Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2103–2108

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

EFFECTS OF PHOSPHOROTHIOATE OLIGODEOXYRIBONUCLEOTIDE AND OLIGORIBONUCLEOTIDES ON HUMAN COMPLEMENT AND COAGULATION

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Received 9 March 1998; accepted 30 June 1998

Abstract: We have synthesized and studied the effects of phosphorothioate (PS) oligodeoxyribonucleotide (DNA) and oligoribonucleotides (RNA, 2'-O-methyl-RNA and 2'-5'-RNA) on complement activation and prolongation of activated partial thromboplastin time (aPTT) *in vitro*. These results suggest that a PS-DNA prolongs aPTT, and inhibits complement lysis more than do the PS-RNA analogs.

Progress in chemical synthesis of nuclease-resistant oligonucleotides and their pharmacological properties have permitted antisense oligonucleotides to advance to human clinical trials.[1-3] Oligodeoxyribonucleotide (DNA) phosphorothioates (PS) are currently at various stages of human clinical trials for viral diseases and cancers.[4-6] PS-DNA is the choice of chemical modification for many preclinical and clinical studies because they are stable against nucleases, efficiently taken up by cells, and elicit RNase H activity upon binding to an mRNA target.[7]

PS-DNAs have been shown to induce prolongation of aPTT (activated partial thromboplastin time) and activation of complement both *in vivo*[8,9] and *in vitro*[9-12] studies. It has been shown that compounds containing sulfur, such as beta-mercaptoethanol, pencillamine, and N-acetylcysteine, also activate complement in a dose-dependent manner.[13] Studies showed that the effects of synthetic oligonucleotides on human complement and coagulation depend on several factors, such as oligonucleotide length and the nature of the chemical modification in the backbone.[11,14] These effects can be neutralized by polycations such as protamine,[11] suggesting that polyanionic nature of the PS-backbone of oligonucleotides is responsible for these effects.

PS-oligoribonucleotides (RNA) have been synthesized[15,16] and studied for their nuclease stability and anti-HIV activity.[15,17] Studies with second-generation antisense oligonucleotides containing two or more chemical modifications (mixed backbone oligonucleotides, MBOs) showed more desirable pharmacokinetic properties and reduced side effects in preclinical studies compared with the first generation PS-DNAs,[7,14,18] suggesting that the modifications that are introduced in MBOs, other than PS-DNAs, have less of an effect on hemolytic complement activation and prolongation of aPTT. However, there are no reports on the direct comparison of effects of PS-DNA and PS-RNA on hemolytic complement and prolongation of aPTT *in vitro*. In this paper we examined the effects of PS-RNA on coagulation and complement activation, and we have also studied the plasma protein binding characteristics of PS-DNA and PS-RNA.

We used a 25-base-long sequence that is complementary to the initiation codon region of the HIV-1 gag mRNA, the pharmacokinetic and safety profiles of which have been studied extensively.[4,19] In addition to normal (3'-5') PS-RNA, we have also studied PS-2'-O-methyl-RNA and 2'-5'-linked RNA (Fig. 1).

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00368-0

The chemical structures of PS-oligonucleotides used in the study are shown in Figure 1. Oligonucleotides were synthesized, purified, and characterized as described earlier.[14,18] The integrity of the PS-internucleotide linkages of oligonucleotides was confirmed by ^{31}P NMR spectroscopy. All oligonucleotides showed the presence of a signal at 56 ppm (95-99% of the signal at 0 ppm). Thermal melting stability of duplexes of oligonucleotides with a complementary RNA strand was measured[20] and the data are presented in Table 1.

1 5'-d(CTC TCG CAC CCA TCT CTC TCC TTC T)-3'
2 5'-CUC UCG CAC CCA UCU CUC UCC UUC U-3'
3 5'-**CUC UCG CAC CCA UCU CUC UCC UUC U**-3'
4 5'-CUC UCG CAC CCA UCU CUC UCC UUC U-2'

