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Synthesis and properties of small interfering RNA duplexes carrying 5-ethyluridine residues.

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Abstract. Oligoribonucleotides carrying 5-ethyluridine units were prepared using solid-phase phosphoramidite chemistry. The introduction of the *tert*-butyldimethylsilyl group at the 2ô-OH position proceeded in good yield and very high 2ô-regioselectivity. RNA duplexes carrying 5-ethyluridine either at the sense or the guide strands display RNAi activity comparable to or slightly better than that of unmodified RNA duplexes. Gene suppression experiments using luciferase targets in SH-SY5Y cells show that the ethyl group is generally well accepted at all positions although a small decrease in RNA interference activity is observed when one 5-ethylU residue is incorporated in the 3ô overhangs.

KEYWORDS: siRNA, RNA interference, 5-ethyluridine, dual luciferase assay, oligonucleotide synthesis.

Abbreviations

as, guide (antisense) strand; ACN, acetonitrile; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, *N*, *N*-dimethylaminopyridine; DMEM, Dulbeccoôs modified Eagle medium; DMT, 4,4ô-dimethoxytrityl; EtOAc, ethyl acetate; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeOH, methanol; siRNA, small interfering RNA; ss, sense strand; TBAF, tetrabutylammonium fluoride; TBDMS, *tert*-butyldimethylsilyl; TEAA, triethylammonium acetate.

INTRODUCTION

The discovery of RNA interference (RNAi) [1] and the subsequent demonstration that RNAi can be triggered by small interfering RNA (siRNA) [2] has generated intense research efforts in the development of new RNA derivatives for the control of gene expression [3, 4]. These small RNA duplexes formed by two RNA strands (known as

sense strand and guide (antisense) strand) of approximately 20-25 nucleotides long complementary to a particular messenger RNA bind to the RNA-induced silencing complex (RISC). After siRNA binding the complex formed by the guide RNA strand and RISC catalyzes the efficient degradation of a specific messenger RNA, thereby lowering the amount of target protein [5].

Some of the problems encountered during the development of nucleic acids as drugs entail the degradation of these by exonucleases under physiological conditions and their low uptake by cells. Most of the improvements in the design of nucleic acid derivatives aim to enhance stability to nuclease and improve cell uptake without affecting the hybridisation properties, which are vital for the efficient inhibitory properties of oligonucleotides. To this end, a large number of nucleic acid derivatives have been developed showing that is possible to improve stability towards nuclease degradation [3, 4, 6]. On the other hand, the delivery problem [7] is being addressed with the development of non-viral carriers such as polymers [8], lipids [9], cell-penetrating peptides [10] or nanoparticles [11].

The use of modifed bases in siRNA has been somehow less studied, but there are several examples in the literature. siRNAs containing 5-bromo, 5-iodouridine and 2,6-diaminopurine nucleoside substitutions can induce RNAi gene suppression although to a level slightly lower to the one observed for the wild type siRNA [12]. 4-Thiouridine [13], 2-thiouridine, dihydrouridine and pseudouridine [14] have also been studied with some promising results. Non-polar nucleosides carrying 2,4-difluorobenzene [15], 2,4-difluorotoluene [16], dichlorobenzene and 4-methylbenzimidazole [17] moieties have been used to determine the effect of nucleobase shape and steric effects on RNA interference. Recently, 5-methyl and 5-propynyl pyrimidine nucleosides (^{m}U and ^{p}U, respectively; Figure 1) have been used to probe major groove steric effects in the active

RISC complex [18]. It was found that the 5-methyl group was well tolerated by the RNA interference machinery, whereas the bulky 5-propynyl group was detrimental to RNA interference activity, despite its stabilization of the duplex.

Here we describe the synthesis and RNA interference activity of oligoribonucleotides carrying 5-ethyluridine (5-ethylU; ^{e}U; Figure 1). The ethyl group is larger than the methyl group but smaller than the propynyl group and it may be a good compromise between methyl and propynyl groups in terms of size, duplex stabilization properties, nuclease resistance to degradation and RNA interference activity.

EXPERIMENTAL SECTION

General experimental methods

All reagents and anhydrous solvents obtained from commercial suppliers were used without further purification. All reactions were carried out under argon atmosphere in oven-dried glassware. Thin-layer chromatography was carried out on aluminium-backed Silica-Gel 60 F_{254} plates. Column chromatography was performed using Silica Gel (60 Å, 230 x 400 mesh). NMR spectra were measured on Varian Mercury-400 or Varian-300 instruments. Chemical shifts are given in parts per million (ppm); *J* values are given in hertz (Hz). All spectra were internally referenced to the appropriate residual undeuterated solvent.

5-Iodo-N^3,$O^{2\delta}$,$O^{3\delta}$,$O^{5\delta}$-tetratoluoyluridine (**2**)

To a solution of 5-iodouridine (**1**) (Pharma-Waldorf) (3.0 g, 8.1 mmol) in dry pyridine (30 mL), DIPEA (3.8 mL, 21.9 mmol) and toluoyl chloride (8.6 mL, 64.9 mmol) were added at 0 ºC. The mixture was allowed to warm to room temperature and stirred for 20 h. Then, 8 mL of water were added and the mixture was allowed to stirr at room temperature for 1 h. The solvents were removed by rotatory evaporation. The residue

that was obtained was dissolved in CH_2Cl_2 and washed with water. The mixture was extracted with CH_2Cl_2, dried with $MgSO_4$, filtered and concentrated. The residue that was obtained was purified by silica gel column chromatography (hexanes/EtOAc 3:1) to give 3.8 g (80%) of **2** as a foam. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.04-7.76 (m, 9H, Ar, C6H), 7.32-7.11 (m, 8H, Ar), 6.38 (d, 1H, 1'H, *J* = 6.4 Hz), 5.87 (dd, 1H, 2'H, *J* = 6.0 Hz, *J* = 2.6 Hz), 5.73 (dd, 1H, 3'H, *J* = 6.0 Hz, *J* = 6.4 Hz), 4.81-4.68 (m, 3H, 4'H, 5'CH_2, 5''CH_2), 2.43, 2.40, 2.36 (3s, 12H, 4CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) 166.9, 166.1, 165.5, 165.3, 158.4, 149.1, 146.5, 144.7-143.5, 130.7-129.2, 128.2, 126.2, 125.8, 125.4, 87.4, 81.3, 73.8, 71.5, 69.5, 63.7, 21.8, 21.7, 21.6. HR-ES-MS $[M + Na]^+$ Calcd. for $C_{41}H_{35}IN_2NaO_{10}$: 865.1234, found: 865.1230.

