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Synthesis, biochemical and biological studies on oligonucleotides bearing a lipophilic dimethoxytrityl group*°

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Dimethoxytritylphosphono-oligonucleotide conjugates have been prepared. They are totally resistant to nucleases present in human serum and do not affect cleavage of a complementary oligoribonucleotide by RNase H. Conjugates possessing a phosphate backbone gave better antisense inhibition of expression of plasminogen activator inhibitor type-1 within endothelial cells as compared with unconjugated oligonucleotides.

Medium size (10–30 mers) synthetic oligonucleotides, due to their specific interaction (hybridization) with complementary fragments of mRNA or pre-mRNA, can be promising, rationally designed, agents for blocking biosynthesis of selected proteins (*antisense strategy*) [1]. Since unmodified oligonucleotides (PO-Oligos) are rapidly degraded by nucleases present in plasma [2] and within cells, more stable analogues, especially oligo(nucleoside phosphorothioate)s (PS–Oligos) [3] have been broadly exploited in *in vitro* and *in vivo* studies. Several PS-Oligos are now in preclinical and clinical trials as drugs against cancer, restenosis and viral diseases. Therapeutic effects of PS-Oligos are, however, limited by their poor cell membrane permeability, inhibitory activity towards human polymerases,

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Abbreviations: PAI-1, plasminogen activator inhibitor; $T_{PDMT}T$, dithymidylyl dimethoxytritylphosphonate moiety; PO-Oligos, unmodified oligonucleotides; PS-Oligos, oligo(nucleoside phosphorothioate)s; $T_{PDMT}T$-Oligos, $T_{PDMT}T$ conjugated to oligonucleotides; for other abbreviations see Materials and Methods.

and some side-effects resulting from undesired interactions with other proteins. One approach to improving pharmacological properties of PO- and PS-Oligos is their attachment to other molecules (*conjugation*) [4].

We present here the results of studies on the synthesis of dithymidylyl dimethoxytritylphosphonates conjugated to oligonucleotides (T_{PDMT}T-Oligos) on resistance of these constructs against serum nucleases, efficacy in promoting RNase H cleavage of the complementary mRNA, and antisense activity leading to inhibition of biosynthesis of plasminogen activator inhibitor type 1 (PAI-1) within endothelial cells. The dithymidylyl dimethoxytritylphosphonate moiety (T_{PDMT}T) was selected because of its highly lipophilic character. It was earlier shown that oligonucleotide conjugates having lipophilic ligands like cholesterol [5], phospholipids [6], or long-chain alcohols [7], exhibited higher antisense activity. Moreover, the dimethoxytrityl group at the 5′ end of an oligonucleotide increases its anti-HIV activity [8], although a major drawback of this modification is the limited chemical stability, especially at pH < 7.

MATERIALS AND METHODS

Oligonucleotides. Oligodeoxynucleotides: d(GAGGGCTGGAGACATC) (PO-16), its phosphorothioate analogue (PS-16) and d(AGATGTTTGAGCTCT) (PO-Sac), and oligoribonucleotide r(AGAGCUCAAACAUCU) (r-Sac), were synthesized by the solid-phase phosphoramidite method [9] on a DNA synthesizer (ABI 380B). Bis-(diisopropoxyphosphinothioyl) disulfide (S-Tetra) was used for sulphurization [10]. 5′-*O*-Dimethoxytrityl dithymidylyl-3′,5′-dimethoxytritylphosphonate 3′-*O*-[*N,N*-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite] (T_{PDMT}T-PA) was obtained by phosphitylation of 5′-*O*-dimethoxytrityl dithymidylyl-3′,5′-dimethoxytritylphosphonate [11] with *N,N,N′,N′*-tetraisopropyl-*O*-(2-cyanoethyl) phosphoramidite [12]. Solid support with attached 5′-*O*-DMT-dithymidylyl-3′,5′-dimethoxyritylphosphonate moiety (T_{PDMT}T-S) was prepared by attachment of 5′-*O*-dimethoxytrityl-dithymidylyl-3′,5′-dimethoxytritylphosphonate to the long chain alkylamine controlled pore glass (LCA CPG) *via* a succinoyl linker [13]. 5′-T_{PDMT}T-Oligos were obtained using 5′-*O*-DMT-T_{PDMT}T-PA at the last step of oligonucleotide assembly. All syntheses were performed on a DNA synthesizer with a DMT-*off* end procedure. 3′ End T_{PDMT}T-Oligos were synthesized by starting chain assembly on a 5′-*O*-DMT-T_{PDMT}T-S with a DMT-*on* end procedure. After standard cleavage from a solid support (28% NH_4OH, 2 h, room temperature) followed by removal of nucleobase-protecting groups (28% NH_4OH, 12 h, 55°C) each construct was purified by means of RP HPLC (ODS Hypersil, 0.1 M ammonium acetate in a gradient of CH_3CN from 0 to 72%). 3′ End conjugates were finally 5′-*O*-detritylated by a treatment with 80% acetic acid for 2 h at room temperature.

