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Synthesis, RNAi activity and nuclease-resistant properties of apolar carbohydrates siRNA conjugates

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ABSTRACT

Oligoribonucleotide conjugates carrying apolar carbohydrates at the 5′-end and the corresponding siRNA duplexes have been prepared using phosphoramidite chemistry. All the carbohydrate-siRNA derivatives were compatible with RNA interference machinery if transfected with oligofectamine. In the absence of a transfection agent, some of them exerted certain reduction of gene expression. Double-tailed permethylated glucose conjugated to siRNA through a long spacer inhibited gene expression up to 26% compared to the scrambled duplex. Such modifications contribute positively to the stability of oligoribonucleotides against 5′-exonuclease degradation.

Oligonucleotides have shown great potential as therapeutic agents by different mechanisms[1] especially small interfering RNA duplex (siRNA)[2]. However, the drug potential of siRNAs is still limited due to low stability and inefficient delivery.[3] To increase its stability towards nuclease degradation and increase cellular uptake, modified oligonucleotides and conjugation of siRNA to different delivery carriers such as antibodies, lipids, polymers, peptides or carbohydrates have been reported.[4] Among them, lipids are being widely studied and cholesterol conjugates have been tested in vivo showing promising results.[5] Conjugation to lipophilic molecules introduces a partial hydrophobic character to the bioconjugates which may enhance their cellular uptake not only by increased membrane permeability but also through receptor mediated endocytosis.[6] Some hydrophobic modifications used on siRNA with some success are shown in Fig. 1.

We recently reported the synthesis of glucose oligonucleotide conjugates where the sugar was attached to the 5′-end of the DNA strand and had different spatial presentations.[7] Cell uptake studies using flow cytometric analysis showed that conjugates with the glucose moiety linked through long spacers (15–18 atom distances) were better internalized than those with short linkers (4 atom distance) or than DNA control strands without sugar modification.

Then we synthesized carbohydrate–siRNA duplexes targeting tumor necrosis factor carrying one, two or four glucose and galactose residues at the 5′-end of the passenger strand. The modified siRNA duplexes have similar inhibitory properties than unmodified RNA duplexes in HeLa cells when transfected with oligofectamine.[8] Without transfection agent, glucose–siRNA conjugates showed no inhibition whereas galactose–siRNA duplexes on HuH-7 cells which possess abundant asialoglycoprotein receptors displayed up to 25% anti-TNF inhibitory properties.

Here, the idea was to examine apolar carbohydrates linked to siRNA duplexes as new hydrophobic platforms that may improve the oligoribonucleotide cell uptake without disrupting the RNAi machinery during gene inhibition. The design consisted of permethylated glucose attached to the 5′-end of the passenger strand of the siRNA via two different spacers, a short ethylene glycol and a long dodecanodiol (Fig. 2). A dendron scaffold was also used since it has been demonstrated that lipid–oligonucleotide conjugates presenting double-tailed lipid modifications show good results in cellular uptake.[9] A spacer–oligonucleotide conjugate was also prepared as a negative control.

Synthesis. We synthesized carbohydrate–RNA conjugates using standard solid-phase oligonucleotide automatic synthesis using the corresponding apolar carbohydrate phosphoramidites (Scheme 1). The preparation of permethylated glucose phosphoramidite derivative **6** containing the short spacer was carried out following the same methodology described previously by our

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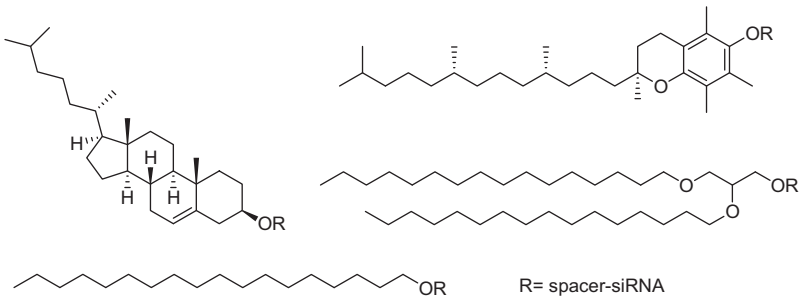

Figure 1. Lipophilic vectors used in the literature to improve siRNA cell uptake. Soutschek et al. (2004) used a cholesterol, Godeau et al. (2008) used a fatty alcohol, MacKellar et al. (1992) used a double aliphatic alcohol and Nishina et al. (2008) used an alpha-tocopherol.

group.[10] This strategy was extended to the apolar sugar with the long dodecanodiol spacer **11**. Briefly, glycosylation of the *O*-benzyl protected dodecanodiol spacer with α-bromoacetoglucose **7** resulted in compound **8** in 62% yield. Deprotection of the acetyl groups followed by methylation under standard conditions produced compound **9** in good overall yield (78%, two steps). Then, hydrogenation and standard phosphoramidite preparation were carried out yielding permethylated glucose phosphoramidite **11** (67% and 74%, respectively). We also prepared a permethylated cellobiose phosphoramidite **12** and a perbutylated glucose phosphoramidite **13**, both with the long spacer following the same synthetic strategy. A dodecanol phosphoramidite control **14** was also synthesized from 1-dodecanol as described previously.[11]

