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Inhibition of HIV Tat–TAR Interactions by an Antisense Oligo-2′-*O*-methylribonucleoside Methylphosphonate

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Abstract—An antisense oligo-2′-*O*-methylribonucleotide having alternating methylphosphonate/phosphodiester linkages, **1676**, whose sequence is complementary to the apical stem-loop of HIV-1 TAR RNA, was prepared to determine its effects on Tat protein–TAR interaction and Tat-mediated gene transactivation in cell culture. This oligomer and its all-phosphodiester analogue, **1707**, were shown to: (1) bind to TAR at 37 °C with K_d's in the low nM concentration range; (2) inhibit Tat-TAR complex formation; and (3) inhibit expression of a chloramphenicol reporter gene under control of the HIV LTR in HeLa HL3T1 cells in culture.

Transcription of HIV proviral DNA is regulated by the trans-acting response element, TAR, a 59-nucleotide stem-loop found at the 5′ untranslated end of all newly transcribed HIV mRNAs.[1] TAR consists of a lower, 20-base pair stem and an upper 4-base pair stem joined by a 3-base bulge and capped by a 6-base loop. A virally encoded protein, Tat, interacts with the 3-base bulge of TAR and recruits a host cell protein kinase complex, TAK, comprised of cyclin dependent kinase 9 (Cdk 9) and cyclin T (Cyc T), which binds to the loop region of TAR.[2–5] As a consequence of these interactions, the C-terminal domain of the stalled RNA polymerase II holoenzyme complex is phosphorylated resulting in an overall 3000-fold increase in the rate of transcription.[3] TAR–Tat–TAK mediated transactivation is essential for viral transcription and disruption of this interaction would be expected to cripple viral mRNA synthesis and thus inhibit virus replication. Indeed a variety of compounds including small organic molecules,[6,7] aminoglycosides,[8–15] oligoureas,[16] peptides,[17] peptide analogues[18] and peptide nucleic acids,[19,20] which can interact with TAR, have been shown to inhibit Tat–TAR interactions and in some cases, inhibit HIV replication in cell culture.[20]

Antisense oligodeoxyribonucleotides and their analogues have been designed that are complementary to the upper stem-loop region of TAR. These oligodeoxyribonucleotides can bind to TAR, although the binding affinities are usually low.[21–24] Better binding has been observed with anti-TAR oligo-2′-*O*-methylribonucleotides, which form complexes with TAR whose dissociation constants are in the low nM range.[24–29] Anti-TAR oligo-2′-*O*-methylribonucleotides and chimeric oligomers that contain either 5-propynyl C or 5-methyl C locked nucleosides have been shown to inhibit Tat–TAR binding and Tat-mediated transcription in vitro.[25,27]

We have recently described anti-TAR oligo-2′-*O*-methylribonucleosides that have alternating methylphosphonate/phosphodiester linkages (mr-AOMP).[28,30] Unlike oligonucleotides that contain only phosphodiester linkages, the internucleotide linkages of mr-AOMPs are resistant to nuclease hydrolysis.[28] Anti-TAR mr-AOMPs form stable complexes with TAR under physiological

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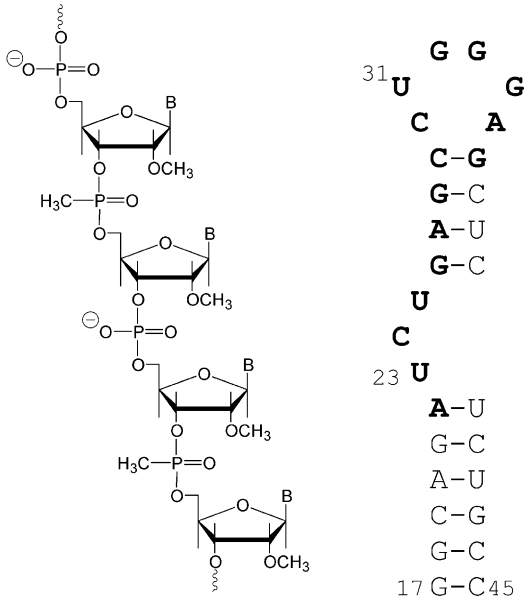
conditions, with low nM dissociation constants. In the present study we show that an mr-AOMP complementary to the TAR apical stem-loop can prevent Tat–TAR interaction in vitro. We also demonstrate that this oligomer is capable of inhibiting expression of a reporter gene under control of TAR in cell culture.

