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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.**

Keywords: Coxsackievirus; Locked nucleic acid; RNA interference; RNA structure; Small interfering RNA

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 emergence 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

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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 transcribed 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

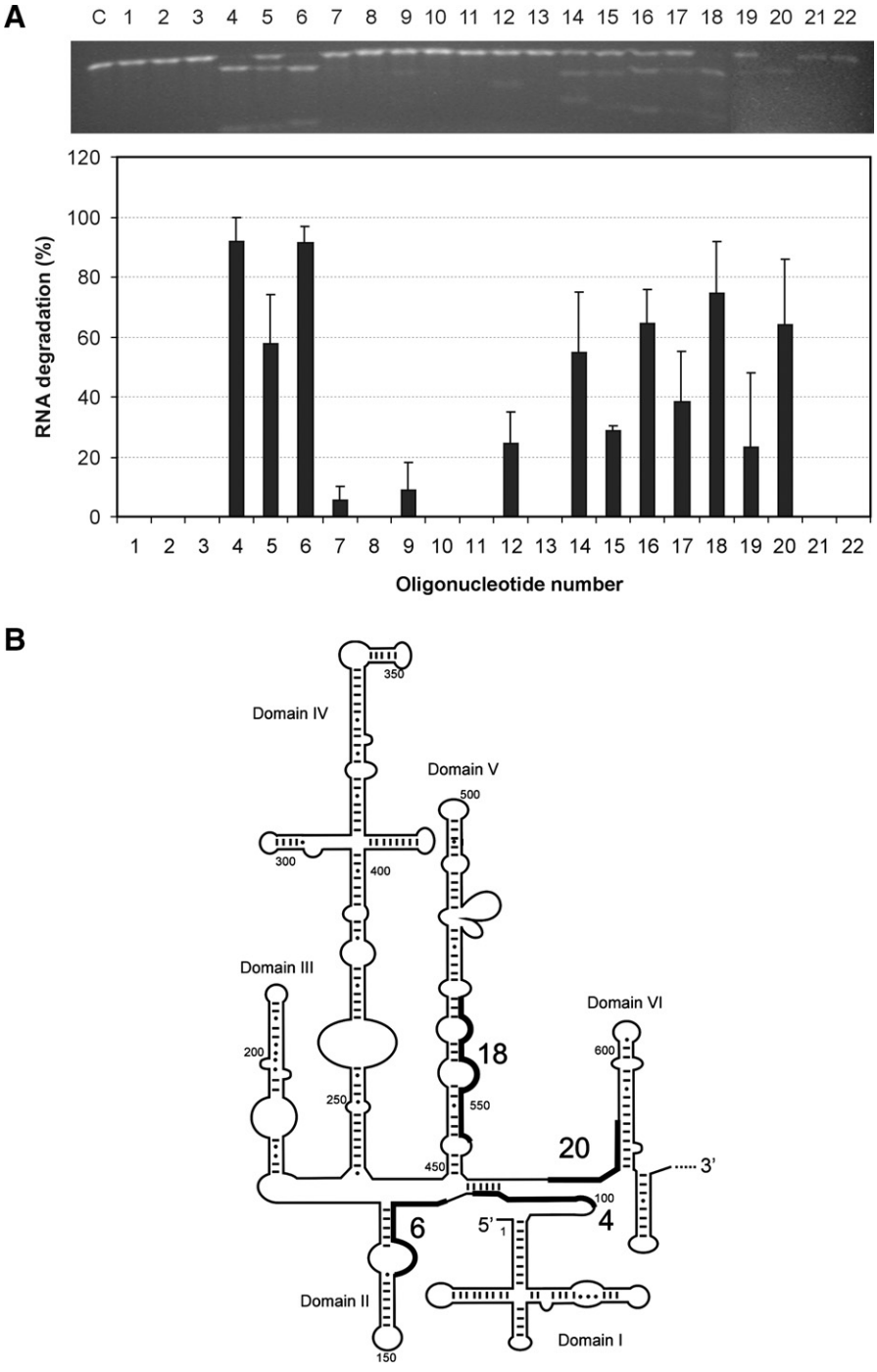

Fig. 1. Identification of accessible sites of the 5′ UTR of CVB-3. (A) Representative agarose gel showing degradation of in vitro transcribed RNA by RNase H. Lanes 1–22 represent RNA cleavage induced by 19mer DNA oligonucleotides against specific sites of the 5′ UTR. (C) Control reaction without DNA 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.