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Synthesis and *in Vitro* Inhibition Properties of siRNA Conjugates Carrying Acridine and Quindoline.

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The synthesis of RNA molecules carrying acridine or quindoline molecules at their 3' termini and 5' termini is reported. These conjugates are fully characterized by MLADI-TOF mass spectrometry. Modified siRNA duplexes carrying acridine or quindoline residues were evaluated in the inhibition of the tumor necrosis factor. The conjugates showed similar inhibitory properties than unmodified RNA duplexes in HeLa cells transfected with oligofectamine. The fluorescent properties of acridine derivatives allow the direct observation of the cytoplasmatic distribution of modified siRNA inside the cells.

Introduction. Chemically modified oligonucleotides have a tremendous potential as therapeutics. Antisense oligonucleotides were used against a wide range of targets and some chemically modified oligonucleotides have entered in the clinic. However, RNA interference (RNAi) has energized the field and the successful use of short interfering RNA (siRNA) for gene silencing has focused most of the attention. The opportunities of siRNA in therapeutics are reduced due to poor cellular uptake [1], degradation by nucleases [2] and side effects such as stimulation of the immune response [3]. Modified siRNA have solved partially some of these obstacles [4]. Conjugation of oligonucleotides with molecules that provide novel properties to the conjugate have been extensively used [5]. Small drug molecules, aptamers, lipids, peptides, carbohydrates or polymers are conjugated to siRNA to improve pharmacokinetic behaviour while maintaining sufficient gene silencing activity [6]. Targeted delivery of siRNA to the desired cells and tissues has been considered a big challenge in the development of therapeutic siRNA. Specific interaction between a

specific ligand and its cellular membrane receptor generally enhances the cellular uptake by the aid of a mechanism of receptor mediated endocytosis [7].

Small molecules considered as groove binders or intercalators have also received considerable interest as potential groups for conjugation to oligonucleotides [8]. These conjugates do not primarily exhibit enhanced cellular uptake or cell-type specific targeting, but they can stabilize nucleic acids structures by π -stacking interactions as they are usually planar aromatic molecules. Conjugation at any terminus of oligoribonucleotides could also prevent degradation by exonucleases. In addition, these molecules are constituted of chromophores that could be used to study intracellular distribution of nucleic acids.

Different strategies are used to introduce these derivatives to the oligonucleotides. One strategy is based on the introduction of the ligands during solid phase synthesis of the oligonucleotides either as a phosphoramidite derivative or as a modified nucleoside or solid support [9]. The second strategy consists of the conjugation of the derivative and the oligonucleotide using a post synthetic approach [10].

Several derivatives including acridine, porphyrin, quindoline and other polycyclic molecules have been reported to bind and stabilize different nucleic acids structures. In particular, oligonucleotide conjugates carrying these compounds have been used in the antisense field showing improved uptake, and biodistribution as well as increased triplex stability [11].

Small interfering RNA (siRNA) is formed of two complementary strands (guide and passenger) so there are four terminal ends for potential conjugation sites. Previous studies have demonstrated that 3' and 5' ends of the sense strand and the 3' end of the

antisense are considered the best sites for conjugation without decreasing RNAi activity [12].

In this paper we have synthesized and evaluated several oligonucleotide conjugates containing single or multiple acridine or quindoline derivatives for siRNA studies (*Scheme 1*). Previously, we have used monomers of these intercalating agents to prepare oligodeoxynucleotide conjugates using solid phase methods [13, 14]. Moreover acridine has been successfully used to increase delivery of peptide nucleic acids [15]. But, to our knowledge, there is no information on the effect of acridines in RNA interference. In this article, siRNA containing acridine and quindoline moieties were tested for the inhibition of tumour necrosis factor (TNF- α) protein which is involved in apoptosis, inflammation and immunity processes [16]. Acridine or quindoline moieties may favour stability of the siRNA or prevent nuclease degradation, thus improving pharmacokinetic properties of these conjugates.

