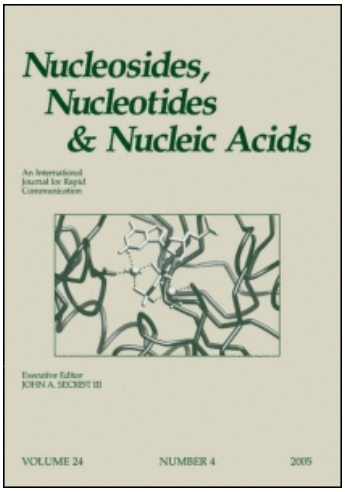

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Prooligonucleotides Exhibit Less Serum-Protein Binding Than Phosphodiester and Phosphorothioate Oligonucleotides

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PROOLIGONUCLEOTIDES EXHIBIT LESS SERUM-PROTEIN BINDING THAN PHOSPHODIESTER AND PHOSPHOROTHIOATE OLIGONUCLEOTIDES

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ABSTRACT: The protein-binding properties of dodecathymidine derivatives (prooligos) bearing either methyl- or *tert*-butyl-S-acylthioethyl (Me- or tBuSATE) protecting groups were evaluated. The dissociation constants (Kd) were estimated for complexes of prooligos with serum blood proteins and lactoferrin using prooligos to compete the binding of the radiolabeled, alkylating probe oligonucleotide $ClRp(T)_{12}$ with the proteins. tBuSATE and MeSATE prooligos have decreased affinity of binding with serum proteins and lactoferrin compared with their parent oligos. These data suggest that prooligos should cause less side effects which combined with their stability to nucleases and enhanced permeability into cells make them potentially useful for design of therapeutics.

INTRODUCTION

Antisense oligodeoxynucleotides have been shown to be very potent inhibitors of gene expression in cell culture and *in vivo* (1,2). The primary interest for synthetically modifying oligonucleotides arose from the instability of phosphodiester oligonucleotides in a biological environment, due to enzymatic digestion by nucleases. In later years, antisense

effects of modified oligonucleotides were demonstrated, and many of oligonucleotide derivatives were shown to be able to inhibit cellular genetic expression by a sequence-specific mechanism (for a review see (2,3)). Furthermore phosphorothioate oligonucleotides, which have now entered clinical trials (4), possess non-sequence-specific effects due to binding with proteins. Oligonucleotides were shown to bind with serum albumin, IgG, IgM both in vitro and in vivo (5,6) form complexes with barrier fluid proteins lactoferrin, lysozyme and sIgA (7) and interact with membrane Ig and Ig-like receptors (8). Binding of oligonucleotides with proteins of biological importance can affect distribution and metabolism of oligonucleotides administered *in vivo* and provoke side effects of oligonucleotides (9).

The prooligonucleotide approach has been developed recently to improve the bioavailability of antisense oligonucleotides (10). This approach consists of a temporary protection of the charged backbones with enzymolabile protecting groups to obtain more lipophilic oligonucleotides which could reverse back once inside the cell to the parent oligo. These prooligonucleotides (prooligos) seem to be rather suitable for therapeutics because of their improved nuclease resistance and enhanced cellular uptake (11). To forecast possible side effects of prooligos the estimation of their binding with biologically active proteins is becoming necessary. We estimated here the dissociation constants of prooligos binding with serum blood proteins and lactoferrin, the main protein of tear fluid. We have shown that prooligos bind with the proteins under investigation with lower affinity compared with their parent oligos.

METHODS

PO and PS oligonucleotides. - All oligodeoxynucleotide analogs (prooligos Fig. 1) were synthesized as previously described (10). Solutions of prooligos in N,N'-Dimethylacetamide (DMAA) were used as stock solutions for further investigations.

