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Effective incorporation of 2′-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol

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ABSTRACT

Cholesterol was linked to 2′-O-methyl-oligoribonucleotides (2′-OMe-RNA) via a disulfide bond by reacting the 3′-(pyridyldithio)-modified 2′-OMe-RNA with thiocholesterol in dichloromethane-methanol solution. This ligation reaction was made possible by a novel strategy in which the highly charged oligonucleotide was rendered soluble in nonaqueous solvent through conversion to a lipophilic amidinium salt. The biodegradable lipophilic modification of 2′-OMe-RNA resulted in a large increase in incorporation of such oligonucleotides into liposomes prepared by reverse-phase evaporation. Furthermore, association of these modified oligonucleotides with cultured TIB 73 cells was 100-fold higher than that seen with unmodified 2′-OMe-RNA in serum-free medium and about 10 to 30-fold higher in the presence of 10% calf serum. During incubation with cells, release of the internalized oligonucleotide from the thiocholesteryl moiety can be demonstrated.

INTRODUCTION

Modified antisense oligonucleotides (ONs) may become important tools for the study of regulation and inhibition of genes (1,2). A substantial limitation of this approach is the inefficient uptake of natural ONs into cells. To enhance the penetration of ONs into cells, non-ionic methylphosphonate (3), phosphoramidate (4) analogs or ONs conjugated to poly(L-lysine) (5) have been designed. A further approach has been to use antibody-targeted liposomes containing antisense ONs (6) that allow cell-specific delivery by endocytosis. This interesting method may be particularly applicable if the encapsulation efficiency of the ONs (about 3%) could be substantially improved. With this aim, we considered the use of lipid-modified ONs for incorporation into liposomes. ONs covalently linked to phospholipid (7), cholesterol (8,9), dodecandiol (10) or undecyl residues (11) have already been described and all show an enhanced association with cultured cells. Permanent trapping of such ON analogs in membranes, however, may limit their action as antisense compounds. In the present communication we describe the synthesis of ON-thiocholesterol conjugates containing a potentially bioreversible disulfide linkage (12), the highly efficient incorporation of such compounds into liposomes, as well as their enhanced affinity for and internalization into cells.

MATERIALS AND METHODS

Quantitative assays

Oligonucleotides were quantified by UV absorbance at 260 nm; correction of the values (if necessary) was made by subtracting the corresponding UV absorption of dithiopyridine or buffer at 260 nm. The amount of dithiopyridine linkers in modified ONs was determined by reducing an aliquot with dithiothreitol and measuring the absorption of released pyridine-2-thione at 343 nm (molar extinction coefficient $\epsilon = 8080\ M^{-1}cm^{-1}$). The amount of PMDBD (Heinzer base, Fluka) was determined by UV absorption at 219 nm; 1 μmol of the hydrochloride in water corresponds to 16.3 AU_{219} (i.e. $\epsilon = 16300\ M^{-1}cm^{-1}$).

Synthesis of 3′-amino-modified 2′-O-methyl-oligoribonucleotides

The 19mer 2′-O-methylated oligoribonucleotides modified with (3-amino-2-hydroxypropyl)phosphate at the 3′-end were synthesized on an ABI 380 B DNA synthesizer (Applied Biosystems) using a modified LCAA controlled-pore glass (CPG) solid support carrying 1-O-DMTr,3-N-[fluorenyl(methoxycarbonyl)]aminopropandiol groups (3′-amine-ON CPG, Clontech). 2′-O-Methyl nucleoside (2-cyanoethyl)-N,N-diisopropylphosphoramidites with the following protecting groups were used: A: N^6-phenoxyacetyl, 5′-O-DMTr; C: N^4-benzoyl, 5′-O-MMTr; G: N^2-phenoxyacetyl, 5′-O-DMTr; U: 5′-O-MMTr (13,14). Standard DNA synthesis methodology with increased coupling time (5 min instead of 3 min) was used. The ON was deprotected and cleaved from the solid support with 25% aqueous ammonia (15 hours at 55°C). The crude ON **1** was purified by ethanol precipitation.