Scheme 1

Results and Discussion. 1. *Synthesis of Oligonucleotides.* Conjugation of oligonucleotides to intercalating compounds has been demonstrated by direct in-line incorporation or by post-synthetic methods [9, 10]. The direct incorporation of the intercalating drug requires the preparation of the appropriate derivative compatible with the phosphoramidite method. In addition the resulting conjugate should be stable to deprotection conditions. In the first studies we used a solid support functionalized with a derivative of acridine that was commercially available. Unfortunately, the resulting conjugate did not survive the ammonia and fluoride treatments. For that we selected the threoninol derivatives of acridine and quindoline (*Scheme 1*) which have been described

to be stable to ammonia [13, 14, 17]. L-threoninol was used as starting material for the preparation of the corresponding phosphoramidite monomers and solid supports containing the intercalating units. Threoninol has been previously described for the introduction of dyes, photoactive azabenzenes and DNA-binding drugs in oligonucleotides [14, 18, 19]. Recently, siRNA derivatives carrying aromatic groups linked through threoninol at their 3'-end were prepared to modulate binding of a passenger strand to the RISC complex [17]. The synthesis of the threoninol derivatives of acridine and quindoline (*Scheme 2*) has been previously described [13].

Scheme 2

Modified oligoribonucleotide sequences of the TNF- α gene carrying acridine and quindoline at the 5'-end or at the 3'-end (**1-6**, *Table 1*) were synthesized using the appropriate phosphoramidites and solid supports of acridine and quindoline (*Scheme 2*). These RNA sequences are the passenger and the guide strands of a siRNA described to downregulate TNF- α [16]. Oligoribonucleotides **1-6** presented a major peak along with some impurities (*Fig. 1*) that were easily separated by HPLC obtaining the desired conjugates in good yields.

Figure 1

The good results obtained in these syntheses proved the stability of the acridine- and quindoline-threoninol derivatives in both ammonia and fluoride deprotection conditions. For these reasons, sequences **7-8** having four acridine or quindoline units were synthesized using the commercially available symmetric doubler (DB). This

phosphoramidite was added two consecutive times at the 5' termini generating four sites for the attachment of acridine or quindoline phosphoramidites. In this way, 5' modified RNA conjugates with four intercalating units were obtained. Yields were moderated as the coupling of the branching units was less efficient than standard phosphoramidites [20]. RNA strands with intercalating units at the end of the sequences were easily purified by HPLC from the truncated sequences as these products are less retained in the chromatographic column. *Figure 2* shows the HPLC profile of oligonucleotide sequence **8** carrying four quindoline units. The desired RNA strand had a retention time of 16.7 min. Mass spectrometry analysis confirmed the expected molecular weight (*Table 1*). This indicates that the acridine- and quindoline-conjugates are stable to oligonucleotide synthesis conditions.

Figure 2

2. *Thermal denaturation studies.* Thermal denaturation studies are summarized in *Table 1*. The presence of the acridine and quindoline moiety induced a small stabilization of the duplex structure. In general, duplexes containing quindoline have a higher melting temperature than acridine conjugates (except siRNA **1** and **2**). This increased stabilization of quindoline derivatives compared to acridine derivatives have also been found in quadruplex structures [13]. Melting temperatures of duplexes containing 3' modifications are similar to those of 5'-modified duplexes. Oligonucleotide duplexes carrying four acridine or quindoline molecules **7** and **8** have lower melting temperatures than the unmodified duplex. Nevertheless, the small destabilization (0.4-0.6 °C) is in the range of the uncertainty of the melting temperatures (0.5 °C). In summary, the presence of acridine and quindoline modifications in the

siRNA duplexes induces a weak stabilization of the siRNA duplex, except in the case of tetrasubstituted siRNA.

Table 1

3. Inhibition of tumor necrosis factor. 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. Consequently inhibition of this protein is of particular relevance. Modified oligoribonucleotides were annealed with equimolar amounts of the unmodified guide or passenger strands and the resulting duplexes were used in the 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 cotransfected 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).