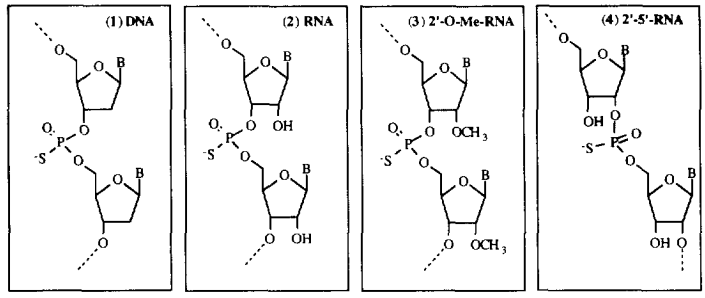

Figure 1. Sequences and chemical modifications used in the study. Sequences **1**: phosphorothioate oligodeoxyribonucleotide; **2**: phosphorothioate oligoribonucleotide; **3**: phosphorothioate oligo (2'-O-methyl) ribonucleotide (bold); and **4**: phosphorothioate oligo (2'-5') ribonucleotide.

Effect of DNA vs RNA Phosphorothioates

PS-oligonucleotides **1-4** were tested for their relative activity in coagulation assays using normal human donor citrated plasma, as described earlier.[11] Figure 2 shows percent prolongation of aPTT as a function of the concentration of each oligonucleotide. Oligonucleotides **1**, **3**, and **4** showed dose-dependent prolongation of aPTT. Both PS-2'-O-methyl-RNA (**3**) and 2'-5'-RNA (**4**) showed a lower ability to prolong aPTT in clotting assays than did PS-DNA **1** (Fig. 2). The estimated concentrations of oligonucleotides **3** and **4** required to prolong aPTT by 50% were about two fold higher than for the PS-DNA **1** (Table 1). The unmodified PS-RNA **2**

Table 1. Effects of PS-DNA and PS-RNA on activated partial thromboplastin clotting time (aPTT) and hemolytic complement[a]

Oligonucleotide	Tm with RNA, °C	aPTT 50% prolongation conc., μM	Complement lysis 50% inhibitory conc., μM
DNA (**1**)	63.5	5.8	23.2
RNA (**2**)	74.6	>30.8	>123.0
2'-O-Me-RNA (**3**)	79.8	11.3	>118.0
2'-5'-RNA (**4**)	55.6	12.1	>123.0

[a]: Values vary from donor to donor, and reagents hence the values given for **1** are slightly different than the earlier reports.[11,14,18].

produced no measurable effect on aPTT at the highest concentration tested. These results suggest that the PS-RNA analogs have reduced effects on prolongation of aPTT compared to PS-DNA **1**.

In a similar experiment, in which PT (prothrombin clotting time) was measured, oligonucleotides **2-4** had an insignificant effect, while PS-DNA **1** had a marginal effect (data not shown). The estimated concentration of PS-DNA **1** required to prolong PT by 50% is 5-6 times higher than that required to prolong aPTT by 50%. These results suggest that the intrinsic pathway (aPTT) is more sensitive to inhibition by PS-oligonucleotides than the extrinsic pathway of the coagulation cascade.[9-11]

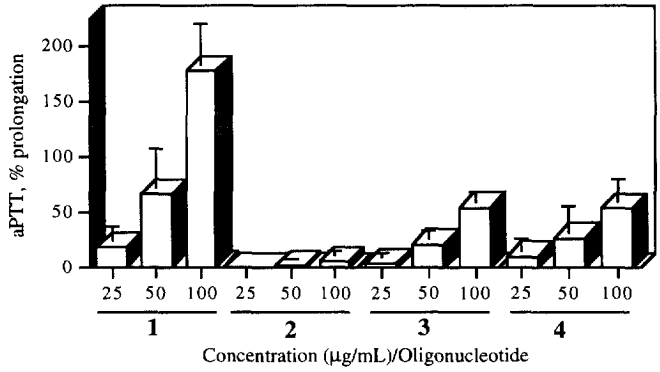

Figure 2. Effect of PS-oligonucleotides (**1-4**) on partial thromboplastin clotting time (aPTT).

The four oligonucleotides (**1-4**) were also examined for inhibition of complement-mediated lysis.[10,11] The results are presented in Figure 3. Pretreatment of normal human serum *in vitro* with PS-DNA **1** produced a reduction in hemolytic complement activity in a dose-dependent manner (Fig. 3). PS-DNAs activate serum complement and consume the complement components necessary for subsequent expression of serum hemolytic activity.[11,12] The present data also confirm the inhibitory effects of PS-DNA (**1**) on complement lysis. The results with the three PS-RNA analogs (**2-4**) suggest that they have minimal inhibitory effects on complement lysis under the same experimental conditions. The estimated 50% inhibitory concentrations for the four oligonucleotides (**1-4**) are shown in Table 1.

