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Stepwise synthesis of RNA conjugates carrying peptide sequences for RNA interference studies.

Anna Aviñó, Sandra M. Ocampo, Clara Caminal, 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), Edifici Helix, Baldori Reixac 15, E-08028 Barcelona, Spain.

[#]Dpt. of Ciències Fisiològiques. School of Medicine. University of Barcelona (Campus Bellvitge). C/ Feixa Llarga, L'Hospitalet de Llobregat. 08907 Barcelona, Spain

Abstract. Oligoribonucleotide conjugates carrying nuclear localization peptide sequences at the 3'-end were prepared stepwise on a single support. The siRNA duplex carrying the nuclear localization peptide sequence at the 3'-end of the passenger strand has similar inhibitory properties as those of unmodified or cholesterol-modified RNA duplexes.

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

INTRODUCTION

In the last two decades new compounds comprising small synthetic nucleic acids have shown promising results as potential drugs [1, 2]. In these cases, nucleic acids are used to inhibit a specific gene by blocking translation or transcription or by stimulating the degradation of a particular messenger RNA. Several strategies can be followed. In the antisense strategy, synthetic oligonucleotides complementary to the messenger RNA of a given gene are used to inhibit the translation of messenger RNA to protein [3, 4]. In the siRNA strategy, small RNA duplexes complementary to messenger RNA bind to a protein complex named RISC. The complex formed by the antisense or guide RNA strand and RISC catalyzes the efficient degradation of a specific messenger RNA, thereby lowering the amount of target protein [5, 6].

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. A large number of nucleic acid derivatives have been developed [5, 7]. The conjugation of lipids to oligonucleotides, such as cholesterol [8, 9], produces molecules with improved inhibitory properties [10, 11].

Oligonucleotide-peptide conjugates are chimeras of oligonucleotides and peptides which are produced to add some of the biological or biophysical properties of peptides to oligonucleotides. Reaction of oligonucleotides to polylysine [12], basic [13, 14],

fusogenic [15, 16] and signal peptides [17, 18] has showed to produce new compounds with improved cellular uptake.

Peptide-oligodeoxynucleotide conjugates are prepared through distinct conjugation chemistries, which include either stable or cleavable linkages. In the post-synthetic conjugation approach, the two moieties are prepared independently on solid-phase and thiols and maleimido groups are specifically introduced to link the two molecules [17, 19]. In the stepwise approach, oligonucleotide-peptide conjugates are prepared by the addition of amino acids and nucleobases using solid-phase protocols on the same solid support [16, 20, 21, 22]. In this case, the problem to be solved is the incompatibility of standard schemes of protection. For example, at the end of the solid-phase peptide synthesis a treatment with acid is usually required, which can lead to partial depurination of DNA. In the synthesis of oligonucleotide 3'-peptides, this effect can be prevented using Boc-protected amino acids with Fm or Fmoc groups for the protection of side chains, a base-labile linker and standard phosphoramidites [16, 20, 21].

In the RNA interference field, cationic cell-penetrating peptides such as TAT-peptide and penetratin, facilitate the cell uptake of siRNA [23-28]. A particularly successful result was obtained with a peptide derived from the glycoprotein of the rabies virus [23]. The incubation of this peptide, which carries a polyarginine tail with siRNA, produced a complex that facilitates the delivery of siRNA to the central nervous system [23]. Most of the results obtained to date using peptides and siRNA were either not covalently linked to RNA or covalently linked by the post-synthetic conjugation method. Solid-phase protocols for the preparation of aminoacyl-tRNA fragments have been described [29, 30]; however to our knowledge there are no data on the preparation of RNA-peptide conjugates for RNA interference studies using the stepwise approach.

Here we describe the stepwise synthesis of oligoribonucleotides carrying nuclear localization peptide sequences. Tumor necrosis factor (TNF- α) was selected as a target. This protein is a major mediator of apoptosis as well as inflammation and immunity. Furthermore, TNF- α has been implicated in the pathogenesis of many human diseases and consequently the inhibition of this cytokine is of particular relevance.

