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QUARTERLY

Inhibition of plasminogen activator inhibitor release in endothelial cell cultures by antisense oligodeoxyribonucleotides with a 5′-end lipophilic modification[★][○]

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A series of conjugates containing residues of lipophilic alcohols covalently bound to 5′ end of oligodeoxyribonucleotides targeted against human plasminogen activator inhibitor (PAI-1) mRNA was synthesized *via* the oxathiaphospholane approach. The highest anti-PAI-1 activity in EA.hy 926 endothelial cell cultures was found for conjugates containing menthyl or heptadecanyl groups linked with an oligonucleotide complementary to a segment of human PAI-1 mRNA. The phosphodiester antisense oligonucleotides, which otherwise exhibit only limited anti-PAI-1 activity, were found to be more active than phosphorothioate oligonucleotides when conjugated to lipophilic alcohol residues. For menthyl conjugates an evidence of antisense mechanism of inhibition was found.

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Abbreviations: DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMT, dimethoxytrityl group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor (Type 1).

In our recent reports [1, 2] results were presented on the use of 24-mer oligo (deoxyribonucleoside phosphorothioate)s for the modulation of expression of type-1 plasminogen activator inhibitor (PAI-1), a protein which plays an important regulatory role in fibrinolysis and thrombus formation [3, 4]. Oligodeoxyribonucleotide PS-24H of sequence 5′-GAGGGCTGGAGACATCTGCATCCT was selected as an antisense probe targeted to a segment of human PAI-1 mRNA coding for a part of its signal peptide (codons from -24 to -17). We have found that PS-24H (used as stereorandom mixture of diastereomers) [5] at a concentration of 2.52 μM, effectively inhibits PAI-1 release in cultured endothelial cells. This inhibition was found to be sequence-, time-, and concentration-dependent. PO-24H, an unmodified phosphodiester oligonucleotide of the same sequence, did not affect PAI-1 release under the same experimental conditions [1, 2]. In the following studies we found that an even higher level of PAI-1 inhibition can be achieved by employing hexadeca(deoxyribonucleoside phosphorothioate) PS-16H (**1**) which has a sequence identical with that of the 5′-portion of the parent 24-mer [6]. Again, the unmodified phosphodiester congener PO-16H (**2**) was found to be much less active. As a generally accepted explanation for the low inhibitory effect of PO-24H and PO-16H we considered the low stability of unmodified oligonucleotides against cellular endo- and 3′-exonucleases [7, 8]. In this paper we wish to present the effect of covalent attachment of lipophilic alcohol residues onto the 5′ end of the antisense oligonucleotides **1** and **2** on their PAI-1 inhibitory activity. The residues of borneol, cholesterol, menthol, and heptadecanol were bound to the 5′ end of **1** and **2** either directly or indirectly (through a linker) *via* a phosphorothioate linkage. The conjugates obtained **3–18** (for detailed structures see Table 1 and Fig. 1) were tested for their efficacy as PAI-1 inhibitors in human hybrid endothelial cell cultures.

MATERIALS AND METHODS

Oligonucleotide conjugates synthesis. Oligodeoxyribonucleotides PS-16H (**1**) and PO-16H (**2**), as well as oligodeoxy- and oligo-

