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Cell Binding, Uptake and Cytosolic Partition of HIV Anti-*gag* Phosphodiester Oligonucleotides 3′-Linked to Cholesterol Derivatives in Macrophages

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Abstract—The purpose of this study is to evaluate the cell interactions of a new class of compounds composed of phosphodiester oligonucleotides linked to the cholesterol group at position 3, 7, or 22 of the steroid structure. The resulting conjugates were assessed for their capacity to bind, penetrate and partition in the cytoplasmic compartment of murine macrophages. The results showed that lipophilic conjugates bind to cells much faster ($t_{1/2} \leq 10$ min) than do underivatized oligomers. Oligomers tethered to the cholesterol at positions 3 and 7 (PO-GEM-3-Chol and PO-GEM-7-Chol) interacted more efficiently with cell membranes and were better internalized than oligomers attached to the cholesterol moiety at position 22 (PO-GEM-22-Chol). The cytosolic fraction of internalized oligomers was studied by a digitonin-based membrane permeabilization method. The recovered fraction of oligomers that can freely diffuse from the cytosol was comparable for GEM-91™, a phosphorothioate congener, and for PO-GEM-7-Chol (50–60% of the internalized oligomers), while that of PO-GEM-3-Chol was less (30% of the internalized oligomers) indicating a higher membrane affinity of the latter derivative as compared to the other investigated compounds. Membrane binding and cell internalization correlated well with the hydrophobicity of the conjugates as characterized by their partition coefficients in a water–octanol system. Due to their capacity of rapid binding and cytosolic partition in cells, cholesterol-derivatized oligonucleotides at position 3 or 7 of the steroid molecule appeared as good candidates for systemic delivery of anti-HIV antisense compounds.

Introduction

The concept of antisense became a reality when a dozen oligodeoxynucleotides (ODN) molecules underwent clinical trials (for a review see ref 1). These advanced stages in the field have encouraged basic studies, especially those involved in viral therapy, to develop new molecules that can bind rapidly and cross efficiently the cell membrane and, once internalized, partition favorably in the cytosol and/or nucleus of cells where host and viral messenger RNAs are processed. Moreover, in view of future applications in antisense therapy, antisense molecules should fulfill additional requirements such as nuclease resistance, long plasmatic half-life and absence of toxicity. Pharmacokinetic studies of oligonucleotides on animals and humans have shown that phosphorothioate derivatives (PS) are superior to phosphodiester congeners (PO) due to a more favorable body distribution and longer plasmatic half-life of the phosphorothioate compounds.[2] However, besides certain toxicological issues related to their chemical structure,[3] the main disadvantage of PS oligonucleotides remains their propensity to bind proteins,[4] a phenomenon that can prevent these molecules from binding to their nucleic acid targets. In vivo, both PS and PO oligomers face the problem of breakdown by nucleases (PS slower than PO), as the primary degradation pathway is found to occur at the 3′ end of the oligomer molecule.[5,6] Within this context, provided that their 3' termini are suitably protected against nucleases degradation, natural phosphodiester oligonucleotides could be reconsidered for in vivo applications as phosphodiester oligonucleotides, unlike phosphorothioate congeners, are also known to exhibit reduced affinity for proteins.[4]

It has been previously shown that cholesteryl-derivatized oligonucleotides whose sequences are complementary to various genes[7–13] including HIV genes[7–9] were more

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efficient as compared to underivatized compounds in vitro. In these studies the antisense oligonucleotide was tethered to the cholesterol moiety at the 3′-OH position. We hypothesized that the attachment site of the oligonucleotide molecule to the cholesterol part could lead to different interaction modes of the resulting conjugates and possibly to a different subcellular distribution. In this study, the 3′ ends of PO-oligomers were linked to the cholesterol derivative at three positions: 3, 7, or 22 via a cleavable disulfide bond containing linker. This type of construction should allow the oligomer to be detached from the steroidal vector by reducing agents present inside cells as shown previously.[14] The conjugates were assessed on inflammatory murine peritoneal macrophages, a blood monocyte-derived cell type. Macrophages were considered as reservoirs and dissemination agents of the virus in the infected organism.[15] Moreover one of us (S.A.) has recently shown that macrophages take up the majority of injected ODN in mice.[16]

