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RNA interference by 2′,5′-linked nucleic acid duplexes in mammalian cells

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Abstract—Synthetic small interfering RNA (siRNA) mediated silencing of a specific gene is emerging as a powerful tool for gene regulation. However, their utility is limited for therapeutic applications primarily due to poor stability. The 2′,5′-linked oligonucleotides are known to be more stable to nucleolytic degradation than 3′,5′-linked oligonucleotides. The 2′,5′-linkage is tolerated in the sense strand of the siRNA duplex. However, the 2′,5′-linkage is not tolerated in the antisense strand of the siRNA duplex.

RNA interference (RNAi) is an evolutionarily conserved process for the control of gene expression where double-stranded RNA guides the degradation of mRNA that are homologous in sequence to the guide sequence.[1,2] The prospect of using this technology for therapeutic applications became a possibility, since the demonstration that synthetic 19–21 nt RNA duplexes (small interfering RNA or siRNA) exhibited RNAi activity in mammalian cells.[2] Several groups have demonstrated the efficacy of siRNA mediated inhibition of clinically relevant genes in vitro.[3] Recently, in vivo activity of nuclease resistant siRNA has also been reported.[4]

Modification of siRNA is important for therapeutic applications.[5] Chemical modifications are necessary to enhance nuclease stability of the siRNAs. Chemical modifications are also needed to improve the bio-distribution and pharmacokinetic properties of the siRNA and specifically deliver siRNA to certain cell types. Recently several chemically modified siRNAs have shown improved activity in cell culture.[5,6]

Extensive biochemical and crystallographic studies have shown that RNA interference is initiated by double-stranded RNA.[7] Recent reports also suggest that fully modified siRNAs with A-form helical conformation exhibit potent siRNA activity.[7] This result may suggest that modified oligonucleotide duplexes with A-form helical conformation should be able to initiate RNA interference.

The 2′,5′-linked nucleic acid (Fig. 1) exhibits very unique hybridization properties to complementary RNA. While 2′,5′-linked nucleic acids form a stable duplex with RNA, they hybridize weakly or not at all to complementary DNA.[8] The duplexes formed between 2′,5′-linked DNA or RNA and 3′,5′-RNA adopt A-form helical structures. This suggests that, these duplexes may be used as siRNAs to inhibit gene expression. Since 2′,5′-

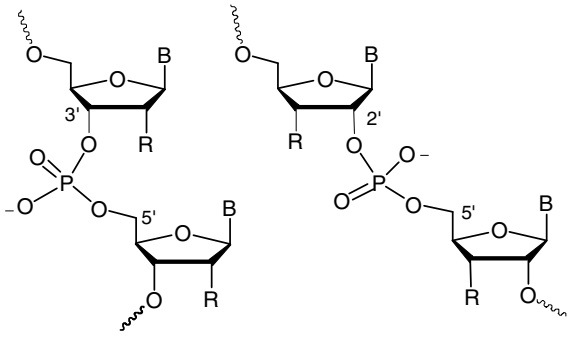

Figure 1. 3′,5′- and 2′,5′-Linked nucleic acids.

Keywords: 2′,5′-DNA; 2′,5′-RNA; RNAi; siRNA; Mammalian cell.
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Table 1. 3′,5′- and 2′,5′-Linked oligonucleotides used for this study

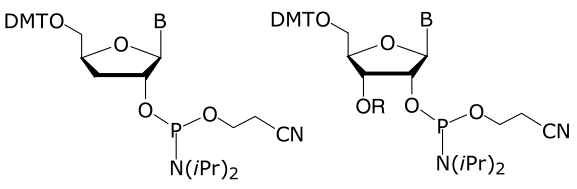
Compound	Sequence[a]	Calcd mass	Found mass
1	5′ r(CAAAUCCAGAGGCUAGCAG) TT 3′	6715.3	6714.8
2	5′ r(CUGCUAGCCUCUGGAUUUG) TT 3′	6600.5	6600.9
3	5′ d(*CAAATCCAGAGGCTAGCAG* TT) 2′	6439.4	6439.8
4	5′ d(*CTGCTAGCCTCTGGATTTG* TT) 2′	6394.9	6393.6
5	5′ r(*CAAAUCCAGAGGCUAGCAG* TT) 2′	6715.3	6714.5
6	5′ r(*CUGCUAGCCUCUGGAUUUG* TT) 2′	6600.5	6600.5