RNA synthesis. The siRNA sequences designed to target Renilla Luciferase gene were 5′-AUCUGAAGAAGGAGAAAAATT-3′ as the passenger sequence and 5′-UUUUUCUCCUUCUUCAGAUTT-3′ as the guide sequence. Oligoribonucleotide conjugates (**1**–**4**) were prepared using a DNA synthesizer. Apolar sugar phosphoramidites were added at the last step on the RNA synthesis. Oligonucleotides presented a major peak in HPLC chromatograms with some impurities but they were easily separated by HPLC obtaining the desired conjugates. However, isolation of siRNA conjugates containing permethylated cellobiose or perbutylated glucose, both with the long spacer, was not successful due to problems either during coupling with the corresponding phosphoramidites or during the cleavage and deprotection steps. Oligoribonucleotide sequences carrying apolar carbohydrates at the 5′-end of the passenger strand were hybridized to the unmodified guide strand.

Dual-luciferase assay. After RNA synthesis, we evaluated the effect of the above described 5′-sense-carbohydrate-modified siRNAs on the ability of the corresponding conjugate derivatives to act as inhibitors of gene expression. Furthermore, we tested the ability of such conjugates to impart cell uptake. We carried out separate gene knockdown experiments in HeLa cells. In a first series of experiments, the cells were cotransfected with two luciferase plasmids (*Renilla* and firefly; target and internal control, respectively), with siRNA duplexes containing each of the five modified passenger strands (**1**–**4**), and with the corresponding unmodified siRNA (wt). Cotransfection of plasmids and siRNAs was carried out by using commercial cationic liposomes (Lipofectamine 2000). Twenty-two hours after transfection, the luciferase activities of the samples were measured by using a luminometer. The results, showing *Renilla* luciferase activity normalized to firefly luciferase, are shown in Fig. 3. Interestingly, the presence of permethylated glucoseC2, and permethylated glucoseC12 at the 5′-end of the passenger strand (**1** and **2**, respectively) did not disrupt RNAi activity. The corresponding siRNA conjugates showed activities comparable to the unmodified siRNA (wt) [(90 ± 1)% knockdown of *Renilla* expression for unmodified wt vs (90 ± 1)% and (89 ± 1)% for **1** and **2**, respectively]. Conjugation of permethylated glucose C12-doubler and C12 spacer to the 5′-end of the sense strand siRNA (conjugates **3** and **4**, respectively) caused a slight decrease in activity, but gene silencing activity of these siRNAs was still quite remarkable: [(71 ± 1)% and (71 ± 3)% for **3** and **4**, respectively, vs (90 ± 1)% for wt]. A sequence scrambled siRNA (as negative control) was used to ensure specificity. Cells cotransfected with the scrambled siRNA

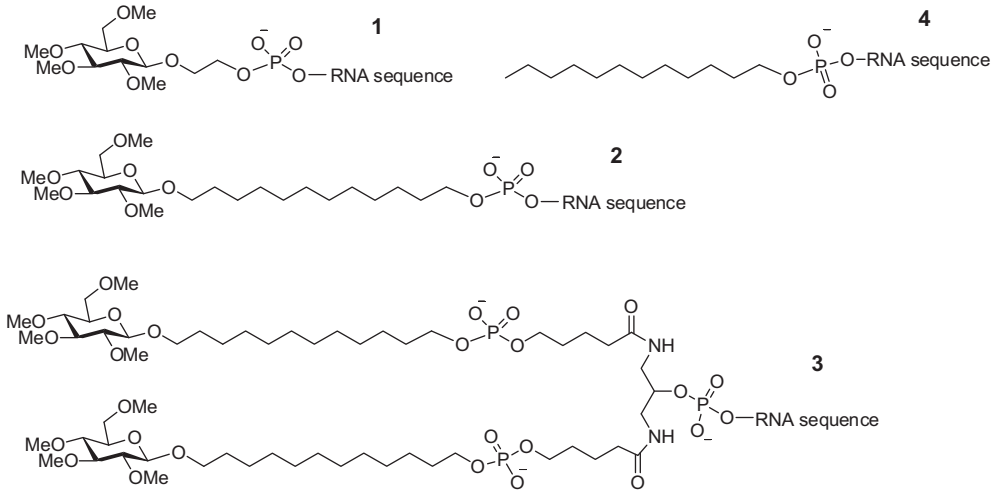

Figure 2. Structure of the carbohydrate oligoribonucleotide conjugates prepared in this study. RNA sequence is the passenger sequence: 5′-AUCUGAAGAAGGAGAAAAATT-3′. The RNA sequence of the guide is: 5′-UUUUUCUCCUUCUUCAGAUTT-3′.