The results showed that cholesteryl conjugates bind and internalize cells more rapidly and more efficiently than do free ODN except for the ODN-22-cholesterol derivative. The amount of internalized cholesteryl-ODN species for the compound that reached the highest uptake value could be as high as 15 pmol/million cells which corresponds roughly to a mean cellular concentration of 15 μM, a value that could be favorably compared to the less than 1 μM for the nonderivatized compound.

Results and Discussion

Most of the previous works related to the ODN-cholesteryl conjugates dealt with cholesterol or other steroidal groups linked to ODN at the 3-OH position of the cholesterol moiety.[5,7–14,17–19] Studies of the antiviral or other biological effects in vitro of the resulting conjugates have shown a more positive effect over the nonmodified compound obtained by tethering the cholesterol group to the antisense derivative. We and others[13,18] have previously shown that cholesterol-ODN derivatives could be transported in vivo by low density lipoproteins (LDL) and part of the cell penetration of these derivatives could be processed via the LDL receptor.[13] Greater uptake was often observed and proposed to explain the gain in efficiency of the cholesterol-ODN conjugates as compared to the free ODN but no extensive study of the interactions of these compounds with cells was carried out. In this study, we hypothesized that changing the linkage site on the cholesterol molecule could result in differences in cell binding, internalization and intracellular distribution of the internalized species. The following results tend to confirm this expectation.

Characterization of the oligonucleotide–cholesterol conjugates

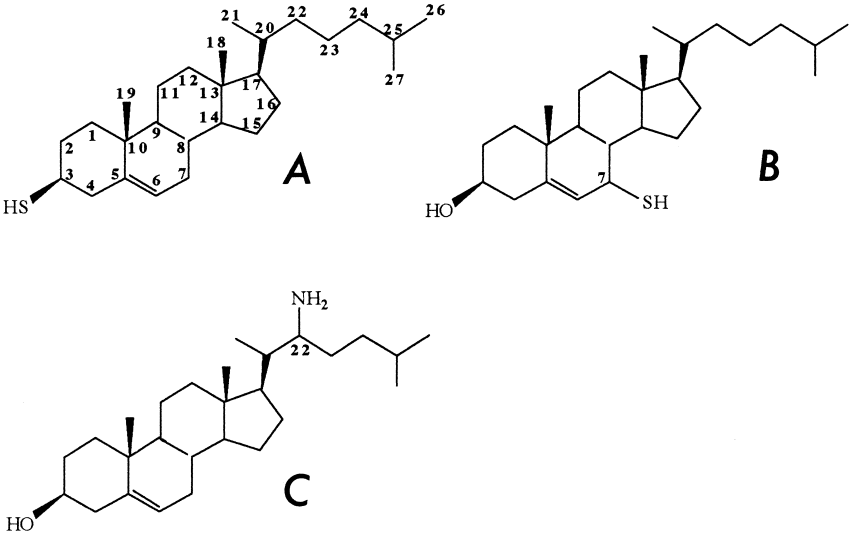
The cholesterol-derivatized anti-*gag* (HIV) ODN and parent compounds are presented in Figure 1. The three cholesteryl derivatives and the PO parent compound (PO-GEM-Pyr) contain a cleavable disulfide bond in their structures. The latter was used as a noncholesterol reference compound. The phosphorothioate congener PS-GEM-91 was included in this study as this oligomer was extensively studied in the past for its anti-HIV properties.[2,3,5] We attempted to carry out characterization of the ODN-cholesteryl conjugates by mass spectroscopy but without success, likely due to poor matrix desorption. We, therefore, turned to reversed-phase HPLC and water-octanol partition measurements for characterization. Based on HPLC retention times on the C18 reversed-phase column (see Experimental), the hydrophobicity of the conjugates decreased as follows: PO-GEM-3-Chol > PO-GEM-7-Chol > PO-GEM-22-Chol > > PS-GEM-91 > PO-GEM-Pyr. The difference in lipophilicity of the PO-GEM-Cholesterol conjugates was further characterized by measuring the partition coefficient of the conjugates in a water-octanol system. Results shown in Table 1 confirm the trend observed with HPLC retention times.