Oligo-2′-*O*-methylribonucleotides with alternating methylphosphonate/phosphodiester backbones, **1676** and **1677**, or with all-phosphodiester backbones, **1707** and **1757**, were synthesized and characterized as previously described.[28] The sequences of the oligomers and the structure of the alternating methylphosphonate/phosphodiester backbone are shown in Figure 1. Oligomers **1676** and **1707** are complementary to the nucleotides 22–36 of TAR, a region that includes the apical stem loop and 3-base bulge of TAR. Oligomers **1677** and **1757** are complementary to the same region, but contain two mismatched bases that target nucleotides 29 and 31 on the 5′ side of the apical stem-loop.

Interactions between these oligomers and the TAR target shown in Figure 1 were studied by gel electrophoretic mobility shift assays. The target contains the minimal sequence that is required for Tat responsiveness in vivo[31] and for in vitro binding of Tat-derived peptides.[32] Unlike the 59-nucleotide TAR target used in our previous studies,[28] the target shown in Figure 1 does not include the full 20-base pair lower stem of the full length TAR target. Rather it contains the upper 4-base pairs of the lower 20-base pair stem and two non-wild-type G:C base pairs at nucleotides 17:45 and 18:44.[33]



1676 mr-pCpUpCpCpCpApGpGpCpUpCpApGpGpApU
1677 mr-pCpUpCpCpCpGpGpApCpUpCpApGpGpApU

1707 mr-pCpUpCpCpCpApGpGpCpUpCpApGpGpApU
1757 mr-pCpUpCpCpCpGpGpApCpUpCpApGpGpApU

Figure 1. Structure of the alternating oligo-2′-*O*-methylribonucleotide methylphosphonate backbone; the sequence of the TAR RNA target; and the sequences of the anti-TAR oligonucleotides. The symbol p is a phosphodiester linkage, p is a methylphosphonate linkage and mismatched bases are shown in italics. The oligomer binding site on TAR is shown in bold.

The target was synthesized chemically or by Sp6 RNA polymerase-mediated run-off transcription of a synthetic DNA template, cloned into the Eco RI and Hind III sites of the polylinker of pGEM3Z.[28] The latter target contained an additional 13 nucleotides (5′ GAAU ACUC AAGC U -) at its 5′end. Binding experiments were carried out in 10 μL of buffer that contained 100 mM sodium chloride, 10 mM Tris–HCl, pH 7.5, 0.5 mM ethylenediaminetetraacetate, 0.1 nM 5′-[^{32}P]-labeled TAR and 0.1–50 μM **1676**, **1677**, **1707** or **1757**. After overnight incubation at 37 °C, the solutions were diluted with 5 μL of 80% glycerol and loaded onto 20×20 cm, native 8% polyacrylamide gels. The gels were run at 400 V at 37 °C until the bromophenol blue tracking dye migrated approximately 15 cm. The ratio of the free TAR and TAR-oligo complex was determined using a phosphorimager.

The apparent dissociation constants were determined from the binding isotherms generated by plotting the % oligomer-target complex versus the log of the oligomer concentration. Average K_d values were determined from at least four separate experiments and the results are shown in Table 1. Oligomer **1676** formed a complex with TAR whose dissociation constant is approximately 47 nM at 37 °C, which is similar to the K_d observed previously for the interaction of this oligomer with the full length, 59-mer TAR RNA.[28] Introduction of two mismatched bases, oligomer **1677**, reduced binding by at least 100-fold. Higher binding affinity, K_d approximately 2 nM, was observed for **1707**, which contains an all-phosphodiester backbone. As was the case for the alternating methylphosphonate oligomer, introduction of two mismatched bases, oligomer **1757**, resulted in loss of binding at 37 °C. Although oligomer **1707** has a higher binding affinity for TAR than does mr-AOMP **1676**, the all- phosphodiester oligomer is rapidly degraded by exonucleases found in mammalian serum, having a half-life of approximately 30 min at 37 °C, whereas **1676** is completely stable even after 18 h incubation.[28]