Phosphodiester RNA is highly susceptible to ubiquitous RNase and undergoes rapid degradation. The PS modification is more stable than the phosphodiester backbone.[15,16] We examined whether the PS-RNA remained intact for the duration of the experiment in human serum. Aliquots of plasma- and serum-treated samples used in these assays were examined by gel electrophoresis. The PS-RNAs were stable under these conditions for the duration of the experiment (data not shown). This result confirms that the little or no effects observed in clotting and complement assays were not due to digestion of PS-RNAs.

Plasma Protein Binding of Oligonucleotides

In order to examine if these effects are related to non-specific binding of PS-oligonucleotides to proteins, we studied the interaction of PS-oligonucleotides with some of the plasma proteins. A small amount of ^{32}P 5'-

end-labeled oligonucleotide (except 2'-5'-linked oligonucleotide) was incubated with increasing concentrations of human plasma proteins, HSA (human serum albumin), γ-globulins, and fibrinogen. Analysis of oligonucleotide-protein complexes on native polyacrylamide gels reveals that the PS-DNA (**1**) binds to all three proteins studied. Figure 4 shows binding of oligonucleotides **1-3** to HSA. PS-DNA **1** showed higher binding to all the three proteins studied than the PS-RNA analogs. The autoradiogram shown in Figure 4 suggests that PS-2'-O-methyl-RNA (**3**) has a lower binding than PS-DNA, and PS-RNA (**2**) shows no binding to HSA. The two PS-RNA analogs tested did not show measurable binding to γ-globulins or fibrinogen (data not shown). Qualitative examination of the gels suggest that the binding affinity of the PS-DNA (**1**) is in the order fibrinogen > γ-globulins > albumin. These results suggest that the observed effects in complement and coagulation assays could be related to binding of PS-DNA to one (at least fibrinogen) or more of the complement and coagulation factors. We were not able to study PS-2'-5'-linked RNA for protein binding as it is not a good substrate for T4 kinase.

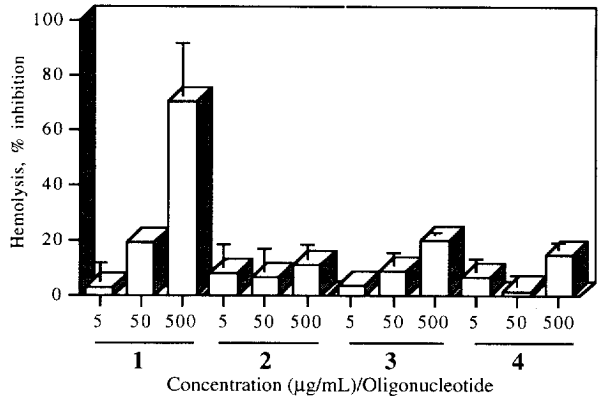

Figure 3. Effect of PS-oligonucleotides (**1-4**) on hemolytic complement inhibition.

Genomic DNA and phosphodiester oligonucleotides show little or no effects on aPTT or complement activation.[11] Certain phosphodiester oligonucleotides containing G-rich sequences that form secondary structures are known to specifically interact with thrombin and inhibit coagulation.[21] A phosphodiester oligodeoxyribonucleotide containing G-rich sequences with a limited number of phosphorothioate linkages on either end showed prolongation of aPTT as a result of secondary structure formation.[22] In contrast, PS-DNAs show effects on coagulation and complement activation *in vitro*[9-12] and *in vivo*[8,9] in a sequence-independent but length-dependent manner. Recently a PS oligonucleotide containing 2'-methoxyethoxyribonucleotide and 2'-deoxyribonucleotide segments has been reported to show similar prolongation of aPTT as that of a PS-DNA of the same sequence and length in monkeys,[23] suggesting that prolongation of aPTT depends on the nature of the substitution at 2'-position and internucleotide linkage.