Considering the percentages of the partitioned fraction in the aqueous phase, it appeared that the two most lipophilic derivatives were the conjugates derivatized at position 3 or 7 of the cholesterol while that derivatized at position 22 was less hydrophobic. The sum of the organic and interfacial phases of the three cholesteryl derivatives exceeded 10% of the radioactive compounds added to the medium. PS-GEM-91 and the noncholesterol PO-GEM-Pyr were definitely aqueous soluble derivatives as more than 95% of the dissolved compounds were found in the water phase. The measure of radioactivity at the interface of the water–octanol system revealed other interesting properties of the amphiphilic molecules. Again the two lipophilic compounds linked at positions 3 and 7 of the cholesterol were found to be present at the interface, since the percentages of these ''interfacial'' species were far from being negligible (28 and 41%, respectively) while those of the two most water soluble derivatives were very low (2 and 4% for PS-GEM-91 and PO-GEM-Pyr, respectively). It can be speculated that, at the interface of the two phases, the amphiphilic conjugate may have its cholesterol head dipping into the octanol phase while the ODN tail remains in the water phase. This is reminiscent of the situation at the cell surface where one can expect similar types of interactions. The loss of products by adsorption on the plastic wall (adsorbed phase) was found to be negligible, between 2 and 4% for the three cholesterol-derivatized oligomers. The calculated partition coefficients ($P_{o/w}$) of the conjugates manifest well the properties of the tested compounds as discussed above, since the $P_{o/w}$ values of the noncholesterol oligomers were close to 4 while those of the cholesterol-derivatized compounds were in the order of 2. The difference in lipophilicity of the cholesteryl-ODN derivatives was better reflected by their concentrations at the interface (PO-GEM-7-Chol > PO-GEM-3-Chol > PO-GEM-22-Chol) of the organic and the aqueous phases.

Stability of the PO-GEM-cholesterol conjugates in the culture medium

To best preserve the integrity of oligomers in the culture medium, we used heat-inactivated serum and added

Functionalized Cholesterol Derivatives



anti-*gag*-oligodeoxynucleotides

5'-CTC-TCG-CAC-CCA-TCT-CTC-TCC-TTC-T-3'-R

	R
PS-GEM-91:	**-OH**
PO-GEM-Pyr:	**-p-(CH2)3-S-S-Pyridyl**
PO-GEM-3-Chol:	**-p-(CH2)3-S-S-3-Cholesterol**
PO-GEM-7-Chol:	**-p-(CH2)3-S-S-7-Cholesterol**
PO-GEM-22-Chol:	**-p-(CH2)3-S-S-(CH2)2-CO-NH-22-Cholesterol**

Figure 1. Structures of the functionalized cholesterol derivatives (A = Thio-3-cholesterol, B = Thio-7-cholesterol, C = Amino-22-cholesterol), the sequence of ODN and the linker by which the two entities are attached to each other. PO: phosphodiester; PS: phosphorothioate; p: phosphate group.

Table 1. Distribution of oligonucleotide derivatives in a DMEM–Octanol system at 37°C

ODN	PO-GEM-3-Chol	PO-GEM-7-Chol	PO-GEM-22-Chol	PS-GEM-91	PO-GEM-Pyr
Aqueous phase	67[a]	56.3	84.4	97.6	95.8
Organic phase	0.8	0.5	0.5	0.01	0.006
Interfacial phase	28.5	41.1	11.1	2.06	4.1
Adsorbed phase	3.7	2.1	3.9	0.4	0.11
Partition coefficient	1.9	2.05	2.2	4.0	4.2

[a] Figures represent the percentages of the radioactivity of the corresponding phase over the total radioactivity of the mixture.