[a] Underline italics represent 2′,5′-linked nucleotides; TT overhangs with 3′,5′-linkages.

linked nucleic acids (Fig. 1) are known to resist enzymatic hydrolysis,[8] one could infer that siRNAs with 2′,5′-linkages would be more stable in plasma than unmodified siRNAs. Chimeras containing 2′,5′-linkages show less nonspecific binding to plasma and cellular proteins in comparison to 3′,5′-phosphorothioate oligonucleotides.[9] This result may suggest that 2′,5′-linked oligonucleotide phosphorothioates may produce fewer side-effect compared to 3′,5′-linked oligonucleotide phosphorothioates. Here, we report the activity of siRNA duplexes with 2′,5′-linked nucleic acids in mammalian cells.

The unmodified siRNAs used in this study were designed to target the coding region of human PTEN mRNA.[10] The corresponding 2′,5′-linked DNA and RNA sequences **3**–**6** (Table 1) were designed and synthesized. The oligonucleotides **3**–**6** were synthesized using commercially available phosphoramidites (Fig. 2) on a solid-phase oligonucleotide synthesizer according to the reported procedure.[8] Oligonucleotides were characterized by mass spectroscopic (ES-MS) analysis (Table 1) and the purity was assessed by capillary gel electrophoresis (CGE).

The siRNA duplexes were formed by combining equivalent molar amount of antisense and sense strands. The solution was mixed and heated at 90 °C for 1 min then incubated at 37 °C for 6 h. Percentage of duplex formed was determined using CGE analysis.[6] Synthetic siRNA duplexes were transfected into cells using Lipofectin reagent. Cells were harvested 18–24 h post-transfection and total cellular RNA was isolated on an RNeasy 3000 BioRobot (Qiagen, Valencia, CA). Reduction of target mRNA expression was determined by real time quantitative RT-PCR. Total RNA for each well was measured using RiboGreen[6] (Molecular Probes, Eugene, OR), and these values were used for sample-to-sample normalization.

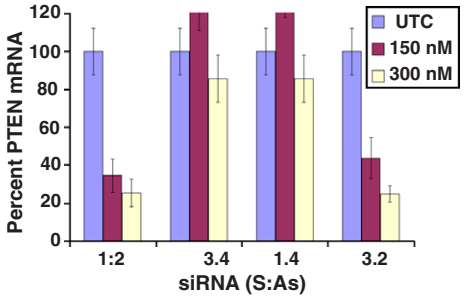


B = Ade^{Bz}, Gua$_{ibu}$, Cyt^{Bz}, Thy or Ura
R = *tert*-Butyldimethylsilyl
DMT =4,4′-(Dimethoxy)triphenylmethyl
Bz = Benzoyl
ibu = Isobutyryl

Figure 2. Nucleoside (2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidites.

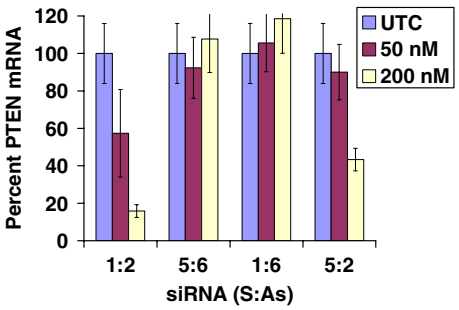
The unmodified siRNA duplex **1**:**2** exhibited dose dependent inhibition of PTEN mRNA expression (Fig. 3). The siRNA duplex **3**:**2** (Fig. 3) with 2′,5′-sense strand DNA and 3′,5′-antisense strand RNA showed similar activity. The siRNA duplex **1**:**4** (Fig. 3) with 3′,5′-sense strand RNA and 2′,5′-antisense strand DNA was inactive. Similar results (Fig. 4) were obtained with siRNA duplexes with 2′,5′-RNA. The siRNA duplex **5**:**2** with 2′,5′-sense strand RNA and 3′,5′-RNA was active. However, siRNA duplex **1**:**6** was inactive.