N^3-Toluoyl-1-[2',3',5'-tri-O-toluoyl-β-D-ribofuranosyl]-5-vinyluracil (**3**)

To a solution of **2** (1.1 g, 1.3 mmol) and tetrakis(triphenylphosphine)palladium (0) (151 mg, 0.13 mmol) in hexamethylphosphoramide (10 mL), tetravinyltin (475 µL, 2.6 mmol) was added. The mixture was allowed to stirr at 60 ºC for 16 h. Then, 100 mL of water were added. The mixture was extracted with diethyl ether, dried with $MgSO_4$, filtered and concentrated. The residue that was obtained was purified by silica gel column chromatography (hexanes/EtOAc from 5:1 to 3:1) to give 716 mg (74%) of **3** as an oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.04-7.77 (m, 8H, Ar), 7.50 (s, 1H, C6H), 7.32-7.12 (m, 8H, Ar), 6.47 (d, 1H, 1'H, *J* = 6.8 Hz), 5.95 (ABX system, 1H, CH_AH$_B$CH$_X$, J_{AB} = 17.6 Hz, J_{AX} = 11.4 Hz), 5.90 (dd, 1H, H-2', *J* = 6.0 Hz, *J* = 3.2 Hz), 5.78 (ABX system, 1H, CH$_A$$H_BCH_X$, J_{AB} = 17.6 Hz, J_{BX} = 1.2 Hz), 5.76 (dd, 1H, 3'H, *J* = 6.8 Hz, *J* = 6.0 Hz), 5.03 (ABX system, 1H, CH$_A$H$_B$CH_X, J_{AX} = 11.4 Hz, J_{BX} = 1.2 Hz), 4.87 (dd, 1H, 4'H, *J* = 2.4 Hz, *J* = 12.4 Hz), 4.71 (m, 1H, 5'CH_2), 4.65 (dd, 1H, 5''CH_2, *J* = 3.2 Hz, *J* = 12.4 Hz), 2.43, 2.40, 2.37 (3s, 12H, 4CH_3). ^{13}C-NMR (100 MHz,

$CDCl_3$): δ (ppm) 167.8, 166.1, 165.5, 165.4, 160.7, 148.7, 146.3, 144.7-144.6, 135.2, 130.6-129.2, 128.7, 127.0, 126.3, 125.9, 125.5, 86.9, 81.1, 73.5, 71.4, 63.7, 21.8, 21.7, 21.7. HR-ES-MS $[M + Na]^+$ Calcd. for $C_{43}H_{38}N_2NaO_{10}$: 765.2424, found: 765.2419.

1-(β-D-Ribofuranosyl)-5-vinyluracil (**4**)

This compound has been previously synthesized by an alternative route [19-20]. Compound **3** (200 mg, 0.27 mmol) was treated with 2.6 mL of 0.5 M sodium methoxide in methanol. After 1 h at room temperature, the reaction mixture was quenched with a solution of 1N HCl (2.6 mL). The solvent was concentrated in vacuo and the residue that was obtained was purified by silica gel chromatography (CH_2Cl_2/MeOH from 95:5 to 90:10) to give 121 mg (68%) of **4** as an oil. ^{1}H-NMR (400 MHz, D_2O): δ (ppm) 7.94 (s, 1H, C6H), 6.31 (ABX system, 1H, $CH_AH_BCH_X$, J_{AB} = 17.4 Hz, J_{AX} = 11.8 Hz), 5.78 (m, 1H, 1'H), 5.67 (ABX system, 1H, $CH_AH_BCH_X$, J_{AB} = 17.4 Hz, J_{BX} = 1.2 Hz), 5.15 (ABX system, 1H, $CH_AH_BCH_X$, J_{AX} = 11.8 Hz, J_{BX} = 1.2 Hz), 4.21 (m, 1H, H2'), 4.13 (m, 1H, 3'H), 4.00 (m, 1H, 4'H), 3.84-3.69 (m, 2H, 5'CH_2, H-5"CH_2). ^{13}C-NMR (100 MHz, D_2O): δ (ppm) 164.7, 151.2, 138.0, 127.4, 116.1, 113.0, 89.8, 84.1, 74.1, 69.1, 60.3.

5-Ethyl-1-(β-D-ribofuranosyl)uracil (**5**)

This compound has been previously synthesized by an alternative route [19-21]. A mixture containing compound **4** (198 mg, 0.73 mmol), Pd/C (10%, 53.5 mg) and methanol (2 mL) was stirred under H_2 for 18 h. The mixture was filtered through Celite and the solvent evaporated from the filtrate in vacuo to give compound **5** (200 mg, quant) as a colorless oil. ^{1}H-NMR (400 MHz, D_2O): δ (ppm) 7.53 (s, 1H, C6H), 5.75 (m, 1H, 1'H), 4.18 (m, 1H, 2'H), 4.09 (m, 1H, 3'H), 3.97 (m, 1H, 4'H), 3.79-3.65 (m,

2H, 5'CH_2, 5"CH_2), 2.14 (q, 2H, CH_2-ethyl, *J* = 7.2 Hz), 0.92 (t, 3H, CH_3-ethyl, *J* = 7.2 Hz). ^{13}C-NMR (100 MHz, D_2O): δ (ppm) 166.6, 152.1, 136.7, 117.2, 89.4, 84.1, 73.9, 69.4, 60.6, 19.7, 12.0.

