [11]. Interestingly, however, a number of siRNAs against the 5′ UTR of CVB-3 have been tested, but none of them exerted significant antiviral activity [8,9,12,13]. Since it is well known that target RNA structures can greatly modulate the efficiency of antisense oligonucleotides and ribozymes [14] it is conceivable that the failure to target the viral UTR by siRNAs is due to the tight structure of the IRES element. While several reports stress the importance of the thermodynamic features of the siRNA for its activity [15,16], others have shown that the structure of the target RNA might be detrimental for the silencing as well [17–19].

Locked nucleic acid (LNA) is a widely used modification to improve the properties of oligonucleotides like aptamers [20] and antisense oligonucleotides [21] due to its high target affinity and nuclease resistance. The incorporation of LNA is tolerated in various positions of the siRNA and can even improve its efficiency [22,23]. A three-stranded construct with two short strands complementary to the antisense strand dubbed small internally segmented interfering siRNAs (sisiRNAs) was recently shown to posses improved silencing properties [24]. Furthermore, LNA-modified siRNAs were successfully used for functional investigations of the silencing mechanism [25] and for in vivo applications [26].

Here, we describe a strategy to obtain active siLNAs targeting the tightly structured 5′ UTR of CVB-3. The approach comprises RNase H experiments, reporter assays in cell culture as well as virus assays and led to the identification of active molecules for the inhibition of virus replication.

2. Materials and methods

2.1. Oligonucleotides

DNA oligonucleotides and siRNAs were purchased from Purimex (Göttingen, Germany). LNA gapmers and LNA-modified siRNAs

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Abbreviations: CVB-3, coxsackievirus B3; IRES, internal ribosome entry site; RNAi, RNA interference; siRNA, small interfering RNA; sisiRNA, small internally segmented interfering RNA

were synthesized by RiboTask (Odense, Denmark). The sequences of all oligonucleotides are summarized in Supplementary Tables 1 and 2.

2.2. RNase H assay

The 5′ UTR of CVB-3 (nucleotides 1–742, GenBank accession No. M33854) was cloned into pcDNA 3.1/CT-GFP (Invitrogen, Karlsruhe, Germany). After linearization of the plasmid, the 5′ UTR was tran-

scribed in vitro using the Ribomax Kit (Promega, Madison, WI). RNase H assays were carried out as described previously [27] with an additional RNA renaturation step before starting the reaction.