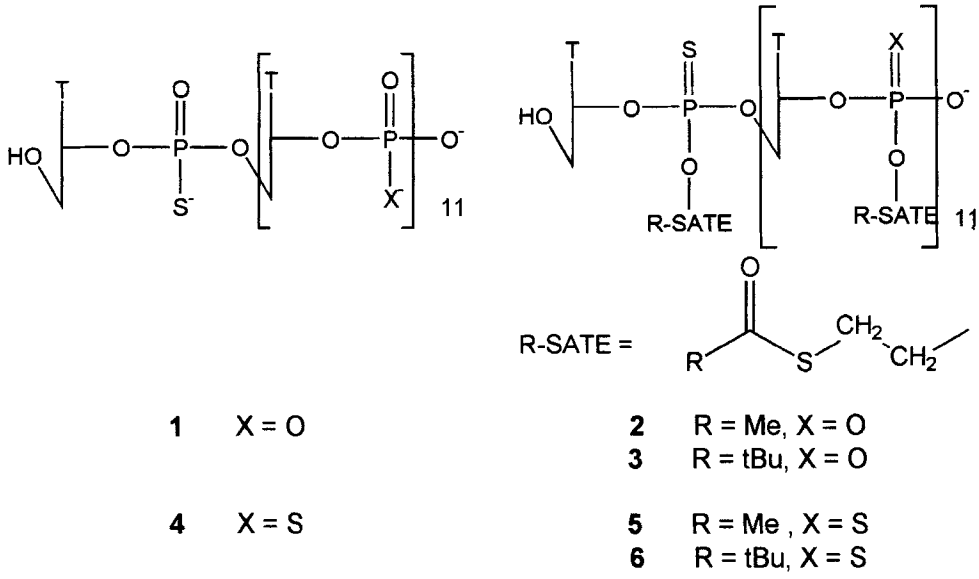
Alkylating, radioactive oligonucleotide. - Oligonucleotide $p(T)_{12}$ was synthesized in a synthesizer ASM-102U (BioSet) by the phosphoramidite method. The 5' end was labeled with [^{32}P] using T4 polynucleotide kinase. Oligonucleotide $p(T)_{12}$ cetrimonium salt was 5'-labeled with an alkylating group 4-[(N-2-chloroethyl-N-methyl)amino]benzylamine in a non aqueous solution (12), thus producing $ClRp(T)_{12}$. The yield of alkylating derivatives by electrophoretic analysis of the reactive mixture was no less than 90% with specific radioactivity up to 50 Ci/mmole.

Modification of serum proteins with ClRp(T)$_{12}$. Kd of oligonucleotide-protein complexes were estimated from the concentration dependence of the modification of purified proteins by [^{32}P]ClRp(T)$_{12}$. Immunoglobulins M and G of mice (BioSAN, Novosibirsk), bovine serum albumin (Sigma) and human lactoferrin (Sigma) (1μM) were incubated in buffered physiological solution (0,14 M NaCl, 0,01 M NaH_2PO_4, pH 7,5), containing 1% DMA, with ^{32}P labeled ClRp(T)$_{12}$ in concentrations ranging from 0.02 to 10 μM. In competition experiments putative competitors (prooligos) of the binding of the reactive ClRp(T)$_{12}$ to proteins were added as solutions in DMAA (1% of the volume of reaction mixture) to give the final concentration as indicated in "Results". After incubation at 37° for 45 min, the equal volume of a buffer containing 20% glycerol, 2% β-mercaptoethanol, 2% SDS and 0,0001% bromophenol blue was added to the reaction and SDS-PAGE (10-20% acrylamide) was performed. The gels were dried and allowed to expose X-ray film for 24 hours. The film was developed and band intensities were quantified by excising the gel band and counting in a β-counter.

Competition experiments: Purified serum proteins were incubated at 37° for 45 min with ^{32}P labeled ClRp(T)$_{12}$. Unlabeled prooligonucleotide derivatives were included as competitors, the reactions were subjected to SDS-PAGE and the products visualized by autoradiography. Bands, which corresponded to protein-oligonucleotide complex, were excised and the radioactivity in each band was determined. Data obtained were used for competition constants (Kc) calculation. The average from three independent experiments ± SD is given.

RESULTS AND DISCUSSION.

