ñSynthesis and in vitro inhibition properties of siRNA conjugates carrying glucose and galactose with different presentationò Aviñó, A., Ocampo, S. M., Lucas, R., Reina, J. J., Morales, J. C., Perales, J. C., Eritja, R. Mol. Divers., 15(3), 751-757 (2011).
doi: 10.1007/s11030-011-9305-6

Synthesis and in vitro inhibition properties of siRNA conjugates carrying glucose and galactose with different presentation.

Anna Aviñó, Sandra M. Ocampo, Ricardo Lucas,[$] José J. Reina,[$] Juan Carlos Morales,[$] José Carlos Perales,[#] and Ramon Eritja

Institute for Research in Biomedicine (IRB Barcelona), Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Networking Centre on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Baldiri Reixac 10, E-08028 Barcelona, Spain.

[#]Dpt of Ciencies Fisiológiques. School of Medicine. University of Barcelona (Campus Belvitge). C/ Feixa Llarga, LÕHospitalet de Llobregat. 08907 Barcelona, Spain

[$]Instituto de Investigaciones Químicas, CSIC − Universidad de Sevilla, 49 Americo Vespucio, 41092 Sevilla, Spain

Corresponding author: Ramon Eritja. Phone 34-934039942, Email: recgma@cid.csic.es

KEYWORDS: siRNA, oligonucleotide-carbohydrate conjugates, RNA interference, tumor necrosis factor, oligonucleotide synthesis.

Abstract. Oligoribonucleotides conjugates and the corresponding siRNA duplexes against tumor necrosis factor carrying one, two or four glucose and galactose residues at the 5ô-end have been prepared using phosphoramidite chemistry. Carbohydrate-modified siRNA duplexes displayed similar or slightly reduced inhibitory properties compared to unmodified RNA duplexes in HeLa cells upon transfection with oligofectamine. When HeLa cells were treated with siRNA carrying one, two or four glucose residues without oligofectamine, no inhibition was observed. The inhibitory properties of siRNA carrying galactose residues without transfecting agent were tested on HuH-7 cells that have abundant asialoglycoprotein receptors. In these cells siRNA carrying galactose residues have slight anti-TNF inhibitory properties (25% in the best case) that are eliminated if the receptors are blocked with a competitor such as α-D-galactopyranosyl-albumine. These results demonstrate receptor-mediated uptake of siRNA carrying galactose residues although the efficacy of the process is not enough for efficient RNA interference experiments.

INTRODUCTION

Chemically synthesized DNAs and RNAs have shown promising results as potential drugs [1, 2]. In these cases nucleic acids are used for the inhibition of a specific gene by blocking gene translation or gene transcription or by stimulating the degradation of a

particular messenger RNA. In the antisense strategy synthetic oligonucleotides complementary to the messenger RNA of a given gene are used to inhibit translation of mRNA to protein [1,3,4]. On the other hand, small interfering RNA duplexes (siRNA) are used to inhibit a specific gene expression by the RNA interference (RNAi) mechanism. This is a cellular post-transcriptional gene silencing mechanism induced by a double stranded siRNA. In this strategy one of these strands binds to a protein called RISC and the complex formed is able to cleave the complementary mRNA sequence so that the corresponding protein is not formed [5-7]. Although large efforts have been devoted in the development of novel siRNA derivatives for gene silencing, the clinical potential of siRNA is still limited mainly due low stability and inefficient delivery of siRNAs.

Chemical modification of DNA or RNA strands has been addressed as one promising strategy to solve these inconvenients [8-10]. Conjugation with different molecules such as lipids, peptides, aptamers or polymers has been used to improve pharmacokinetic properties of siRNA without decreasing activity [9]. Synthesis of carbohydrate conjugates is a potential alternative to enhance oligonucleotide uptake by using sugar binding membrane receptors to mediate cell entry [10,11]. Oligonucleotide delivery in the form of covalent conjugates with carbohydrates is not as well explored as in the form of non covalent complexes [10]. Because the interaction between individual carbohydrates and lectins is rather weak, multivalent carbohydrate moieties linked to oligonucleotides may enhace the affinity. In the antisense strategy, galactosylated PEG-conjugates of oligonucleotides phosphorothioates have been shown to enhance uptake by galactose-specific asialoglycoprotein receptor-mediated endocytosis [12]. Multivalent carbohydrate conjugates have been described in order to increase the interaction of carbohydrates with cellular receptors [13-16]. Oishi *et al* described the

synthesis of siRNA conjugates with lactose and polyethyleneglycol at the 3ô-end of the passenger strand. This modification improved gene inhibition in hepatoma cells [17]. Glycotargeting is considered a valuable method to enhance delivery of therapeutic oligonucleotides. Moreover, selectivity between different cell types can be achieved if different carbohydrate binding receptors are expressed. This is the case with a membrane-active polymer with N-acetyl galactosamine covalently attached, which preferentially accumulated the oligonucleotides in hepatocyte cells whereas if mannose was attached to the polymer the dsDNA went preferentially to nonparenchymal and Kupffer cells, which possess mannose receptors, and away from hepatocytes [18].