2.3. Cell culture reporter assays

For reporter assays, a fusion construct of GFP and the viral 5′ UTR was used as described in [12]. Cos-7 cells were cotransfected with this plasmid and the respective oligonucleotide using Lipofectamine 2000
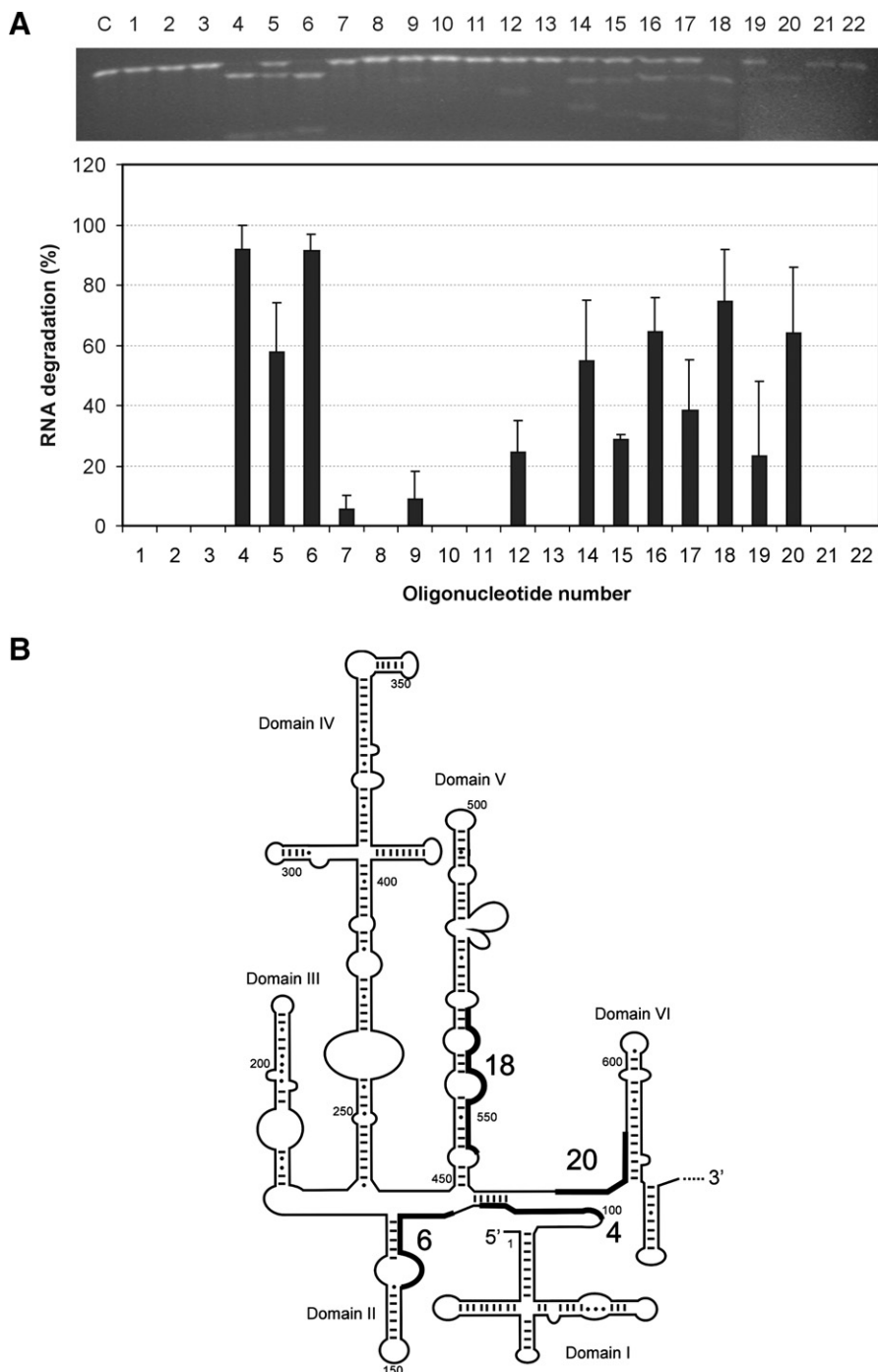

Fig. 1. Identification of accessible sites of the 5′ UTR of CVB-3. (A) Representative agarose gel showing degradation of in vitro transcribed RNA by RNase H. Lanes 1–22 represent RNA cleavage induced by 19mer DNA oligonucleotides against specific sites of the 5′ UTR. (C) Control reaction without DNA oligonucleotide. The diagram shows the extent of target RNA degradation. Average and S.D. of three independent experiments are given for each oligonucleotide. (B) Secondary structure model of the 5′ UTR of CVB-3 according to Bailey et al. [29]. Target sites of most efficient oligonucleotides according to the screening shown in (A) are indicated.

(Invitrogen). Twenty-four hours after transfection cells were harvested and a Western blot with rabbit GFP antiserum (Invitrogen) was carried out. The procedure is described in detail in [28] except that the ECL Kit (Pierce, Rockford, IL) was used for chemoluminescence.

2.4. Plaque reduction and cell viability assay

Plaque reduction and cell viability assays were performed as described before [12]. Briefly, HeLa cells were transfected with the respective LNA gapmer, siRNAs or LNA-modified siRNA and subsequently infected with CVB-3 (Nancy, ATCC No. VR-30). For plaque reduction assays, cells were overlaid with agar containing Eagle's MEM. Cells were stained with neutral red and the plaque number was counted after 3 days of incubation at 37 °C. Cell viability was determined with the Cell Proliferation Kit II 48 h post-inoculation (Roche, Mannheim, Germany).

3. Results and discussion

3.1. Identification of accessible target sites

For the development of an efficient siRNA against the 5′ UTR of CVB-3 we first aimed at identifying accessible target sites. Based on a recently published secondary structure model [29], we designed a set of 22 DNA antisense molecules being 19 nucleotides in length (Supplementary Table 1). Since these DNA oligonucleotides were supposed to help identifying suitable target sites for siRNAs, well-known thermodynamic features of siRNAs (relative stability of the ends, preference for bases in certain positions according to Reynolds et al. [30]) against these target sites were taken into consideration and sequences that would result in highly structured antisense strands of the siRNA were excluded, since this feature was also found to be detrimental to silencing [31]. Furthermore, regions which are prone to point mutations were omitted.