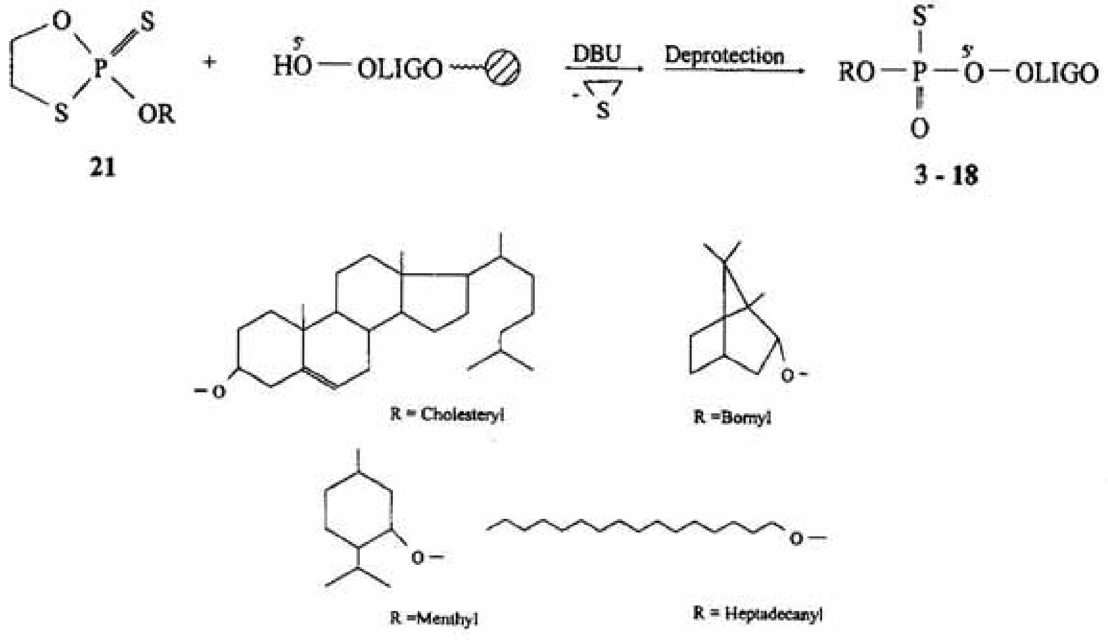

Figure 1. The synthesis and structure of oligodeoxyribonucleotide conjugates.
3–6, OLIGO = PO-16H; **7–10**, OLIGO = PS-16H; **11–14**, OLIGO = Link1-PO-16H; **15–18**, OLIGO = Link2-PS-16H.

Table 1. Yields and analytical data of oligonucleotides and oligonucleotide conjugates

Comp. No.	Oligonucleotide constructs	HPLC R.T. (min)	Molecular mass^{a} (for free acids) (Da) calculated	Molecular mass^{a} (for free acids) (Da) observed	Yield^{b}
1	PS-16H 5′-$G_SA_SG_SG_SG_SC_ST_SG_SG_SA_SG_SA_SC_SA_ST_SC$	27.31^{e}	5208	5210	24.0
2	PO-16H 5′-$G_PA_PG_PG_PG_PC_PT_PG_PG_PA_PG_PA_PC_PA_PT_PC$	25.30^{e}	4968	4969	16.0
3	BORN-PO-16H	17.77; 18.68^{d}	5200.5	5202	22.8
4	CHOL-PO-16H	21.51; 22.04^{c}	5433	5437	10.0
5	MENTH-PO-16H	19.27; 19.72^{d}	5202.5	5203	11.6
6	HEPT-PO-16H	26.07^{c}	5302.5	5305	9.0
7	BORN-PS-16H	18.43^{d}	5440.5	5443	27.1
8	CHOL-PS-16H	21.83^{c}	5673	5676	12.0
9	MENTH-PS-16H	19.29^{d}	5442.5	5445	18.4
10	HEPT-PS-16H	26.36^{c}	5542.5	not determined	25.6
11	BORN-Link1-PO-16H	15.10^{c}	5969	5969	10.2
12	CHOL-Link1-PO-16H	29.41^{c}	6202	6203	14.3
13	MENTH-Link1-PO-16H	16.33^{c}	5971	5973	15.9
14	HEPT-Link1-PO-16H	26.94^{c}	6071	6072	11.7
15	BORN-Link2-PS-16H	16.07^{c}	6256	6257	11.0
16	CHOL-Link2-PS-16H	29.60^{c}	6489.5	6491	11.5
17	MENTH-Link2-PS-16H	17.07^{c}	6259	6262	11.6
18	HEPT-Link2-PS-16H	27.32^{c}	6359	6360	4.0
19	PS-16H SENSE 5′-$G_SA_ST_SG_ST_SC_ST_SC_SC_SA_SG_SC_SC_SC_ST_SC$	27.14^{e}	5048	5033	11.0
20	PS-16H SCRAMBLE 5′-$T_SG_SA_SG_SA_SG_SC_SG_SA_SA_SG_ST_SC_SG_SG_SC$	28.52^{e}	5208	5210	18.2

P, phosphate; S, phosphorothioate; Link1, $(OCH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2O_P)_3$; Link2, $(OCH_2CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2O_S)_3$; BORN, bornyl; MENTH, menthyl; CHOL, cholesteryl; HEPT, heptadecanyl; ^{a}measured by ESI-MS; ^{b}measured in A_{260} units for one micromole synthesis; ^{c}0.1 M ammonium acetate, 2.0% CH_3CN/min; ^{d}0.1 M ammonium acetate, 1.3% CH_3CN/min; ^{e}0.1 M triethylammonium bicarbonate, 1.3% CH_3CN/min (Hamilton PRP-1 column).

