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APTAMERS CONTAINING THYMIDINE 3'-O-PHOSPHORODITHIOATES: SYNTHESIS AND BINDING TO NUCLEAR FACTOR-κB

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Abstract: Aptamers targeting NF-κB containing thymidine 3'-O-phosphorodithioates in selected positions of an oligonucleotide duplex were synthesized. Binding affinities to NF-κB varied with the number and positions of the dithioate backbone substitutions. One of the aptamers showed specific binding to a single NF-κB dimer in cell culture extracts.

The NF-κB/Rel transcription factor family mediates inducible transcriptional activity of HIV-1 as well as a multitude of immune and acute phase response genes in a variety of cell types.[1-4] Several family members have been identified based on sequence, and structural and functional homology.[3] Members of this protein family can be divided into two groups: one group consists of p50 and p52 homodimers, a second group includes p65(Rel-A), c-Rel and v-Rel, among others. Homo- and heterodimers containing at least one member from this group are strong transcriptional activators. Recently, X-ray crystallography disclosed the structure of NF-κB bound to DNA, in which the patterns of specific subunit sidechain interactions with the DNA backbone and base pairs have begun to be elucidated.[5-9]

Modulation of NF-κB mediated inflammation can in principle be achieved by selective binding of DNA aptamers to either homodimers or heterodimers. Unmodified oligonucleotides (ODNs), however, are susceptible to nuclease digestion and are not stable for intravenous or oral administration. Oligonucleotide phosphoromonothioate analogues ([S]-ODNs) are nuclease resistant, but the diasteromeric phosphoromonothioate mixtures (due to the new chiral phosphorus center) have variable biochemical, biophysical and biological properties. While stereocontrolled synthesis of P-chiral [S]-ODNs[10-12] represents one possible solution to this problem, another lies in the synthesis of modifications which are achiral at phosphorus. Several groups of researchers have synthesized oligonucleotide phosphorodithioate analogues ([S_2]-ODNs) that have been shown to be relatively nuclease resistant and effective as antisense agents.[13-23] In addition it has been generally observed that the increased thioation of the phosphoryl oxygens of ODNs often leads to their enhanced binding to numerous proteins. Thus, single-stranded [S_2]-ODNs are 36–600 times more effective in inhibiting HIV reverse transcriptase than normal antisense ODN or the [S]-ODN.[23] ODNs possessing high monothio- or dithiophosphate backbone substitutions appear to be "stickier" towards proteins than normal

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phosphodiesters, often attributed to "non-specific interactions". One needs, therefore, to optimize the total number of dithioated phosphates in order to minimize non-specific protein binding while enhancing specific binding to the protein of interest.

Since the initial reports on the solution, and solid-phase synthesis of [S_2]-ODNs,[13–16] there have been numerous synthetic efforts directed toward the development of this chemistry.[13–24] Perhaps the most currently satisfactory method is solid-phase synthesis based on nucleoside phosphorothioamidite chemistry.[13–18,20] Efforts have been mainly focused on investigation of various active nucleoside phosphorothioamidite building blocks and base deblocking conditions in order to lower the levels of the undesired side products of [S]-ODNs .[20,24–25]

In this communication we describe our results on the solid-phase synthesis of aptamers containing thymidine 3'-O-phosphorodithioates free of phosphoromonothioates, and their binding to NF-κB. The oligonucleotide sequence 5'-CCAG GAGA TTCC AC-3', (1, Scheme 1), was chosen from the wild-type CK-1 duplex sequence comprising three tandem repeats of a 14mer NF-κB consensus like sequence 5'-CCAG GAGA TTCC AC CCAG GAGA TTCC AC CCAG GAGA TTCCAC-3' identified by Sharma et al.[26] to be an efficient aptamer binding to NF-κB. The thymidine 3'-O-phosphodiesters of 1 and its complementary sequence of 5'-GTGG AATC TCCTGG-3' were replaced with thymidine 3'-O-phosphorodithioates in two or four positions. Binding to NF-κB was examined.