Synthesis of thiocholesterol-modified ONs

a) Modification with dithiopyridine groups: A solution of 14.1 AU_{260} (about 450 μg; 67 nmol) of a 2′-O-methylated oligoribonucleotide 19mer **1** with the sequence 5′-CUA AAA GAG CUG UAA CACU-3′ modified with (3-amino-2-hydroxypropyl)phosphate at the 3′-end, in 0.75 ml 50 mM HEPES buffer (pH 7.9) was treated with a solution of 4.2 mg (13.4 μmol, 200 equivalents) of N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, Pharmacia) in 300 μl ethanol for 4 hours at room temperature. The mixture was then subjected to gel filtration on Sephadex G25 (PD10 column, Pharmacia) with 20 mM HEPES buffer (pH 7.3) as eluent. The first fractions containing ON were evaporated to dryness in a Speedvac (Savant), dissolved in 200 μl

of water, and further purified by reverse phase HPLC (Nucleosil RP-18 column, 250×4 mm; buffer A: 100 mM triethylammonium acetate pH 6.5; buffer B: acetonitrile; gradient: 0–40 min, 0–40% B). This procedure gave 12.2 AU_{260} ON **2a** (corresponding to 57 nmol, 85% yield, **Fig. 1**) modified with about 52 nmol dithiopyridine groups (according to the UV assay at 343 nm described above).

PMDBD-salt formation: A solution of 8.1 AU_{260} (about 255 μg, 37 nmol) of **2a** in 250 μl water was added to 250 μl of a solution of 4 mg PMDBD (3,3,6,9,9-pentamethyl-2,10-diazabicyclo [4.4.0]dec-1-ene, = Heinzer base, Fluka, (15)) carbonate salt in 50% aqueous methanol. The solution was then diluted with water to a volume of 3 ml and freeze-dried. The lyophylisate was dissolved in 1 ml of methanol/water (1:4) and subjected to gel filtration on Sephadex G25 (PD10 column; elution with methanol/water 1:4). The fractions containing ON (7.1 AU_{260}, due to absorbance of the nucleotides; 15 AU_{219}, mainly due to absorbance of ca 0.65 μmol PMDBD; 88% yield of **2b**) were concentrated in the Speedvac to remove methanol, lyophilized and then dissolved in 1.0 ml of methanol/dichloromethane (1:2).

Conjugation to thiocholesterol: To half of the solution (0.5 ml, 16 nmol of ON **2b**), 200 μl methanol, 30 μl of 180 mM methanolic PMDBD trifluoroacetate buffer (pH 9) and 600 μg (1.5 μmol) thiocholesterol (Sigma) in 300 μl dichloromethane were added. The reaction mixture was kept under argon for 20 h at room temperature. The solution was evaporated to dryness in the Speedvac, dissolved in 400 μl methanol/chloroform (1:1), and extracted with 8×100 μl of water. The aqueous extracts (containing 2.3 AU_{260} of ON) were combined and concentrated in the Speedvac to a volume of about 500 μl to remove methanol. Fractionation by reverse phase HPLC (Nucleosil RP-18 column, 250×4 mm; buffer A: 100 mM triethylammonium acetate pH 7; buffer B: acetonitrile; flow: 1 ml/min; gradient: 0–50 min, 0–50% B; 50–70 min, 50–100% B) yielded 0.84 AU_{260} (24%) of ON in cholesterol-free form at a concentration of about 20% acetonitrile, and 1.5 AU_{260} (42%) of the thiocholesterol-modified ON **3** at a concentration of 55–68% acetonitrile.

b) In an analogous fashion a 2′-O-methylated oligoribonucleotide 19mer containing an amino-modified 3′-end with the sequence 5′-GAG CAC ACU UCA UGC AGUG-3′ was linked to thiocholesterol. HPLC purification of the intermediates was omitted in order to improve the overall yield.

The pyridyldithiopropionate-modified ON was obtained as a crude product after reaction of the amino-modified ON with 200 equivalents of SPDP and conversion to the PMDBD salt (12 AU_{260}; about 60 nmol). Reverse phase HPLC analysis (see **Fig. 2**) showed that the crude product contained about 75% dithiopyridine-modified product (eluting at 20% acetonitrile) contaminated with 25% of non-modified material eluting at 19% acetonitrile. The crude product was treated with 100 equivalents of thiocholesterol in a total volume of 2 ml methanol/dichloromethane (2:3) containing 5 mM PMDBD trifluoroacetate pH 8.5. Workup and HPLC purification yielded 4.7 AU_{260} (about 160 μg; 39%) of ON in thiocholesterol-free form (eluting at about 20% acetonitrile) and 5.7 AU_{260} (about 190 μg; 48%) of the thiocholesteryl-modified form (eluting at 55–68% acetonitrile).