Synthesis of oligonucleotide conjugates with carbohydrates has been reviewed [19,20]. There are three main protocols reported for the solid phase synthesis of glycoconjugates. Carbohydrate phosphoramidite coupling is the oldest and one of the most used so far [13-15, 21-23]. Synthesis of oligonucleotide glycoconjugates can also be performed using a 1,3-dipolar cycloaddition (ñclick reactionò) of sugar azides and support-anchored alkynylated oligonucleotides [24]. Moreover, sugar oligonucleotide conjugates have also been prepared by solid phase oximation with carbohydrate mono-substitution [25] or displaying sugar multivalency [26]. Few examples for carbohydrate-RNA conjugates have been described. In these cases sugar attachment has been carried out using an amide bond [27], an oxime bond [28], a 3,4-diethoxy-3-cyclobutene-1,2-dione as linker [29], or a long functional spacer built with an amide bond (containing sugar and spacer) and a Michael reaction between the sugar acrylate derivative and a 5ô-thiol-modified RNA [17].

In this paper we describe, for the first time, the synthesis of carbohydrate conjugates of oligoribonucleotides (**1**-**6**) using phosphoramidite chemistry (scheme 1). These modifications are introduced to the passenger strand of the siRNA for the inhibition of

tumor necrosis factor (TNF-Ŭ) protein which is involved in apoptosis, inflammation and immunity processes [30]. We envisioned that the introduction of multiple carbohydrates (glucose or galactose) at the 5ô-terminus of the passenger strand may favour cellular uptake by receptor-mediated endocitosis as the number of carbohydrate residues is increased.

EXPERIMENTAL SECTION

Oligonucleotides.

The following RNA sequences were obtained from commercial sources (*Sigma-Proligo*, *Dharmacon*): sense or passenger scrambled 5ô-CAGUCGCGUUUGCGACUGG-dT-dT-3ô, antisense or guide scrambled 5ô-CCAGUCGCAAACGCGACUG-dT-dT-3ô, antisense or guide anti-TNFα: 5ô-GAGGCUGAGACAUAGGCAC-dT-dT-3ô and sense or passenger anti-TNFα: 5ô-GUGCCUAUGUCUCAGCCUC-dT-dT-3ô. RNA monomers in capital letters, dT represents thymidine. The anti-TNFα siRNA has been previously described to efficiently downregulate murine TNFα mRNA [30].

3-Hydroxypropyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (7). To a solution of the tetra-*O*-acetylglucopyranosyl bromide [31] (730 mg, 1.78 mmol) in anhydrous CH_2Cl_2 (40 mL) was added Na_2SO_4 (two lips of spatula) and 1,3-propanediol (1.43 mL, 17.8 mmol). Alter stirring at room temperature for 15 min, under argon atmosphere, Ag_2CO_3 (4.9 g, 17.8 mmol) was added to the reaction mixture and was stirred for 96 hours (TLC hexane-ethyl acetate, 2:3). The mixture was then filtered over celite and the crude was purified by silica gel column chromathography using as eluent (hexane-ethyl acetate, 2:3) to give **7** (390 mg, 54%) as a syrup. $[Ŭ]_D^{20}$ -12.3 (c 1 in $CHCl_3$); ^{1}H-NMR

($CDCl_3$, 300 MHz) ŭ (ppm) 5.15 (t, *J* = 9.5 Hz, 1H,), 5.01 (t, *J*= 9.6 Hz, 1H), 4.91 (m, 1H,), 4.47 (d, *J*= 7.9 Hz, 1H, H_1), 4.18 (dd, *J*= 12.3 & 4.7 Hz, 1H, H_6), 4.09 (dd, *J*= 12.3 & 2.3 Hz, 1H, H_6), 3.93 (td, *J*= 9.6 & 5.9 Hz, 1H), 3.63 (m, 4H, 2 x CH_2O-), 2.18 (s, 1H, OH), 2.03, 1.98, 1.96, 1.94 (4s, 12H, 4 x $OCOCH_3$), 1.75 (dd, *J*= 5.7 & 4.7 Hz, 2H, -$OCH_2C\mathit{H}_2CH_2O$-). ^{13}C-NMR ($CDCl_3$, 75 MHz) ŭ (ppm) 171.1 (C=O), 170.6 (C=O), 169.9 (C=O), 169.8 (C=O), 101.1 (C_1), 73.1, 72.2, 71.6, 68.8, 68.0, 62.3, 60.2, 32.5 ($CH_2\underline{C}H_2CH_2$), 21.1 ($OCOCH_3$), 21.0 ($OCOCH_3$), 20.9 ($OCOCH_3$), 20.9 ($OCOCH_3$). HRMS (ES^+) Calcd. for $C_{17}H_{26}O_{11}$ (M+H): 407.1553, found; 407.1555.

3-[(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyloxy)]propyl (2-cyanoethyl) (*N*,*N*-diisopropyl) phosphoramidite (8). Diisopropylethylamine (DIEA) (0.040 mL, 0.228 mmol) and 2-cyanoethyl-*N*,*N*ô-diisopropylamino-chlorophosphoramidite (20 µL, 0.092 mmoL) were added to a solution of compound **7** (25.0 mg, 0.061 mmol) in anhydrous CH_2Cl_2 (2 mL) at room temperature under an argon atmosphere. After 1.0 h no starting material was observed. Solvent was then removed and the crude was purified by silica gel column chromatography by using hexane-ethyl acetate (1:1 with 5% of triethylamine) to give compound **8** (35 mg, 95%) as a syrup. ^{1}H-NMR ($CDCl_3$, 300 MHz) (mix of isomers) ŭ (ppm) 5.13 (dt, *J* =4.2 & 9.5 Hz, 1H, H_3), 5.01 (t, *J*= 9.6 Hz, 1H, H_4), 4.90 (t, *J*= 8.6 Hz, 1H, H_2), 4.45 (t, *J*= 8.3 Hz, 1H, H_1), 4.20 (dd, *J*= 12.1 & 4.0 Hz, 1H, H_6), 4.05 (d, *J*= 12.1 Hz, 1H, H_{66}), 3.93-3.84 (m, 1H, H_5), 3.83-3.44 (m, 8H, 2 x CH_2O- + -$OC\underline{\mathit{H}_2}CH_2CN$ + $2CH_{isopropyl}$), 2.63-2.53 (m, 2H, -$OCH_2C\underline{\mathit{H}_2}CN$), 2.01, 1.98, 1.95, 1.93 (4s, 12H, 4 x $OCOCH_3$), 1.80 (m, 2H, -$OCH_2C\mathit{H}_2CH_2O$-), 1.11 (d, 12H, *J*=6.5 Hz, $4CH_{3isopropyl}$). HRMS (ES^+) Calcd. for $C_{26}H_{44}N_2O_{12}P$ (M+H): 607.2632, found; 607.2638.