1-[5'-O-(4,4'-Dimethoxytrityl)-β-D-ribofuranosyl]-5-ethyluracil (**6**)

To a solution of triol **5** (191 mg, 0.71 mmol) in pyridine (3.6 mL), DIPEA (185 µL, 1.1 mmol) and DMTrCl (288 mg, 0.85 mmol) were added at 0 ºC. After 5 min the reaction was allowed to warm to room temperature and stirred for 2 h. The mixure was then quenched with a solution of $NaHCO_3$ (5%), dried with $MgSO_4$, filtered and concentrated. The residue that was obtained was purified by silica gel column chromatography (CH_2Cl_2/MeOH 98:2) to give compound **6** (190 mg, 47%) as a foam. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 10.24 (bs, 1H, NH), 7.56 (s, 1H, C6H), 7.41-7.21 (m, 9H, Ar), 6.82 (d, 4H, Ar, *J* = 8.0 Hz), 5.99 (d, 1H, 1'H, *J* = 4.4 Hz), 5.34 (m, 1H, OH), 4.40-4.35 (m, 2H, 2'H, 3'H), 4.21 (m, 1H, 4'H), 3.77 (s, 6H, 2CH_3O-dimethoxytrityl), 3.57 (m, 1H, OH), 3.53 (dd, 1H, 5'CH_2, *J* = 2.4 Hz, *J* = 10.8 Hz), 3.36 (dd, 1H, 5"CH_2, *J* = 2.8 Hz, *J* = 10.8 Hz), 1.98 (m, 1H, CH_2-ethyl), 1.75 (m, 1H, CH_2-ethyl), 0.81 (m, 1H, CH_3-ethyl). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) 163.9, 158.6, 151.3, 144.3, 135.4, 135.3, 135.2, 130.1, 130.1, 128.2, 127.9, 127.0, 117.0, 113.2, 89.7, 86.8, 84.0, 75.2, 70.7, 62.8, 55.2, 20.2, 13.1. HR-ES-MS $[M + Na]^+$ Calcd. for $C_{32}H_{34}N_2NaO_8$: 597.2213, found: 597.2208.

1-[2'-O-tert-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-5-ethyluracil (**7**)

Diol **6** (175 mg, 0.31 mmol) was dissolved in THF (3.2 mL) followed by the addition of pyridine (91 µL, 1.1 mmol) and silver nitrate (62.2 mg, 0.34 mmol). The solution was

stirred (20 min) until all the silver nitrate was dissolved and then *tert*-butyldimethylsilyl chloride (59.8 mg, 0.40 mmol) was added. The mixture was stirred at room temperature for 5 h. The solution was then filtered into a 5% $NaHCO_3$ solution (3 mL). This solution was extracted with CH_2Cl_2 and the extractes were dried and evaporated. After silica column chromatographic purification (hexanes/EtOAc from 70:30 to 60:40), 128 mg (62%) of the 2'-*O*-TBDMS protected isomer (**7**) and 3 mg (2%) of the 3'-*O*-TBDMS protected isomer were obtained as colorless oils. ^{1}H-NMR of compound **7** (g-COSY, 400 MHz, $CDCl_3$): δ (ppm) 8.63 (bs, 1H, NH), 7.52 (s, 1H, C6H), 7.43-7.29 (m, 9H, Ar), 6.87 (d, *J* = 8.8 Hz, 4H, Ar), 6.11 (d, 1H, 1'H, *J* = 6.0 Hz), 4.51 (m, 1H, 2'H), 4.27 (m, 1H, 3'H), 4.21 (m, 1H, 4'H), 3.82 (s, 6H, $2OCH_3$), 3.57 (dd, 1H, 5'CH_2, *J* = 2.4 Hz, *J* = 10.8 Hz), 3.38 (dd, 1H, 5''CH_2, *J* = 2.4 Hz, *J* = 10.8 Hz), 2.80 (bs, 1H, 3'OH), 1.86-1.81 (m, 2H, CH_2-ethyl), 0.95 (s, 9H, *t*Bu-TBDMS), 0.82 (t, *J* = 7.4 Hz, 3H, CH_3-ethyl), 0.17, 0.15 (2s, 6H, $2CH_3$-TBDMS). ^{13}C-NMR of compound **7** (100 MHz, $CDCl_3$): δ (ppm) 162.9, 158.7, 150.3, 144.2, 135.2, 135.1, 135.0, 130.1, 128.1, 128.0, 127.2, 117.4, 113.2, 87.4, 87.0, 83.8, 75.4, 71.5, 63.4, 55.2, 25.6, 20.2, 18.0, 13.5, -4.8, -5.1. HR-ES-MS of compound **7** $[M + Na]^+$ Calcd. for $C_{38}H_{48}N_2NaO_8Si$: 711.3078, found: 711.3072.

*1-[2'-O-tert-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]-5-ethyluracil 3***ô***-(2-cyanoethyl diisopropylphosphoramidite)* (**8**)

To a solution of alcohol **7** (131 mg, 0.19 mmol) in CH_2Cl_2 (2.2 mL) at 0 ºC, DIPEA (183 µL, 1.1 mmol), DMAP (6.8 mg, 0.06 mmol) and 2-cyanoethyl diisopropylphosphoramidochloridite (85 µL, 0.38 mmol) were added. After 15 min the reaction was allowed to reach room temperature and stirred for 2 h. The reaction was quenched with 5% $NaHCO_3$, extracted with CH_2Cl_2, dried with $MgSO_4$ and