We have probed the interaction of oligonucleotide p(T)$_{12}$ and of the 4 prooligo analogues (Fig. 1) with lactoferrin and serum IgG, IgM and albumin. Initially, the dissociation constants (Kd) of binding of p(T)$_{12}$ oligonucleotide to investigated proteins were determined. 5'-^{32}P-labeled phosphodiester (PO) oligodeoxynucleotide that have been covalently coupled at the 5'-end to an alkylating reagent, capable of alkylating DNA and proteins (13) was used for estimation of Kd of oligonucleotide-protein complexes in affinity modification experiments. The purified protein was incubated with alkylating



1 X = O

2 R = Me, X = O
3 R = tBu, X = O

4 X = S

5 R = Me , X = S
6 R = tBu, X = S

FIGURE 1 : Structure of oligonucleotides **1** to **6**.

radiolabeled oligonucleotide derivative $CIRp(T)_{12}$, the reaction products were subjected to SDS-PAAG electrophoresis and exposed to X-ray film. The band seen on the film corresponded to the product of chemical modification of the protein by the radiolabeled oligonucleotide, the band intensities were quantified by excising the gel band and counting in a β-counter.

We examined the concentration dependence of the modification of purified IgM, IgG, albumin and lactoferrin by ^{32}P labeled $CIRp(T)_{12}$. The concentrations of the probe oligonucleotide were plotted as a function of gel band intensities (in cpm). The data for every protein-oligonucleotide binding fitted a single site binding model and dissociation constants could be determined from the double-reciprocal plot.

The data obtained indicate that oligonucleotide $CIRp(T)_{12}$ has higher affinity to IgM and IgG than to albumin. The Kd for protein - oligonucleotide binding were estimated as 5 μM for IgM, 7 μM for IgG and 25 μM for albumin, which are similar with our data obtained earlier for the immunoglobulins and albumin binding with $CIRp(T)_{16}$ (5). It should be noted, that the Kd of $CIRp(T)_{12}$ binding with lactoferrin was estimated as 1 μM,

which is one order of magnitude lower than that for IgM, indicating that the PO thymidine homooligomer binds to lactoferrin with greater affinity than to other proteins under investigation.

In order to evaluate the dissociation constants of the proteins binding with the prooligos, we used them to compete the binding of the radiolabeled, alkylating probe oligonucleotide $ClRp(T)_{12}$ with the serum proteins and lactoferrin. We analyzed the modification of proteins with saturating amounts of $ClRp(T)_{12}$ as described above using unlabeled oligonucleotide analogs as competitors of $ClRp(T)_{12}$ binding to proteins. The values of Kc may be calculated from Equation 1 (14), which permits calculation of Kc for any competitor of $ClRp(T)_{12}$ interaction with the protein, if the value of Kd for the latter is known.

$$Kc/Kd = [C_c]/[\ ClRp(T)_{12}][1/(R_o/R_c) - 1] \qquad (Eq.1)$$

C_c represents concentration of competitor; R_o and R_c are the intensities of the gel bands (in cpm), in the absence and presence of competitor, respectively.

To ascertain the accuracy of Kc determination obtained with a single concentration of competitor according to Equation 1, Kc for every oligonucleotide derivative was estimated in three independent experiments with different concentrations of competitors. The concentrations of the "all PS" competitors used in experiments were 3, 10 and 30 μM, while oligonucleotide analogs with the single 5'-PS internucleoside bond were added to the reaction in 5, 20 and 100 μM concentrations. The data obtained are listed in the Table1.

As is seen from the Table, there is a strong decrease in the value of Kc of the "all PS" prooligos binding with proteins compared with their congeners bearing the single 5'-PS internucleoside bond (compared in pairs: 1 with 4, 2 with 5, 3 with 6). These data imply that prooligos with all phosphorothioate backbone bind with the investigated proteins with greater affinity than their congeners with the single 5' -PS bond. The data obtained correlate with previous observations, which demonstrated that phosphorothioate oligonucleotides form more durable complexes with proteins than phosphodiester oligonucleotides (9).

tBuSATE and MeSATE modifications of the charged backbones change oligonucleotide-protein interaction dramatically, but somewhat differently for the

Table 1: Competition constants of prooligonucleotide binding with proteins.