Synthesis of oligoribonucleotide-carbohydrate conjugates.

Oligoribonucleotide carrying carbohydrates at the 5ô-end were synthesized by a DNA synthesizer (*Applied Biosystems 3400*) using 2-cyanoethyl phosphoramidites and polystyrene solid supports (LV200). The following sequences were prepared: Glucose-C3-5'-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô, $(Glucose-C2)_2$-DB-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô, $(Glucose-C2)_4$-DB-DB-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô, Galactose-C2-5'-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô, $(Galactose-C2)_2$-DB-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô, $(Galactose-C2)_4$-DB-DB-GUG CCU AUG UCU CAG CCU C-dT-dT-3ô. DB stands for the symmetric doubler phosphoramidite obtained from commercial sources (*Glen Research*); C3 stands for $-CH_2CH_2CH_2-OPO_2^-$-; C2 for $-CH_2CH_2-OPO_2^-$-; and dT for thymidine. The synthesis of carbohydrate phosphoramidites of Glucose-C2 (**9**) and Galactose-C2 (**10**) is described in [14, 15] whereas details for the preparation of Glucose-C3 phosphoramidite (**8**) are described above. Guanosine was protected with the dimethylaminomethylidene group, cytidine was protected with the acetyl group and adenosine with the benzoyl group. The 2ô-OH protecting group for the RNA monomers was the *t*-butyldimethylsilyl (TBDMS) group. The phosphoramidites were dissolved in dry acetonitrile (0.1M) and a modified cycle was used with increased coupling time (10 min). The solid supports were treated with concentrated aqueous ammonia-ethanol (3:1) for 1hr at 55ºC. After filtration of the solid supports, the supports were washed with ethanol and the combined solutions were evaporated to dryness. Sequences were treated with 0.15 ml of triethylamine hydrofluoride / triethylamine / *N*-methylpyrrolidone (4:3:6) for 2.5 hours at 65 ºC to remove the *t*-butyldimethylsilyl groups. The reactions were stopped by addition of 0.3 ml of isopropoxytrimethylsilane and 0.75 ml of ether. The resulting mixtures were mixed with the vortex and cooled at 4

ºC. A precipitate was formed that was centrifugated at 7000 rpm, 5 min at 4ºC. The precipitates were washed with ether and centrifuged again. The residues were dissolved in water and the conjugates were purified by HPLC. HPLC conditions were: column: Nucleosil 120-10 C_{18} (250x4 mm); 20 min linear gradient from 0% to 50% B (DMT off conditions); flow rate 3 ml/min; solution A was 5% acetonitrile in 0.1 M aqueous triethylammonium acetate (TEAA) buffer and B 70% acetonitrile in 0.1 M aqueous TEAA. The purified products were analyzed by MALDI-TOF mass spectrometry. MALDI-TOF spectra were performed using a *Perseptive* Voyager DETMRP mass spectrometer, equipped with nitrogen laser at 337 nm using a 3ns pulse. The matrix used contained 2,4,6-trihydroxyacetophenone (THAP, 10 mg/ml in ACN/ water 1:1) and ammonium citrate (50 mg/ ml in water). Glucose-C3-RNA **1** [M] = 6861 (expected M= 6858). $(Glucose\text{-}C2)_2$-DB-RNA **3** [M] = 7476 (expected M= 7478). $(Glucose\text{-}C2)_4$-DB-DB-RNA **5** [M] = 8755 (expected M= 8754). Galactose-C2-RNA **2** [M] = 6847 (expected M= 6841). $(Galactose\text{-}C2)_2$-DB-RNA **4** [M] = 7461 (expected M= 7478). $(Galactose\text{-}C2)_4$-DB-DB-RNA **6** [M] = 8746 (expected M= 8754). Yields (0.2 µmol scale synthesis) were between 15-20 OD units at 260 nm. Melting temperatures of siRNA duplexes (15 mM HEPES-KOH pH 7.4; 1 mM $MgCl_2$, 50 mM potassium acetate) : Unmodified 83.3 ºC; **1** 83.7 ºC; **2** 84.7 ºC; **5** 82.1 ºC; **6** 83.0 ºC.

Cell culture, transfection and cellular assays.

HeLa cells were cultured under standard conditions (37ºC, 5% CO_2, Dulbeccoôs Modified Eagle Medium, 10% fetal bovine serum, 2mM L-glutamine, supplemented with penicillin (100 U/ml) and streptomycin (100mg/ml). All in vitro experiments were conducted at 40-60% confluence. HeLa cells were transfected with 250 ng of murine expressing TNF-α plasmid using lipofectin (*Invitrogen*) according to the manufacturerôs

instructions. One hour following transfection murine TNF-α expressing HeLa cells were transfected with 50nM of siRNA (5ô-GUGCCUAUGUCUCAGCCUC-dT-dT-3ô / 5ô GAGGCUGAGACAUAGGCAC-dT-dT-3ô, [30]) against TNF-α, using oligofectamine (*Invitrogen*). TNF-α concentration was determined from cell culture supernatant by enzyme-linked immunoabsorbent assay kit (*Bender MedSystems*) according to the manufacturerôs instructions.