The following conjugates were obtained:

1.	T_{PDMT}T-GAGGGCTGGAGACATC,	Bl-PO-16
2.	GAGGGCTGGAGACATC-T_{PDMT}T,	PO-16-Bl
3.	T_{PDMT}T-$G_SA_SG_SG_SG_SC_ST_SG_SG_S$-$A_SG_SA_SC_SA_ST_SC$,	Bl-PS-16
4.	$G_SA_SG_SG_SG_SC_ST_SG_SG_SA_SG_SA_SC_S$ A_ST_SC-T_{PDMT}T,	PS-16-Bl
5.	T_{PDMT}T-AGATGTTTGAGCTCT,	Bl-PO-Sac
6.	AGATGTTTGAGCTCT-T_{PDMT}T,	PO-Sac-Bl

Stability 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 withdraw leaving packed cells untouched. PO-16 and PO-16-Bl were 5′-*O*-phosphorylated by [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase [14]. Labeled PO-16 or PO-16-Bl (5 μl, 0.3 nmol) were incubated in a mixture of human plasma and phosphate buffered saline (1:1, v/v, 60 μl) for 1, 2, 4, 6 and 8 h. After incubation the digestion mixture was heat-denatured (2 min at 95°C). Each sample was

diluted with water (100 μl) and centrifuged at 10000 × *g* for 15 min. The obtained supernatants were concentrated, redissolved in formamide (10 μl) and subjected to electrophoresis on 20% polyacrylamide gel with 7 M urea in Tris/borate (TBE) buffer. The bands were visualized by autoradiography.

RNase H induced digestion of complementary oligoribonucleotide. The oligoribonucleotide r-Sac complementary to PO-Sac was labeled at 5′ end with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. PO-Sac, PO-Sac-Bl, and Bl-Sac-PO were hybridized to this RNA fragment (35 pmol, molar ratio 1:3) in a total volume of 10 μl (3 min at 90°C and slow cooling to room temperature). The hybridized sample was supplemented with 5 ×RNase H buffer (4 μl: 100 mM Hepes/KOH, pH 8.0, 250 mM KCl, 100 mM $MgCl_2$ and 5 mM dithiotreitol), RNasin (1 μl, 20 U, Amersham), RNase H (1 μl, 1 U, Boehringer) and diluted with water to a total volume of 20 μl. The mixture was incubated at 37°C for 30 min and then ethanol (60 μl) and glycogen (80 μg) were added.

The appearing precipitate was dissolved in the loading buffer (formamide, bromophenol blue, 0.03%, xylene cyanol, 0.03%) and the products of enzymatic digestion were electrophoresed on 20% polyacrylamide denaturing gel (7 M urea) in TBE buffer. The bands were visualized by autoradiography. The unhybridised RNA was used as a control.

PAI-1 inhibition. Cultured endothelial cells [15] were incubated in the presence of antisense oligonucleotides at a 2.5 μM final concentration for 4 and 24 h. Post-culture medium (serum free) was assayed for PAI-1 by a functional test of the ability of PAI-1 to inhibit tissue plasminogen activator (t-PA) which catalyses plasminogen conversion to plasmin. Plasmin activity was measured by spectral analysis of the rate of fibrin digestion [16].

