Application of small interfering RNAs modified by unlocked nucleic acid (UNA) to inhibit the heart-pathogenic coxsackievirus B3

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A B S T R A C T

This study describes the first application of unlocked nucleic acid (UNA)-modified small interfering RNAs (siRNAs) directed against a medically relevant target, the coxsackievirus B3. We systematically analyzed the impact of different siRNA modification patterns and observed good compatibility of the introduction of UNA with the maintenance of high antiviral activity. Additionally, the polarity of an siRNA was successfully reversed by modulating the relative stability of the termini with locked nucleic acid (LNA) and UNA as shown in a reporter assay. The potency of the reversed siRNA against the full-length target was, however, too low to inhibit the infectious virus. Altogether, combined modification of siRNAs with LNA und UNA provides a promising approach to alter and improve properties of an siRNA.

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1. Introduction

RNA interference (RNAi) has emerged as one of the most important technologies of biomedical research within just a few years. This evolutionary conserved mechanism of post-transcriptional gene silencing is triggered by small double-stranded RNAs, which induce sequence-specific degradation of a target RNA (for recent reviews see [1–3]). Double-stranded small interfering RNAs (siRNAs) of ~21 nucleotides in length become incorporated into a multimeric complex called the RNA-induced silencing complex (RISC) with a member of the argonaut family nucleases as its main component. While the sense strand of the siRNA is degraded, the antisense strand acts as a guide for RISC to its complementary target mRNA. After binding of activated RISC, cleavage of target mRNA is initiated. Depending on the thermodynamic parameters of the siRNA duplex, the strand containing the less stable 5′-end is preferred to guide RISC [4,5].

The potency and efficacy of siRNAs can be optimized by incorporation of chemically modified RNA analogues. Locked nucleic acid (LNA) nucleotides are conformationally restricted due to a methylene bridge connecting the 2′-oxygen with the 4′-carbon of the ribose ring. Due to their nuclease resistance and low toxicity, LNAs improve the properties of antisense oligonucleotides (for reviews, see [6,7]) and aptamers [8]. Incorporation of LNA is tolerated in various positions of the siRNA and can improve its efficacy [9,10]. Similarly, silencing by the modified strand is inhibited when LNA monomers are introduced at specific sites of an siRNA [9].

Unlocked nucleic acid (UNA), an acyclic analogue of RNA, has an incomplete ribose ring open between the 2′- and 3′-carbon (Fig. 1). The structural flexibility of UNA monomers destabilizes duplexes [11] and thermal denaturation studies demonstrated the potential of UNA monomers to decrease or increase mismatch discrimination depending on their sites of incorporation [12]. Modification of siRNA with both UNA and LNA nucleotides showed highly potent silencing activity. Importantly, the high efficacy of UNA-modified siRNAs was accompanied by low cell toxicity [13].

Coxsackievirus B3 (CVB-3) belongs to the picornavirus family and is one of the most frequent causes of heart muscle infections. The small, non-enveloped virus possesses a single-stranded RNA genome in positive orientation that is used directly as mRNA in infected cells [14]. After entering the host cell, the genomic RNA is transcribed into a complementary negative RNA strand by the viral RNA dependent RNA polymerase (RdRP), which is used as a template for generation of new viral mRNA [15]. Despite improvements in virological and medical research, there is still no specific...
clinical therapy available against CVB-3. Among other applications, RNAi has been found to efficiently inhibit viruses [16]. We and others have described the successful inhibition of CVB-3 by means of RNAi in cell culture [17–21] and in vivo [22–24]. By using LNA-modified siRNAs, RNAi-silencing was shown to be mediated via the genomic plus-strand rather than the intermediate minus-strand [25]. Furthermore, LNA-modified siRNAs were successfully employed to target the highly structured internal ribosome entry site of CVB-3 [26].