ribonucleotide constructs used in complementary studies were prepared on solid support by a phosphoramidite approach [9] using an ABI-394 DNA Synthesizer with S-TETRA sulfurization for the phosphorothioate sequences [10]. One micromole of nucleoside 3′-*O*-succinyl-sarcosinyl-LCA-CPG [11] was used for each synthesis. For preparation of the conjugates, lipophilic alcohol residues were connected *via* phosphorothioate linkages either directly with the 5′-hydroxyl group of oligonucleotide (compounds **3–10**), or *via* a linker containing tri(tetraethylene glycol phosphate) (Link1, compounds **11–14**) or tri(tetraethylene glycol phosphorothioate) (Link2, compounds **15–18**) [12, 13]. After completion of the oligonucleotide chain assembly 5′-protecting DMT groups were removed by standard acid treatment. The attachment of the linker (Link1 or Link2) was performed with PO-16H or PS-16H, while still bound to the solid support, by treatment with protected TEG-phosphoramidite [MMT-OCH_2-$CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2O$-P($OCH_2CH_2CN$)$N^iPr_2$] followed by iodine oxidation (Link1) or sulfurization with S-TETRA (Link2). This linker extension procedure was repeated three times. The final introduction of the lipophilic alcohol phosphorothioate was achieved by treating the oligonucleotide construct (with or without linker), still bound to the solid support, with CH_2Cl_2 solution (190 μl) containing a 2-thiono-1,3,2-oxathiaphospholane derivative of the corresponding alcohol (**21**, 0.15 M) and DBU (2 M) (prepared from more concentrated stock solutions and premixed [14], see Fig. 1).

The condensation was carried out for 40 min at room temperature in an ABI DNA Synthesis column connected to a syringe, with occasional swirling. After standard cleavage from the solid support (28% NH_4OH, 2 h, room temperature) and further removal of nucleobase-protecting groups (28% NH_4OH, 12 h, 55°C) the conjugates were purified by reversed phase high performance liquid chromatography (RP HPLC, ODS Hypersil column, CH_3CN gradient from 0 to 80% in 0.1 M ammonium acetate). The structure of the isolated products was confirmed by electrospray ionization mass spectrometry (ESI-MS) and their purity, in addition to the HPLC data, was confirmed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (7 M urea).

2-Alkoxy-2-thiono-1,3,2-oxathiaphospholanes (**21**) were obtained according to the procedure described in detail elsewhere [14, 15]. Each alcohol was reacted with 2-*N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane [16, 17] in the presence of tetrazole in CH_2Cl_2 solution, followed, after 3 h, by addition of elemental sulfur. Crude **21** were isolated by column chromatography on Silicagel 60 (elution system: toluene/chloroform, 3:1, v/v) as a mixture of diastereomers (for cholesteryl, bornyl, and menthyl) or as the racemate (for heptadecanyl derivative). Control dinucleotide conjugates (**22–25**) were prepared in an identical manner as the above mentioned oligonucleotide conjugates **3–10** starting from support-bound dithymidine and the corresponding oxathiaphospholanes **21**. The details have been described elsewhere [14].

The effect of antisense oligonucleotide conjugates on PAI-1 expression in the EA.hy 926 human hybrid endothelial cell line. The human hybrid endothelial cell line EA.hy 926, derived by fusion of human umbilical vein endothelial cells with the continuous human lung carcinoma cell line A549, was a gift from Professor Cora-Jean S. Edgell (Pathology Department, University of North Carolina at Chapel Hill, NC, U.S.A.). The cells were cultured in Dulbecco's modified Eagle medium with high glucose, supplemented with 10% fetal calf serum, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) in a 90–95% humidified atmosphere of 5% CO_2 at 37°C. Before performing the assays, the serum-containing medium was changed to a serum-free medium and the cells were incubated with 1.25 μM of the relevant bioconjugate for 24 h. After incubation, supernatants were collected, centrifuged to remove cells and cellular debris at 200 × ***g*** for 5 min, and stored in aliquots at −70°C until use. Post culture medium (serum-free) was assayed for PAI-1 by measuring the inhibition of activation of plasminogen by t-PA as described by Levin [18] and Lewis *et al.* [19]. Briefly, 10 μl samples of the medium were incubated with 10 μl of t-PA solution (2 U/ml) in standard wells (96-well flat bottom microwell plates, Nunc, Denmark) for 20 min at 37°C. Then, 8 μl of thrombin solution (20 U/ml) and 10 μl of plasminogen solution (0.25 mg/ml) were added to each well. All reagents were mixed by adding 190 μl of fibrinogen solution (6 mg/ml) prewarmed to 37°C. After 30 min incubation at room temperature the wells were read at 405 nm using an EL 340 microplate reader from Bio-Tek Instruments equipped with the KinetiCalc program. Activation of latent PAI-1 by treatment with SDS was done as described by Levin [18]. Each measurement of PAI-1 level was repeated 6–10 times and average values are presented (see Fig. 2).