The effect of the oligonucleotides on the interaction between Tat protein and TAR was also investigated using a gel mobility shift assay. A solution of 40 nM 5′-[^{32}P]-labeled TAR and 200 nM Tat in buffer containing 50 mM Tris–HCl, pH 7.4, 20 mM potassium chloride and 0.1% Triton X-100 was prepared. Increasing concentrations, 16, 33, 66 or 100 nM, of oligomer **1676**, **1677**, **1707** or **1757** were added and the solution was incubated at room temperature for 1 h. The TAR-Tat complexes as well as the TAR-oligomer complexes

Table 1. Apparent dissociation constants of complexes formed between anti-TAR oligonucleotides and TAR RNA at 37 °C

Oligonucleotide	K_d (nM)
1676	47.3 (±9.7)
1677	> 5000
1707	1.9 (±0.8)
1757	> 50,000

were separated from free TAR by electrophoresis on native 12% polyacrylamide gels that contained 0.1% Triton X-100. The gels were run in at 4 °C at 500 V for 3 h and the products were visualized by phosphorimaging.

As shown in Figure 2A and B, lane 2, the TAR-Tat complex is well separated from free TAR. Addition of **1676** or **1707** results in disappearance of the band corresponding to TAR–Tat complex and appearance of a new band that corresponds to the TAR-oligomer complex. The result shows that 16 nM **1676** or **1707** almost completely inhibit TAR–Tat complex formation. Although mismatched oligomers **1677** and **1757** each have very low affinities for TAR at 37 °C, as shown by the binding experiments described above, they were able to competitively inhibit TAR–Tat complex formation at 4 °C, lanes 3–6. However, inhibition by these mismatched oligomers was less effective than that observed for the completely complementary oligomers, **1676** or **1707**. Thus inhibition was only complete at concentrations above 33 nM **1677** or 66 nM **1757**.

The ability of the anti-TAR oligonucleotides to inhibit Tat-mediated transcription of a reporter gene in cell culture was also investigated. A HeLa cell line, HL3T1, that contains a chloramphenicol acetyl transferase (CAT) reporter gene under the control of an integrated HIV-1 LTR promoter was transfected with pSV2Tat, an SV40-derived plasmid that expresses Tat protein, and with pAL, a control plasmid that expresses luciferase, in the presence of increasing concentrations of anti-TAR oligomer. HL3T1 cells were grown in 60 mm dishes in 2 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C in a 5% carbon dioxide atmosphere. The old media was replaced with 2 mL of fresh DMEM just prior to transfection. Solutions containing 15 μg of pSV2Tat, 15 μg of pAL, 0–94 nM anti-TAR oligomer and 20 μg of Cytofectin GS (Glen Research) were prepared in 400 μL of serum-free DMEM and incubated at room temperature for 10 min. The solution was then diluted with 1.6 mL of DMEM containing 10% fetal calf serum. Transfection was initiated by replacing the cell culture medium with the plasmid/oligomer solution. The cells were incubated for 5 h at 37 °C; fresh DMEM containing 10% FCS was added and incubation was continued for 10–16 h. Finally, the cells were harvested, lysed in reporter lysis buffer (Promega, Inc.), and aliquots of the lysate were used to measure CAT and luciferase activities as previously described.[17] Both activities were normalized to the protein concentration as determined by a modified Bradford assay (Bio-Rad, Inc.). CAT and luciferase activities were determined from multiple experiments and normalized to 100% at 0 μM oligomer concentration.

As shown in Figure 3, expression of the CAT reporter gene, which is under control of an integrated HIV-1 LTR promoter in HL3T1 cells, is upregulated by transfection of the cells with pSV2Tat, which encodes the Tat gene. Expression of CAT is inhibited in a concentration dependent manner by oligomer **1676** (see Fig. 3A). CAT expression was also inhibited by oligomer **1707**, which contains an all-phosphodiester backbone, as shown in Figure 3B. In contrast, expression of luciferase, which is not under control of the HIV-1LTR promoter, is not affected by the presence of either oligomer **1676** or **1707** These results are consistent with a mechanism whereby interaction of **1676** or **1707** with TAR prevents formation of the TAR–Tat complex, which is necessary for transactivation of the CAT reporter gene. It is unlikely

