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LNA® incorporated siRNAs exhibit lower off-target effects compared to 2'-OMethoxy in Cell Phenotypic Assays and Microarray Analysis

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ABSTRACT

Despite the promise of short interfering RNAs (siRNA), contending with off-target is a challenge for RNAi users. To alleviate these problems, we have developed locked nucleic acid (LNA®) modified siRNAs and optimized performance using cellular phenotypic assays as well as microarray analysis. During development, we compared LNA® and 2'OMethoxy (2'OMe) chemistries placed strategically throughout the siRNA molecule and found a novel pattern of LNA® placement that greatly improved the specificity of the siRNA and reduced it's toxicity in culture while preserving the potency of the siRNA. The improvements in specificity made by LNA®-modified siRNAs were developed and validated by measuring the phenotypic signatures in a high content cell-based screening assay as well as comparison of the level of differentially expressed genes observed in microarray analysis between modified and unmodified siRNAs. HT screening of a collection of genes demonstrated that the LNA®-modified siRNAs exhibits the best overall rate to elicit the expected phenotype, reduced toxicity and achieved an improved coherence of phenotype compared to 2'OMe-modified or unmodified siRNAs.

INTRODUCTION

RNA interference (RNAi) has become one of the most important research tools in functional genomics analysis ever since the discovery of the phenomenon. In order to identify disease relevant biomarkers, it is important to address specificity of mRNA targeting by siRNAs. Chemical modifications incorporated in siRNAs have been reported to enhance thermal stability, nuclease stability as well as improve biodistribution and cellular uptake[1]. A recent report[2] has highlighted the use of 2'-OMe in reducing off-target transcript silencing by siRNAs. We here compare off-target effects by cellular phenotypic assays and microarray analysis between siRNAs partially modified by LNA® and 2'-OMe residues. LNA®-modified siRNAs have been reported[3] to enhance siRNA functionality in vitro and in vivo.

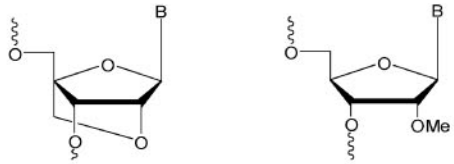
MATERIALS AND METHODS

Global Gene profiling: HeLa cells were reverse transfected with gene-specific *Silencer*® Select (LNA® modified) siRNA or 2'-OMethoxy modified siRNA or *Silencer*® Select Negative Control #2 siRNA, using siPORT™ *NeoFX*™ Transfection Agent. 48 hours post-transfection, RNA was isolated, quantitated and amplified using Ambion MessageAmp™ II aRNA Amplification kit. Affymetrix Human Genome U133 Plus 2.0 Arrays (>47,000 transcripts) were used to evaluate the off-target gene changes following siRNA transfection.

Cell Based Assays: U2-OS osteosarcoma cells were transfected with siRNA using optimized transfection protocols. 48 hours post transfection, cells were fixed and immunofluorescence was performed using the following antibodies: anti-phospho histone H3 (NEB) to identify mitotic cells, and cleaved lamin A (NEB) to detect apoptotic cells. Immunofluorescence was detected using the ImageXpressMICRO Automated Acquisition and Analysis System

RESULTS AND DISCUSSION

To identify the optimal format, greater than 25 different siRNA formats were manufactured with partial modifications with LNA® and 2'-OMe chemistries (Figure 1).



LNA 2'-OMethoxy

Figure 1 LNA® and 2'-OMethoxy structures incorporated in siRNAs.

These modified siRNAs were tested for efficacy, potency and strand bias. The optimal format displayed no loss of potency and a statistically significant bias for targeting by

the guide strand as compared to the passenger strand. To determine the off-target footprint of modified siRNAs via microarray analysis, the lead format was manufactured in the LNA® and 2'-OMe chemistries for two siRNA targeting FDFT1.

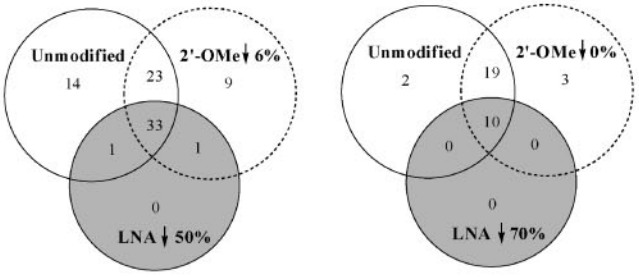


Figure 2 Microarray analysis on two FDFT1 siRNAs transfected into HeLa cells comparing unmodified siRNA to 2'OMe-modified and LNA®-modified siRNA.

The LNA® and 2'-OMe siRNAs against FDFT1 were transfected, RNA harvested at 24 hours post transfection and analyzed for over 47,000 transcripts. As compared to the unmodified siRNAs, the 2'-OMe siRNA displayed a modest decrease (Figure 2) in off target transcripts being differentially expressed. However the LNA® modified siRNAs eliminates up to 70% of the 2-fold ($p<0.001$) differentially expressed genes (DEG) compared to the unmodified and 2'OMe modified siRNA.

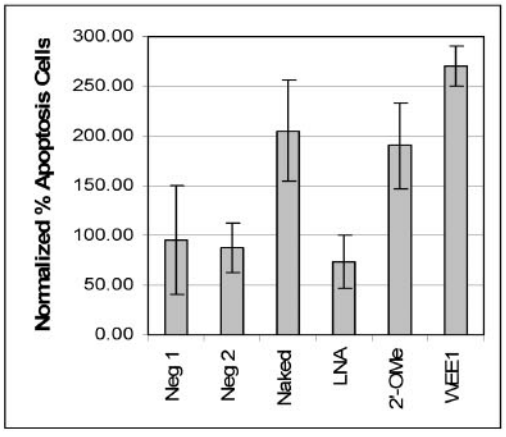


Figure 3 Normalized levels of apoptosis in cells treated with LDLR siRNAs in optimal LNA and 2'-OMe format as well as unmodified format. The Wee1 siRNA is a positive control that illicit apoptosis while *Silencer*® Neg1 and Neg2 were used for normalization.

Next, the two chemistries were compared for their ability to limit off-target phenotypes in cellular phenotypic assays. The siRNA used was one shown to give an apoptosis signature as an off-target effect, as measured in cells transfected with siRNAs that target LDLR in U2OS osteosarcome cells. Knockdown of LDLR results in the expected decrease in LDL uptake (data not shown). However in U2OS cell, the unmodified siRNA design also induced a dramatic increase in off target apoptosis. Apoptosis was measured by immunofluorescence assays using anti-cleaved lamin A antibodies. The 2'-OMe-modified siRNA exhibited similar apoptotic phenotype as compared to the unmodified siRNA and the positive control. The LNA®-modified siRNAs however reduced the off-target phenotype to the levels generated by the negative controls.

CONCLUSION

Our comparison of chemically modified siRNAs demonstrated that LNA®-modified siRNA is superior to unmodified and 2'-OMe-modified siRNA for eliminating off-target effects, as measured by global gene profiling and elimination of off-target phenotypes in cell based assays, without reducing siRNA potency.

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