EDTA and potassium phosphate, (1 mM each), to inactivate phosphatases and nucleases.[20] Under these conditions and in the presence of macrophages, oligomer degradation as monitored by polyacrylamide gel electrophoresis (results not shown) was found to be limited, less than 20% at 2 h of incubation for the most degraded compound (PO-GEM-7-Chol). The most stable derivative was PS-GEM-91 which remained practically intact for 6 h under our incubation conditions. Consequently, the incubation time was limited to

2 h in order to compare the internalization of oligomers under conditions where the majority (more than 80%) were intact in the culture medium.

Binding of PO-GEM-Cholesterol conjugates to cell membranes

After determined incubation times, cells were first rinsed with PBS (see Materials and Methods) and then washed by 2×1 mL of PBS containing 10% fetal calf serum, with each wash lasting 30 min at 4°C. The amount of desorbed oligomer was taken as a parameter for measuring the affinity of the conjugates for the cell surface. The two washes were counted and the radioactivities were totaled, converted into oligomer equivalents, expressed as pmol/1 million cells, and represented as a function of the incubation time. The results are presented in Figure 2.

Again, the two most lipophilic compounds as already characterized by their HPLC retention times or their $P_{w/o}$ values exhibited the greatest amount of species desorbable from the cell surface by serum proteins. The amount of these detachable oligomers decreased with the incubation time suggesting that, for a given cell number on the dish, the cholesteryl-oligomers externally-bound at definite sites of the cell membranes were reshaped with time, a process that could be linked to the endocytosis process of the adsorbed species to the cell surface. In contrast, the amount of PO-GEM-22-Chol that could be displaced by the serum proteins was much smaller than those of the two other cholesterol derivatives. Linking the oligonucleotide to the aliphatic tail of the cholesterol moiety (i.e. position 22) endowed a particular structure to the conjugate consisting of a central hydrophobic core flanked by two polar regions, the OH group on one side and the ODN on the opposite side. This nonpolarized structure could prevent the molecule from interacting efficiently with the membrane bilayer. An NMR study of the NH_2-22-cholesterol precursor (Fig. 1, C), showed an unusual structure consistent with a complete folding of the hydrocarbon chain towards the polycyclic part of the molecule (Letourneux, personal communication). PO-GEM-Pyr that do not bear any hydrophobic ligand showed a similarly low membrane interaction as did the PO-GEM-22-Chol derivative. Finally, PS-GEM-91 exhibited a higher membrane affinity as compared to the PO-GEM-Pyr congener probably due to the higher lipophilicity of the sulfurized phosphate groups along the PS oligomer backbone.

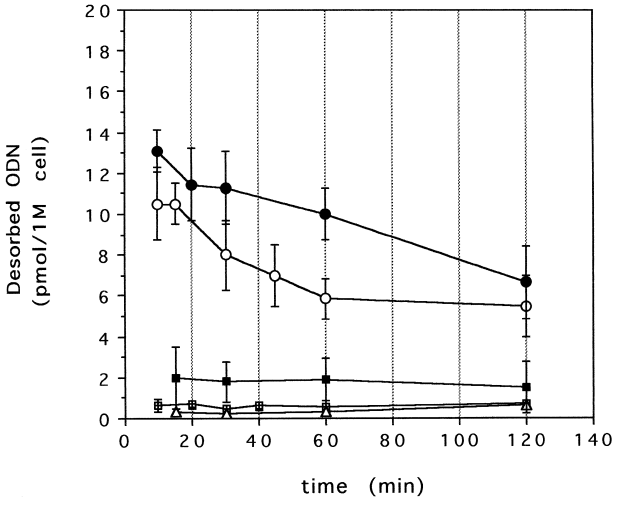


Figure 2. Desorption of ODN-cholesteryl conjugates from cell membranes as derived from the radioactivity collected in the two washes with PBS containing 10% fetal calf serum (for details see Experimental). Results are expressed as number of pmol of ODN desorbed per million of cells. The data are presented as means ± SD. ○ = PO-GEM-3-Chol; ● = PO-GEM-7-chol; □ = PO-GEM-22-chol; △ = PO-GEM-Pyr; ■ = PS-GEM-91.