In order to assess accessibility of the target RNA for hybridization to complementary oligonucleotides, cleavage of an in vitro transcript of the 5′ UTR mediated by RNase H in the presence of either of the 22 DNA oligonucleotides was determined (Fig. 1A). Interestingly, only seven oligonucleotides were capable of inducing more than 50% target RNA cleavage and only two of them led to more than 90% degradation. The
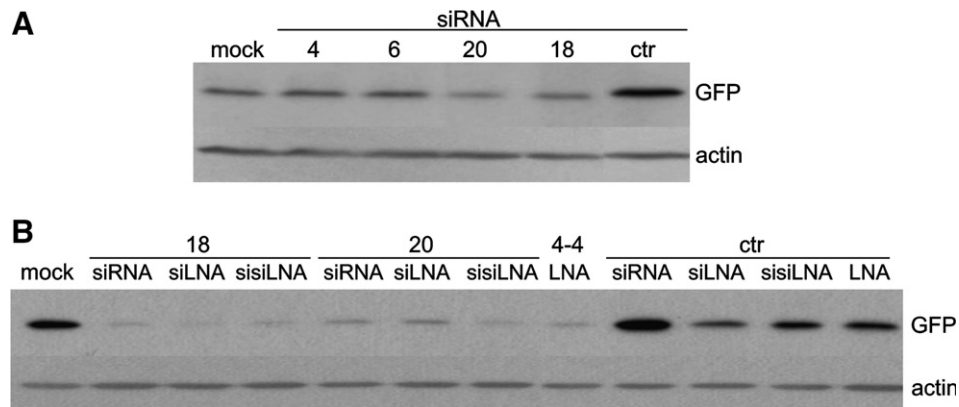

Fig. 2. Western blots showing downregulation of GFP reporter by LNA gapmers, siRNAs and modified variants thereof. (A) siRNAs Nos. 4, 6, 18 and 20 (at 100 nM concentration). (B) siRNAs Nos. 18 and 20 as well as LNA modified variants siLNA and sisiRNA at 100 nM concentration. LNA 4-4 was used at 20 nM concentration to minimize toxicity. Controls (ctr) are shown for each type of oligonucleotide at the respective concentration. Actin is shown as a loading control.
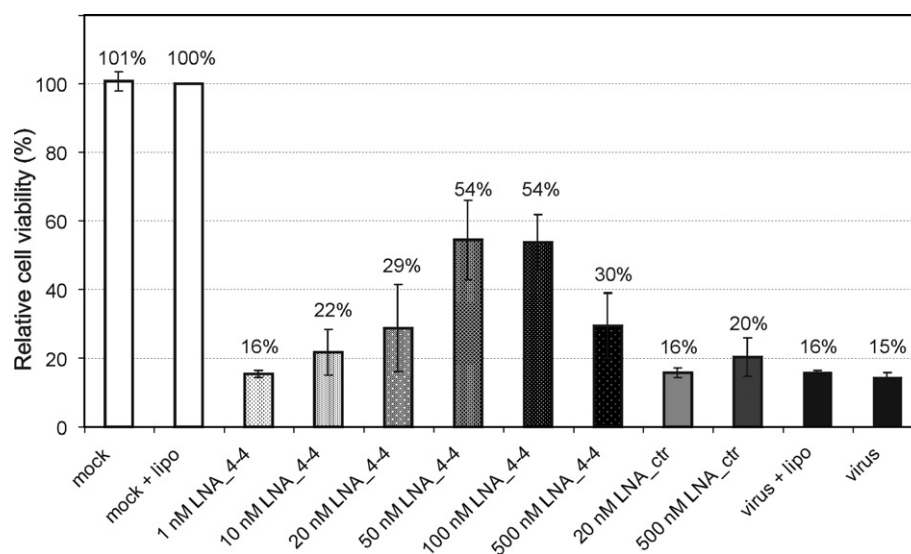

Fig. 3. Viability of HeLa cells 48 h after inoculation with CVB-3. Cells were pre-treated with increasing concentrations of the LNA gapmer 4-4. Mock-cells were neither transfected nor inoculated, mock + lipo-cells were treated with the transfection reagent only. LNA ctr: unrelated control oligonucleotide; virus and virus + lipo: cells inoculated with the virus in the presence or absence of the transfection agent. Average and S.D. of four independent experiments are given.

location of the four sites with highest accessibility to complementary 19mer oligonucleotides is depicted in the schematic secondary structure model in Fig. 1B.