^{32}P-Labeling of modified ONs

Thiocholesteryl-modified 2′-O-methyl-oligoribonucleotide and a non-modified 2′-O-methyl-oligoribonucleotide as a control were 5′-^{32}P-labeled using an incubation buffer with a minimum amount of reducing agents. One pmol ON was incubated in 10 μl buffer [50 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol (DTE)] with 10 units T4 polynucleotide kinase (Boehringer Mannheim) and 2 pmol γ-^{32}P-ATP for 30 min at 37°C. The labeled ON was then purified by gel filtration on Sephadex G-25 (1.8 ml bed volume, 50 mM HEPES pH 7.3) after addition of 10 pmol unlabeled ON to minimize unspecific absorption. The first peak of radioactivity appearing in the void volume was collected.

Reductive cleavage of thiocholesteryl-modified ONs

A solution of 0.1 AU_{260} (46 nmol) thiocholesteryl-modified ON (as prepared in b) in 900 μl 25 mM HEPES buffer (pH 7.3) was brought to a concentration of 10 mM dithiothreitol by addition of 1.4 mg DTT and kept under argon at 37°C for 18 h. Analysis by reverse phase HPLC under the same conditions described above showed complete cleavage of the thiocholesteryl-modified ON. A similar experiment was performed with β-mercaptoethanol (BME) as reducing agent and resulted in the same cleavage product, which eluted at a gradient concentration of about 18% acetonitrile (**Fig. 2**, lane 4). The other earlier eluting peaks derive from the reducing agent.

Incorporation of thiocholesteryl-modified 2′-O-methyl-oligoribonucleotides into liposomes

Liposome preparation: Liposomes were prepared by the REV (reverse-phase evaporation) method (16,17). The aqueous phase consisted of 175 μl 50 mM HEPES (pH 7.3), 1 mM calcein and 1 μM 2′-OMe-RNA (thiocholesteryl-modified or unmodified as control), spiked with a trace amount of 5′-^{32}P-labeled material. The organic phase was a solution of 5 μmol L-α-lecithin (from egg yolk, mainly palmitoyl-oleoylphosphatidylcholine; Avanti Polar Lipids) in 600 μl diethylether (extracted with 50 mM HEPES buffer). The two phases were thoroughly mixed in a glass tube by vortexing and sonicated 5 min at 0°C in a bath-type sonicator. The resulting stable emulsion was slowly evaporated on a rotary evaporator with occasional vortexing. After complete removal of the diethylether (30 min at 200 mbar) the liposome

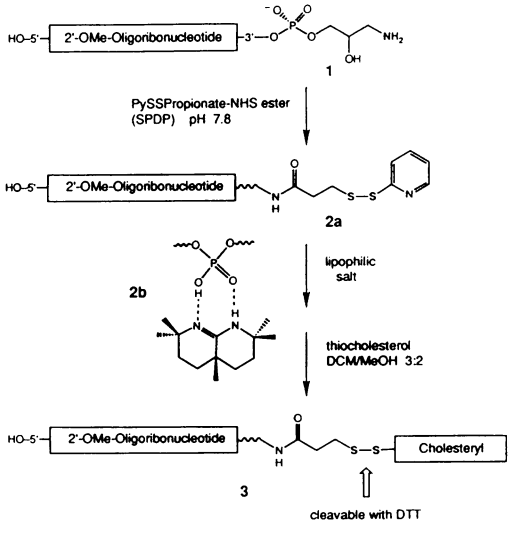

Figure 1. Synthesis of thiocholesteryl-modified oligonucleotides.

solution was then further sonicated for 20 min at 0°C. The resulting solution was diluted with 175 µl 50 mM HEPES pH 7.3 and stored at 2°C.

Gel filtration of liposomes: The liposomes (an aliquot of 200 µl) were separated from unincorporated material by gel filtration on Sephadex G-75 Superfine (10 ml bed volume, 50 mM HEPES pH 7.3). Fractions of 600 µl were collected and subjected to UV analysis and Cerenkov counting. The calcein absorbance (493 nm) was corrected for the unspecific absorbance of the liposomes measured at 600 nm. As a control an aliquot of labeled ON not coupled to thiocholesterol was subjected to gel filtration under the same conditions. The efficiency of encapsulation of the aqueous phase was calculated from the calcein absorbance associated with the liposome fractions (2.5–4.5 ml, see **Fig. 3**) and the non-incorporated calcein eluting with the low molecular weight fractions (7–14 ml). In the case of the unmodified ON incorporation efficiency was determined by comparing the radioactivity in the liposome fractions (2.5–4.5 ml) with the total eluted radioactivity. Because of some degradation of the label during liposome preparation in the presence of thiocholesteryl-modified ON, the liposome-incorporated radioactivity (2.5–4.5 ml) was compared to the radioactivity in the high molecular weight fractions only (2.5–9.0 ml; see **Fig. 3a**). The value of the liposome-associated radioactivity was corrected for the amount of low molecular weight ^{32}P from orthophosphate and/or nucleotides in the liposomes due to simple volume inclusion.