Cell uptake and cytosolic partition

The profile of the kinetics of ODN uptake by macrophages (Fig. 3) reflected the properties of the investigated compounds as described above.

From the results presented in Figures 2 and 3A, one can state that the more the oligomer binds to the membrane

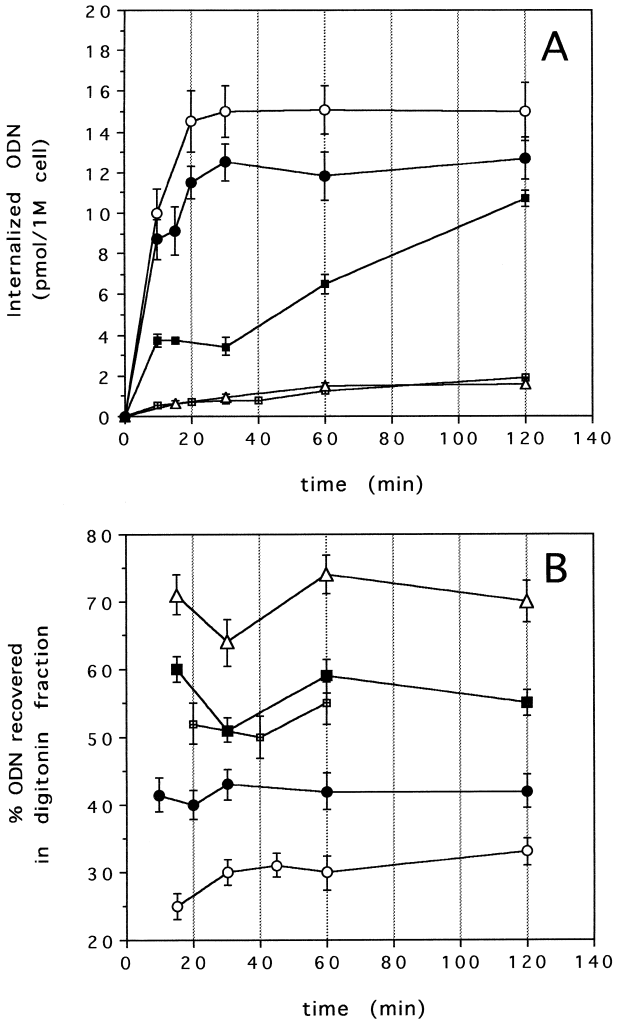


Figure 3. (A) Internalization of ODN-cholesteryl conjugates in macrophages expressed as number of picomoles of ODN internalized per million of cells. The outside ODN concentration was 1 μM. (B) Fraction of freely diffusible ODN recovered following the permeabilization of cells by digitonin. Symbols as in Figure 2. The data are presented as means ± SD.

the more it internalizes cells. The uptake rates were fast for the PO-GEM-3- and 7-cholesterol derivatives, as the plateau was reached after 30 min of incubation. In contrast, the internalization rate was much slower for the PO-GEM-Pyr and PO-GEM-22-cholesterol derivatives. The low membrane binding of these two latter compounds may account for their weak uptake rate and yield. PS-GEM-91 exhibited an internalization profile between the two groups of compounds. Assuming an average cell volume of 1 pL, the plateau value reached by the two most lipophilic compounds corresponded to an internal oligonucleotide concentration of 12–15 μM which was far above the values of 0.7 and 3.5 μM at 30 min for PO-GEM-Pyr and PS-GEM-91, respectively, the external ODN concentration was 1 μM for all the tested compounds.