3.2. Reporter assays

Four siRNAs and three LNA gapmers against the most accessible sites 4, 6, 18 and 20 (Supplementary Table 2) were tested in further assays. For the LNA gapmers a design with three LNA monomers at each end and a total length of 16 nucleotides was chosen. Since each 19mer sequence contains four 16mer sequences, further RNase H assays with 16mer DNAs were performed to identify the best target site for the LNA gapmer (data not shown). For the chosen LNA gapmers 4-4, 6-3 and 18-2 the first number reflects the target site, while the second indicates the first position of the 16mer within the 19mer sequence.

As a first qualitative test of the activity of various siRNAs and LNAs, GFP-reporter assays were carried out. To this end, the viral 5′ UTR was cloned downstream of GFP and co-transfection assays with the reporter plasmid and the

respective siRNA were performed. As can be seen in Fig. 2A, siRNAs Nos. 18 and 20 were found to be superior as compared to Nos. 4 and 6. This is somewhat surprising with regard to the results of the RNase H assay. It should be mentioned that a large scale comparison of siRNAs and antisense oligonucleotides binding the same position on the target mRNA revealed a good correlation of their activity, but some exceptions were noted [32]. A possible explanation was given recently, when Westerhout and Berkhout elegantly demonstrated that an accessible target 3′ end to be most important for RNAi-mediated inhibition [17], thus showing that siRNAs and antisense oligonucleotides not necessarily follow the same design rules in all cases.

To further improve the efficiency of the siRNAs, LNA monomers were incorporated into the sense strand and the 3′ end of the antisense strand. Furthermore, sisiLNAs with a segmented sense strand were synthesised (Supplementary Table 2). The modifications have previously been shown to enhance nuclease resistance, to increase efficiency by improving RISC loading and to reduce off-target effects [23,24]. Fig. 2B shows
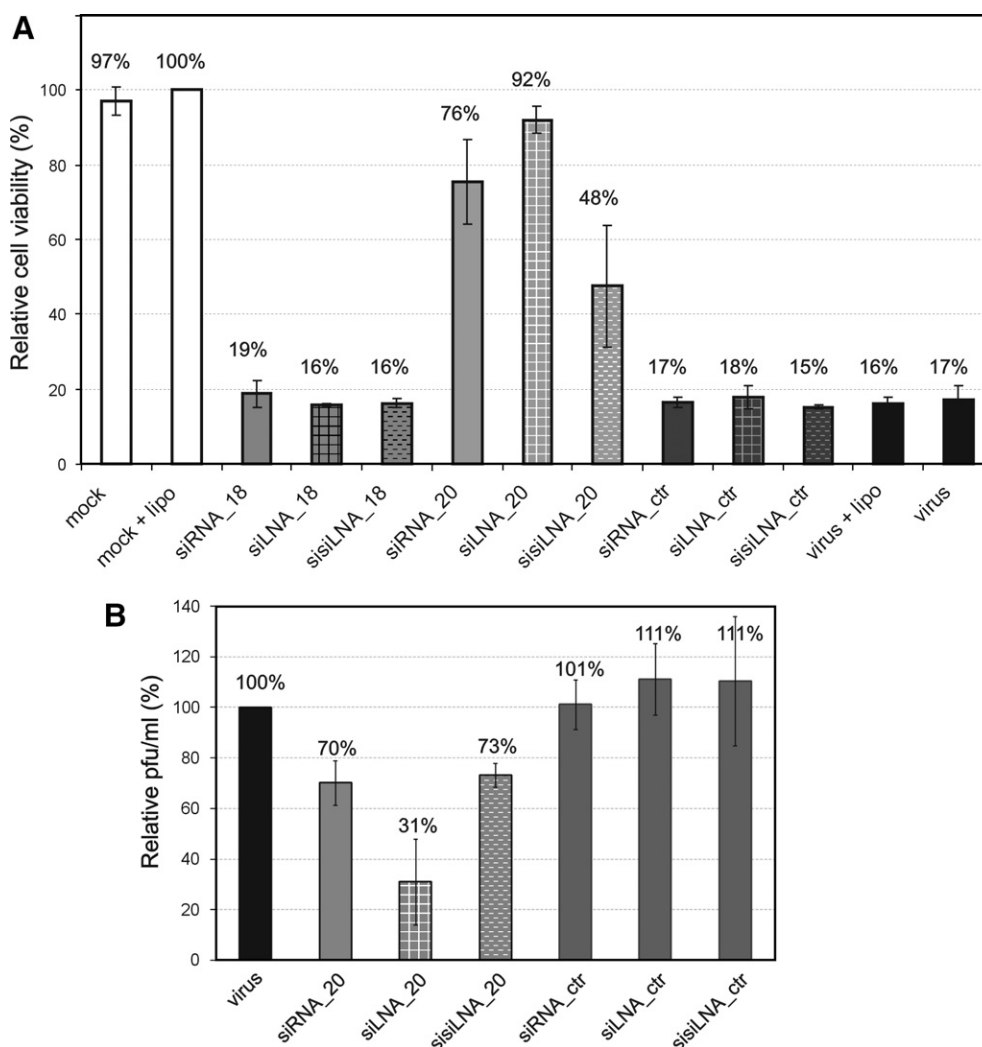