EXPERIMENTAL SECTION

Abbreviations: ACN, acetonitrile; AmHex, 6-aminohexylsuccinyl; Boc, *t*-butoxycarbonyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMT, 4, 4 ϕ -dimethoxytrityl; Fm, fluorenylmethyl; Fmoc, fluorenylmethoxycarbonyl; PEG-PS, polyethyleneglycol-polystyrene; PyBOP, (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate, TBDMS, *t*-butyldimethylsilyl; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid.

Oligonucleotides.

The following RNA sequences were obtained from commercial sources (*Sigma-Proligo, Dharmacon*): sense or passenger scrambled 5 ϕ -CAGUCGCGUUUGCGACUGG-dT-dT-3 ϕ antisense or guide scrambled 5 ϕ -CCAGUCGCAAACGCGACUG-dT-dT-3 ϕ antisense or guide anti-TNF α : 5 ϕ -GAGGCUGAGACAUAGGCAC-dT-dT-3 ϕ and sense or passenger anti-TNF α : 5 ϕ -GUGCCUAUGUCUCAGCCUC-dT-dT-3 ϕ RNA monomers in capital letters, dT represents 2 ϕ -deoxythymidine. The 3 ϕ -cholesterol passenger anti-TNF α : strand 5 ϕ -GUGCCUAUGUCUCAGCCUC-dT-dT-3 ϕ -cholesterol

was prepared using the cholesterol-tetraethyleneglycol (TEG)-3 ϕ -CPG support from commercial sources (*Glen Research*). The anti-TNF α siRNA has been previously described to efficiently downregulate murine TNF α mRNA [31].

Synthesis of oligonucleotide-peptide conjugates.

Oligonucleotide-peptide conjugates were synthesized by the stepwise approach [32]. The following sequences were prepared I) 5'-uggauucgu-3 ϕ -p-O-(CH₂)₅CONH-^NAla-Lys-Lys-Lys-Lys-Leu-Asp-Lys^C-CONH-(CH₂)₆-OH; II) 5' CUGAC-dT-ps-dT-3 ϕ -ps-O-(CH₂)₅CONH-^NAla-Lys-Lys-Lys-Lys-Leu-Asp-Lys^C-CONH-(CH₂)₆-OH; and III) 5'-GUGCCUAUGUCUCAGCCUC-dT-dT-p-O-(CH₂)₅CONH-^NAla-Lys-Lys-Lys-Lys-Leu-Asp-Lys^C-CONH-(CH₂)₆-OH. The following abbreviations are used: 2 ϕ -O-methyl-RNA monomers in low case, RNA monomers in capital letters, dT: 2 ϕ -deoxythymidine, p: phosphate, ps: phosphorothioate linkages. Guanosine was protected with the dimethylaminomethylidene group, cytidine was protected with the acetyl group and adenosine with the benzoyl group. The 2 ϕ -OH protecting group for the RNA monomers was the *t*-butyldimethylsilyl (TBDMS) group. Boc-aminohexanol was reacted with succinic anhydride to form the corresponding hemisuccinate [20], which was reacted with amino functionalized PEG-PS support. Peptide chain was elongated manually in DMF using a 5-fold excess of amino acid Boc-protected amino acid and a 5-fold excess of PyBOP and a 10-fold excess of DIEA for 1 h. Lysine was protected with the Fmoc group and aspartic acid was protected with the Fm group. Once the peptide sequence was completed, the resulting support was reacted with *p*-nitrophenyl 6-(4,4'-dimethoxytrityloxy)hexanoate [20]. The addition of this building block introduces an hydroxyl group to the existing peptide. This hydroxyl group is required for the assembly of the oligonucleotide. The linker was coupled to the peptide-support using PyBOP and