RESULTS AND DISCUSSION

In continuation of earlier studies [11, 17] on the synthesis of dimethoxytritylphosphonate oligonucleotide analogues *via* reaction of intermediary internucleotide *O*-methyl phosphites with dimethoxytrityl chloride efforts were undertaken towards adaptation of this procedure to the requirements of solid phase synthesis to get 5′-*O*-dimethoxytritylphosphono-derivatives of oligonucleotides. Phosphitylation of the 3′-*O*-solid-bound oligonucleotide d(GAGGGCTGGAGACATC) with *O,O*-dimethyl *N,N*-diisopropylphosphoramidite in the presence of *H*-tetrazole, followed by addition of dimethoxytrityl chloride and ammoniolytic deprotection and cleavage from the support did not provide the expected 5′-*O*-dimethoxytritylphosphonylated oligonucleotide. Most probably, prolonged treatment of a preformed 5′-*O*-phosphitylated oligonucleotide with an excess of dimethoxytrityl chloride led to alkylation of exocyclic amino groups of adenine and guanine. That modification of assembled oligonucleotide was deleterious, because depurination during ammoniolytic deprotection had to occur.

Therefore, another mode of introduction of dimethoxytritylphosphonic moiety at 5′ and 3′ ends of presynthesized oligonucleotide has been elaborated. 5′-*O*-Dimethoxytrityldithymidylyl-3′,5′-dimethoxytritylphosphonate was prepared, as the mixture of two diastereomers, as described earlier [11]. This key intermediate was either phosphitylated by means of *N,N,N′,N′*-tetraisopropyl-*O*-2-cyanoethyl phosphorodiamidite to get phosphoroamidite T_{PDMT}T-PA, or it was attached to long-chain alkylamino controlled pore glass to produce the modified support T_{PDMT}T-S (Fig. 1).

Synthesis of T_{PDMT}T-Oligos was performed on a DNA synthesizer using T_{PDMT}T-PA at the

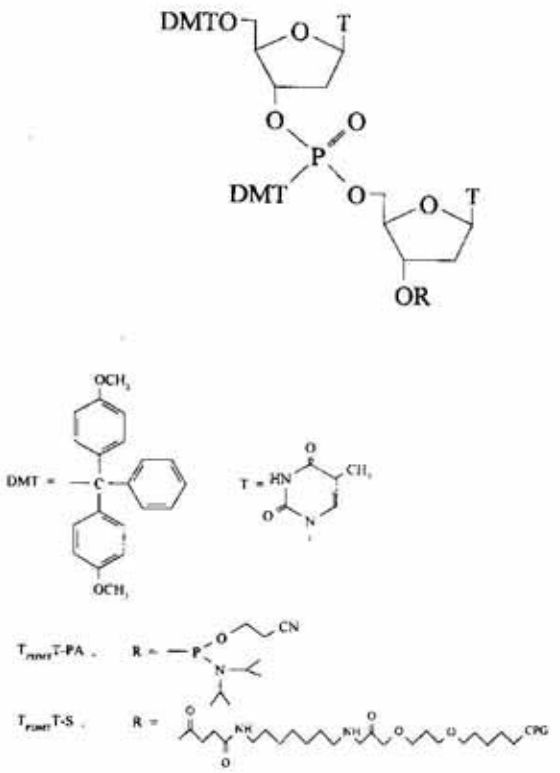

Figure 1. Structures of T_{PDMT}T-PA and T_{PDMT}T-S.

last step of assembly of the oligonucleotide. 3′ End modified conjugates were obtained, if oligonucleotide syntheses were performed with the use of solid T_{PDMT}T-S. Both phosphate and phosphorothioate constructs were prepared in this way. Purity (> 95%) of the synthesized conjugates was confirmed by RP-HPLC and PAGE methods. As expected, retention times of T_{PDMT}T conjugates were longer than those of parent oligonucleotides. During electrophoresis conjugates migrated slower than standards. Unlike 5′-*O*-dimethoxytrityl oligonucleotides [8], phosphono-conjugates having a dimethoxytrityl group linked to the oligonucleotide *via* carbon-phosphorus bond are resistant to prolonged treatment with weak acids.