Fig. 4. Protective effect of siRNAs and LNA modified variants thereof. (A) Viability of HeLa cells 48 h after inoculation with CVB-3. Cells were transfected with 100 nM siRNA, siLNA and sisiLNA as indicated prior to inoculation. (B) Plaque reduction assay of cells treated with siRNA_20, siLNA_20, sisiLNA_20 and the respective controls. The titre of CVB-3 is expressed as plaque-forming units (p.f.u.)/ml of cells pre-treated with oligonucleotides relative to virus-only treated cells. All data are means and S.D. from four independent experiments each performed in quadruplicate (A) or in duplicate (B).

that the siRNAs, LNA-modified siRNAs and the LNA gapmer 4-4 were capable of inhibiting expression of the fusion construct almost to completion.

3.3. Virus assays

The crucial test for the siRNAs and LNAs was, of course, their antiviral activity. We therefore performed cell viability assays in HeLa cells infected with CVB-3 (Nancy strain). The LNA gapmer 4-4 exhibited partial inhibitory activity increasing cell viability to approximately 55% (Fig. 3). A plateau for the inhibitory activity way reached at 50 nM and at very high concentrations (500 nM) cell viability was even reduced most likely due to toxic effects of the oligonucleotide (results not shown).

Interestingly, siRNA_18 and LNA-derivatives thereof did not have any antiviral effect despite being very efficient in the reporter assay (Fig. 4A). The lack of activity can be explained either by the formation of different local structures in the GFP-fusion construct and the infectious virus or by binding of proteins to the viral RNA. This finding clearly demonstrates that results from reporter assays cannot always be transferred to the native situation.

More important, however, was the finding, that siRNA No. 20 exerted good antiviral activity. In cell viability assays, the siLNA protected cells against lysis by the virus by more than 90% (Fig. 4A). The protective effect of the siRNA and sisiLNA was approximately 80% and 50%, respectively. The superiority of the siLNA in comparison to the siRNA and sisiLNA against the best target site identified for the 5′ UTR of CVB-3 was confirmed in plaque reduction assays, which demonstrated successful reduction of the virus titer (Fig. 4B). In addition to the shown reduction of the plaque number, the plaque morphology was characterized by a smaller diameter in comparison to virus controls in the absence of siRNAs. The small plaque morphology also represents reduced virus reproduction.

While genome-wide RNAi-libraries exist for several mammalian organisms that allow silencing of any chosen endogenously expressed gene, the development of siRNAs with good antiviral potency still remains challenging. This is particularly true for highly structured target regions like the 5′ UTR of picornaviruses. We describe in our present study a stepwise approach to inhibit replication of CVB-3. This strategy comprises computer-based and experimentally validated secondary structure models, in vitro RNase H assays, GFP-reporter assays and the use of LNA-modified siRNAs. We finally succeeded in efficiently inhibiting CVB-3. To the best of our knowledge this is the first report on an efficient siRNA against the highly conserved 5′ UTR of CBV-3 which is an attractive target region for siRNAs since it is unlikely to easily accumulate mutations that allow viral escape.

The strategy described here might not only help to design siRNAs against any highly structured virus RNA but might also offer help for difficult to silence endogenously expressed genes. Kruger et al. recently published their experience with large-scale siRNA screening [33]. While 70% of the targets could easily be silenced with siRNAs and the expression of further genes was successfully inhibited after testing a larger number of siRNAs, 6% of the targets turned out to be refractory to silencing even with up to 10 different, well-designed siRNAs. This failure is likely to be due to structural features of the targeted RNAs. We suggest that initial mapping of accessibility

with a large set of DNA oligonucleotides and improvement of the siRNA by introducing LNA monomers might help to overcome these problems. More comprehensive studies, however, will be necessary to confirm, whether this approach is generally applicable.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.051.

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