The nucleoside phosphorothioamidite approach was used to synthesize the [S_2]-ODNs. Thus, thymidine S-(β-thiobenzoylethyl)pyrrolidinophosphorothioamidite was prepared in ca. 80% yield.[20] The purity (ca. 80%) was assayed via ^{31}P NMR (δ^{31}P 161.1, 164.7 CD_2Cl_2). In initial experiments, oligonucleotide 2 containing two thymidine 3'-O-phosphorodithioate linkages was first prepared on a Gene Assembler Plus (Pharmacia) (1.3 μmole). A modification of the normal coupling cycle for the phosphorothioamidite DMT yielded a coupling efficiency of ca. 98-99%. Sulfurization was carried out with 3H-1,2-benzodithiole-3-one, 1,1-dioxide (Beaucage Reagent), and the normal phosphoramidite DMT-efficiency was ca. 99%. The crude oligonucleoside phosphorodithioate (DMT on) was cleaved from the support and deblocked by treatment with 28–30% aqueous ammonia (ca. 1.5 mL) in a tightly stoppered vial at 55 °C for 16 h. After removal of the support, the ammonia solution was concentrated and subjected to ^{31}P NMR analysis, which showed the correct ratio of phosphorodithioate linkages (δ^{31}P ca. 113 ppm) to phosphate linkages (δ^{31}P ca. 0 ppm). However, ^{31}P NMR analysis of this oligonucleotide also showed some small amounts of nucleoside phosphoromonothioates (δ^{31}P ca. 58 ppm) as a previously noted contaminate.[19–20] The 5'-O-DMT-oligonucleotide 2 was purified by reverse-phase HPLC (Hamilton PRP-1 column), desired fractions were collected and evaporated. Detritylation was accomplished with 75% acetic acid for 15 min at 0 °C. After three diethyl ether extractions, the solution was neutralized with aqueous ammonia, followed by lyophilization. ^{31}P NMR showed increased amounts of phosphoromonothioates, suggesting that the deprotection step leads to some desulfurization. However, if the final 5'-O-DMT protecting group was removed on the synthesizer while still on the solid support,

desulfurization during detritylation should not be increased. The crude oligonucleotides were dissolved in ca. 1.0 mL of water, and purified by FPLC ion exchange (Pharmacia Mono Q 5/5) chromatography.[27] ^{31}P NMR showed that the oligonucleotide phosphoromonothioate impurities were not present. This suggests that a Mono Q ion exchange column can potentially remove these impurities.

Prompted by the above observation and the data described below, we designed and prepared its complementary sequence containing two or four thymidine 3'-O-phosphorodithioate linkages. The purity of each oligomer (3-5) was assayed by ^{31}P NMR. Figure 1 is a representative ^{31}P NMR spectrum of an oligonucleotide 5 containing four 3'-O-phosphorodithioate linkages showing the absence of any

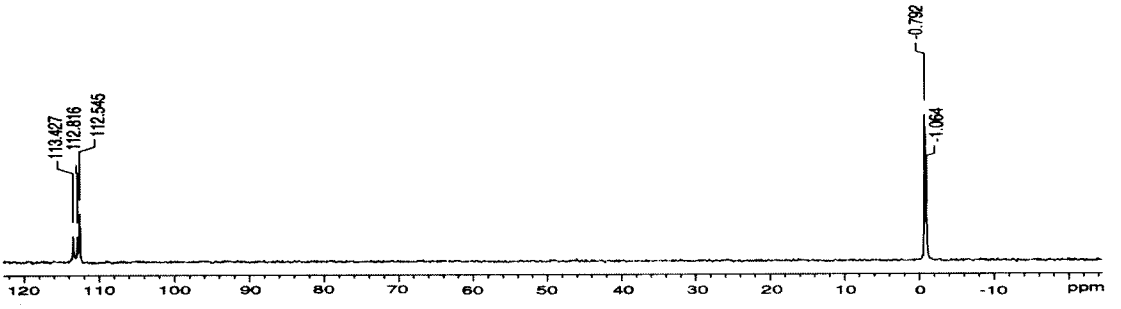

Figure 1. ^{31}P NMR spectrum of 5 was recorded on a Varian Unity plus spectrometer operating at 242 MHz. The sample contained ca. 40 OD A_{260} units dissolved in 500 μL of D_2O. Chemical shifts were referenced to 85% phosphoric acid.