Binding of ONs to liposomes or to cultured cells

Liposomes were prepared by REV from an aqueous phase containing 10 mM HEPES pH 7.3, 150 mM NaCl and 100 mM calcein. After purification by gel filtration using HEPES/NaCl buffer, 200 µl of liposomes was mixed with ^{32}P-labeled thiocholesteryl-modified 2'-OMe-RNA (0.5 µM). After agitating for 10 min the solution was analyzed by gel filtration on Sephadex G-75 Medium (Pharmacia, 13 ml bed volume) as described above.

The mouse embryonic liver cell line ATCC TIB 73 (BNL CL.2 (18)) was grown at 37°C in 5% CO_2 in high glucose DMEM (0.45% glucose) supplemented with 10% heat-deactivated FCS. For binding studies the cells were seeded into 24-well plates (1.5 cm diameter). After growing to a density of 1.2×10^5 cells/well (30% confluency) 250 µl of fresh medium supplemented with 0.5 µM ^{32}P-labeled thiocholesteryl-modified 2'-OMe-RNA or unmodified 2'-OMe-RNA was added. At the indicated times the medium was removed and the cells were washed 3 times with 2 ml PBS and finally with 250 µl serum-containing medium. Then the cell layer was lysed with 250 µl proteinase K lysis buffer (0.1 µg/µl proteinase K, 0.5% SDS, 150 mM NaCl, 20 mM HEPES, pH 7.4) and counted (**Fig. 5**).

HPLC analysis of cell-associated and internalized oligonucleotides: After a 5 h treatment of TIB 73 cells with 0.5 µM ^{32}P-labeled thiocholesteryl-modified 2'-OMe-RNA in serum-free medium as described above, the cells were washed and lysed with 250 µl 1% SDS in PBS. The DNA was sheared by a short sonification and the diluted extract (ca. 5-fold, PBS) was filtered through a Centricon centrifugal microconcentrator (10 000 MW cutoff, Amicon) to remove membranes, proteins and cellular nucleic acids. During this procedure the thiocholesteryl-modified ONs were retained due to adsorption to the membrane and/or the retained cellular components. An aliquot of the flow-through was subjected to reverse phase HPLC. The collected fractions (4 ml) were analyzed by scintillation counting (**Fig. 6**).

Reversed phase C-18 cartridge analysis of oligonucleotides: To measure the amount of thiocholesteryl-modified ONs in cell extracts, 250 µl of medium containing 10% FCS, 200 µl 100 mM triethylammonium acetate pH 6.5 and 300 µl acetonitrile were added to 250 µl of extract made in proteinase K lysis buffer. The

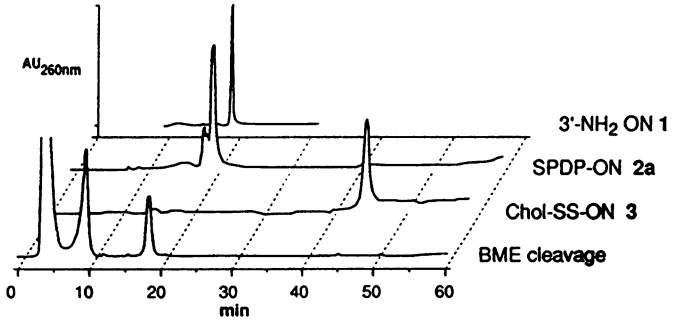

Figure 2. Reverse phase HPLC analysis of modified oligonucleotides on a Nucleosil RP-18 column (250×4 mm; buffer A: 100 mM triethylammonium acetate pH 6.5; buffer B: acetonitrile; gradient: 0–40 min, 0–50% B; 40–60 min, 50–100% B). **Lane 1**: 3'-amino modified 2'-OMe-RNA **1** (3'-NH_2 ON); **lane 2**: SPDP modified 2'-OMe-RNA **2a** (SPDP-ON); **lane 3**: thiocholesteryl coupled 2'-OMe-RNA **3** (Chol-SS-ON); **lane 4**: cleavage of ON **3** with BME (see Materials and Methods).