RESULTS AND DISCUSSION

Synthesis of oligoribonucleotide carrying glucose and galactose

Among the different approaches described to prepare carbohydrate oligonucleotide conjugates [19,20] we selected the protocol based on the coupling of a sugar phophoramidite to oligonucleotides on a solid support. This approach has been previously used in our group to introduce several sugars in DNA [14,15]. We have extended this approach to prepare RNA-carbohydrate conjugates that possess one, two and four glucose or galactose moieties. Glucose and galactose phosphoramidites carrying C2 and C3 spacers (scheme 2) were synthesized in two steps: classical glycosylation from their corresponding bromo peracetylated sugars and ethylene glycol or propylene glycol and subsequent standard phosphoramidite chemistry as described in [14,15].

Oligoribonucleotide carrying glucose and galactose at the 5ô-end were synthesized. using the appropriate carbohydrate phosphoramidites (Scheme 2) and the commercial dendrimer symmetric doubler phosphoramidite. We selected the 21mer RNA sequence 5'-GUGCCUAUGUCUCAGCCUC-dT-dT-3ô for the introduction of one or several

glucose or galactose moieties at the 5ô-end . This RNA sequence is the passenger strand of a siRNA described to downregulate TNF-α [30]. Oligonucleotides presented a major peak with some impurities (Figure 1) that were easily separated by HPLC obtaining the desired conjugates in good yields after HPLC purification. As described in [14] some side products were observed after the synthesis of the branched oligonucleotides that lack one or two glucose or galactose residues. It is described that phosphoramidite couplings after branching are somehow less efficient [32] but this fact did not prevent obtaining pure RNA conjugates with two and four glucose or galactose units. Mass spectrometry analysis confirmed the expected molecular weight. Denaturation melting curves of siRNA duplexes formed by mixing unmodified guide strand and passenger RNA strands **1**, **2**, **5** and **6** shown little diferences (experimental section) compared with unmodified siRNA. These results indicate that the presence of carbohydates at the 5ô-end of the passenger strand do not affect hybridization properties of modified RNA strands.

Inhibition of TNF-α.

Tumor necrosis factor (TNF-α) was selected as a target for RNA interference studies. This protein is a major mediator of apoptosis as well as inflammation and immunity, and it has been implicated in the pathogenesis of a wide spectrum of human diseases. Therefore, the inhibition of TNF-α is of special interest especially for the development of antiflammatory drugs. Oligoribonucleotide sequences carrying carbohydrates at the 5ô-end of the passenger strand were annealed with equimolar amounts of the unmodified guide strand and the resulting duplexes were used for inhibition of the expression of the TNF-α gene. HeLa cells were transfected first with the murine TNF-α plasmid using lipofectin and one hour later were co-transfected with the siRNA duplex (50 nM) using

oligofectamine. After 24 hrs the amount of TNF-α produced by the cells was analyzed by enzyme-linked immunoabsorbent assay (ELISA). Figures 2 and 3 show the amount of TNF-α produced after 24 hrs of transfection of 50nM siRNA unmodified duplex (Unmodified), 50 nM of the same siRNA duplex carrying one, two or four glucose (Figure 2) or galactose (Figure 3) residues at the 5ô-end of the passenger strand and 50 nM of a scrambled RNA duplex control sequence (Scr). Both, single modified and unmodified siRNA against TNF-α produced an 80-90% inhibition of the production of TNF-α compared with the scrambled control siRNA duplex. However, the presence of either two or four carbohydrate residues produced a decrease of the inhibitory properties. These results indicate that the introduction of either glucose or galactose at the 5ô-end of the passenger strand of an RNA duplex is well tolerated by the RNAi machinery in HeLa cells although branching somehow reduces the inhibitory properties of the resulting siRNA duplex about 2-fold.

In addition we have analyzed the inhibition of the TNF-α production in Hela cells by the siRNA duplexes carrying glucose without using oligofectamine (Figure 4). In this case no inhibition of the TNF-α production was noted indicating that the siRNA duplexes carrying one, two or four glucose residues are unable to enter HeLa cells.

In order to evaluate the potential uptake of siRNAs carrying galactose, human hepatoma HuH-7 cells were used. These cells have abundant asialoglycoprotein receptors as shown by incubation of α-D-galactopyranosyl-albumine labelled with tetramethylrhodamine phenylisotiocyanate (α-D-galactopyranosyl-TRITC-albumine, *Sigma*) (supplementary data). HuH-7 cells were transfected with the murine TNF-α plasmid using lipofectin and one hour later were treated with the corresponding siRNA duplexes (100 nM) without transfecting agent. After 48 hrs the amount of TNF-α

produced by the cells was analyzed by ELISA (Figure 5). A small inhibition of TNF-α (up to 25%) was observed with the siRNAs carrying galactose with no significant differences between the siRNAs having 1, 2 or 4 galactoses. It has been described that transfection of galactose derivatives may be inhibited by galactosylated proteins such as asialofetuin [33]. In order to confirm that the small inhibitory properties of siRNAs carrying galactoses were due to asialoglycoprotein receptor mediated uptake we analysed the amount of TNF-α produced by the HuH-7 cells in the presence of a large excess of α-D-galactopyranosyl-TRITC-albumine that is a ligand for asialoglycoprotein receptor. Figure 6 shows that in the presence of the competitor no inhibition of TNF-α is observed. These results indicate that, most probably, the inhibition of TNF-α by siRNA carrying galactose is mediated by asialoglycoprotein receptor uptake. Unfortunately the efficacy of the uptake is low for the practical use of these siRNA conjugates without transfecting agent. Ideally the carbohydrate moiety should be separated from the oligonucleotide to optimize binding efficiency [34, 35]. However, results from antisense studies using oligonucleotide conjugates with carbohydrates similar to our work showed good results in antisense inhibition of gene expression [36].