The effect of oligonucleotide conjugates on PAI-1 mRNA level in EA.hy 926 cells. Total mRNA was purified from 10^7 EA.hy 926 cells using Total RNA Prep Plus Kit (A&A Biotechnology, U.S.A.). First strand cDNA was synthesized according to the manufacturer's instruction using a $(dT)_{20}$ primer. Reverse transcription was carried out for 60 min at 30°C and stopped at 0°C. The PAI-1 mRNA assay employed the following oligodeoxyribonucleotide primers: 5′-GCTGAATTCCTGGAGCTCAG and 5′-CTGCGCCACCTGCTGAAACA. The conditions of PCR assay were as follows: 5 μl of cDNA sample, 10 × Taq polymerase buffer (Epicentre Technologies, U.S.A.), 2 mM $MgCl_2$, 10 × PCR Enhancer, dNTP mix, 25 pmol of each primer and 1.25 U of Taq DNA Polymerase (Epicentre Technologies) in a 50 μl reaction volume. Samples were denatured at 95°C for 5 min, then cooled on ice. After the enzyme was added, 30 primer extension cycles consisting of a 30 s denaturation step at 94°C, a 30 s annealing step at 60°C, and a 40 s polymerase extension step at 72°C were performed. Finally, each reaction was terminated with a 10 min elongation step at 72°C. To detect mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control the same protocol was used with oligodeoxyribonucleotide primers 5′-GAGAGATGATGACCCTTTTGGC and 5′-CCATCACCATCTTCCAGGAGCG. The final products for PAI-1 mRNA and GAPDH mRNA were separated by electrophoresis in 7% polyacrylamide gels in TAE buffer using as size marker a 100 bp DNA Ladder (Promega). Bands were visualized by UV light and the results were recorded photographically. The cells used for mRNA semi-quantitation were treated for 24 h before harvesting with appropriate oligodeoxyribonucleotide constructs at 1.25 μM concentration.

Stability of conjugates in human plasma. Plasma was isolated from human blood containing 0.38% sodium citrate by centrifugation at 16000 × ***g*** for 5 min. Plasma aliquots were withdrawn carefully, leaving packed cells untouched. A sample of oligonucleotide conjugate (or unconjugated oligonucleotide) (2.0 nmol) was dissolved in 200 μl of PBS buffer, added to an equal volume of plasma and incubated at 37°C for 4 h. The samples were then extracted twice with phenol/chloroform (1:1, v/v) and once with chloroform. After ethanol precipitation, the oligonucleotides/conjugates were resuspended in 10 μl of formamide containing 0.03% Bromophenol Blue and Xylene Cyanol, and analyzed by PAGE under denaturing conditions (20% polyacrylamide, 7 M urea in TBE buffer). The bands were visualized with Stains All reagent (Aldrich). Gels were scanned using an LKB Ultrascan XL densitometer.

The inhibitory effect of oligonucleotide conjugates toward plasma nucleases was assayed by 2 h pre-incubation of the corresponding conjugate (0.2 to 1.0 nmol) in 50% human plasma at 37°C, followed by addition of the

PO-16H oligonucleotide (**2**, 0.2 nmol) radioactively labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham). Ten μl samples were taken after 2, 4, and 6 h and heat-denatured for 2 min at 95°C. Each sample was diluted with 100 μl of water and centrifuged for 15 min at 10000 × ***g***. The supernatant was electrophoresed on a 20% denaturing polyacrylamide gel. Bands were visualized by autoradiography and scanned with LKB Ultrascan XL densitometer.