DIEA as described above. The unreacted amino groups of the resulting support were blocked with acetic anhydride and DIEA. Oligonucleotides were prepared on a DNA synthesizer (*Applied Biosystems 3400*) using 2-cyanoethyl phosphoramidites. The following solutions were used: 0.4 M 1*H*-tetrazol in ACN (activation); 3% trichloroacetic acid in DCM (deprotection), 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 phosphoramidites were dissolved in dry DCM (0.1 M). A modified cycle was used: Coupling time was increased to 5 min, capping and oxidation times to 1 min, and deprotection time to 2 min (4 x 30 s). In the synthesis of the 2'-*O*-methyl-RNA sequence I, the last DMT-protecting group was not removed. In the RNA sequences II and III, the last DMT group was removed because the DMT is partially lost during the fluoride treatment. The average coupling yield was around 97-98% per step for RNA monomers and 99% for 2'-*O*-methyl-RNA monomers. The solid supports containing the oligonucleotide-peptide conjugates were washed with acetonitrile, treated with a 0.5 M DBU solution in ACN for 5 min, washed with ACN, and dried. The resulting supports were then treated with concentrated aqueous ammonia-ethanol (3:1) for 1 h at 55 °C. After filtration of the solid supports, the supports were washed with ethanol and the combined solutions were evaporated to dryness. Sequences II and III were treated with 0.15 ml of triethylamine.tris(hydrofluoride) / triethylamine / *N*-methylpyrrolidone (4:3:6) for 2.5 h at 65 °C to remove the TBDMS groups. The reactions were stopped by adding 0.3 ml of isopropoxytrimethylsilane and 0.75 ml of ether. The resulting mixtures were stirred and cooled at 4 °C. A precipitate was formed which was then centrifuged at 7000 rpm for 5 min at 4 °C. The precipitates were washed with ether and centrifuged again. The residues were dissolved in water and the conjugates were purified by HPLC. Column:

Nucleosil 120-10 C₁₈ (250x4 mm); 20 min linear gradient from 15% to 80% B and 5 min 80% B (DMT-on conditions, sequence I); 20 min linear gradient from 0% to 50% B (DMT-off conditions, sequences II and III); 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 purified products were analyzed by MALDI-TOF mass spectrometry. Sequence I [M] = 4161.0 (expected M= 4165.3). Yield (0.5 μmol scale synthesis) was 12 OD units at 260 nm (120 nmol, 24%). Sequence II [M] = 3376.5 (expected M= 3377.2). Yield (0.5 μmol scale synthesis) was 1.7 OD units at 260 nm (25 nmol, 5%). Sequence III [M+Na⁺] = 7785.7 (expected M= 7766.6). Yield (0.2 μmol scale synthesis) was 2.1 OD units at 260 nm (10 nmol, 5%). The purified conjugates were judged to be homogeneous (purity >90%) by analytical HPLC and denaturing polyacrylamide gel electrophoresis.

Analytical HPLC was performed using XBridgeTM OST C18 (*Waters*), 2.5 μm, 4.6 x 50 mm column using a 10 min linear gradient from 0% to 35% B, flow rate 1 ml/min; solution A was 5% ACN in 0.1 M aqueous TEAA and B 70% ACN in 0.1 M aqueous TEAA.

Denaturing polyacrylamide gel electrophoreses were performed in a SE-600 *Hoefler Scientific* apparatus. The gels (8 M urea, 20%, acrylamide/ bisacrylamide ratio 19:1), 14 x 16 x 0.1 cm in size were run for 5 h at 400 V. Three micrograms of RNA were loaded. The gel was stained with Stains-all (*Sigma*).

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).

Melting experiments.

Oligonucleotides (1 μ M each) were dissolved in 100 mM potassium acetate, 2 mM magnesium acetate, and 30 mM HEPES-KOH at pH 7.4. The solutions were heated to 90 °C, allowed to cool slowly to room temperature, and stored at 4°C until UV was measured. UV absorption spectra and melting experiments (absorbance vs. temperature) were recorded in Teflon-stoppered 1-cm path length quartz cells using a spectrophotometer, with a temperature controller and a programmed temperature increase rate of 1 °C/min. Melting curves were run in duplicate at 260 nm. In all cases the complexes displayed sharp, apparently two-state transitions. The data were analyzed by the denaturation curve-processing program.

Cell culture, transfection and cellular assays.

HeLa cells were cultured under standard conditions (37°C, 5% CO₂, Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 2mM L-glutamine, supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). All experiments were conducted at 40-60% confluence. HeLa cells were transfected with 250 ng of murine expressing the TNF- α plasmid using lipofectin (*Invitrogen*), following the manufacturer's instructions. One hour after transfection, m-TNF- α expressing HeLa cells were transfected with 50nM of siRNA (5'-GUGCCUAUGUCUCAGCCUC-dT-dT-3' / 5'-GAGGCUGAGACAUAGGCAC-dT-dT-3' [31]) against TNF- α , using oligofectamine (*Invitrogen*). The TNF- α concentration was determined from cell culture supernatant by enzyme-linked immunosorbent assay kit (*Bender MedSystems*) following the manufacturer's instructions.