To assess the effect of T_{PDMT}T ligand on stability of the obtained conjugates, PO-16-Bl was 5′-labelled with ^{32}P and then exposed to 50% human plasma for different periods of time. In this experiment unmodified PO-16 was used as a reference. Progress of enzymatic degradation of PO-16-Bl and PO-16 was analyzed by gel electrophoresis. It was found that within the unmodified PO-16 a phosphodiester bond is hydrolysed stepwise from the 3′ end, leading to formation of shorter oligonucleotides. After 4 h of incubation no intact PO-16 was left. Under the same conditions PO-16-Bl was completely stable even after 8 h.

The antisense effect of PO- and PS-Oligos is attributed mainly to their induction of ribonuclease H cleavage of that portion of mRNA which is involved in hybridization with an oligonucleotide [18]. Thus, changes in efficiency of this cleavage may alter the biological activity of analyzed Oligos. The influence of T_{PDMT}T ligand on the cleavage of complementary oligoribonucleotide (r-Sac) by RNase H was analyzed using both Bl-PO-Sac and PO-Sac-Bl with a standard PO-Sac as a reference. The obtained results proved that T_{PDMT}T ligand located either on 3′ or 5′ end of oligonucleotide does not distrub hybridization with a complementary oligoribonucleotide, and such a duplex is recognized by RNase H.

In our earlier studies [19] it was found that phosphorothioate oligonucleotide d(GAGGGC-TGGAGACATC) (PS-16), complementary to the human PAI-1 mRNA coding a part of its signal peptide, inhibits effectively PAI-1 release in cultured endothelial cells. The antisense activity of conjugates PO-16-Bl, Bl-PO-16, PS-16–Bl, and Bl-PS-16 has been studied under the same experimental conditions (Fig. 2).

It was found that inhibition of PAI-1 release by PO-16-Bl and Bl-PO-16 constructs, measured after 4 and 24 h of incubation, was higher than that of parent PO-16. However, inhibition of biosynthesis of PAI-1 by PS-16-Bl and Bl-PS-16 was slightly lower as compared with that caused by PS-16. There was also no major difference in PAI-1 release inhibition between conjugates with T_{PDMT}T ligand attached to the 3′ or those with attached ligand at 5′ end of the oligonucleotide. High antisense activity of PO-16-Bl and Bl-PO-16 is also in line with the results of kinetics of digestion of RNA/PO-Sac heteroduplexes by RNase H. The most probable reason of stronger inhi-

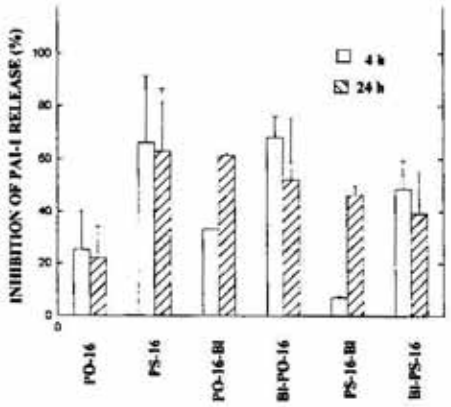

Figure 2. Inhibition of PAI-1 release from endothelial cells incubated for 4 and 24 h with antisense oligonucleotides at 2.5 μM concentration.

bition of PAI-1 biosynthesis by PO-16-Bl and Bl-PO-16, as compared to parent PO-16, is their observed resistance to degradation by nucleases, and/or better uptake by endothelial cells. The results on antisense activity, nuclease stability, and promotion of RNase H cleavage, obtained for conjugates of $T_{PDMT}T$ with oligonucleotides, are in good agreement with the results obtained for oligonucleotide conjugates possessing other types of lipophilic ligands, e.g. cholesteryl, bornyl, menthyl, and heptadecanyl (unpublished results).

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