In conclusion we have studied the effect of the 5ô-insertion of one, two or four glucose or galactose units on the inhibitory properties of siRNA duplexes. siRNA duplexes covalently linked to carbohydrates can be efficiently delivered to HeLa cells and these conjugates enter the RNAi pathway to silence gene expression with similar or slightly reduced efficiency compared to unmodified siRNA duplexes. When HeLa cells were treated with siRNA carrying glucose residues without oligofectamine, no inhibition was observed. On the contrary, some inhibition of the expression of TNF-α was found when galactose modified siRNAs are used without transfection agent in HuH-7 cells that have abundant asialoglicoprotein receptors. This inhibition was eliminated when the

receptors are blocked with a competitor. These results indicate a possible mediation of cellular asialoglicoprotein receptors in the uptake of galactose-siRNA conjugates.

Acknowledgements. This work was supported by the CSIC (PIF06-045); the Spanish Ministry of Education (grants BFU2007-63287, CTQ2006-01123, CTQ2010-20541, CTQ2009-13705), Generalitat de Catalunya (2009/SGR/208). CIBER-BBN is an initiative funded by the VI National R&D&i Plan *2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions* and financed by the Instituto de Salud Carlos III with assistance from the *European Regional Development Fund.* RL thanks CSIC for a JAE contract.

References.

[1] Uhlmann E, Peyman A (1990) Antisense oligonucleotides: a new therapeutic principle. Chem Rev 90: 543-584. doi:10.1021/cr00102a001

[2] Wilson C, Keefe AD (2006) Building oligonucleotide therapeutics using non-natural chemistries. Curr Opin Chem Biol 10: 607-614. doi:10.1016/j.cbpa.2006.10.001

[3] Aboul-Fadl T (2005) Antisense oligonucleotides: the state of the art. Curr Med Chem 12: 2193-2214. doi:10.2174/0929867054864859

[4] Chan JH, Lim S, Wong WS (2006) Antisense oligonucleotides: from design to therapeutic application. Clin Exp Pharmacol P 33: 533-540. doi:10.1111/j.1440-1681.2006.04403.x

[5] Bumcrot D, Manoharan M, Koteliansky V, Sah DWY (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol 2: 711-719. doi:10.1038/nchembio839

[6] De Fougerolles A, Manoharan M, Meyers R, Vornlocher HP (2005) RNA interference in vivo: toward synthetic small inhibitory RNA-based therapeutics Method Enzymol 392: 278-296. doi:10.1016/S0076-6879(04)92016-2

[7] Sioud M, Iversen PO (2005) Ribozymes, DNAzymes and small interfering RNAs as therapeutics. Curr Drug Targets 6: 647-653. doi: 10.2174/1389450054863653

[8] Urban E, Noe CR (2003) Structural modifications of antisense oligonucleotides. Farmaco 58: 243-258. doi:10.1016/S0014-827X(03)00022-3

[9] Jeong JH, Mok H, Oh YK, Park TG (2009) siRNA conjugate delivery systems. Bioconjugate Chem 20: 5-14. doi:10.1021/bc800278e

[10] Yan H, Tram K (2007) Glycotargeting to improve cellular delivery efficiency of nucleic acids. Glycoconjugate J 24: 107-123. doi:10.1007/s10719-006-9023-y

[11] Zhang H, Ma Y, Sun XL (2010) Recent developments in carbohydrate-decorated targeted drug/gene delivery. Med Res Rev 30: 270-289. doi:10.1002/med.20171

[12] Zhu L, Ye Z, Cheng K, Miller DD, Mahato RI (2008) Site-specific delivery of oligonucleotides to hepatocytes after systemic administration. Bioconjugate Chem 19: 290-298. doi:10.1021/bc070126m

[13] Dubber M, Fréchet JM (2003) Solid-Phase synthesis of multivalent glycoconjugates on a DNA Synthesizer. Bioconjugate Chem 14: 239-246. doi:10.1021/bc0256244

[14] Ugarte-Uribe B, Pérez-Rentero S, Lucas R, Aviñó A, Reina JJ, Alkorta I, Eritja R, Morales JC (2010) Synthesis, cell-surface binding, and cellular uptake of fluorescently

labeled glucose-DNA conjugates with different carbohydrate presentation. Bioconjugate Chem 21: 1280-1287. doi:10.1021/bc100079n

[15] Morales JC, Reina JJ, Díaz I, Aviñó A, Nieto, PM, Eritja R (2008) Experimental measurement of carbohydrate-aromatic stacking in water by using a dangling-ended DNA model system. Chem-Eur J 14: 7828-7835. doi:10.1002/chem.200800335

[16] Maier MA, Yannopoulos CG, Mohamed N, Roland A, Fritz H, Mohan V, Just G, Manoharan M (2003) Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. Bioconjugate Chem 14:18-29. doi:10.1021/bc020028v

[17] Oishi M, Nagasaki Y, Itaka K, Nishiyama N, Kataoka K (2005) Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. J Am Chem Soc 127: 1624-1625. doi:10.1021/ja044941d