Interactions of heteroduplexes: conjugate/complementary RNA with RNase H. An oligoribonucleotide fragment complementary to PO-16H (5′-GAUGUCUCCAGCCCUC) was synthesized and labeled at the 5′ end with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. Oligonucleotide conjugates (or oligonucleotides) were hybridized to this RNA fragment (35 pmol, molar ratio 1:1 or 1:3) in a total volume of 10 μl (3 min at 90°C with slow cooling to room temperature). The hybridized sample was supplemented with 5 × RNase H buffer, pH 8.0 (4 μl: 100 mM Hepes/KOH, 250 mM KCl, 100 mM $MgCl_2$ and 5 mM dithiothreitol), RNasin (1 μl, 20 U, Amersham), RNase H (1 μl, 1 U, Boehringer, Mannheim, Germany) and diluted with water to 20 μl. The mixture was incubated at 37°C for 30 min and precipitated with ethanol in the presence of glycogen. The precipitate was resuspended in formamide containing dyes, electrophoresed on a 20% polyacrylamide denaturing gel (7 M urea) in TBE buffer and analyzed as above. Unhybridized RNA was used as a control.

t_m measurements. The t_m values were measured for duplexes formed by equimolar amounts of oligonucleotide conjugates and the complementary synthetic oligoribonucleotide [sequence as above, total concentration (*per* nucleotide) 162 μM in 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl]. The mixture was heated to 90°C for 2 min and then slowly (1 h) cooled to room temperature. Melting profiles were recorded at 260 nm using GBC UV/VIS 916 spectrophotometer equipped with a Peltier unit. The insulated cell compartment was warmed from 20°C to 90°C at a rate of 0.3°C/min.

CD measurements. The CD spectra of PO-16 conjugates were recorded on a Dichrograph CD6 (Jobin Yvon) in the same buffer as for t_m measurements as the single stranded species (concentration 243 μM *per* nucleotide) and in the form of 1:1 duplexes with complementary oligonucleotides (concentration 486 μM *per* nucleotide). The samples were prepared from stock solutions directly before measurement.

RESULTS AND DISCUSSION

Numerous literature reports indicated an enhancement of the efficacy of antisense oligonucleotides *via* their conjugation [20, 21] with biomolecules such as lipids [22], cyclodextrins [23], steroids [24], porphines [25], long-chain alcohols [26] or peptides [27, 28]. The modification of phosphodiester oligonucleotides by a cholesteryl moiety at the 5′-terminus [29] attracted special attention due to their enhanced cellular uptake [30]. The 5′-cholesteryl modified oligonucleotides were shown to be sequence-specific (antisense) inhibitors of expression of the MCF-7 protein [30] and non-sequence-specific inhibitors of HIV-1 reverse transcriptase [31]. Similar enhancement of the antiviral activity of antisense oligonucleotides bearing a highly lipophilic DMT group bound to the 5′ end *via* ether linkage has been recently reported by Agatsuma *et al.* [32]. Therefore, we decided to conjugate the previously selected hexadecamers PS-16H (**1**) and PO-16H (**2**) [6] with natural alcohols such as borneol, cholesterol, or menthol, and with heptadecanol. The alcohols were linked at the 5′ end of **1** or **2** either directly *via* a phosphorothioate linkage, or through a phosphorothioate group and a linker comprising three tetraethylene glycol units combined *via* phosphate or phosphorothioate linkages [12, 13]. The structural fea-

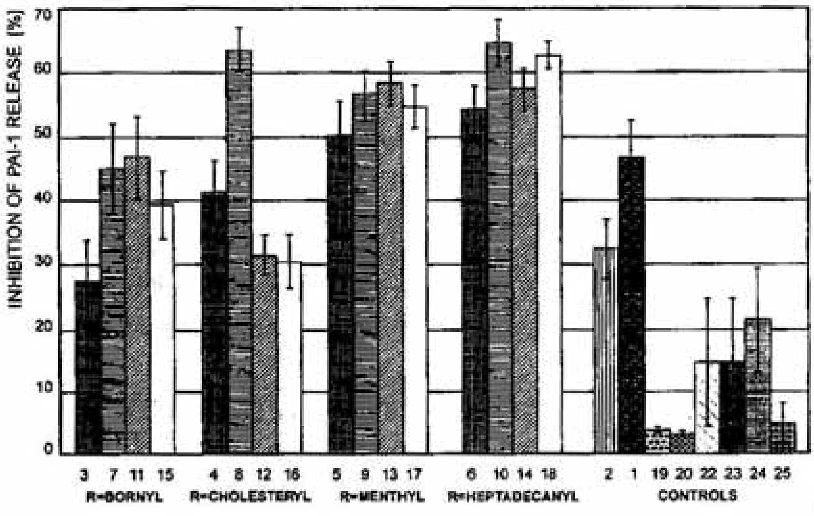

Figure 2. The effect of oligodeoxyribonucleotide conjugates of borneol, cholesterol, menthol and heptadecanol (3–18) and controls (1, 2, 19, 20 and 22–25) on PAI-1 expression in the human hybrid endothelial cell line EA.hy 926.