The unmodified siRNA duplex produced 87% inhibition of TNF- α compared with the scrambled siRNA sequence, cholesterol-modified siRNA produced 81% inhibition and nucleoplasmine-modified siRNA produced 89% inhibition (Fig. 3).

RESULTS AND DISCUSSION

Synthesis of oligonucleotide-peptide conjugates

Oligonucleotide-peptide conjugates carrying the nucleoplasmic nuclear localization sequence (AKKKKLDK) were synthesized following the stepwise approach. Polyethyleneglycol-polystyrene (PEG-PS) was selected as solid support since it gave the best results for the coupling of amino acids and nucleoside phosphoramidites [20, 32, 33]. Scheme 1 shows the outline of the synthesis of the oligonucleotide-peptide conjugates on the same support [20, 32, 33]. To avoid the use of strong acids in the presence of the oligonucleotide, the peptide part was first synthesized using the acid-labile Boc group to protect the α -amino function. The protective groups of the side chain of the amino acids (Fmoc for Lys and Fm for Asp) and the linker of the first amino acid to the support were base-labile so that they would be removed at the same time as the protective groups of the nucleobases. To this end, the 6-aminohexylsuccinyl (AmHex, [34]) was used. This linker yields the peptide with an aminohexylamide group at the C-terminal.

Once the peptide had been synthesized, the *p*-nitrophenyl 6-(4,4'-dimethoxytrityloxy)hexanoate spacer molecule [20] was added to connect the oligonucleotide. This molecule introduces an hydroxyl group protected by a DMT group at the *N*-terminal position of the peptide. After the addition of the linker, oligonucleotide synthesis was studied in a small 2 ϕ -*O*-methyl-RNA sequence (I: 5'-uggauucgu-3' ϕ -*p*-O-(CH₂)₅CONH-^NAKKKKLDK^C-CONH-hexyl-OH) using a slightly modified synthesis cycle and 1*H*-tetrazole activation. The average coupling yield was around 99% per step. The last DMT group was not removed in order to facilitate the

purification of the conjugate by reversed phase HPLC (Fig. 1). The desired compound was purified in good yield and was characterized by mass spectrometry.

We next studied the assembly of a short RNA sequence (II: 5' CUGAC-dT-ps-dT-3' ϕ -ps-O-(CH₂)₅CONH-^NAKKKKLDK^C-CONH-hexyl-OH). This sequence contains 5 ribonucleotides, 2 thymidine residues (dT) and 2 phosphorothioate (ps) linkages at the 3' ϕ -end. These are usual modifications in RNA interference studies. RNA monomers were protected with the *t*-butyldimethylsilyl (TBDMS) group. The same modified cycle for the synthesis of the 2' ϕ -O-methyl-RNA conjugate was used. In this case the coupling yield was slightly lower 97-98% as a result of steric hindrance of the TBDMS. The last DMT group was removed before deprotection because partial loss was detected during fluoride treatment. For cleavage and deprotection, three consecutive treatments were applied: first a 0.5 M DBU solution in ACN; second, concentrate aqueous ammonia-ethanol 3:1 at 55°C, and third, triethylamine tris(hydrofluoride) in *N*-methylpyrrolidone at 65°C. HPLC purification gave a major product that had the mass expected. The yield obtained after purification (5%) was lower than that achieved in the synthesis of the 2' ϕ -O-methyl-RNA conjugate (24%). This difference is attributed to the manipulation steps followed when removing the TBDMS groups. In order to eliminate the excess of fluoride, precipitation of the conjugate with ether is required and this may lower the recovery of the desired conjugate as a result of the small size of the oligomer (7mer).

Finally, the 21mer sequence III) 5'- GUGCCUAUGUCUCAGCCUC-dT-dT-p-O-(CH₂)₅CONH-^NAKKKKLDK^C-CONH-hexyl-OH was assembled using the same protocol described for sequence II. In this case the desired conjugate was isolated in a 5% after HPLC purification (Fig. 2). The yield of the 21mer obtained after purification was the same as that of the 7mer but the former was three times longer, thereby indicating a more efficient recovery of the longer conjugate. The purified conjugate was

analyzed by HPLC and polyacrylamide gel electrophoresis and characterized by MADI-TOF mass spectrometry.