concentrated. The residue that was obtained was purified by silica column chromatography (hexanes/EtOAc 60:40 + 2% Et_3N) to give 159 mg (90%) of **8** as a mixture of diastereoisomers. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 7.96 (bs, 1H, NH), 7.53 (s, 1H, NH), 7.51 (s, 1H, C6H), 7.42-7.26 (m, 18H, Ar), 6.85 (d, 4H, Ar, *J* = 8.8 Hz), 6.83 (d, 4H, Ar, *J* = 8.8 Hz), 6.13 (d, 1H, 1'H, *J* = 6.8 Hz), 6.07 (d, 1H, 1'H, *J* = 6.8 Hz), 4.54 (dd, 1H, 2'H, *J* = 6.8 Hz, *J* = 4.8 Hz), 4.46 (dd, 1H, 2'H, *J* = 6.8 Hz, *J* = 5.2 Hz), 4.34-4.21 (m, 4H, 3'H, 4'H), 4.02-3.91 (m, 2H, $POCH_2$), 3.92 (s, 6H, $2OCH_3$-dimethoxytrityl), 3.81 (s, 6H, $2OCH_3$-dimethoxytrityl), 3.66-3.62 (m, 7H, $POCH_2$, 2CH-^{i}Pr, 5'CH_2), 3.29-3.25 (m, 2H, 5''CH_2), 2.69-2.64 (m, 2H, CH_2CN), 2.32-2.28 (m, 2H, CH_2CN), 1.89-1.84 (m, 4H, $2CH_2$-ethyl), 1.31-1.28 (m, 24H, $8CH_3$-^{i}Pr), 0.92, 0.90 (2s, 18H, 2*t*Bu-TBDMS), 0.90-0.81 (m, 6H, $2CH_3$-ethyl), 0.15, 0.12, 0.11, 0.08 (4s, 12H, $4CH_3$-TBDMS). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) 162.8, 162.7, 158.7, 150.3, 150.2,144.2, 144.1, 135.3, 135.2, 135.1, 135.1, 135.0, 130.2, 130.1, 130.0, 130.0, 128.2, 128.1, 128.0, 127.9, 127.2, 117.7, 117.2, 113.3-113.2 (m), 87.1 (d, J_{CP} = 4.6 Hz), 87.0, 86.7, 83.9 (m), 83.7 (m), 77.2, 75.2, 74.6 (d, J_{CP} = 6.2 Hz), 73.5, 72.3 (d, J_{CP} = 13.8 Hz), 63.5, 63.2, 59.0 (d, J_{CP} = 14.9 Hz), 57.4 (d, J_{CP} = 19.1 Hz), 55.3, 55.2, 46.8, 45.3 (d, J_{CP} = 6.3 Hz), 43.4 (d, J_{CP} = 12.8 Hz), 42.8 (d, J_{CP} = 12.4 Hz), 29.7, 25.7, 25.6, 24.7-24.6 (m), 22.9 (dd, J_{CP} = 2.4, J_{CP} = 8.7, Hz), 20.5 (d, J_{CP} = 5.8 Hz), 20.3 (d, J_{CP} = 5.2 Hz), 19.2, 18.1, 17.9, 13.5, 13.4, -4.4, -4.9. ^{31}P-NMR (110 MHz, $CDCl_3$): δ (ppm) 151.1, 148.8. HR-ES-MS $[M + Na]^+$ Calcd. for $C_{47}H_{65}N_4NaO_9PSi$: 911.4156, found: 911.4146.

RNA synthesis and purification methods

RNA oligonucleotides were synthesized on the 0.2 µmol scale on an Applied Biosystems 394 synthesizer using 2'-*O*-TBDMS protected phosphoramidites.

Acetonitrile (ACN) (synthesis grade) and the 2'-*O*-TBDMS protected phosphoramidite monomers of A, C, G and U were from commercial suppliers. The following solutions were used: 0.4 M 1*H*-tetrazole in ACN (activation); 3% trichloroacetic acid in DCM (detritylation), acetic anhydride / pyridine /tetrahydrofuran (1: 1: 8) (capping A), 10 % *N*-methylimidazole in tetrahydrofuran (capping B), 0.01 M iodine in tetrahydrofuran/ pyridine /water (7: 2: 1) (oxidation). The coupling time was 15 min. All oligonucleotides were synthesized in DMT-ON mode. After the solid-phase synthesis, the solid support was transferred to a screw-cap glass vial and incubated at 55 ºC for 1 h with 1.5 mL of NH_3 solution (33%) and 0.5 mL of ethanol. The vial was then cooled on ice and the supernatant was transferred into a 2 mL eppendorf tube. The solid support and vial were rinsed with 50% ethanol (2 x 0.25 mL). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue that was obtained was dissolved in a total volume of 85 µL of 1M TBAF in THF and rocked at room temperature for 12 h. Then, 85 µL of 1M triethylammonium acetate (TEAA) and 330 µL of water were added to the solution. The oligonucleotide was desalted on a NAP-5 column using water as the eluent and evaporated to dryness. The oligonucleotide was purified by HPLC (DMT-ON). Column: Nucleosil 120-10 C_{18} (250x4 mm); 20 min linear gradient from 15% to 80% B and 5 min 80% B, flow rate 3 mL/min; solution A was 5% ACN in 0.1 M aqueous TEAA and B 70% ACN in 0.1 M aqueous TEAA.

The pure fractions were combined and evaporated to dryness. The residue that was obtained was treated with 1 mL of 80% AcOH solution and incubated at room temperature for 30 min. The deprotected oligonucleotide was desalted on a NAP-10 column using water as the eluent. All oligonucleotides were quantified by absorption at 260 nm and confirmed by MALDI 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).

UV-monitored thermal denaturation

Absorbance versus temperature curves of duplexes were measured at 1 µM strand concentration in 15 mM HEPES-KOH (pH 7.4), 1 mM $MgCl_2$ and 50 mM potassium acetate buffer. Experiments were performed in Teflon-stoppered 1 cm path lenght quartz cells on a JACSO V-650 spectrophotometer equipped with thermoprogrammer. The samples were heated to 90 ºC, allowed to slowly cool to 25 ºC, and then warmed during the denaturation experiments at a rate of 1 ºC/min to 85 ºC, monitoring absorbance 260 nm. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures (T_m) were determined by computerfit of the first derivative of absorbance with respect to 1/T.

RNAi methods

SH-SY5Y were grown at 37 ºC in Dulbeccoôs modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were regularly passaged to mantain exponential growth. Twenty-four hours before transfection at 50ï80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1ï3 x 10^5 cells/mL) and transferred to 24-well plates (500 µL per well). Two luciferase plasmids, *Renilla* luciferase (pRL-TK) and Firefly luciferase (pGL3) from Promega, were used as a reporter and control, respectively. Cotransfection of plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for

adherent cell lines. Per well, 1.0 µg pGL3-Control, 0.1 µg pRL-TK and 0.03ï 26 nM siRNA duplex formulated into liposomes, were applied; the final volume was 600 µL per well. The cells were harvested 22 h after transfection, and lysed using passive lysis buffer, 100 µL per well, according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a MicroLuma*Plus* LB 96V (Berthold Technologies) with a delay time of 2 s and an integrate time of 10 s. The following volumes were used: 20 µL of sample and 30 µL of each reagent (Luciferase Assay Reagent II and Stop and Glo Reagent). The inhibitory effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter (*Renilla*) luciferase gene and the control (Firefly) luciferase gene.

Stability assays in 50% human serum

Unmodified or modified double-stranded siRNA samples (20 µM; 24 µL) were incubated in human serum (24 µL) at 37 ºC. At appropriate periods (0, 0.5, 1, 2, 1, 7 and 9 hours), 6 µL aliquots of the reaction mixture were added to 54 µL of a 1% sodium dodecyl sulphate aqueous solution and the mixtures were heated-denatured for 5 min at 90 ºC. siRNAs were isolated by hot phenol extraction followed by ethanol precipitation. After re-suspension in 20 µL of loading buffer (90% formamide, 10% 1X TBE), the samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide. RNA bands were visualized with the SYBR Green II reagent (Sigma-Aldrich) according to the manufacturerôs instructions.