Figure 3 shows the amount of TNF- α produced after 24 hrs of transfection of 50nM siRNA unmodified duplex (Unm), 50 nM of the same siRNA duplex carrying acridine or quindoline derivatives at the 3' end and 50 nM of a scrambled RNA duplex control sequence (Scr). A siRNA carrying cholesterol at the 3' end of the passenger strand (Cho) was also included for comparison purposes. In figure 3A, siRNA modified with acridine or quindoline at the 3' end of the passenger strand produced an inhibition of the production of TNF- α around 90% compared to the Scr RNA duplex control and slightly better than unmodified siRNA. The inhibition potency is similar to the siRNA

carrying cholesterol at the 3' end of the passenger strand. By contrast, the situation changes with siRNAs containing acridine or quindoline in the 3' end of the guide strand (Figure 3B). In this case, a small decrease in the inhibitory activity is found compared to unmodified siRNAs although the inhibitory activity is still good compared to Src (80-85%). This result is in agreement with previous observations on other aromatic derivatives at the 3' end of the guide strand in the inhibition of a luciferase gene [17]. In general it can be concluded that the introduction of acridine or quindoline at the 3' end of the passenger or guide strand of an RNA duplex is well tolerated by the RNAi machinery and these derivatives do not interfere in the recognition of siRNA by RISC complex.

Figure 3

In addition we have analyzed the inhibition of the TNF- α production by the siRNA duplexes carrying acridine or quindoline in the sense or antisense strand without using oligofectamine in HeLa cells (data not shown). In this case there is no inhibition of the TNF- α indicating that intercalating modified siRNA duplexes are unable to enter HeLa cells.

Figure 4 shows the amount of TNF- α produced when siRNA unmodified duplex (Unmodified), siRNA duplex carrying acridine or quindoline in the 5' end of sense strand and a scrambled RNA duplex control sequence (Scr) were transfected with oligofectamine. Moreover, siRNA modified in the sense strand with four acridines or quindolines in the 5' end is also included in this work. Inhibition of TNF- α is around 75% in the latter case. These results indicate that conjugates of intercalating agents at the 5' end of the sense strand are less effective than unmodified siRNA but they do not interfere in the recognition of the RISC complex. Unfortunately, conjugation of these

compounds does not enhance cell delivery because the inhibition of TNF- α without using oligofectamine in HeLa cells turned out to be similar to unmodified siRNA (data not shown). In addition, a small loss of inhibition is detected in siRNA containing intercalating agents at the 5' end of the sense strand compared to the 3' end of the sense strand. In summary, acridine and quindoline derivatives are well tolerated in both ends of the sense strand and in the 3' end of the antisense strand. The addition of four acridine or quindoline derivatives in the 5' end of the sense strand do not enhance inhibitory activity compared with a single modification.

Figure 4

4. *Cellular uptake.* When HeLa cells were incubated with acridine and quindoline siRNA duplexes, the cells became highly fluorescent especially cells treated with acridine siRNA duplexes. In order to distinguish between the fluorescence due to membrane absorption or the fluorescence due to cellular uptake, confocal microscopy studies were performed. Fluorescence was only observed when acridine siRNA was transfected with lipofectamine. As seen in *Figure 5A*, transfection of acridine-modified siRNA is highly efficient and the fluorescence is localized in the cytoplasm where the siRNA mechanism is active indicating that the fluorescent properties of acridine can be used for the direct observation of cellular uptake of siRNA.

Figure 5

Conclusions. We have reported an efficient strategy for the introduction of one or several acridine and quindoline molecules at the 3' or 5' position of RNA molecules that are stable to oligonucleotide synthesis conditions. We have analyzed the effect of

3' or 5' insertions on the inhibitory properties of TNF- α in HeLa cells. Modified siRNAs containing intercalating compounds are as efficient as unmodified siRNA using oligofectamine. Multiple intercalating agents have been introduced in the 5'-end of the sense strand. This large structure does not interfere in the RNAi pathway to silence gene expression but has similar efficiency compared to single modifications. No inhibition of siRNA conjugates was observed when cells were transfected without oligofectamine. The fluorescent properties of acridine were used to study cell delivery. Transfection of siRNAs modified with acridine showed a clear cytoplasmatic localization of siRNAs in HeLa cells.