We examined the effect of the peptide on siRNA duplex stability. The thermal stability of the modified and unmodified duplexes was measured in 100 mM potassium acetate, 2 mM magnesium acetate, and 30 mM HEPES-KOH at pH 7.4. The melting temperature (T_m) of the duplex containing nucleoplasmine was 83.8 °C while the T_m of the unmodified duplex was 83.5 °C. A slightly higher stabilization of the duplex has been described for this peptide on DNA duplexes [32] and it has been attributed to a possible interaction between lysine side chain and DNA phosphates. In the present study two unpaired thymidines separated the peptide from the DNA backbone, therefore it is reasonable to expect no differences between modified and unmodified duplexes.

Inhibition of TNF- α .

Oligonucleotide-peptide sequence III was annealed with equimolar amounts of the unmodified guide strand and the resulting duplex was used to inhibit the expression of the TNF- α gene. HeLa cells were transfected first with plasmid expressing murine TNF- α using lipofectin and 1 h later were co-transfected with the siRNA duplex (50 nM) using oligofectamine. After 24 h the amount of TNF- α produced by the cells was analyzed by enzyme-linked immunosorbent assay (ELISA). Fig. 3 shows the amount of TNF- α produced after 24 h of transfection of 50nM siRNA unmodified duplexes. 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. These results indicate that the introduction of the peptide sequence at the 3' end of the passenger strand of an RNA duplex does not affect the inhibitory properties of the

resulting siRNA duplex in HeLa cells. Further work is required to determine whether the presence of the peptide improves the delivery of the siRNA into cells.

In conclusion, we have demonstrated that RNA carrying nucleoplasmine peptide can be synthesized using the stepwise approach. siRNA duplexes covalently linked to the nucleoplasmine peptide can be efficiently delivered to HeLa cells and these conjugates enter the RNAi pathway to silence gene expression as efficiently as unmodified and 3' cholesterol modified siRNA duplexes.

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Scheme 1. Outline of the synthesis of oligonucleotide-3 ϕ -peptide conjugates.