Recently, a large number of modified siRNAs including LNA- and UNA-modified siRNAs targeting the Green Fluorescent Protein (GFP) were analysed with respect to their potency and specificity [13]. Our present study describes the first application of LNA- and UNA-modified siRNAs to the medically relevant target CVB-3. Based on the above mentioned study, we systematically analysed the impact of different siRNA modification patterns and screened for the best variant with respect to its inhibitory activity against CVB-3. In experiments with reporter assays, LNA and UNA monomers were introduced at the ends of an siRNA and succeeded in reversing its polarity. These experiments add to our knowledge about the relevance of the thermodynamic features of an siRNA for its silencing activity.

### 2. Materials and methods

#### 2.1. Oligonucleotides

The LNA- and UNA-modified as well as unmodified RNA oligonucleotides were synthesized by RiboTask (Odense, Denmark). Both, siRdRP (target sequence CUA AGG ACC UAA CAA AGU U) and siRev (target sequence UCU CAU AGC AUU UGA UUA C), are directed against the 3D RNA dependent RNA polymerase (RdRP) of CVB-3 (GenBank Acc. No. M33854; target nucleotides 6315–6333 and 6594–6612, respectively). Both siRNAs were already used in an earlier study [25] where siRev was dubbed siRev1 and siRdRP was dubbed siRdRP2.

As a control, we used an siRNA designed by Qiagen (Hilden, Germany) with no known homology in the human and viral genome: UUC UCC GAA CGU GUC ACG U. The modification patterns of all oligonucleotides are summarized in Tables 1 and 2. DNA oligonucleotides used for cloning procedure were purchased from TibMolBiol (Berlin, Germany).

#### 2.2. Cells and virus

Vero (ATCC, Manassas, VA) and COS7 (DSMZ, Braunschweig, Germany) cells were propagated in monolayer culture in MEM (GIBCO-Invitrogen, USA) containing 5% heat inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic, gentamicin and non-essential amino acids, at 37°C in a humidified atmosphere with 5% carbon dioxide. CVB-3 (strain Nancy; ATCC No. VR-30) was propagated in Vero cells.

#### 2.3. Luciferase reporter assay

The reporter constructs psiCheck2-RdRP(+) and psiCheck2-RdRP(−) containing the cDNA of the viral RNA dependent RNA polymerase (RdRP) in plus- and minus-strand orientation, respectively, were described previously [25]. To obtain a plasmid producing the

### Table 1

Sequences and modification patterns of classical 21-mer siRNAs with 2 nt overhangs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Pictogram</th>
<th>Sequence and modification pattern</th>
<th>Nucleotides</th>
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<td></td>
<td>CUAAGGACCUCUAACAAUGUUU</td>
<td>21</td>
</tr>
<tr>
<td>siRev 2_2</td>
<td></td>
<td>UUGAUUCUCUGAUUGUUCA</td>
<td>21</td>
</tr>
<tr>
<td>siCtr 2_2</td>
<td></td>
<td>UCCUAACAGAUUGAUACUU</td>
<td>21</td>
</tr>
<tr>
<td>mod A</td>
<td></td>
<td>UUCUGGAUCUAACCA</td>
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</tr>
<tr>
<td>mod B</td>
<td></td>
<td>L-------------------------------</td>
<td>21</td>
</tr>
<tr>
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</tr>
<tr>
<td>mod F</td>
<td></td>
<td>L-------------------------------</td>
<td>21</td>
</tr>
</tbody>
</table>

Top strand depicts the sense strand in 5’→3’ direction (complementary to viral minus-strand). Bottom strand depicts the antisense strand in 3’→5’ direction (complementary to viral plus-strand). Pictogram: open circle = UNA; filled square = LNA. Modification pattern: L = LNA; U = UNA.
Table 2
Modified siRNAs containing two and three nucleotide 3′-overhangs.