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Experimental Part

Mass spectrometry (MALDI-TOF) was recorded on a Fisons VG Tofspec spectrometer and ESI mass spectra on a Fisons VG Plattform II spectrometer. Oligonucleotide sequences were prepared using solid phase methodology. The syntheses were carried out on an Applied Biosystems Model 3400 DNA synthesizer using 1 μ mol scale. Oligoribonucleotides were purified by DMT on- based protocols and by reversed-phase HPLC.

Oligonucleotides. The following RNA sequences were obtained from commercial sources (*Sigma-Proligo*, *Dharmacon*): sense or passenger scrambled 5'-CAG UCG CGU UUG CGA CUG GTT-3' antisense or guide scrambled 5'-CCA GUC GCA AAC GCG ACU GTT-3' antisense or guide anti-TNF α : 5'-GAG GCU GAG ACA UAG GCA CTT-3' and sense or passenger anti-TNF α : 5'-GUG CCU AUG UCU CAG CCU CTT-3'. RNA monomers in capital letters, T represents thymidine. The 3'-cholesterol passenger anti-TNF α : strand 5'-GUG CCU AUG UCU CAG CCU CTT-3'-cholesterol was prepared using the cholesterol-tetraethyleneglycol 3'-Cholesteryl-TEG-CPG support from commercial sources (*Glen Research*).

Synthesis of oligoribonucleotide conjugates carrying acridine or quindoline. Oligoribonucleotide carrying acridine (Act) or quindoline (Qut) either at the 3' or the 5' end were synthesized by a DNA/RNA synthesizer (*Applied Biosystems 3400*) using 2-cyanoethyl phosphoramidites. Sequences with modifications at the 3' end were synthesized with controlled pore glass (CPG) solid supports (0.2 μ mol) functionalized with the threoninol derivatives of acridine or quindoline (*Scheme 2*) prepared as previously described [13, 14]. Sequences carrying one acridine or one quindoline were synthesized on polystyrene solid supports (LV200) using the acridine (Act) or quindoline (Qut) phosphoramidites (*Scheme 2*) prepared as previously described [13]. Sequences carrying four acridine or quindoline units were prepared assembling two units of the symmetric doubler (DB) phosphoramidite (*Glen Research*) between the RNA sequence and the acridine or quindoline units. The sequences synthesized are shown in *Table 1*.

Guanosine was protected with the dimethylaminomethylidene group, cytidine was protected with the acetyl group and adenosine with the benzoyl group. The 2'-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 an increased coupling time of 10 min. The solid supports were treated with concentrated aqueous ammonia-ethanol (3:1) for 1hr at 55°C. The supports were filtered and washed with ethanol and the combined solutions were evaporated to dryness. Sequences were treated with 0.15 ml of triethylamine.tris(hydrofluoride) / triethylamine / *N*-methylpyrrolidone (4:3:6) for 2.5 hours at 65 °C 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 °C. A precipitate was formed that was centrifuged at 7000 rpm, 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. HPLC conditions were: column: Nucleosil 120-10 C₁₈ (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 (*Table 1*). Yields (0.2 μmol scale synthesis) were between 10-15 OD units at 260 nm except for sequences **7** and **8** that were around 5 OD units.

Thermal denaturation studies. The thermal denaturation curves were performed monitoring the absorption change at 260 nm for duplexes formed by oligonucleotides **1-8** and their unmodified complementary strands. Samples were heated from a temperature of 20°C to 90°C, with a linear temperature ramp of 0.5°/min in a JASCO

V-650 spectrophotometer equipped with a Peltier temperature control. Concentration of the samples were around 2 μ M. All the measurements were repeated three times, conducted in 15 mM HEPES 1 mM Mg(OAc)₂ mM 50 mM KOAc pH 7.4

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 (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 manufacturer's instructions. One hour following transfection murine-TNF- α expressing HeLa cells were transfected with 50nM of siRNA (5'-GUG CCU AUG UCU CAG CCU CTT-3' / 5'-GAG GCU GAG ACA UAG GCA CTT-3') 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 manufacturer's instructions.