Statistical analysis

Data were analyzed by GraphPad Prism 5 program (GraphPad Software). Significant differences were assessed by ANOVA to compare three or more groups followed by Bonferroni test. In all figures, * represents p values < 0.05.

RESULTS AND DISCUSSION

Study of the conformational flexibility of the 5-ethyl substitution

In previous studies carried out in our laboratory, we evaluated the conformational flexibility of an ethyl group located at the C-5 position of uridine by means of computer-based modeling (which consisted in a molecular mechanics conformational search and geometry optimization of the most energetically stable conformers (Spartan ’04)). To carry out these studies, the sugar ring was kept frozen while the nucleobase and the ethyl group were allowed to move freely during the calculations. The results of these studies suggest that this system has two energy minima that are quite close in relative energies, in which (i) the ethyl group is nonlinear and has the CH_2-CH_3 bond perpendicular to the nucleobase plane, and (ii) the ethyl group is linear (see Figure 9 in the Supporting Information). Our results suggest that in order to prevent possible steric clashes, the ethyl group could fold and adopt a nonlinear conformation during the process of recognition of the ^{e}U-modified siRNA guide strand by RISC. In this conformation, the ethyl substitution would behave more like a methyl group than like a propynyl group (Figure 1), as only the methylene hydrogen atoms of the ethyl group would point toward the amino acid residues of RISC.

Synthesis of 5-ethyluridine phosphoramidite

5-Ethyluridine can be prepared either (i) by condensation of a mercury derivative of 5-ethyluracil with a poly-*O*-acylglycosyl halide [21], (ii) by condensation of silylated 5-ethyluracil with protected 1-*O*-acetyl ribofuranose using the Vorbrüggen glycosylation

procedure [22], or (iii) by catalytic hydrogenolysis of 5-vinyluridine, which can be obtained by ethylene coupling to organopalladium intermediates derived from uridine [19, 20]. In this work we have prepared 5-ethyluridine following an alternative method described for the preparation of 2ô-deoxy-5-ethyluridine [23], which is based on the cross-coupling of protected 2ô-deoxy-5-iodo-uridine with tetravinyltin in the presence of tetrakis(triphenylphosphine)palladium (0) (scheme 1). 5-Iodouridine (**1**) (Pharma-Waldorf) was treated with p-toluoyl chloride and the resulting protected nucleoside (**2**) was converted into the 5-vinyluridine derivative **3** in good yield by reaction with tetravinyltin in the presence of tetrakis(triphenylphosphine)palladium (0) in hexamethylphosphoric triamide at 60 ºC [23]. Removal of the protecting toluoyl groups with sodium methoxide in methanol at room temperature followed by catalytic hydrogenolysis gave the desired 5-ethyluridine **5**. The 5ô hydroxyl group was protected by a 4,4ô-dimethoxytrityl group (DMT) and the resulting 5ô-*O*-DMT-protected nucleoside **6** was treated with *tert*-butyldimethylsilyl (TBDMS) chloride using silver nitrate as catalyst [24] to give the desired 2ô-*O*-TBDMS-5ô-*O*-DMT-protected nucleoside **7** with excelent 2ô-regioselectivity (> 90%; as evaluated by ^{1}H NMR of the crude; see the Supplementary Data). The g-COSY ^{1}H-NMR spectrum of the major regioisomer (**7**; which could be isolated by column chromatography) confirmed that the TBDMS group was on the 2ô-OH. To the best of our knowledge there are no reports on the direct protection of the 2ô-OH group of 5ô-*O*-DMT-protected nucleosides with such high regioselectivity. For example, in the report of Hakimelahi *et al.* [24], 2ô-*O*-TBDMS-protection of 5ô-*O*-DMT-protected uridine under the above described conditions proceeded with 70 : 15 regioselectivity (2ô versus 3ô, respectively). Our results bring new data on the effect of an alkyl substituent at the 5 position of pyrimidine nucleosides on the selectivity of silylation, since the incorporation of an

ethyl group has given a remarkable improvement in 2ô-selectivity with respect to natural uridine. Finally the 2ô-*O*-TBDMS-5ô-*O*-DMT-protected nucleoside **7** was converted into the desired phosphoramidite (**8**) by reaction with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

Synthesis and thermal stability of siRNA duplexes containing 5-ethyluridine

The 5-ethyluridine (^{e}U) monomer was incorporated into different positions in place of natural U along a 21-mer siRNA that targets an A-rich site (501ï509) of *Renilla* luciferase mRNA (Table 1) [15,16,25]. This sequence allowed us to make a large number of U to ^{e}U replacements into the guide strand, which has been reported to be more sensitive to chemical modification compared to the sense strand [12]. Moreover, it is known that different regions of siRNA (as defined by the guide strand) play distinct roles in target recognition, cleavage and product release. Thus, the nature of the chemical modification, as well as their placement within the guide strand can provide useful information on the RNAi mechanism and on guide siRNA-RISC interactions. First, we prepared the unmodified siRNA **11**. Then, to localize possible steric effects in the RISC complex, we made single U (or T) to ^{e}U exchanges at different positions in the guide strand. Among the positions that were tested (Table 1) we compared 5ô-substituted siRNAs (position 2, siRNA **12**; position7, siRNA **13**) with 3ô-substituted siRNAs (position 13, siRNA **14**; position 20, siRNA **15**), since it is known that the 5ô-half of the siRNA duplex (as defined by the guide strand) is functionally different from the 3ô-half, due to the asymetric nature of siRNA recognition for initiation and unwinding [12]. siRNA **15** (containing a single ^{e}U substitution at the second position of the 3ô dinucleotide overhang) was also designed to study the effect of the ^{e}U modification on nuclease resistance. Moreover, we prepared siRNA **16**, with a single substitution at position 11; it is known that positions 10 and 11 play an important role in

the RNAi process, since mRNA clevage occurs between these two positions. Next, in order to study the effect of the number of ^{e}U substitutions on RNAi activity, we prepared siRNAs **17** and **18**, containing two substitutions at both ends of the guide strand (positions 2 and 20, siRNA **17**) and two substitutions at internal positions of the guide strand (positions 7 and 13, siRNA **18**). Finally, in order to study the effect of the modification of the dinucleotide overhangs on serum stability and the effect of the modification of the sense strand on RNAi activity, we prepared siRNA **19**, which contains a single ^{e}U substitution at position 20 of the sense strand. The desired RNA strands were purified by HPLC and characterized by MALDI-TOF mass spectrometry (Table 1).