<table>
<thead>
<tr>
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<th>Pictogram</th>
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<th>Nucleotides</th>
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<td>siCtr 3_3</td>
<td><img src="image" alt="Pictogram" /></td>
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<td>22 22</td>
</tr>
<tr>
<td>mod 1</td>
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</tr>
<tr>
<td>mod 2</td>
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<td>21 22</td>
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(continued on next page)
19mer plus-strand siRev target site, the corresponding sense and antisense oligodeoxynucleotides containing overhangs for XhoI and NotI restriction sites were annealed and cloned into the multiple cloning site of psiCheck2 (Promega, Mannheim, Germany) downstream of the Renilla luciferase stop codon.

For reporter assays, COS7 cells were seeded in 96-well plates at a density of 7000 cells per well in medium without antibiotics and incubated in a humidified atmosphere. The next day, cells were co-transfected with 0.1 μg vector and 10 nM of the respective siRNAs by using 0.3 μl Lipofectamine™ 2000 (Invitrogen, Karlsruhe, Germany) per well according to manufacturer’s protocol. Following an incubation for 24 h, cells were lysed with 20 μl passive lysis buffer (Promega) and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions on a Centro LB 960 luminometer (BertholdTech, Bad Wildbach, Germany). The Renilla luciferase signals were normalized to the firefly luciferase signals.

2.4. SiRNA transfection and virus inoculation

For transfection, Vero cells were seeded at a density of 7500 cells per well in 96-well plates in medium without antibiotics. The next day, cells were incubated for 4 h with different amounts

Table 2 (continued)

<table>
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<td>22</td>
</tr>
</tbody>
</table>

Top strand depicts the sense strand in 5’-3’ direction. Bottom strand depicts the antisense strand in 3’-5’ direction. Pictogram: open circle = UNA; filled square = LNA, a short separate line indicates a three nucleotide 3’ overhang. Modification pattern: L = LNA; U = UNA.

Fig. 2. Titre of the virus propagated in Vero cells after transfection with modified (siRdRP mod. A), unmodified (siRdRP 2_2) and non-silencing (siCtr) siRNA. Cells were pre-treated with siRNAs in the range of 1–100 nM, followed by virus inoculation. The virus titer was determined by plaque forming assay 19 h after infection. The mean and S.D. of at least three independent experiments, each performed in duplicate are shown.
of siRNA as indicated and 0.3 μl Lipofectamine™ 2000 per well, following the manufacturer’s instructions. Cells were then incubated with CVB-3 at a multiplicity of infection (m.o.i.) of 0.1 in medium without FBS for 30 min and maintained in cell culture medium for additional 19 h. Further details and variations of the standard procedure are described in Section 3.

2.5. Determination of virus titer

The amount of infectious virus in lysed cells was determined on Vero cells by an agar overlaid plaque forming assay according to the procedure described elsewhere [20]. Briefly, the serially diluted samples were incubated for 30 min on Vero cells. Subsequently, cells were overlaid with agar containing Eagle’s MEM. After incubation in a humidified atmosphere for 3 days, cells were stained with neutral red and virus titers were determined by counting plaques.

3. Results and discussion

In the present study, we evaluated the impact of incorporation of UNA monomers into a previously identified, highly efficient siRNA, dubbed siRdRP, against the heart-pathogenic CVB-3. UNA monomers are very flexible due to an open structure of the ribose ring, leading to a gradual decrease in the thermodynamic stability of duplexes [12]. In contrast, the widely used LNA monomers enhance the target affinity and nuclease resistance [9,25,26]. As published by Bramsen et al. [13], the insertion of UNA and LNA monomers at specific positions of an siRNA against GFP lead to an improvement of the silencing activity.