The effects of PS-DNAs on coagulation and complement could be related to their enhanced binding affinity to proteins compared with the phosphodiester backbone analogs. The formation of a complex between nucleic

acids and proteins involves charge, hydrogen bonding, stacking and hydrophobic interactions. In the case of PS-DNAs, charge interaction could be the primary force leading to complex formation with proteins, while subsequent stacking interactions between the heterocyclic nucleobases and aromatic amino acid side chains further stabilize the complex. PS-RNAs also possess a backbone charge similar to that of a DNA. But the PS-RNAs show lower or no binding to proteins (at least to the plasma proteins examined in this study), and lower or no effects on coagulation and complement activation, indicating that the PS-backbone alone is not responsible for clotting prolongation and complement activation.

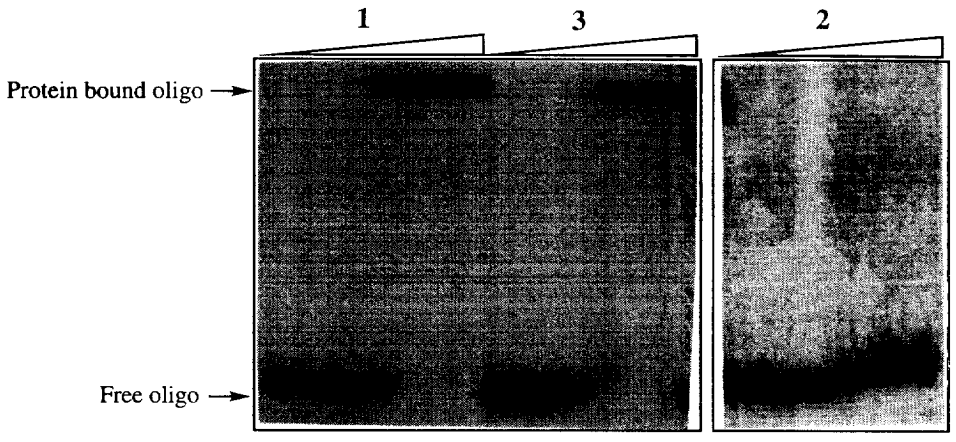

Figure 4. An autoradiogram showing binding of PS oligonucleotides **1-3** to HSA at different concentrations of HSA. The concentration of HSA (from left to right) is 0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0 mg in a final volume of 20 μL and the concentration of oligonucleotide is about 1 nM.

It appears that in addition to the charge on phosphorothioate backbone, the differences in the repeat (charge) distance of phosphates (intraphosphate distance) in RNA and DNA chains play a role in eliciting effects on clotting and complement. Single-stranded sequences (without self complementarity) do not form helical structures. The stacking interactions between the adjacent bases in single-stranded oligo-, poly- and mononucleotides could, however, lead to the formation of self-ordered structures in solution.[23] The RNA and DNA nucleotides adopt different conformations, and in general, RNA adopts a 3'-endo conformation and DNA adopts a 2'-endo conformation in aqueous solutions. As a result of different conformations RNA (~5.9 Å) and DNA (~7.0 Å) chains will have different intraphosphate distances.[23] A negative charge on the PS-backbone with the appropriate intraphosphate (repeat charge) distance is probably necessary for binding to proteins and to elicit effects on coagulation, complement, etc. We hope that the ongoing modeling and pharmacokinetic studies will lead to a better understanding of the different biological effects of DNA and RNA phosphorothioates.

In conclusion, the present study suggests that a PS-backbone associated with deoxysugar moieties affects coagulation and complement activation. PS-RNA, 2'-OMe-RNA, or 2'-5'-RNA oligonucleotides have lower or negligible effects on coagulation and complement activation than does PS-DNA. The incorporation of two modifications into antisense oligonucleotides (MBOs) could lower side effects (coagulation prolongation and

complement activation; RNA analogs of the kind described here) and retain antisense efficacy (RNase H activity properties; dexyribonucleotide segments).[14,18] Several of such second generation antisense oligonucleotides are in preclinical and clinical trials currently. In addition, it would be interesting to understand the role of PS-backbone stereochemistry (Rp *vs* Sp)[25] on coagulation, complement and plasma protein binding. Such studies with stereoregular PS oligonucleotides are currently in progress in our laboratory.

Acknowledgments

We would like to thank Ms. Adrienne Manning (Hybridon) and Ms. Connie Jenkins (University of Alabama at Birmingham) for excellent technical assistance.

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