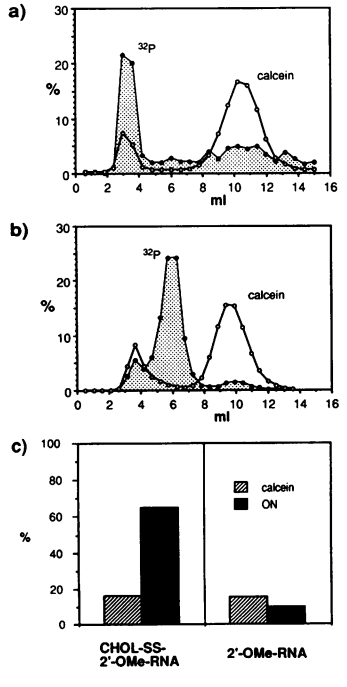

Figure 3. Incorporation of oligonucleotides into liposomes: Liposomes prepared with thiocholesteryl-modified 2'-OMe-RNA (**a**) or with unmodified 2'-OMe-RNA (**b**) were subjected to gel filtration on Sephadex G-75 as described under Materials and Methods. [Open circles: calcein absorbance at 493 nm (% of total eluted absorbance); filled circles: cpm of ^{32}P (% of total eluted radioactivity)]. **c**) Incorporation efficiencies of thiocholesteryl-modified ONs and unmodified ONs compared to the inclusion of fluid-phase marker (calcein). Values were calculated from the data in Fig. 3a and 3b as described under Materials and Methods.

mixture was passed through a C-18 cartridge (Sep-Pack, Waters) which was pretreated with 30% acetonitrile in 100 mM triethylammonium acetate pH 6.5 and the flow-through was collected. The cartridge was washed with 2 ml of the same buffer and the combined solutions, which would contain unmodified 2'-OMe-RNA and free phosphate, were counted for ^{32}P. The adsorbed radioactivity representing thiocholesteryl-modified ON was detected by counting the packing material. Analysis of the ONs in the conditioned medium was performed analogously after adjusting the total protein and lipid content by adding 250 µl of extract from untreated control cells to 250 µl of conditioned medium. For calibration the same analysis was performed after mixing thiocholesteryl-modified and unmodified 2'-OMe-RNA with control cell extract and medium (76% resp. 2% retention on the cartridge).

RESULTS

Synthesis of thiocholesteryl-modified 2'-O-methyl-oligoribonucleotides

The conjugation of modified 2'-O-methyl-oligoribonucleotides with thiocholesterol was accomplished as shown in **Fig. 1** through ligation via disulfide bond formation. Reaction of ON **1**, modified with a (3-amino-2-hydroxypropyl)phosphate group at the 3'-end, with an excess of SPDP, and subsequent isolation by gel filtration and reverse phase HPLC yielded the pyridyldithiopropionate-modified compound **2a**. To enable the coupling to thiocholesterol, a compound which has very limited solubility in polar solvents like water or methanol, the ON was converted to the lipophilic PMDBD salt. PMDBD, a highly methylated bicyclic amidinium base developed by the group of A. Eschenmoser (15), has been reported to solubilize sulfate and phosphate ions in apolar solvents such as dichloromethane. The ON-PMDBD salt **2b** was readily soluble in dichloromethane/methanol (2:1). The ligation with thiocholesterol via disulfide bond formation occurred in the presence of 5 mM PMDBD trifluoroacetate buffer (pH 9); workup and purification by HPLC gave the desired thiocholesteryl-modified ON **3**.

The increased lipophilicity of the conjugate is indicated by its behavior during reverse phase HPLC (**Fig. 2**). Conjugate **3** eluted at ca. 60% acetonitrile (lane 3), compared to ca 20% acetonitrile for unconjugated ONs (lanes 1 and 2). As a consequence of the disulfide group within the conjugate, the ON moiety could be released from the lipophilic anchor by treatment with reducing agents such as dithiothreitol or BME (**Fig. 2** lane 4).