We examined the effect of the 5-ethyluridine substitutions on siRNA duplex stability. The thermal stability of the modified and unmodified duplexes was measured in 50 mM potassium acetate, 1 mM magnesium acetate, and 15 mM HEPES-KOH at pH 7.4. Compared to the unmodified siRNA (**11**), most of the modified siRNAs had greater duplex stability (Table 2). The melting temperature (T_m) of the unmodified duplex was 67.8 ºC while the T_m of the 5-ethyluridine modified duplexes **12-15** and **17-19** were 0.6-1.4 ºC higher than unmodified duplex. Our results indicate a small but clear stabilization of the duplex induced by the 5-ethyl- uridine substitutions. This stabilization effect depends on the position of the substitution. The stabilization is similar to the one described for 5-methylU but smaller than that of 5-propynylU [18]. In contrast, the replacement of uracil by 5-ethyluracil in DNA has been described to produce a slight destabilization of the duplex [26,27].

Stability of modified siRNA in human serum

An important issue on nucleic acids drug development is the stability of the nucleic acid derivatives in serum. In DNA, oligonucleotides carrying 2ô-deoxy-5-ethyluridine have

shown a high stability to exonucleases [28] and to several restriction endonucleases [26]. In RNA oligonucleotides the presence of 5-methyluridines induced a moderate increase on the stability of siRNA in human serum [18]. A higher stability was found when uridine was replaced by 5-propynylU [18].

Unmodified and modified 5-siRNAs (**11**-**19**) were incubated in 50% human serum. At various time points, siRNAs were extracted, analyzed on a 20% polyacrylamide gel under denaturing conditions and visualized by staining. Figure 2 shows the results obtained with three representative examples: unmodified oligoribonucleotide **11** and siRNAs **15** and **17**. Contrary to what has been described for DNA oligomers we could not observe any significant difference in the degradation rates.

Inhibition of Luciferase.

To evaluate whether 5-ethyluridine-modified siRNAs are accepted by RISC and regulate gene expression via the RNAi pathway we carried out a first series of RNAi studies in SH-SY5Y cells with siRNAs containing each of the seven ^{e}U-modified guide strands (**12**ï **18**), siRNA **19** (containing a single ^{e}U substitution into the sense strand), as well as with the unmodified (wild type) RNA (**11**). In a previou work, Terrazas and Kool found that the presence of bulky substitutents at the C-5 position of uridine could be detrimental to RNAi activity and, in some cases, siRNA concentrations higher than 2.6 nM were needed in order to observe significant activity [18]. Thus, in order to avoid a great loss of activity (when compared with the unmodified siRNA), we used a relatively high siRNA concentration in the cell media (26 nM). The cells were transfected with dual reporter plasmids that express *Renilla* luciferase (the target) and nontargeted firefly luciferase as an internal nontargeted control, and with siRNAs **11**-

19. The effects of the different RNAs on luciferase expression were evaluated measuring luminescence responses after 22 h

Figure 3 shows the inhibition of the expression of *Renilla* luciferase caused by modified siRNAs at a concentration of 26 nM. Significant differences were assessed by ANOVA analysis followed by Bonferroni test. *Renilla* luciferase gene silencing was 75-90% for modified siRNAs **12-19**, and 87% for unmodified oligoribonucleotide **11**. The less active siRNAs were compounds **12**, **15** and **19**. In particular, siRNA **15** displayed RNAi activity significantly lower than the unmodified siRNA (**11**) ($p < 0.01$, Bonferroni test). These siRNAs contain a single ^{e}U at the dangling end (**15**, **19**) or at the 5ô-end of the guide strand (**12**). These results indicate that the ^{e}U substitution is less tolerated at the ends of the duplex. The inhibitory properties of siRNAs **13**, **16** and **17** were very similar to the unmodified siRNA. The most active duplexes were siRNA **14** (with a single ^{e}U modification at position 13) and siRNA **18** (with two ^{e}U modifications at positions 7 and 11).

In order to confirm these results the most active siRNAs (**14**, **17**, and **18**) were studied together with unmodified siRNA **11** in a dose-response experiment (after dosing with 0.21-210 ng of double stranded siRNAs in the cell media; concentrations of siRNA ranged from 0.03 nM-26 nM). The modified ^{e}U-siRNA **18** was slightly more active than the unmodified siRNA as shown in Figure 4 and in the Supplementary Figure 10 (at siRNA concentrations of 0.1 nM, 1.3 nM and 26 nM, $p < 0.05$; Bonferroni test). All the results confirm that 5-ethyluridine is well tolerated by the RNA machinery in a similar manner to what has been described for 5-methyluridine [18]. Based on our computational studies, we could speculate that in the presence of the RISC complex, the ethyl group could fold and adopt a nonlinear conformation, in order to avoid possible steric clashes with RISC (see Panel A in the Supplementary Figure 9). Although this

idea needs further exploration, it may provide an explanation to the observation that 5-ethylU-modified siRNAs and 5-methylU-modified siRNAs have similar biological activity. This is in contrast with previous studies that described a slight decrease of the inhibitory properties when uridine is substituted by 5-bromouridine and 5-iodouridine [12] and a large decrease when uridine is substituted by the propynyl group [18].

In conclusion, we have evaluated the effect on RNAi activity of uridine residues modified with an ethyl group at the 5-position. We described the synthesis of the appropriate phosphoramidite showing that the introduction of the *tert*-butyldimethylsilyl group at the 2ô-OH position proceeded in good yield and very high 2ô-regioselectivity. The presence of 5-ethylU residues provoked a moderate stabilization of the duplex but this stabilization did not increase the stability to degradation by serum nucleases. The ethyluridine substitution was well accepted by the RNAi machinery. In most cases although we observed a small decrease in the silencing properties when 5-ethylU was located at the dangling ends. Thus our study will support the use of 5-ethyluridine as uridine substitute for RNA interference studies.

