Tc-DNA modified siRNA

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ABSTRACT

We investigated the biological activity of siRNAs carrying tc-DNA modifications at various positions in the sense strand. The siRNAs were directed to the coding region of the enhanced green fluorescent protein (EGFP) mRNA. HeLa cells were transfected with the EGFP plasmid and variable concentrations of siRNA duplexes. The antisense effect was quantified on the protein level by fluorescence activated cell sorting (FACS). We found that 3'-end modification as well as modifications at 3'-4 further positions either in the 3'- or the 5'-region of the sequence were well tolerated, some of them leading to higher repression of gene expression than wild type RNA.

![Chemical structure of tc-DNA](image)

**Figure 1.** Chemical structure of tc-DNA

INTRODUCTION

The recently discovered RNA interference (RNAi) pathway1,2 which leads to potent and selective inhibition of gene expression in a variety of mammalian cells, has rapidly developed into an important tool for determining gene function in vitro. Besides this there is considerable potential for application of small interfering RNA (siRNAs) duplexes in oligonucleotide based therapy, provided that the major obstacles as e.g. low biostability and cellular uptake can be overcome.3

Tricylo-DNA, was developed in our group earlier on4,5 and shows increased thermal and thermodynamic RNA duplex stability with increases in Tm of 2-4°C per modification. The incorporation of tc-DNA substantially enhances serum stability of antisense oligonucleotides, which is a key requirement for in vivo use. In vitro applications of fully modified tc-DNA (steric block oligonucleotides) targeting pre-mRNA splicing lead to increases in antisense efficacy compared to other nucleotide analogues as e.g. fully modified 2'-OMe-PS-RNA or fully modified LNA.6,7

Given these properties it was now of interest to evaluate the biological properties of tc-DNA spiked siRNA duplexes in the context of the RNAi mechanism. As a biological target we have chosen the enhanced green fluorescence protein (EGFP).8

RESULTS AND DISCUSSION

siRNAs can either be modified on the sense (passenger) or the antisense (guide) strand. It has been shown before that the RNA activity is more sensitive to antisense strand modification compared to sense strand modification.9 We therefore first investigated whether tc-DNA modifications in the sense strand are accepted by the RNAi machinery. We synthesized siRNA single strands which carry modifications in the 3'-overhangs and three to four pyrimidine exchanges either in the 3' or 5' region of the sense strand oligonucleotides. The antisense strand carried a 3'-dT overhang. The sequences are depicted in Table 1.

**Table 1.** Sequences of siRNAs used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>siRFP</td>
<td>5'-GUGGGAGCGCGUGAUGAACdTDdT -3'</td>
</tr>
<tr>
<td></td>
<td>3'-dTdTCACCCUCGCGACAUACUG -5'</td>
</tr>
<tr>
<td>siGFP</td>
<td>5'-GCAGCAGACUCUCUCAAGdTdT -3'</td>
</tr>
<tr>
<td></td>
<td>3'-dTdTGUCUGCGUAGAAGAUUC -5'</td>
</tr>
<tr>
<td>siYY</td>
<td>5'-GCAGCAGACCUUUCUCAAG tt -3'</td>
</tr>
<tr>
<td></td>
<td>3'-dTdTGUCUGCGUAGAAGAUUC -5'</td>
</tr>
<tr>
<td>siYY3</td>
<td>5'-GCAGCAGACCUU c t t cAGA tt -3'</td>
</tr>
<tr>
<td></td>
<td>3'-dTdTGUCUGCGUAGAAGAUUC -5'</td>
</tr>
<tr>
<td>siYY5</td>
<td>5'-G cAG cA cGACUUCCUCAAG tt -3'</td>
</tr>
<tr>
<td></td>
<td>3'-dTdTGUCUGCGUAGAAGAUUC -5'</td>
</tr>
</tbody>
</table>

Top strand depicts the sense stand in 5'-3' direction. Bottom strand depicts the antisense strand in the 3'-5' direction (complementary to the target). RNA, uppercase; tc-DNA, bold lower case.
siGFP, carrying a 3′-dT overhang in the sense strand was used as a positive control and siRFP, targeting the red fluorescence protein, was used as a sequence unrelated, negative control. HeLa cells were simultaneously co-transfected with 1μg of plasmid encoding EGFP and variable amounts of siRNAs (20nM-100nM) using lipofectamine as transfecting agent. The cells were analyzed after 48h by FACS.

As shown in Figure 2, all sense strand tc-DNA modified siRNAs effectively and selectively reduced the EGFP activity whereas an unrelated siRFP control was essentially without effect.

**Figure 2.** siRNA mediated downregulation of EGFP expression (experiments were performed in triplicate).

\[
\text{EGFP down-regulation (\%)}
\]

Introducion of tc-DNA modifications in the 3’ overhang (siYY) revealed a slight gain in inhibitory effects compared to wild type siGFP. siYY5’ and siYY3’ were also fully compatible showing EGFP down-regulation activities very similar to that of wild type. We found no change in inhibitory activity between 20 and 100 nM siRNA concentration, indicating that maximum inhibition concentration may be substantially lower than 20 nM.

**CONCLUSION**

We were able to successfully incorporate tc-DNA into siRNA sense strands and test them in vitro on the example of EGFP expression inhibition. The tc-DNA modification was well accepted by the RISC machinery and showed similar to slightly enhanced activites compared to natural RNA.

With these promising results in hand, the next steps to be taken are now the determination of a complete dose dependence profile in the low nanomolar range, as well as the evaluation of scope and limitations of tc-DNA containing siRNAs, where the chemical modifications are located on the antisense (guide) strand.

To investigate into the silencing mechanism we will isolate and quantify the EGFP mRNA by quantitative PCR and determine the biostability of tc-DNA modified siRNA duplexes in human serum.

**REFERENCES**


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