The inhibitory effect was calculated with regard to the PAI-1 activity in untreated cells. Oligonucleotide construct concentration 1.25 μM. Incubation time 24 h.

tures of all conjugated constructs are shown in Table 1 and Fig. 1. Their inhibitory activity towards PAI-1 release was demonstrated in the human hybrid endothelial cell line (EA.hy 926) as a lowering of PAI-1 levels in cell culture media after oligonucleotide treatment (Fig. 2).

Unconjugated antisense oligonucleotides **1** and **2** as well as sense (**19**) and scrambled (**20**) oligo(nucleoside phosphorothioate)s were used as controls. As additional controls, dithymidine conjugates **22–25** were employed. Of all bioconjugates studied, the most effective appeared to be the conjugates of **1** and **2** with heptadecanol and menthol. There was no substantial difference between conjugates of **1** and conjugates of **2** with alcohols (except cholesterol) attached directly *via* a phosphorothioate linkage and those attached *via* phosphorothioate linkage and tetraethylene glycol-based linkers. Most interesting, however, was a similar activity observed for 5′-conjugates of menthol and heptadecanol with oligonucleotide **1** (phosphorothioate) and oligonucleotide **2** (phosphodiester), thus confirming earlier observations by Stein *et al.* [31] and Krieg *et al.* [30] concerning the increased inhibitory activity of cholesteryl 5′-conjugates. For menthyl conjugates with $(TEG)_3$ linker (**13, 17**) and for parent oligonucleotides (**1, 2**) the dose-dependent PAI-1 inhibition was measured at 0.625 and 1.25 μM concentrations (24 h incubation at 37°C) (Table 2). In each case an increase of concentration within the above

Table 2. Dose-dependent inhibition of PAI-1 release by antisense oligonucleotides and their menthyl conjugates

Comp. No.	Inhibition of PAI-1 release (%)[a]	
	Oligonucleotide or conjugate concentration 0.625 μM	Oligonucleotide or conjugate concentration 1.25 μM
1	32 ± 2	47 ± 6
2	22 ± 2	33 ± 5
13	51 ± 3	58 ± 4
17	43 ± 2	55 ± 4

[a] EA.hy 926 cells were treated with oligonucleotides for 24 h at 37°C. The extent of inhibition was calculated by comparison of PAI-1 levels in oligonucleotide treated cells with respect to untreated cells.

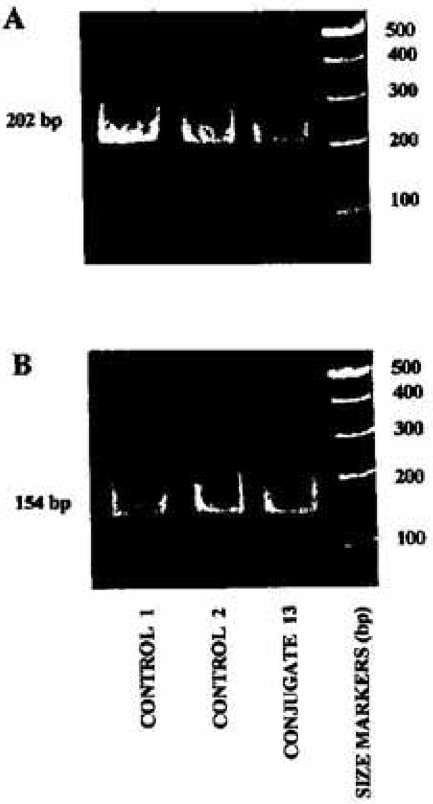

Figure 3. The effect of MENTH-Link1-PO-16H (13) on PAI-1 mRNA (A) and GAPDH mRNA (B) in EA.hy 926 cells.