A: Single Strands:

1. 5'-CCAGGAGATTCCAC-3'
2. 5'-CCAGGAGAT$_{S2}$T$_{S2}$CCAC-3'
3. 5'-GT$_{S2}$GG AATCTCCT$_{S2}$GG-3'
4. 5'-GTGGAAT$_{S2}$CT$_{S2}$CCTGG-3'
5. 5'-GT$_{S2}$GGAAT$_{S2}$C T$_{S2}$CCT$_{S2}$GG-3'

B:Duplexes:

6. 5'- CC A GGAGATTCC A C-3'
   3'- GG$_{S2}$ T CC TCTAAGG $_{S2}$T G-5'
7. 5'-CCAGG AG ATTCCAC-3'
   3'-GGTCC$_{S2}$ TC$_{S2}$ TAAGGTG-5'
8. 5'- CC AGG AG ATT CC AC-3'
   3'- GG$_{S2}$ TCC$_{S2}$ TC$_{S2}$ TAAGG$_{S2}$TG-5'
9. 5'- CC AGGAGAT$_{S2}$T$_{S2}$CC AC-3'
   3'- GG$_{S2}$ TCCT CTA A GG$_{S2}$TG-5'
10. 5'- CCAGG AG AT$_{S2}$ T$_{S2}$ CCAC-3'
    3'- GGTCC$_{S2}$TC$_{S2}$TA A GGTG-5'
11. 5'-CC AGG AG AT$_{S2}$T$_{S2}$CC AC-3'
    3'-GG$_{S2}$TCC$_{S2}$TC$_{S2}$ TA A GG$_{S2}$TG-5'

Scheme 1. **(A)** Single-stranded oligonucleotides 1-5 synthesized are shown, in which thymidine 3'-O-phosphorodithioate was incorporated in two or four positions. **(B)** Duplex aptamers were annealed at 15.75 μM of each strand in 10 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA.

phosphoromonothioate linkages. The molecular structure of oligonucleotide 5 was further confirmed by MALDI-MS, the calculated molecular weight is 4422.92 and the molecular weight observed was 4426.13. We did not observe peaks corresponding to M-16 or M-32 confirming the absence of significant quantities of oligomers with phosphoromonothioate linkages as described in the literature,[19] which is consistent with our ^{31}P NMR results.

In the following studies we investigated duplex [S_2]-ODNs, aptamers 6–11, binding to NF-κB. In standard competitive binding assays, ^{32}P end-labeled IgκB promoter element oligonucleotide (5'-AGTT GAGG GGAC TTTC CCAG GC-3') was incubated with recombinant p65[28] and varying amounts of competitor aptamers (6–11). The relative binding affinity of the unlabeled aptamers is determined by the concentration needed to effectively compete with the standard labeled aptamer. When only one strand of the aptamers contain thymidine 3'-O-phosphorodithioate (6–8), the inhibition/binding of the aptamer to protein is similar to that of the unsubstituted aptamer. With increased dithioate substitution on both strands, binding by the [S_2]-ODNs increases dramatically (Figure 2).