[18] Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, Bertin SL, Reppen TW, Chu Q, Blokhin AV, Hagstrom JE, Wolff JA (2007) Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. P Natl Acad Sci USA 104: 12982-12987. doi:10.1073/pnas.0703778104

[19] Zatsepin TS, Oretskaya, TS (2004) Synthesis and applications of oligonucleotide-carbohydrate conjugates. Chem Biodivers 1: 1401-1417. doi:10.1002/cbdv.200490104

[20] Lönnberg H (2009) Solid-phase synthesis of oligonucleotides conjugates useful for delivery and targeting of potencial nucleic acid therapeutics. Bioconjugate Chem 20: 1065-1094. doi:10.1021/bc800406a

[21] Akhtar S, Routledge A, Patel R, Gardiner JM (1995) Synthesis of mono- and dimannoside phosphoramidite derivatives for solid-phase conjugation to

oligonucleotides. Tetrahedron Lett. 36: 7333-7336. doi:10.1016/0040-4039(95)01512-G

[22] Sheppard TL, Wong C-H, Joyce GF (2000) Nucleoglycoconjugates: design and synthesis of a new class of DNA-carbohydrate conjugates. Angew Chem Int Ed 39: 3660-3663. doi:10.1002/1521-3773(20001016)39:20

[23] Adinolfi M, De Napoli L, Di Fabio G, Iadonisi A, Montesarchio D (2004) Modulating the activity of oligonucleotides by carbohydrate conjugation: solid phase synthesis of sucrose-oligonucleotide hybrids. Org Biomol Chem 2: 1879-1886. doi:10.1039/b401819b

[24] Pourceau G, Meyer A, Vasseur JJ, Morvan F (2009) Synthesis of mannose and galactose oligonucleotide conjugates by bi-click chemistry. J Org Chem 74:1218-1222. doi:10.1021/jo802536q

[25] Forget D, Renaudet O, Defranq E, Dumy P (2001) Efficient preparation of carbohydrate-oligonucleotide conjugates (COCs) using oxime bond formation. Tetrahedron Lett. 42: 7829-7832. doi:10.1016/S0040-4039(01)01682-3

[26] Katajisto J, Virta H, Lönnberg H (2004) Solid-phase synthesis of multiantennary oligonucleotide glycoconjugates utilizing on-support oximation. Bioconjugate Chem 15: 890-896. doi:10.1021/bc049955n

[27] Matulic-Adamic J, Serebryany V, Haeberli P, Mokler VR, Beigelman L (2002) Synthesis of N-acetyl-D-galactosamine and folic acid conjugated ribozymes. Bioconjugate Chem 13: 1071-1078. doi:10.1021/bc025525q

[28] Edupuganti OP, Renaudet O, Defrancq E, Dumy P (2004) The oxime bond formation as an efficient chemical tool for the preparation of 3ô,5ô-bifunctionalised oligodeoxyribonucleotides. Bioorg Med Chem Lett 14: 2839-2842. doi:10.1016/j.bmcl.2004.03.053

[29] Zhao Y, Tram K, Yan H (2009) Synthesis and characterization of mannosylated oligoribonucleotides Carbohyd Res 344: 2137ï 2143. doi:10.1016/j.carres.2009.08.033

[30] Sorensen DR, Leirdal M, Sioud MJ (2003) Gene silencing by systemic delivery of synthetic siRNA in adult mice. J Mol Biol 327: 761-766. doi:10.1016/S0022-2836(03)00181-5

[31] Karjala S, Link KP (1940) Synthesis of Glycol Glucosides. J Am Chem Soc 62: 917-920. doi:10.1021/ja01861a058

[32] Grimau M, Iacopino D, Aviñó A, de la Torre B G, Ongaro A, Fitzmaurice D, Wessels J, Eritja R (2003) Synthesis of branched oligonucleotides as templates for the assembly of nanomaterials. Helv Chim Acta 86: 2814-2826. doi:10.1002/hlca.200390232

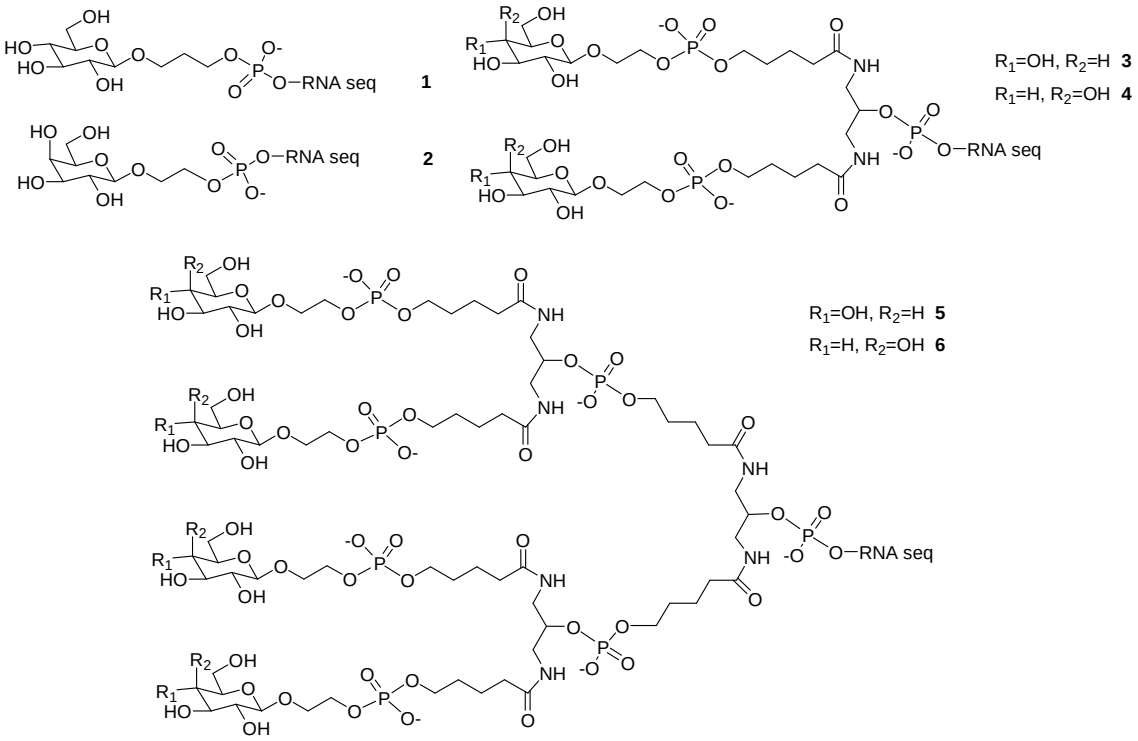
[33] Ren T, Zhang G, Liu D (2001) Synthesis of galactosyl compounds for targeted gene delivery. Bioorg Med Chem 9: 2969-2978. doi:10.1016/S0968-0896(01)00203-6