Control 1, oligonucleotide untreated cells. Control 2, cells treated with MENTH-Link1 conjugated to *sense* phosphodiester oligonucleotide 5′-GATGTCTCCAGCCCTC.

mentioned range had a positive effect on PAI-1 inhibition, however, the effect of concentration was much more emphasized for oligonucleotides (47–50% increase of PAI-1 inhibition) than for their conjugates (14–28% increase). It should be emphasized that the aforementioned anti-PAI-1 activity of conjugates **3**–**18** is not affected by any cytotoxic effect as shown in a separate study [33].

In addition to determining the changes in protein level caused by antisense oligonucleotide constructs, semiquantitative RT-PCR of mRNAs for PAI-1 (Fig. 3A) and, as a control, for GAPDH (Fig. 3B) was performed for mRNAs isolated from EA.hy 926 cells untreated (control 1) and treated with menthyl antisense conjugate **13** or menthyl sense conjugate (control 2). A strong decrease in intensity of the PAI-1 mRNA-related band in cells treated with antisense construct **13**, with respect to controls (Fig. 3A), and the lack of effect of the same construct on GAPDH mRNA concentration (Fig. 3B) indicates that PAI-1 inhibition occurs at the mRNA level and seems to support an antisense mechanism of action of oligonucleotide constructs used in this work (at least for the menthyl derivative).

The enhanced anti-PAI-1 activity of PO-16H conjugates in comparison with the unconjugated oligonucleotide prompted us to perform additional studies of the bioconjugates, including assays evaluating their resistance to plasma exonuclease [34], RNase H induction, and hybridization to complementary RNA fragments. Thus, the conjugates MENTH-Link1-PO-16H (**13**) and HEPT-Link1-PO-16H (**14**) were incubated at 37°C for 4 h in 50% human plasma with unconjugated PO-16H (**2**) as a control (incubated separately under identical conditions). The analysis by gel electrophoresis demonstrated that under these conditions the conjugates **13** and **14** and oligonucleotide **2** were readily degraded, although the extent of hydrolysis of the conjugates was about 20% lower. Conjugates **13** and **14** were further assayed in competitive inhibition experiments toward **2**. Neither **13** nor **14** were found to be competitive inhibitors of plasma exonuclease, as evaluated by incubation of **2** in 50% plasma in the presence of **13** (or **14**) at a concentration of 5 or 25 μM. The unprotected oligonucleotide **2** was easily digested even when 50% human plasma was pre-incubated for 2 h with either of the conjugates (**13** or **14**).

The RNase H induction experiments were performed with duplexes formed by **2**, **13** and **14** with a complementary synthetic oligoribonucleotide labeled with ^{32}P at its 5′ end, with an unhybridized oligoribonucleotide serving as a control. The decay of radioactivity in the band of the starting heteroduplex after electrophoresis (denaturing PAGE) was a measure of RNase H induction. Comparative experiments revealed that conjugates **13** and **14** have iden-

Table 3. The comparative stability of duplexes formed by 2 and its conjugates 11–14 with DNA and RNA templates

Oligonucleotide constructs	t_m (°C)[a]	
	DNA template	RNA template
2	70.0	72.0
11	70.5	72.0
12	71.0	72.0
13	70.0	73.0
14	70.0	73.0

[a] Standard deviation ± 0.5°C.

tical (within experimental error) RNase H induction properties as the unconjugated oligonucleotide **2**.

The hybridization properties of oligonucleotide 5′-conjugates **3, 12, 13** and **14** were assayed and compared with those of the unconjugated oligonucleotide **2** by melting point (t_m) measurements of their duplexes formed with a complementary 16-mer oligoribonucleotide (5′-GAUGUCUCCAGCCCUC) and a 20-mer oligodeoxyribo-nucleotide (5′-GATGTCTCCAGCCCTCATGC). The t_m values obtained (Table 3) clearly show that the hybridization properties of the conjugates are almost identical and do not differ from that of the parent oligonucleotide **2**. All constructs seem to hybridize slightly stronger (higher t_m values) to the RNA template than to the corresponding DNA template.

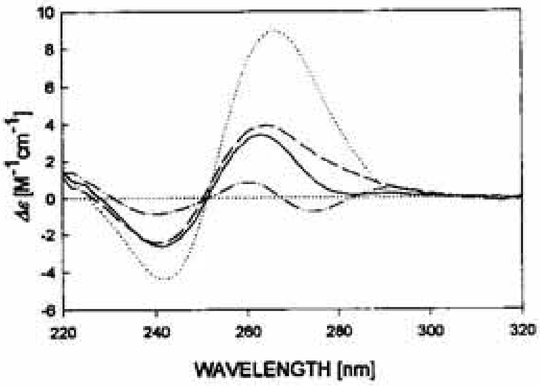

Figure 4. The CD spectra of oligodeoxyribonucleotide conjugates.