Incorporation of thiocholesteryl-modified ONs into liposomes

Liposomes were prepared by the reverse-phase evaporation method (16) in the presence of 1 mM calcein as a low molecular weight fluid phase marker and 1 µM 5'-^{32}P-labeled thiocholesteryl-modified or unmodified ONs. Separation from unincorporated material was performed by gel filtration (see **Fig. 3**). In the case of unmodified 2'-OMe-RNA, 10–15% of both calcein and ON were encapsulated into liposomes and eluted in fractions 2.5–4.5 ml (**Fig. 3b**). Most of the ^{32}P appeared as free ON in fractions 4.5–7.5 ml. By contrast, incorporation of thiocholesteryl-modified ONs into the liposome fractions was much higher (65%). Only a small fraction of free ON, eluting as a broad peak due to unspecific absorption to the chromatographic support, in combination with small amounts of free, unincorporated phophate was seen (**Fig. 3a**). These results cannot be explained by a mechanism of pure fluid phase encapsulation, as only 10–15% of the calcein was incorporated into the same liposomes (**Fig. 3c**). Liposomes containing these lipid-modified ONs were subjected to alkaline phosphatase treatment and a further gel filtration (**Fig. 4**). The ^{32}P label of approximately half of the membrane-associated ON was removed and eluted as ^{32}P-phosphate in fractions 7–12 (**Fig. 4a**). This

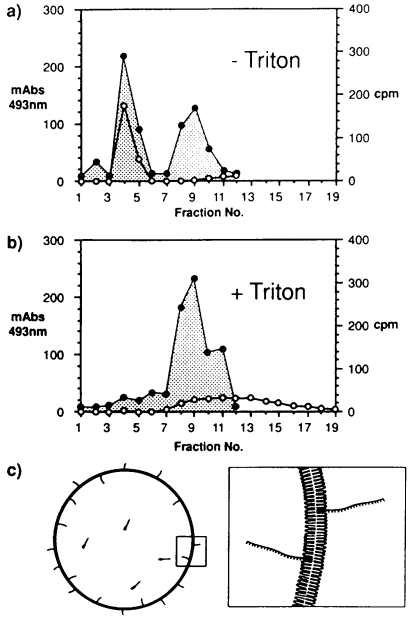

Figure 4. Alkaline phosphatase digest: An aliquot (400 µl) of liposomes containing thiocholesteryl-modified ON from the Sephadex G-75 column in Fig. 3a (fraction 2.5–4.5 ml, in 50 mM HEPES pH 7.3) was treated with 5 units of calf intestine alkaline phosphatase (Boehringer Mannheim) for 2 h at 37°C (**a**). As a control the same amount of liposomes was treated with 5 units alkaline phosphatase in the presence of 1 µl Triton X-100 for 1 h at 37°C (**b**). The digests were subjected to gel filtration on Sephadex G-25 PD-10 columns and 800 µl fractions were analyzed by UV absorption and Cerenkov counting. [Open circles: calcein absorbance at 493 nm; filled circles: ^{32}P (cpm)]. **c**) A cartoon consistent with the finding that most, if not all thiocholesteryl-modified ONs are incorporated into the phospholipid-bilayer.

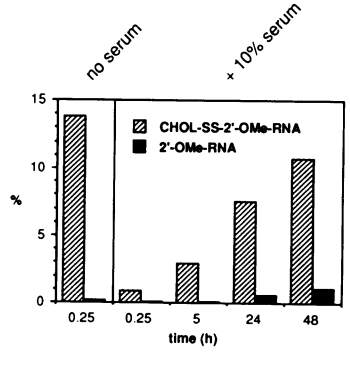

Figure 5. Time course of association of thiocholesteryl-modified 2'-O-methyl-RNA (dashed bars) or unmodified 2'-O-methyl-RNA (dark bars) with TIB 73 cells in the absence (left panel) or presence (right panel) of serum. TIB 73 cells were incubated with ^{32}P-labeled thiocholesteryl-modified or unmodified 2'-OMe-RNA. At the indicated times the medium was removed, cells were washed and the radioactivity in the lysates was counted (see Materials and Methods). During the course of the experiment (48 h) the cells grew from 30% to 100% confluency.

is consistent with the explanation that half of the ON is inserted into the outside of the liposomal membrane (**Fig. 4c**). Alkaline phosphatase treatment was also done in the presence of Triton X-100, which causes liposome breakdown (**Fig. 4b**). All of the radioactivity appeared in the phosphate fractions 7–12, indicating that all the ONs are accessible and dephosphorylated by the phosphatase under these conditions. Calcein was also released from the liposomes and eluted as a broad peak in the low molecular weight fractions 7–19 (**Fig. 4b**).