[34] Biessen EAL, Beuting DM, Roelen HCPF, van der Marel GA, van Boom JH, Van Berkel TJC (1995) Synthesis of cluster galactosides with high affinity for the hepatic asialoglycoprotein receptor. J Med Chem 38: 1538-1546. doi:10.1021/jm00009a014

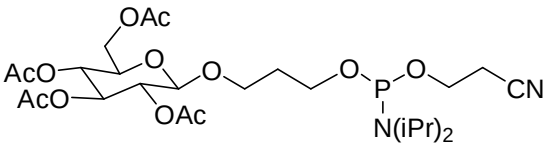
[35] Moni L, Pourceau G, Zhang J, Meyer A, Vidal S, Souteyrand E, Dondoni A, Morvan F, Chevolot Y, Vasseur JJ, Marra A (2009) Design of triazole-tethered glycoclusters exhibiting three different spacial arrengements and comparative study of their affinities towards PA-IL and RCA120 by using a DNA-based glycoarray. Chembiochem 10: 1369-1378. doi: 10.1002/cbic.200900024

[36] Hangeland JJ, Flesher JE, Deamond SF, Lee YC, Tsô POP, Frost JJ (1997) Tissue distribution and metabolism of the [^{32}P]-labeled oligodeoxynucleotide methylphosphonate-neoglycopeptide conjugate [YEE(ah-GalNAc)$_3$]-SMSS-AET-pUmpT7 in the mouse. Antisense Nucleic A 7: 141-149. doi:10.1089/oli.1.1997.7.141

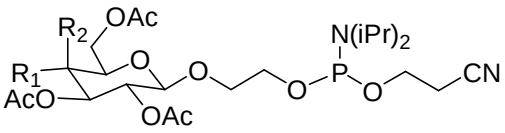
Scheme 1. Structure of the carbohydrate oligodeoxyribonucleotide conjugates prepared. RNA seq is passenger anti-TNF-α sequence: 5ô-GUGCCUAUGUCUCAGCCUC-dT-dT-3ô



Scheme 2. Sugar phosphoramidites (**8**-**10**) used in this work for the preparation of oligoribonucleotides carrying carbohydrates (**1**-**6**).



Glucose-C3 phosphoramidite **8**



R_1=OAc, R_2=H Glucose-C2 phosphoramidite **9**

R_1=H, R_2=OAc Galactose-C2 phosphoramidite **10**

Figure 1. HPLC profile of the product obtained after the synthesis of the oligoribonucleotide bearing glucose (5'- Glucose-C3-GUGCCUAUGUCUCAGCCUC-dT-dT-3ô) using DMT off conditions (see experimental section). Product eluting at 8 min is the desired oligonucleotide.

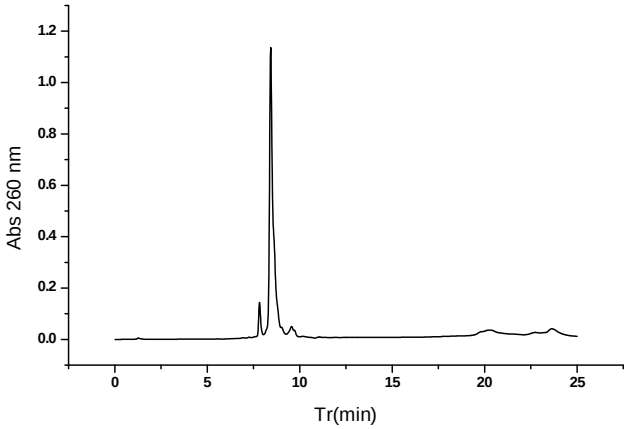


Figure 2. In vitro potency of glucose modified siRNAs against murine TNF–α in Hela cells using oligofectamine as transfecting agent for siRNA. The amount of murine TNF-α produced after 24 hrs of transfection of 50nM siRNA unmodified duplex (Unm), 50 nM of the same siRNA duplex carrying a glucose C3 molecule at the 5ô-end of the passenger strand (**1**), 50 nM of the same siRNA duplex carrying two (**3**) and four (**5**) glucose C2 molecules at the 5ô-end of the passenger strand and 50 nM of a scrambled RNA duplex control sequence (Scr). Error bars represent the s.d of the mean. Statistical analysis was by ANOVA with Bonferroni post-test, one tailed. *** P<0.001 compared with scrambled siRNA.