Cholesteryl (**12**) – dashed line; heptadecanyl (**6**) – dotted line; bornyl (**3**) – solid line; menthyl (**13**) – dot/dashed line.

In addition to t_m measurements CD spectra were recorded for the single stranded conjugates **3, 12, 13** and **14** as well as for their duplexes with complementary DNA and RNA templates. The CD spectra of duplexes formed by the conjugates or **2** with RNA (not shown) are similar to each other and typical for the A-conformation, with the RNA strand being the dominating element. In the case of duplexes with a DNA template which was 4 nucleotides longer, and therefore generated a 5′-protruding end, more variability was observed with regard to the relative intensity of the spectral maxima (not shown). The spectra are characteristic for the B-conformation. The highest variability of the CD spectra was observed for solutions of single stranded constructs (see Fig. 4). The CD spectra of heptadecanol conjugate (**6**) showed a strong positive maximum at 264 nm, characteristic for a tetraplex structure [35], whereas the spectra of unconjugated phosphodiester oligonucleotide (**2**) and menthyl conjugate (**13**) exhibited the pattern typical for a non-associated single-stranded structure [35]. The spectra of conjugates of borneol (**3**) and cholesterol (**12**) are examples of an intermediate situation, with partial tetraplex formation. The CD spectra of **3, 6** and **12** changed dramatically with an increase of temperature from 20 to 90°C showing evident dissociation of the tetraplex. However, upon cooling the tetraplex structure was not reconstituted after incubating a sample for 7 days at 5°C.

The evident enhancement of anti-PAI-1 inhibitory activity of antisense phosphodiester hexadecadeoxyribonucleotide PO-16H (**2**) after its 5′-conjugation with menthol (**5**, **13**) or heptadecanol (**6**, **14**) (an increase of PAI-1 inhibition from 33% to 51–58%) suggests that it may be possible to replace oligo(deoxyribonucleoside phosphorothioate)s as protein biosynthesis inhibitors (for phosphorothioate analogue PS-16H, **1**, 47% of PAI-1 inhibition was observed under identical conditions), thus avoiding the problem of polydiastereomerism [5] and possible side-effects such as non-specific binding to proteins [36], inhibition of polymerases [37], or interactions with regulatory factors [38]. The origin of this enhancement is not fully understood and one can only speculate that it may be a result of better cellular uptake of conjugates [30], or their increased intracellular stability. The noticable (about 20%) increase in stability of conjugates **13** and **14** in 50% human plasma with regard to parent phosphodiester oligonucleotide **2** may speak in favor of this conclusion although the origin of this effect is unclear.

Another interesting point is whether the observed anti PAI-1 inhibitory activity of conjugates **3**–**18** is a real antisense effect. One may argue that the presence of the polypurine tract GAGGG within the conjugated oligonucleotide sequence makes it prone to attaining the four-stranded tetraplex structure *via* guanine-guanine Hoogsteen base pairing [39] especially in view of the fact that 5′-conjugation to lipophilic alcohols (cholesterol) has been found to stabilize such structures [40]. Oligonucleotides capable of tetraplex formation might possess special biological properties, as implicated by the discovery of proteins that promote the formation of such structures [41] and induce specific cleavages in their vicinity [42]. In the case of conjugates **3**–**18** the situation seems to differ depending on the nature of the conjugated alcohol molecules. For menthyl conjugates the inhibition of PAI-1 release proceeds most probably *via* an antisense mechanism as evidenced by sequence-selective decrease of PAI-1 mRNA level by **13** and by the appearance of its CD spectrum, characteristic for single-stranded form. This conclusion is further supported by the observed dose-dependence of PAI-1 inhibition by **13** and its phosphorothioate analog **17**. The remaining conjugates of borneol, cholesterol and heptadecanol may inhibit PAI-1 biosynthesis *via* a more complex mechanism, including the participation of tetraplex structures, as evidenced by analysis of their CD spectra.

Further studies of anti-PAI-1 activity of conjugates of PO-16H oligonucleotide with lipophilic groups other than those described in this paper are in progress, including *in vivo* animal experiments with appropriate menthyl conjugate.

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