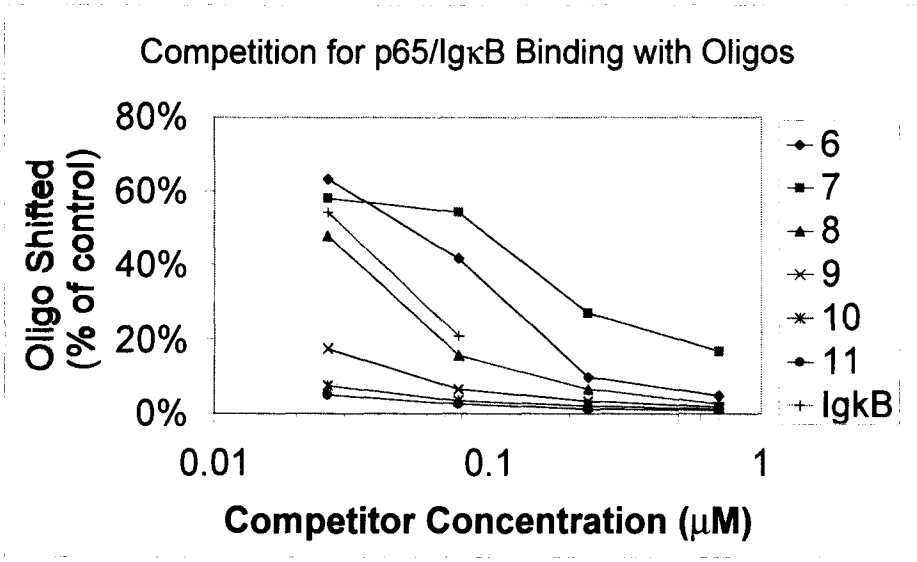

Figure 2. Inhibition of p65 homodimer binding by dithioate substituted aptamers 6–11.

Our lead aptamer 11 contains six dithioate linkages on the two strands, and unlike the fully monothio substituted aptamer, these dithioated aptamers bind more tightly to recombinant p65/p65 (5– to 15– fold) than to the recombinant p50 homodimer in vitro (data not shown). Significantly, the aptamer 11 also binds to a single NF-κB dimer in cell extracts (Figure 3), while the standard phosphodiester shows no NF-κB specific binding in extracts shown in lane 3, Figure 3. As shown in lane 4, Figure 3, aptamer 11 shifts one complex in nuclear

extracts from the murine pre-B cell line, 70Z/3. By using specific antibodies to supershift the complex, we have identified p50 as one component of the complex, but are uncertain at this point if the complex is a p50 homodimer or p50 containing heterodimer. This band does not co-migrate with either the p50/p50 or p50/p65 bands, but the change in the altered chemical structure changes the mobility of the oligonucleotide (data not shown). Only one major band is seen with aptamer 11, however, even though the lysate contains at least two major distinguishable NF-κB complexes (p50 homodimers and p50/p65 heterodimers). Since the p50/p65 heterodimer acts as an activator of transcription whereas the p50 homodimer acts as a suppressor, the aptamer 11 could modulate the immune and inflammatory systems in vivo in a distinct manner.

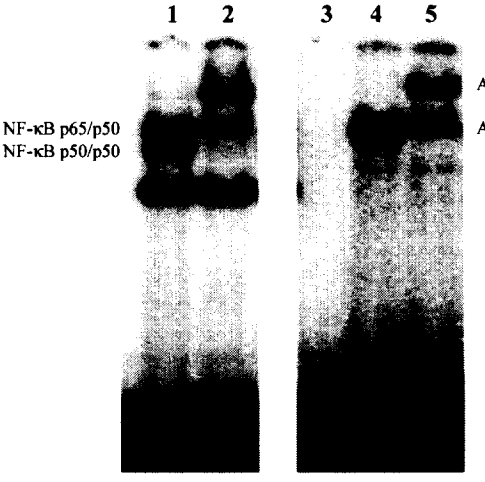

Figure 3. Identification of NF-κB subunit binding to aptamer 11. Radiolabeled oligonucleotide was incubated with 70Z/3 cell nuclear extract in the presence (lanes 2 and 5) or absence (lanes 1,3,4) of anti-p50 antibody. Protein bound ODN duplex was separated on a non-denaturing gel. Lanes 1 and 2: ^{32}P-IgκB promoter element. Lane 3: ^{32}P-phosphodiester CK-1. Lane 4 and 5: ^{32}P-aptamer 11.

In conclusion, we demonstrate that oligonucleotides containing selected phosphorodithioate linkages free of phosphoromonothioate impurities can be isolated by Mono Q ion exchange purification. The binding studies demonstrate the feasibility of altering the binding specificity by dithio-substituting only at a limited number of internucleoside linkages. We also appear to have generated an aptamer which can distinguish among various NF-κB dimers within the cell. Work is in progress to obtain minimally substituted dithioated oligonucleotides with high affinity, high binding specificity and increased nuclease resistance in vitro and in vivo.

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