Binding of thiocholesteryl-modified ONs to liposomes and to cultured cells

When ONs were incubated with preformed liposomes or with cultured cells, the thiocholesterol modification resulted in highly increased binding. Upon addition to liposomes almost all of the modified ONs eluted in the liposome fraction of a gel filtration column, whereas in the absence of liposomes the ONs nearly quantitatively adhered to the column material under these elution buffer conditions (data not shown). In cell culture with the mouse embryonic liver cell line TIB73 in serum-free medium, 14% of the modified ON was rapidly adsorbed by the cell layer after 15 min (**Fig. 5**). In a control experiment only 0.15% of an unmodified 2'-OMe-RNA was adsorbed. Similar findings of enhanced association of thiocholesteryl-ON conjugates have also been found with HL60 or HepG2 cells (data not shown).

The serum content in the tissue culture medium had a striking effect on the association of ONs with cells (see **Fig. 5**). The presence of 10% fetal calf serum significantly reduced the initial binding of thiocholesteryl-ON conjugates to only 0.9% after a 15 min incubation (compared to 0.07% with the control 2'-OMe-RNA). A longer incubation time, however, resulted in increased association with cells that reached a value of 11% after 48 h (compared to 1% with the control 2'-OMe-RNA). Correction of the values for the increasing cell number during the course of the experiment reveals a 3-fold increase in binding during the first 5 h, and thereafter the values remain constant.

To distinguish internalized from cell-surface bound ONs, cells were analyzed by treatment with alkaline phosphatase. In a control experiment, where endocytosis was prevented by pretreating the cells with glutaraldehyde, 97% of the cell-bound radioactivity could be removed (**Table 1**). During incubation of living TIB 73 cells with thiocholesteryl-modified ON, the fraction of internal, phosphatase-inaccessible ON increased from an initial 17% (15 min) to 38% (5 h) of cell-bound ONs . After 5 h incubation in serum-free medium with either cholesteryl-modified or unmodified 2'-OMe-RNA, there was 15-fold higher binding (18.7% vs. 1.3% of total radioactivity) and 8-fold higher internalization (7.1% vs. 0.9%) of the thiocholesteryl-modified compound.

Table 1. Ratio of internalized to surface-bound ONs.

		serum (%)	time	$^{32}P_{tot}$ (%)	$^{32}P_{int}$ (%)	$\frac{^{32}P_{int}}{^{32}P_{tot}}$
a	CHOL-ON fixed cells	0	5 min	6.0	0.2	0.03
b	CHOL-ON	0	15 min	7.6	1.3	0.17
c	CHOL-ON	0	5 h	18.7	7.1	0.38
d	control-ON	0	5 h	1.3	0.9	0.69
e	CHOL-ON	10	5 h	4.5	1.7	0.38

Thiocholesteryl-modified 2'-OMe-RNA (**a-c, e**) or 2'-OMe-RNA (**d**), ^{32}P-labeled at the 5'-end, was incubated with TIB 73 cells in serum-free medium (**a-d**) or in medium containing 10% FCS (**e**) as described in *Material and Methods*. In a control experiment (**a**) the cells were fixed with glutaraldehyde prior to incubation (1.2% in PBS, 10 min, 25°C; inactivation of excess aldehyde groups with 100 mM glycine pH 7.7, 150 mM NaCl, 1 h, 37°C). After removing the medium and thoroughly washing with PBS, the cells were treated 3 times with 10 units alkaline phosphatase in 250 µl HBS (supplemented with 20 mM NaN_3) for 30 min at 37°C. The individual supernatants were collected and counted after removal of some detached cells by centrifugation. The remaining cells together with the detached cells were lysed with proteinase K lysis buffer and counted as described in *Materials and Methods* ($^{32}P_{tot}$, total cell-associated radioactivity in% of total amount added to the cells; $^{32}P_{int}$, radioactivity which is not removed by alkaline phosphatase treatment).

Cleavage of the oligonucleotides

To determine the fate of the thiocholesteryl-modified ONs associated with cells, TIB 73 cell lysates were subjected to analysis on a C-18 cartridge after 7 h and 24 h of incubation. The amount of lipophilic-modified ONs, which was calculated as the percentage of ^{32}P that bound to the cartridge (see Materials and Methods), was 51% at 7 h and decreased to 25% at 24 h. These results could be due to reductive cleavage of the thiocholesteryl moiety and/or to dephosphorylation of the ON by phosphatases. To distinguish between these two possibilities a reverse phase HPLC analysis of cell-associated radioactivity was performed using extracts of TIB 73 cells that had been incubated with thiocholesteryl-modified ON for 5 h in serum-free medium. After removal of thiocholesteryl-modified ONs together with membranes and other macromolecular cellular components by ultrafiltration, HPLC analysis (**Fig. 6**) of the soluble fraction revealed that 48% of the radioactivity eluted at a position corresponding to orthophosphate and/or nucleotides and 52% eluted in the thiocholesterol-free ON fraction at 20–35% acetonitrile. Thus, loss of ^{32}P end-labeled thiocholesteryl-modified ON is due to reduction of the disulfide bond and release of the free ON into the cell as well as to the action of phosphatases to the same extent.