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Table 1. MALDI-TOF mass spectra of synthesized oligonucleotides

Oligonucleotide	Sequence	MW calcd.	MW found
ss (11, wt)	3'-TTAAAAAGAGGAAGAAGUCUA-5'	6790.0	6787.6
as (11, wt)	5'-UUUUUCUCCUUCUUCAGAUTT-3'	6423.0	6426.4
12	5'- U^{e}**U**UUUCUCCUUCUUCAGAUTT-3'	6451.9	6455.6
13	5'- UUUUUC^{e}**U**CCUUCUUCAGAUTT -3'	6451.9	6451.8
14	5'- UUUUUCUCCUUC^{e}**U**UCAGAUTT -3'	6451.9	6454.4
15	5'- UUUUUCUCCUUCUUCAGAU^{e}**U**T-3'	6453.8	6456.5
16	5'- UUUUUCUCCU^{e}**U**CUUCAGAUTT -3'	6474.9 (+Na)	6470.0 (+Na)
17	5'- U^{e}**U**UUUCUCCUUCUUCAGAU^{e}**U**T -3'	6481.8	6484.2
18	5'- UUUUUC^{e}**U**CCUUC^{e}**U**UCAGAUTT -3'	6481.8	6484.9
19	3'-T^{e}**U**AAAAAGAGGAAGAAGUCUA-5'	6821.4	6824.9

Table 2. Sequences of unmodified and modified RNAs (1-9) and T_m data

				Tm (ºC)[a]	ΔTm (ºC)
native	ss	TTAAAAAGAGGAAGAAGUCUA 5'	**11 (wt)**	67.8	--
	as	5ô UUUUUCUCCUUCUUCAGAUTT			
antisense modification		TTAAAAAGAGGAAGAAGUCUA U^{e}**U**UUUCUCCUUCUUCAGAUTT	**12**	69.0	1.2
		TTAAAAAGAGGAAGAAGUCUA UUUUUC^{e}**U**CCUUCUUCAGAUTT	**13**	68.4	0.6
		TTAAAAAGAGGAAGAAGUCUA UUUUUCUCCUUC^{e}**U**UCAGAUTT	**14**	68.6	0.8
		TTAAAAAGAGGAAGAAGUCUA UUUUUCUCCUUCUUCAGAU^{e}**U**T	**15**	68.6	0.8
		TTAAAAAGAGGAAGAAGUCUA UUUUUCUCCU^{e}**U**CUUCAGAUUT	**16**	67.9	0.1
		TTAAAAAGAGGAAGAAGUCUA U^{e}**U**UUUCUCCUUCUUCAGAU^{e}**U**T	**17**	68.7	0.9
		TTAAAAAGAGGAAGAAGUCUA UUUUUC^{e}**U**CCUUC^{e}**U**UCAGAUTT	**18**	69.2	1.4
sense modification		T^{e}**U**AAAAAGAGGAAGAAGUCUA UUUUUCUCCUUCUUCAGAUTT	**19**	69.0	1.2

[a] Errors in T_m are estimated at ± 0.5 ºC.

Scheme 1 Synthesis of the 5-ethyl-U nucleoside TBDMS phosphoramidite

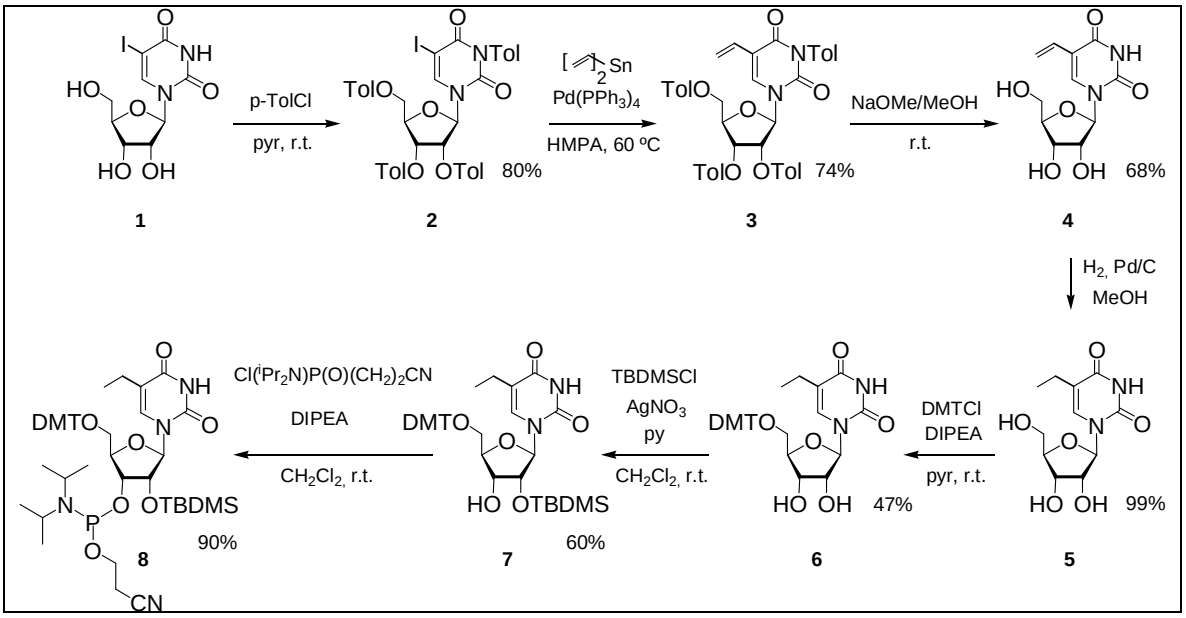
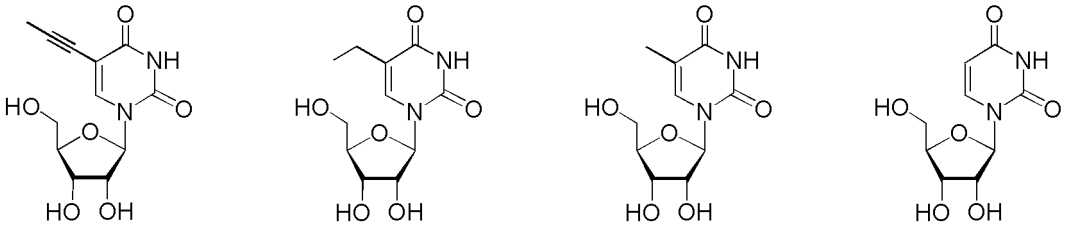