proteins	IgM		IgG	
Competitor oligo	K_c (μM)	K_cn/K_c1	K_c (μM)	K_cn/K_c1
1	17 ± 3.8	1.0	35 ± 7.7	1.0
2	21 ± 3.9	1.2	74 ± 10.3	2.1
3	72 ± 8.4	4.2	148 ± 28	4.2
Competitor oligo	K_c (μM)	K_cn/K_c1	K_c (μM)	K_cn/K_c1
4	3 ± 0.6	1.0	10 ± 1.9	1.0
5	7 ± 2.1	2.3	31 ± 6.8	3.1
6	25 ±4.5	8.3	63 ± 12	6.3

proteins	BSA		Lactoferin	
Competitor oligo	K_c (μM)	K_cn/K_c1	K_c (μM)	K_cn/K_c1
1	110 ± 20	1.0	1 ± 0.7	1.0
2	27 ± 5.1	0.2	69 ± 8.4	53.1
3	90 ± 17	0.8	4 ± 1.7	3.1
Competitor oligo	K_c (μM)	K_cn/K_c1	K_c (μM)	K_cn/K_c1
4	17 ± 3.5	1.0	1 ± 0.7	1.0
5	22 ± 6	1.3	5 ± 3.6	5.0
6	35 ±5.1	2.1	1.7 ± 0.9	1.7

investigated proteins. Both protecting groups lead to decrease of the prooligos competition in the alkylating reaction probably because of their size, which is increased significantly and could prevent the prooligonucleotide-protein complex formation, especially for tBuSATE modified oligonucleotides.

Different polyanions, as heparin and dextran sulfate were shown earlier to be effective competitors of the IgM and IgG binding with the alkylating derivative of thymidine homooligomer $ClRp(T)_{16}$, implying that oligonucleotides bind to some polyanionic binding site of immunoglobulins (5). tBuSATE and MeSATE carrying prooligos are characterized by more lipophilic properties compared with the native oligonucleotides, which could interfere with their binding with polyanionic site of immunoglobulins.

On the contrary, the Kc of MeSATE-bearing prooligos binding with serum albumin was found to be comparable (Kc5/Kc4 = 1,3) and even lower than the Kc of their congeners (Kc2/Kc1 = 0,2), reflecting that the lipophilic modification of the phosphate backbone could improve the oligonucleotide-albumin binding. To note, albumin is known to interact specifically with lipophilic substances and is a major carrier protein for fatty acids in blood, possessing 4 sites of specific binding for them (15). Nevertheless, *tert*-butyl SATE modified thiono $p(T)_{12}$ binding with albumin was characterized by lower affinity (Kc6/Kc4 = 2,1), implying the large size of protecting group to prevent the interaction.

It becomes popular now to use the undamaged routes for the oligonucleotide delivery *in vivo*, which makes the investigation of oligonucleotide-protein binding in barrier fluids rather actual. It has been shown recently, that lactoferrin is one of the main oligonucleotide-binding proteins of saliva and tear fluid (7). Lactoferrin, an iron-containing protein, is known to bind heparin, dextran sulfate and DNA (16). Our data indicate, that the affinity of prooligos bearing MeSATE protecting groups binding with lactoferrin is lower compared with their congeners, the same as for MeSATE prooligonucleotide binding with immunoglobulins. However, on the contrary with immunoglobulins, tBuSATE prooligos were shown to have higher affinity of binding with lactoferrin compared with MeSATE prooligos, implying that the bulk of *tert*-butyl protecting group don't prevent prooligonucleotide-lactoferrin interaction. These data demonstrate that different proteins differ from each other in delicate organization of oligonucleotide-binding sites.

During the last few years the progress occurred in therapeutic antisense oligonucleotides development. Nevertheless, *in vivo* studies showed some side effects of oligonucleotides, *occurring from* oligonucleotide interactions with cellular and tissue proteins. Decreased affinity of tBuSATE and MeSATE prooligos binding with serum proteins and lactoferrin compared with their parent propose them to have no undesirable side effects. These properties together with their stability to nucleases of biological fluids (10), increased cellular uptake and low toxicity (11) make them useful for therapeutics. Based on these observations, we anticipate that other differences between phosphodiester, phosphorothioate and prooligos will eventually be observed. Determination of biological meaningful of prooligonucleotides will be aided by studies on the pharmacology, pharmacocinetics, and in vivo stability and interactions of these oligo classes

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