DISCUSSION

A lipophilic anchor attached to ONs facilitates their uptake into cells (7), but such ONs may remain trapped in membranes. Furthermore, modification with lipid may result in unspecific (not sequence-specific) side effects (7,19). To obtain a potentially bioreversible linkage of lipid to ONs, 2'-O-methyl-

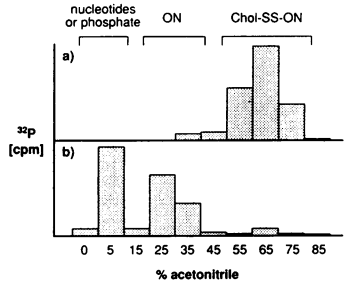

Figure 6. HPLC analysis of ^{32}P-labeled thiocholesteryl-modified ON **3** on a Nucleosil RP-18 column (250×4 mm; buffer A: 100 mM triethylammonium acetate pH 6.5; buffer B: acetonitrile; flow: 0.8 ml/min; gradient: 0–50 min, 0–100% B; the sample was injected in buffer containing 50% ethanol) (**a**). Analysis of the soluble fraction of a TIB 73 cell extract (obtained by ultrafiltration) after 5 h incubation with thiocholesteryl-modified ON (**b**).

oligoribonucleotides modified with an amino-linker at the 3′-end were coupled to thiocholesterol via a disulfide bond. The key step of the synthesis was conversion of the ON to the 'Heinzer base' salt (15) which we found was soluble in dichloromethane containing small amounts of methanol . This allowed reaction of the ON with thiocholesterol in homogeneous solution using an apolar nonaqueous solvent system. 2′-O-Methyl-oligoribonucleotides were used in the present study, as such analogues are resistant to a variety of DNases and RNases (20). These compounds were found to be about 300 times more effective than antisense oligodeoxynucleotides in an *in vitro* antisense inhibition assay (14).

Upon liposome formation in the presence of thiocholesteryl-modified ONs a highly efficient loading of liposomes with the ONs was observed (**Fig. 3c**). The incorporation was about six times higher than the fluid-phase encapsulation of corresponding thiocholesteryl-free ONs. The lipid-modified ONs seem to be incorporated into the liposome membranes both on the inside and outside. This was supported by our finding that the phosphate label of half the ONs, i.e. those which presumably were exposed on the outer surface of the liposomes, could be removed by treatment with alkaline phosphatase. In addition to the high incorporation efficiency these new ON-bearing liposomes may be interesting candidates for the delivery of antisense compounds into cells.

In comparison to unmodified 2′-OMe-RNA, the thiocholesteryl-modified ONs showed a strongly enhanced affinity both for preformed liposomes and cultured cells. In the presence of serum, cell association was initially reduced, presumably resulting from absorption of the ON to serum proteins; for example, cholesteryl-modified ONs bind to lipoproteins (our unpublished results). Upon further incubation, there is a significant increase in cell association of lipid-modified ONs (**Fig. 5**). This serum effect is not due to a rapid cleavage of the ON from the thiocholesteryl moiety by serum components: reverse phase HPLC analysis of the cell culture medium after 5 h of incubation showed that about 80% of the 5′-^{32}P-labeled ONs still contain the thiocholesteryl moiety (data not shown). A large fraction of the cell-associated oligonucleotides is internalized as shown by protection from alkaline phosphatase digestion (**Table 1**).

We have demonstrated that the thiocholesteryl moiety is released from the ON conjugate under mild reducing conditions such as treatment with DTT *in vitro*. The cleavage of disulfide bonds in macromolecule conjugates upon endocytosis into cultured cells has been reported (12). A similar reductive processing also occurs with thiocholesteryl-modified ONs in cells. We were able to detect considerable amounts of thiocholesteryl-free ONs in a cellular extract (**Fig. 6**) which is consistent with cleavage of the disulfide bond. This release of the ONs from the membrane-bound lipophilic anchor may be beneficial for the interaction of the ONs with their cellular targets when applying these compounds as antisense reagents.

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