The fraction of oligomer conjugates that can diffuse out the cytoplasmic compartment (ODN recovered in the digitonin fraction) was expressed as a percentage of the internalized ODN and represented in Figure 3B as a function of incubation time. The corresponding values for the reference compound, PO-GEM-Pyr and the phosphorothioate GEM-91 were found in the range of 60–70% while the two cholesterol derivatives (PO-GEM-3- and-7-cholesterol) exhibited a lower cytosolic availability (30 and 40%, respectively). The PO-GEM-22-cholesterol exhibited behavior close to that of PS-GEM-91 while its properties of membrane binding and uptake were close to that of PO-GEM-Pyr. The digitonin extract could be a good measure of the cytosolic content of molecules if the latter could be considered as a good aqueous soluble compound which also exhibited a minimal affinity for proteins or membranes. This is exemplified by the highly water-soluble lactate dehydrogenase (LDH), a cytosolic protein where more than 80% of this protein could be recovered from the cytosol by this method. This is also true for the water-soluble PO-GEM-Pyr compound (see Table 1 and Fig. 3B). But for amphiphilic compounds such as ODN-cholesteryl conjugates, hydrophobic (and electrostatic) interactions with structures such as membranes or organelles inside the cell cannot be avoided. These bound species will be considered as immobilized species. On the other hand, the disulfide bond in conjugates could be cleaved intracellularly as shown by Oberhauser and Wagner in TIB 73 cells,[14] or could remain intact as shown in T24, a bladder carcinoma cell line.[19] This problem is still debated and reductive cleavage of the S–S bond inside cells likely depends on cell type and culture conditions (temperature and incubation duration). Therefore the diffusible fraction of the internalized cholesteryl-oligomers following the digitonin treatment as displayed on Figure 3B may reflect only a fraction (represented by the freely diffusible ODN species, cleaved or not cleaved) of the internalized compounds.

Conclusion

Linking the ODN moiety at different positions of the cholesterol molecule in fact modifies the properties of cell binding and internalization of the resulting conjugates. We observed that the more the compound is lipophilic the more it binds and internalizes cells. Membrane binding and uptake were fast (less than 30 min) for conjugates of ODN linked at position 3 or 7 and very slow and inefficient for ODN linked at position 22 of the cholesterol. The plasma disappearance of PS-GEM-91 in humans was measured and corresponded to a biphasic exponential decay with half-life values of 0.18 and 27 h.[21] The cholesteryl-ODN conjugates presented in this work could be interesting candidates for rapid capture and internalization by the target blood cells following injection within clinically relevant time frames. Future studies are needed for evaluating these properties in vivo and their correlation with anti-HIV activity.

Experimental

Oligonucleotides and chemicals

The sequence of the 25mer directed to the AUG region of the HIV-*gag* protein used in this work was identical to that of GEM-91™:

5′ – CTC-TCG-CAC-CCA-TCT-CTC-TCC-TTC-T

The phosphodiester oligomer (PO-GEM) derivatized at the 3′ side with a-p-$(CH_2)_3$-SH functional linker (p = phosphate group) was synthesized by Eurogentec (Belgium).

Thio-3-cholesterol was purchased from Sigma and thio-7-cholesterol was a gift from Dr. Guetté (CNAM, Paris). The NH_2-22-cholesterol was synthesized according to a published procedure.[22] Dimethylsulfoxide (DMSO) and pyridine were from Aldrich, the solvents were stored under argon atmosphere in sealed cap flasks. All solvents used throughout this work were of reagent grade.

Details of the functionalized oligonucleotides and cholesterol derivatives are given in Figure 1.

Synthesis, purification and characterization of cholesteryl-ODN conjugates

SH-derivatized oligomers were first converted to the *S*-*S*-pyridyl form by reaction with dithiodisulfide (DTDS, Aldrich) in an acetonitrile-water medium (15:85% by volume) overnight at room temperature. The exchange reaction with thio-(3 or 7)-cholesterol was carried out overnight at room temperature, in pyridine in which the oligomer was complexed by cetyltrimethylammonium bromide (CTAB) for dissolution in the organic medium, as already described.[23] The NH_2-22-cholesterol was first converted to the *S*-*S*-pyridyl form by reaction with succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Boehringer Mannheim). The amino-22-cholesterol derivative (10 μmol) was dissolved in 700 μL chloroform while SPDP (16 μmol) was dissolved in 300 μL of methanol. The two solutions were mixed and triethylamine (14 μmol) was added. The mixture was left overnight at