Cellular uptake studies. For analysis of cellular uptake and distribution, HeLa cells were cultured in 24-well plates with glass cover slip bottoms. siRNA carrying acridine was transfected into HeLa cells using oligofectamine (*Invitrogen*) according to the manufacturer's instructions. The nucleus was stained by Topro-3 (red) 24-h after transfection. A series of images were obtained by using a confocal laser scanning microscope (*BioRad*). Acridine fluorescence was detected by exciting at 300 nm and emission spectrum was recorded from 350 to 560 nm. The spectrum peak at 358 nm represented the fluorescence intensity of acridine and was an indicator of siRNA uptake.

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LEGENDS

Scheme 1. *Structure of oligoribonucleotides carrying acridine and quindoline residues.*

RNA sequences correspond to guide or passenger anti-TNF- α sequences (*Table 1*).

Scheme 2. *Structure of the phosphoramidites and solid supports used in this study for the preparation of oligoribonucleotides carrying acridine and quindoline residues.*

Fig. 1. *Reverse HPLC profiles of 5' and 3'-modified oligoribonucleotides. A) Acridine-5'guide (passenger) strand, 5. B) Quindoline-3'guide (passenger) strand, 2.*

Fig. 2. *Reversed phase HPLC profiles of oligonucleotide conjugate 8 carrying four quindoline units.*

Fig. 3. *Plot of gene-specific silencing activities for 3'-acridine and 3'-quindoline oligonucleotide conjugates with transfecting agent at 50 nM. A) Modifications at the 3'-end of the passenger strand. B) Modifications at the 3'-end of the guide strand. Unm: unmodified siRNA duplex, Scr: scrambled siRNA duplex, Cho: siRNA carrying a cholesterol molecule at the 3'-end of the passenger strand.*

Fig. 4. *Plot of gene-specific silencing activities of 5'-acridine and 5'-quindoline oligonucleotide conjugates with transfecting agent at 50 nM. In all cases modifications at the 5'-end were in the passenger strand.*

Fig. 5. *Cellular uptake of siRNA conjugate carrying acridine.* A) HeLa cells were transfected with siRNA carrying acridine (**1**) using lipofectamine. B) HeLa cells were transfected with siRNA (**1**) without using lipofectamine. After 24 hr, HeLa cells were fixed in 10% paraformaldehyde at room temperature for 10 min and then subjected to TOPRO-3 staining. Localization of the duplex siRNA was monitored by Leica confocal microscopy. Overlay image of siRNA (blue) and nucleus (red) (TOPRO-3) is shown here.

Table 1. *Oligoribonucleotide Sequences Used for the Preparation of the Corresponding siRNA Conjugates for RNA Interference studies.*

#	Sequence (5'3') ^{a)}	MS ^{b)}	T _m ^{c)} (ΔT_m) ^{d)}
1	GUGCCUAUGUCUCAGCCUCTT	6931/6926	85.0 (1.7)
2	GUGCCUAUGUCUCAGCCUCTT-Qut	6970/6968	85.0 (1.7)
3	GAGGCUGAGACAUAGGCACTT-Act	7144/7146	83.5 (0.2)
4	GAGGCUGAGACAUAGGCACTT-Qut	7183/7184	84.8 (1.5)
5	Act-GUGCCUAUGUCUCAGCCUCTT	6931/6929	83.7 (0.4)
6	Qut-GUGCCUAUGUCUCAGCCUCTT	6970/6968	84.9 (1.6)
7	(Act) ₄ -DB-DB-GUGCCUAUGUCUCAGCCUCTT	9105/9101	82.7 (-0.6)
8	(Qut) ₄ -DB-DB-GUGCCUAUGUCUCAGCCUCTT	9261/9251	82.9 (-0.4)

^{a)} Act: acridine threoninol, Qut: Quindoline threoninol, DB: doubler; ^{b)} Mass spectra (expected/found); ^{c)} uncertainty $\pm 0.5^\circ\text{C}$; condition: 15 mM HEPES 1 mM Mg(OAc)₂ mM 50 mM KOAc pH 7.4. Melting temperature of unmodified siRNA is 83.3 $^\circ\text{C}$; ^{d)} ΔT_m is the difference between the melting temperature of the modified siRNA minus the melting temperature of the corresponding unmodified siRNA.

Scheme 1.

Scheme 2.

Figure 1.

A)

B)

Figure 2

Figure 3

A)

B)

Figure 4

Figure 5

A)

B)

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