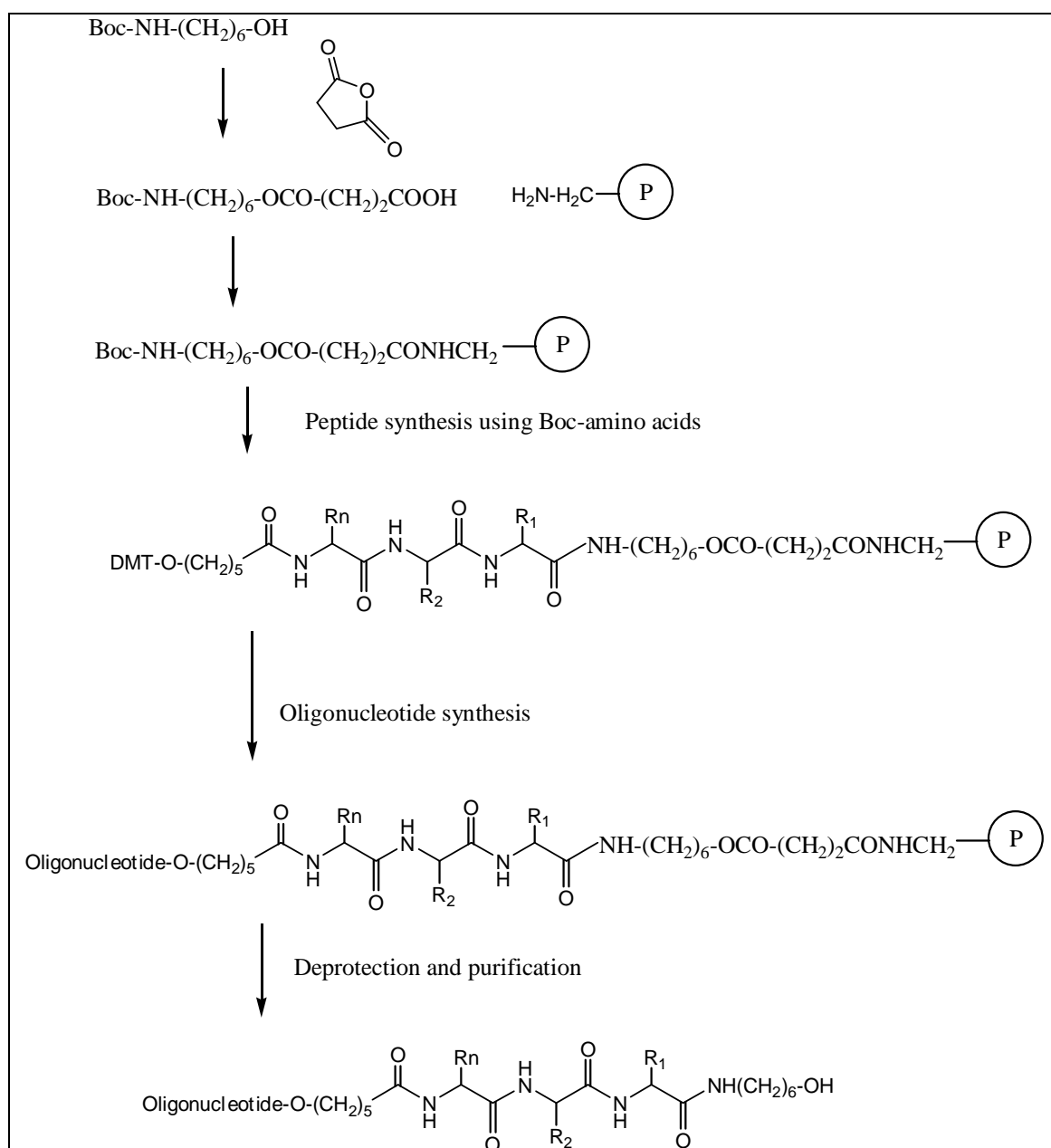


Fig. 1. HPLC profile of the product obtained after the synthesis of the nucleoplasmine-oligo-2'-O-methyl(ribonucleotide) conjugate I (5'-uggauucgu-3'-p-O-(CH₂)₅CONH-^NAla-Lys-Lys-Lys-Lys-Leu-Asp-Lys^C-CONH-(CH₂)₆-OH) using DMT-on conditions. Product eluting at 12 min is the desired conjugate. Inset: analytical HPLC of the purified product (see experimental section).