room temperature. The reaction was stopped by adding 1 mL of phosphate buffer (0.1 M, pH 7) to extract the nonreacted SPDP. The organic phase containing the cholesterol derivative was washed by 2×1 mL of water, evaporated by a stream of argon and dried under vacuum. The resulting cholesterol derivative containing the activated disulfide group (-*S*-*S*-Pyr) was allowed to react with the thiol-terminated oligomer as described above. The conjugates were extracted by phase transfer in 1 mL of ($CHCl_3$-H_2O, 50% each by volume) and purified by ethanol precipitation. The ODN was redissolved in water containing 20% ethanol and the cholesteryl-ODN conjugates were isolated from the nonreacted ODN by reversed-phase HPLC (C18 Nucleosil column 250×4.6 mm, 100 Angstrom from Interchim, France). The gradient profile used for the analysis of the cholesteryl-ODN derivatives was as follows:

0–2 min	0%B	A = 95% TEAA 0.1 M pH 7
2–17 min	0–60%B	5% ACN
17–37 min	60–100%B	
37–40 min	100%B	B = 20% TEAA 0.1 M pH 7
40–45 min	100–0%B	80% ACN

TEAA represents an aqueous solution of triethylammonium acetate and ACN is acetonitrile (HPLC grade from Merck).

The noncholesterol compound bearing the *S*-*S*-pyridyl group at the 3′ side was eluted at 10.5 min, the GEM-91 at 12.3 min while the ODN-*S*-*S*-*n*-cholesteryl derivatives were eluted at 25, 21, and 18 min for $n = 3$, 7, and 22, respectively. The collected ODN solutions were concentrated by evaporation under vacuum and precipitated by ethanol. The yield was calculated from the absorbances of HPLC fractions. The final yields for the 3 ODN-cholesterol conjugates were 66, 73, and 32% for the derivatives attached at $n = 3$, 7, and 22 position, respectively. In internalization studies, the PO-GEM-Pyr oligomer was used as a noncholesterol derivatized compound for comparison with the cholesteryl-tethered compounds. Oligomers were 5′-labeled with ^{32}P–ATP and polynucleotide kinase as already described.[23] It may be noted that for this particular labeling reaction we used a kinase buffer without addition of reducing agents such as dithiothreitol or beta-mercaptoethanol to preserve the disulfide linkage in the ODN-3′-cholesterol conjugate.

The octanol–water partition coefficient of the above oligonucleotide derivatives was determined in a DMEM–octanol system. The experiment was carried out in an Eppendorf tube (polypropylen) containing 600 μL of each medium pre-equilibrated at 37 °C. A small amount of the 5′-^{32}P-Oligo (50 pmol) was added, the mixture vortexed vigorously and allowed to stand in a 37°C bath for 30 min. Radioactivity counting was performed in an LKB Wallac 1215, Rackbeta Liquid Scintillation Counter. Counting was carried out on 400 μL of each phase and the remaining 400 μL containing the interface phase. The radioactivity of the interface phase was calculated by subtracting the value of radioactivity of the interface containing fraction (400 μL) from the values of the corresponding radioactivity of each phase (200 μL). The amount of adsorbed oligomers on the wall of the plastic tube was measured by counting the emptied tube. The partition coefficient $P_{w/o}$ was taken as −log(radioactivity of the octanol phase/radioactivity of the water phase).