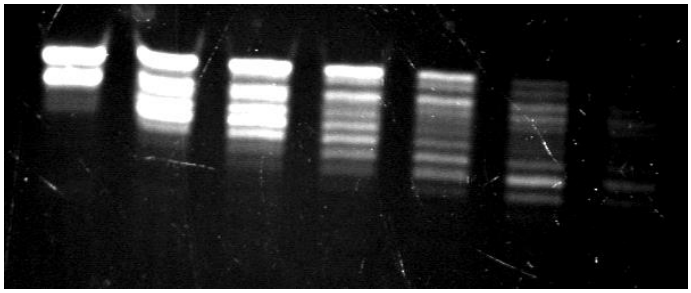
Fig. 1 Uridine derivatives containing substitutions of different size at the C-5 position



^{p}U ^{e}U ^{m}U U

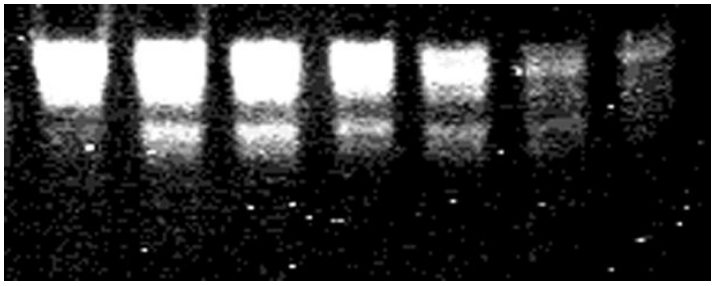
Fig. 2 Stability assays in 50% human serum

0 1/2 1 2 4 7 9 hours



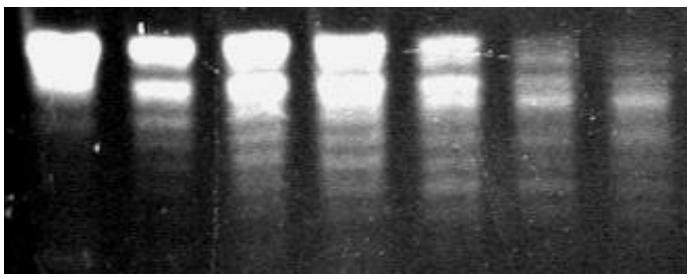
ss TTAAAAAGAGGAAGAAGUCUA 5' **11 (wt)**
as 5'-UUUUUCUCCUUCUUCAGAUTT

0 1/2 1 2 4 7 9 hours



ss TTAAAAAGAGGAAGAAGUCUA 5' **15**
as 5'-UUUUUCUCCUUCUUCAGAU^{e}**U**T

0 1/2 1 2 4 7 9 hours



ss TTAAAAAGAGGAAGAAGUCUA 5' **17**
as 5'-U^{e}**U**UUUCUCCUUCUUCAGAU^{e}**U**T

Fig. 3 Plot of RNAi activities for unmodified (**11**), and modified (**12-19**) siRNAs (at an siRNA concentration of 26 nM per well) targeting the *Renilla* luciferase gene expressed in SH-SY5Y cells. Experiments were carried out in triplicate. Bars indicate standard error. A Bonferroni test was conducted to evaluate T^N modifications to the unmodified control (**1**). ** indicates a significant change in *Renilla* luciferase expression from unmodified siRNA **1** ($p < 0.01$).

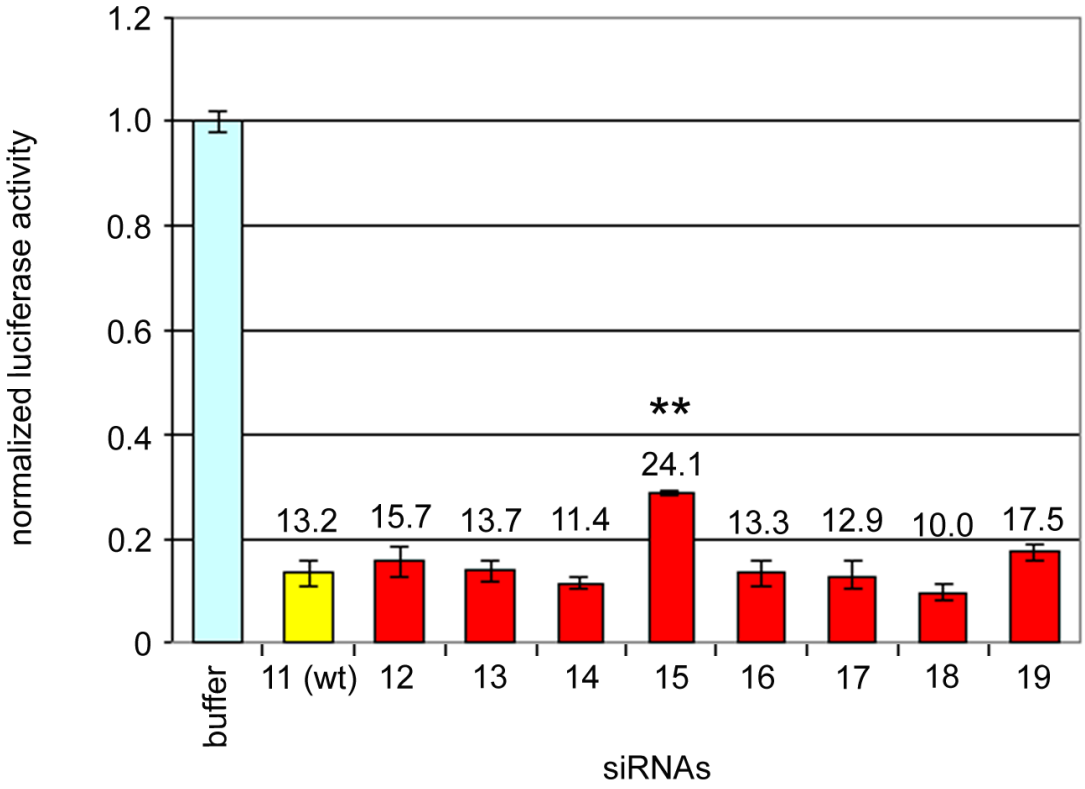


Fig. 4 Plot of gene-specific RNAi activity for unmodified siRNA (**11**) and 5-ethyl substituted siRNAs **14**, **17** and **18** expressed in SH-SY5Y cells. Varied concentrations of siRNA (0.03 nM, 0.1 nM, 1.3 nM, 26 nM) were used. Experiments were carried out in triplicate. Bars indicate standard error.