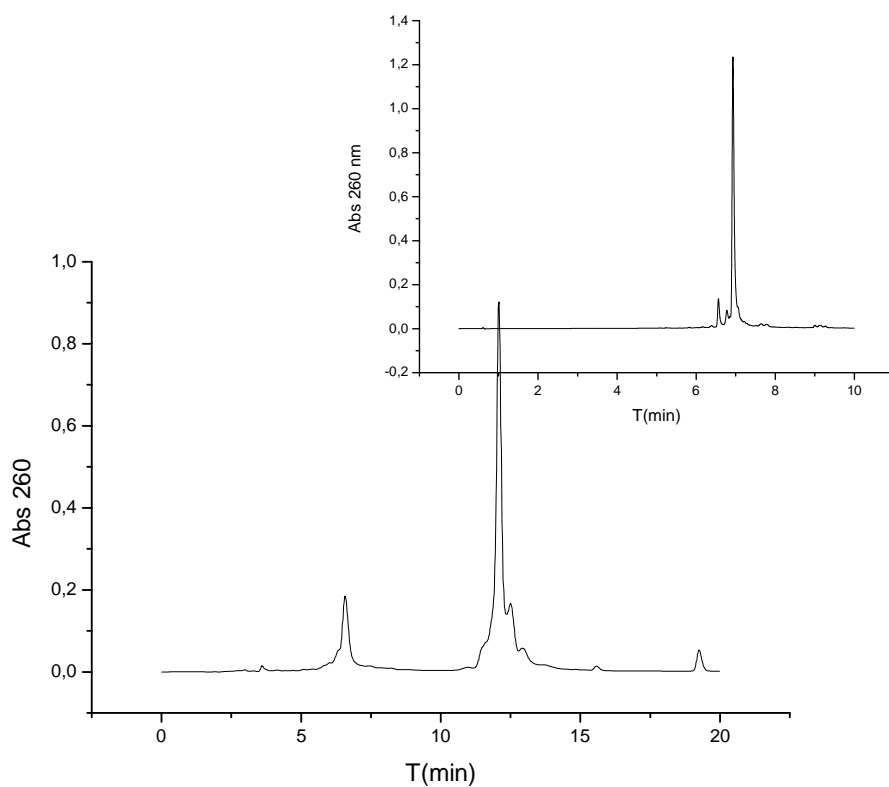


Fig. 2. HPLC profile of the product obtained after the synthesis of the nucleoplasmine-oligoribonucleotide conjugate III (5'- GUGCCUAUGUCUCAGCCUC-dT-dT-p-O-(CH₂)₅CONH-^NAla-Lys-Lys-Lys-Lys-Leu-Asp-Lys^C-CONH-(CH₂)₆-OH) using DMT-off conditions. Product eluting at 10 min is the desired conjugate. Inset: analytical HPLC of the purified product (see experimental section).

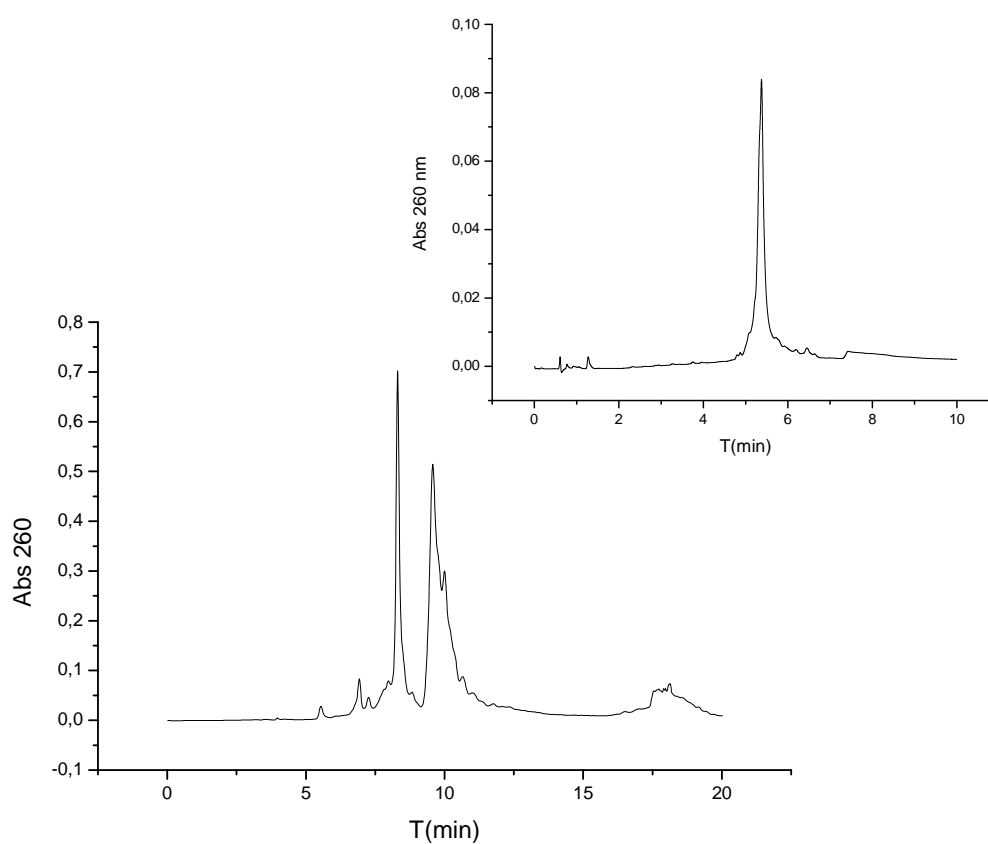


Fig. 3. Potency *in vivo* of several chemically modified siRNAs against TNF- α . Amount of TNF- α produced after 24 h of transfection of 50nM siRNA unmodified duplex (Unmodified), 50 nM of the same siRNA duplex carrying a cholesterol molecule at the 3' end of the passenger strand (Cholesterol), 50 nM of the same siRNA duplex carrying the nucleoplasmine peptide at the 3' end of the passenger strand (sequence III, Nucleoplasmine) 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. ***or ### P<0.001 compared with scrambled siRNA.