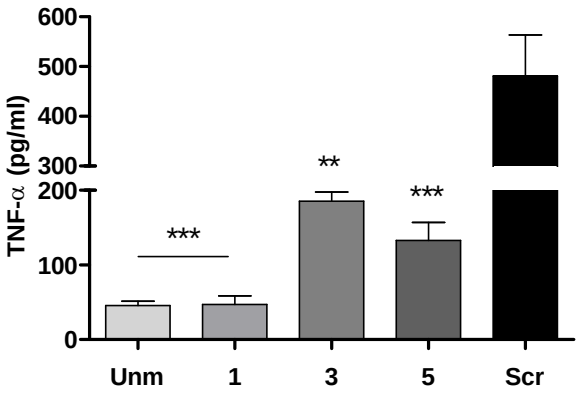


Figure 3. In vitro potency of galactose modified siRNAs against murine TNF–α in Hela cells using oligofectamine as transfecting agent for siRNA. The amount of murine TNF-α produced after 24 hrs of transfection of 50nM siRNA unmodified duplex (Unm), 50 nM of the same siRNA duplex carrying a galactose C2 molecule at the 5ô-end of the passenger strand (**2**), 50 nM of the same siRNA duplex carrying two (**4**) and four (**6**) galactose C2 molecules at the 5ô-end of the passenger strand and 50 nM of a scrambled RNA duplex control sequence (Scr). Error bars represent the s.d of the mean. Statistical analysis was by ANOVA with Bonferroni post-test, one tailed. *** P<0.001 compared with scrambled siRNA.

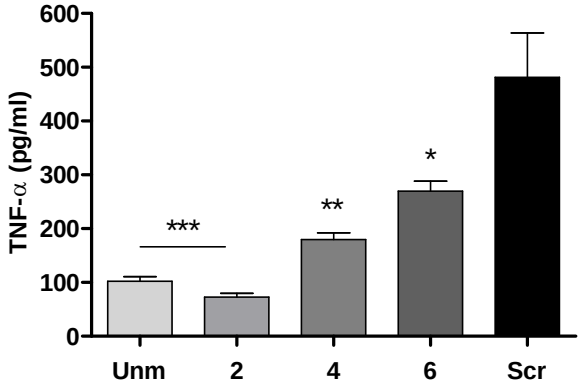


Figure 4. In vitro potency of glucose modified siRNAs against murine TNF–α in Hela cells without using oligofectamine as transfecting agent for siRNA. The amount of murine TNF-α produced after 24 hrs of transfection of 100nM siRNA unmodified duplex (Unm), 100 nM of the same siRNA duplex carrying a glucose C3 molecule at the 5ô-end of the passenger strand (**1**), 100nM of the same siRNA duplex carrying two (**3**) and four (**5**) glucose C2 molecules at the 5ô-end of the passenger strand and 100 nM of a scrambled RNA duplex control sequence (Scr). Error bars represent the s.d of the mean.

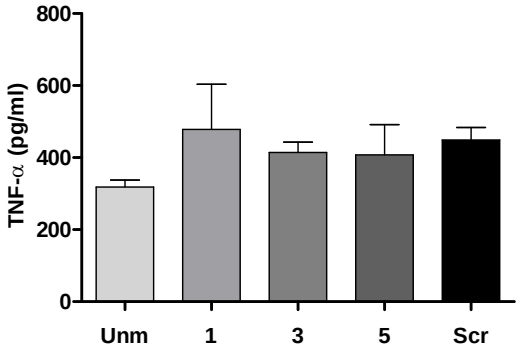


Figure 5. In vitro potency of galactose modified siRNAs against murine TNF–α in HuH-7 cells without using oligofectamine as transfecting agent for siRNA. The amount of murine TNF-α produced after 24 hrs of transfection of 100nM siRNA unmodified duplex (Unm), 100 nM of the same siRNA duplex carrying a galactose C2 molecule at the 5ô-end of the passenger strand (**2**), 100nM of the same siRNA duplex carrying two (**4**) and four (**6**) galactose C2 molecules at the 5ô-end of the passenger strand and 100 nM of a scrambled RNA duplex control sequence (Scr). Error bars represent the s.d of the mean.

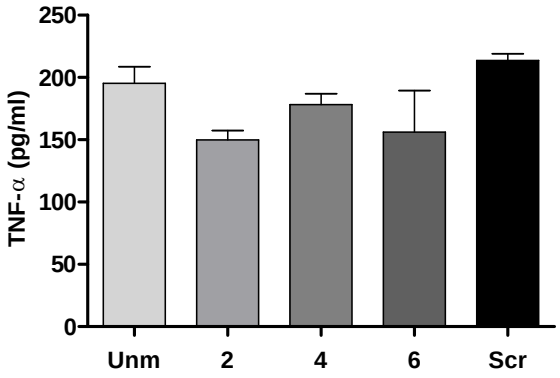


Figure 6. Competition assay between α-D-galactopyranosyl-TRICT-albumine and galactose-siRNAs for the asialoglycoprotein receptor. HuH-7 cells were transfected with pCAm TNF-α plasmid using lipofectine. One hour later 100 mM of the corresponding siRNA was added together with 100 times excess of the α-D-galactopyranosyl-TRICT-albumine ligand directly to the cells without using transfecting agent. After 48 hours the amount of TNF-α was measured by ELISA. Error bars represents the s.d of the mean.