Our highly potent antiviral siRdRP 2_2 composed of a 19mer duplex and 2 nt overhangs in accordance with conventional siRNA design, was modified by the incorporation of UNA and LNA monomers based on the modification pattern reported by Bramsen et al. [13] (siRdRP mod A; Table 1). The CVB-3 titer after siRNA transfection...
Fig. 5. Reversal of the siRNA polarity by incorporation of UNA and/or LNA monomers. (A and C) Luciferase reporter knockdown by modified siRev. Shown are the mean and S.D. of three independent experiments determined from measurements of the luminescence of Renilla luciferase normalized to the firefly luciferase expressed from the same vector. (A) Reporter-construct containing the 19 nt siRev target sequence in plus-strand orientation. (B) Schematic representation of the siRNA modification patterns (open circle: UNA; filled square: LNA). The top strand depicts the sense strand, complementary to the viral minus-strand; the bottom strand depicts the antisense strand, complementary to the viral plus-strand. (C) Reporter constructs containing the full length RdRP target sequence in plus-strand (light gray) or minus-strand (dark gray) orientation. (D) Virus propagation in Vero cells transfected with 100 nM of the indicated siRNA followed by incubation with CVB-3 for 19 h. The mean and S.D. of six independent experiments, each performed in duplicate are shown. siCtr: Non-silencing siRNA; virus: without transfection.
tion and subsequent virus infection of cells clearly revealed a poten-
tant and concentration-dependent inhibition of virus propagation
of the UNA-modified siRdRP (Fig. 2). Compared to the unmodified
counterpart, the UNA-modified siRNA was only slightly less potent.
This finding demonstrates siRdRP tolerated the introduction of
UNA without significant loss of antiviral activity.

The next aim was to analyse in more detail the impact of the
introduction of LNA and UNA at different positions in the siRNA
on its antiviral activity. To this end, a set of sense and antisense
strands with three nucleotide overhangs and UNA as well as LNA
modifications at different positions was designed. Annealing of
each of the 22 nt antisense strands with each of the 22 nt sense
strands, as well as with 21 nt sense strands resulted in 18 modified
siRNAs (shown in Table 2). To evaluate the antiviral potential of
these modified siRNAs, the generation of new infectious virus dur-
ing the first viral replication cycle was determined. For this pur-
pose, cells were transfected with 10 nM of each siRNA and
infected with CVB-3 for 8 h on the next day. Virus titer was deter-
minal by titration of cell lysates on confluent cells. As shown in
Fig. 3, each of the siRNAs was capable of reducing the virus titer
at least 10-fold. This finding demonstrates that LNA and UNA
monomers can be introduced into certain positions of the siRNA
without detrimental effects on its silencing activity.

Modified control siRNAs were then used to further investigate
the specificity of the approach. Some of the most potent modifica-
tion patterns were selected and the corresponding control siRNAs
without homology to the host or viral genome were synthesized.
Fig. 4 shows that neither the modified nor the unmodified 22 nt or
21 nt control siRNAs (light grey bars) had any significant effect on
CVB-3, even at a comparably high concentration of 50 nM. This
result clearly confirms that the observed reduction of virus
propagation is a specific RNAi effect rather than an unspecific
sequence of the transfected siRNAs or the incorporated modifications.

Prominent properties of LNA and UNA monomers are their high
and low affinity towards complementary nucleotides, respectively.
The next aim of our study was to make use of these features to fur-
ter investigate the relevance of the thermodynamic design of an
siRNA for its activity. It has previously been shown that the relative
stability of the two ends of an siRNA determines which of strands
of the duplex will be incorporated into RISC as the guide strand
[4,5]. The Dicer associated double-stranded RNA binding protein
interaction and subsequent virus infection of cells clearly revealed a po-
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[4,5]. The Dicer associated double-stranded RNA binding protein
was identified as the protein sensor for the strand asymmetry
[27]. We now wanted to investigate whether the affinity differ-
cences of LNA and UNA can be employed to reverse the polarity
of an siRNA. In a previous study, we purposely designed siRNAs
which were exclusively active against the CVB-3 RNA in either
plus- or minus-strand orientation [25]. The experiments with
infectious virus revealed that only the plus-strand virus RNA is
amenable to silencing by siRNA, while the minus-strand is resis-
tant against RNAi. We now chose one siRNA, which is exclusively
active against the minus-strand orientation of the CVB-3 RNA, re-
ferred to as siRev. UNA and LNA monomers were then introduced
into the 3’ and 5’ ends of the siRev sense strand, respectively (Table
1 and pictograms in Fig. 5B). These modifications can be expected
to destabilize base pairing at the 5’-end of the antisense strand,
while enhancing the strength of base pairing at its 3’-end. As a con-
sequence, polarity of the siRNAs should be reversed.