ODN uptake by macrophages

Macrophages were elicited by i.p. injection of mice (C57B1/6X DBA2-F1, Iffa-Credo, France) with a thioglycolate broth, a sterile irritant from the Institut Pasteur, France. Four days later, inflammatory macrophages were harvested from the peritoneal cavity, centrifuged, washed and seeded in 35 mm Petri dishes at 2×10^6 cells/dish. Cells were allowed to adhere for 48 h in RPMI 1640 (Sigma Cell Culture) containing penicilline 50 UI/mL, streptomycin (ICN biomedicals) 50 μg/mL, and 5% fetal calf serum (FCS, Boehringer Manheim). Thirty minutes before starting the uptake experiment, the medium was replaced by a new DMEM medium containing 5% heated FCS, EDTA and potassium phosphate, each at 1 mM, to prevent degradation and dephosphorylation reactions.[20] Oligomers were then added to a final concentration of 1 μM, peaked with 5′-^{32}P- labeled ODN at 1–5 million cpm (Cerenkov Counting) and Dextran-Rhodamine (MW 10000, from Sigma) at a concentration of 1 mg/mL. At determined times, the supernatant was removed and cells were washed six times with 1 mL of PBS containing 5 mg/mL of 40 kDa Dextran DT40. All cell washings were performed at 4°C, and the dish was kept on crushed ice to prevent efflux processes. The fraction of ODN adsorbed on plasma membrane proteins was desorbed with 1 mL PBS containing 10% heated FCS for 30 min at 4°C, followed by an additional washing with 1 mL PBS-FCS for 20 min. Cells were then washed at 4°C with 2×1 mL of a protective buffer (0.25 M sucrose, 20 mM HEPES, pH 7.2, 0.5 mM potassium phosphate, 0.24 mM ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid, EGTA and 10 mM $MgCl_2$). Finally, the washed cell layer was recovered in 1 mL of 0.1% Triton×100 (Fluka) and counted to determine the amount of internalized ODN.

Recovery of the cytosolic content by permeabilization of the plasma membrane with digitonin

Cell permeabilization was carried out by adding 1 mL of 0.005% (w/v) digitonin (Merck) in the protective buffer over the washed cell layer and incubating for 12 min at 4°C, followed by a washing with 1 mL of the protection buffer for 30 min at 4°C. This treatment results in the release of freely diffusible cytosolic macromolecules of up to 200 kDa in the supernatant (digitonin extracts), with minimal damage to intracellular vesicles such as endosomes and lysosomes as described previously.[24–26] The radioactivity of the digitonin extracts represents the part of the soluble and diffusible oligomer species that are in equilibrium with the fraction of bound oligomers to macromolecules (proteins, RNA...) or structures such as internal membranes, nucleus and other organelles. The cytosolic lactate dehydrogenase (LDH) was used as a cytosolic marker,[26]

endocytosed dextran–rhodamine (DEX) and β-hexosaminidase (HAM) as endosomal and lysosomal markers, respectively.[25] A correct permeabilization experiment should present the following pattern: > 80% of recovery of LDH while the percentages of vesicles markers (DEX and HAM) should lie below 15% of the total cell content of markers.[24] After collection of the digitonin supernatant, MPM was treated with 1 mL of 0.1% Triton X100 in the protective buffer, to recover the rest of intracellular (nondiffusible) ODN and markers. The Triton fraction contains ODN entrapped in endocytic vesicles as well as those adsorbed on membrane structures and in the nucleus. The sum of the digitonin and the triton fractions was taken as 100% of the internalized compounds.

LDH was measured using the oxidation reaction of β-NADH in the presence of pyruvate. The disappearance of β-NADH in the medium was monitored by its linear absorbance decrease at $\lambda = 340$ nm, with reaction time using a Sigma kit. β-Hexosaminidase was determined by using the chromogenic substrate *p*-nitrophenyl-β-D-hexosaminide (Sigma). The reaction was carried out at pH 5.2 and the appearance of *p*-nitrophenol was monitored by its absorbance increase at $\lambda = 400$ nm after alcalinization of the reaction mixture. The relative amounts of dextran–rhodamine were determined by measuring the relative fluorescence intensities with a Jobin-Yvon spectrofluorimeter (excitation = 540 nm, emission = 580 nm). Systematic measurements of the protein content (Bradford method, Biorad kit) of the digitonin and the triton fractions were performed to detect if dishes had been incidentally overloaded or if the washing procedure had cause cell loss. All measurements performed along the fractionation procedure were thus normalized to take into account the variability of cell density within the batch of dishes.

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