These results suggest that 2′,5′-linkage is tolerated in the sense strand of the siRNA duplex but not in the antisense strand. The differences in activity of siRNA with 2′,5′-linked antisense verses sense strand may suggest that 2′,5′-linkage on the sense strand drives selective loading of the antisense stand to the RISC and elicits activity. Recent crystallography studies suggest that the 5′-end of the antisense strand is critical not only for siRNA loading to RISC, but positioning antisense strand for nucleation with mRNA and subsequent mRNA cleavage.[11] It appears that in addition to A-form duplex, the 5′-end of antisense strand should adopt correct geometry to be able to interact productively with



1:2	**S**	5’ r(CAAAUCCAGAGGCUAGCAG) TT 3’
	As	3’ TT r(GUUUAGGUCUCCGAUCGUC) 5’
3:4	**S**	5’ d(*CAAAUCCAGAGGCUAGCAG*) TT 2’
	As	2’ TT d(*GTTTAGGTCTCCGATCGTC*) 5’
1:4	**S**	5’ r(CAAAUCCAGAGGCUAGCAG) TT 3’
	As	2’ TT d(*GTTTAGGTCTCCGATCGTC*) 5’
3:2	**S**	5’ d(*CAAATCCAGAGGCTAGCAG*) TT 2’
	As	3’ TT r(GUUUAGGUCUCCGAUCGUC) 5’

Figure 3. Reduction of endogenous PTEN mRNA in T-24 cells by 2′,5′-DNA/3′,5′-RNA duplexes; S, sense strand; As, antisense strand. 2′,5′-Linked residues in underline italics; TT overhangs with 3′,5′-linkages.



1:2	S	5' r(CAAAUCCAGAGGCUAGCAG) TT 3'
	As	3' TT r(GUUUAGGUCUCCGAUCGUC) 5'
5:6	S	5' r(*CAAAUCCAGAGGCUAGCAG*) TT 2'
	As	2' TT r(*GUUUAGGUCUCCGAUCGUC*) 5'
1:6	S	5' r(CAAAUCCAGAGGCUAGCAG) TT 3'
	As	2' TT r(*GTTTAGGTCTCCGATCGTC*) 5'
5:2	S	5' r(*CAAAUCCAGAGGCUAGCAG*) TT 2'
	As	3' TT r(GUUUAGGUCUCCGAUCGUC) 5'

Figure 4. Reduction of endogenous PTEN mRNA in HeLa cells by 2′,5′-RNA/3′,5′-RNA duplexes; S, sense strand; As, antisense strand; 2′,5′-linked residues in underline italics; TT overhangs with 3′,5′-linkages.

PIWI domain of RISC complex. The 5′-end of 2′,5′-linked antisense strand might not adopt conformation compared to natural 3′,5′-linked RNA, thereby inhibiting RNAi activity. These results are further supported by the fact that several 2′-modifications such as 2′-*O*-Me and 2′-*O*-MOE have similar effect on siRNA activity when incorporated at the 5′-end of antisense strand.[12]

In conclusion, we have designed and synthesized siRNA duplexes with 2′,5′-DNA and RNA strands. 2′,5′-Linkage is tolerated in the sense strand and is not tolerated in the antisense strand of the siRNA duplex. siRNA duplex where 2′,5′-DNA/RNA sense strand is paired with 3′,5′-RNA antisense strand showed activity in reducing the message in cultured human cells. The known nuclease resistant properties[8] of 2′,5′-linked nucleic acid and the result of current investigation suggest that this could be a very useful sense strand modification for siRNA therapeutics.

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