To test this hypothesis, the target site of the siRNA was cloned
in positive orientation downstream of Renilla luciferase. As can be
seen in Fig. 5A, the siRNA without UNA or LNA monomers at the
termini of the duplex does not reveal any silencing activity against
the target site in positive orientation. This result confirms our pre-
vious findings with the unmodified siRNA [25]. In sharp contrast,
siRNAs with the same sequence but with reversed relative stability
of the two ends by the introduction of LNA und UNA into the 5’
and 3’-end of the sense strand, respectively, gained significant
activity against the target site in plus-strand orientation (mod.
B–F). Interestingly, a single UNA at the 3’-end (mod. E) as well as
a single LNA at the 5’-end (mod. B) of the sense strand was suffi-
cient to reverse the polarity of siRev. The combination of LNA
monomers to stabilize the 3’-end of the antisense strand and
UNA monomers to destabilize its 5’-end at the same time slightly
increased the silencing potency (mod. C).

Next, we wanted to investigate the activity of the modified
siRNAs against the full length RNA encoding RdRP. To this end,
the RdRP sequence was cloned downstream of Renilla luciferase
in either plus- or minus-strand orientation. As reported previously
[25], siRdRP was exclusively active against the plus-strand orienta-
tion, whereas siRev 2_2 inhibited expression of the reporter con-
struct with the minus-stranded RdRP-RNA, but not the plus-strand
orientation (Fig. 5C).

In line with data reported by Elmen et al. [9], a single LNA
monomer in the first position completely abrogates silencing activity
of so modified strand (mod. B–D). The experiments presented
here revealed that UNA at the 3’-end of the sense strand (mod. E
and F) also impairs the inhibitory potency of this stand. Further-
more, the modified siRNAs which had the relative stability of their
ends reversed showed silencing of the RdRP-RNA plus-strand, as
observed for the isolated target site (Fig. 5A). However, the siRNA
had a lower efficacy against the full-length target as compared to
the efficiency of silencing the isolated target site. A possible reason
for this discrepancy might be the differences in the environment
of the target site, as we and others have previously shown that the
structure of the target RNA has a great influence on siRNA-mediated
silencing [28–30].

In experiments with infectious virus (Fig. 5D) the siRdRP 2_2,
which is directed against an accessible target site in the viral
plus-strand RNA, showed high antiviral potency as described pre-
viously [20]. Since the modified siRNAs already had a decreased
activity against the full-length target, it was not unexpected that
no pronounced inhibitory effect on virus replication was observed
with any of the siRNAs.

In summary, our study clearly showed that introduction of a
limited number of UNA and LNA monomers into an siRNA is com-
patible with maintenance of high silencing activity. UNA-modified
siRNAs have been found to have very low toxic side effects on cell
viability [13]. To the best of our knowledge, the data presented
here show for the first time the potential of UNA- and LNA-modi-
fied siRNAs to inhibit a medically relevant target, the coxsackieve-
rus B3, a typical representative of the picornavirus family.
Furthermore, UNA nucleotides can be employed to investigate
the mechanism of RNAi in more detail, as for example their intro-
duction at the ends of an siRNA duplex can reverse its polarity.

Note added in proof

After submission of the present manuscript, a study was pub-
lished, in which UNA monomers were systematically introduced
into each position of an siRNA guide strand [31]. UNA modifi-
cations at various positions were found to be detrimental to siRNA
activity. Particularly, UNAs at positions 1 and 2 prevented phos-
phorylation of the siRNA and thereby abrogated its binding to
Ago2.

Acknowledgements

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Wade for proofreading and valuable comments. This work was
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