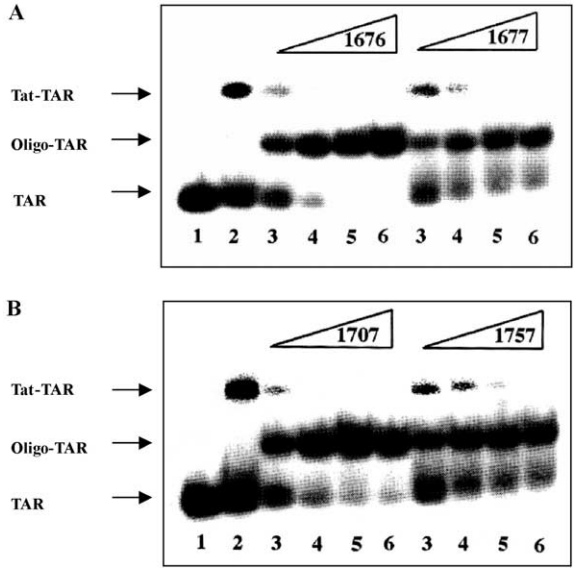


Figure 2. Effects of oligomers **1676** or **1677** (panel A) and **1707** or **1757** (panel B) on Tat–TAR complex formation. Samples containing TAR (lane 1); Tat and TAR (lane 2); or Tat, TAR and 16, 33, 66 or 100 nM oligonucleotide (lanes 3–6) were electrophoresed on 12% native polyacrylamide gels at 500 V for 3 h at 4 °C.

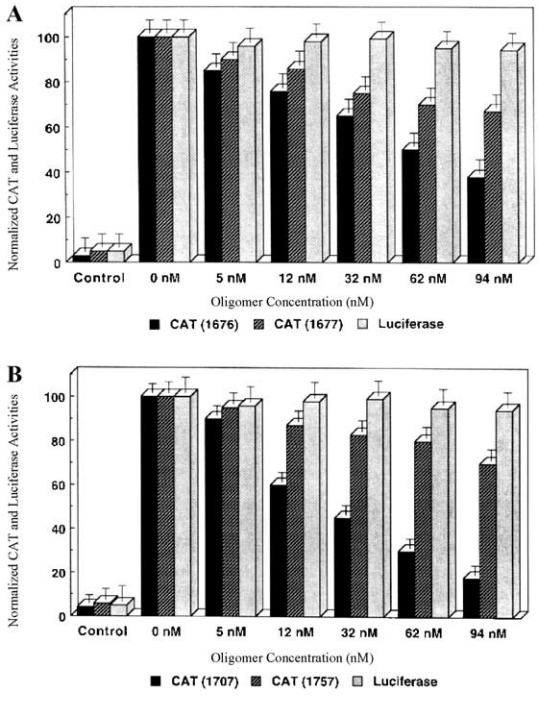


Figure 3. Effects of oligomer **1676** (panel A) or **1707** (panel B) on CAT and luciferase expression and effects of oligomer **1677** (panel A) or **1757** (panel B) on CAT expression in HL3T1 cells in culture. Controls were transfected with oligomer in the absence of pSV2Tat.

that the oligomers interfere with plasmid transfection of the cells. If this were the case, expression of luciferase should be inhibited as well. Although previous experiments have shown that **1707** is rapidly hydrolyzed in serum-containing medium,[28] it appears that such degradation did not occur under the conditions used in the transfection experiment. Most likely complexation with Cytofectin, a cationic lipid, protects the oligomer from the action of serum nucleases in the cell culture medium.

Both mismatched oligomers, **1677** and **1757**, inhibited CAT expression in a similar concentration dependent manner, although the extent of inhibition was less than that seen for the corresponding antisense oligomers, **1676** and **1707**. This result is in contrast to that seen in the in vitro binding experiments at 37 °C and suggests that the stringency of binding discrimination may be reduced in vivo, although why this should occur is unclear. Possibly in addition to binding to TAR, the oligomers interfere with recruitment of TAK components to TAR RNA in a non-sequence dependent manner.

Oligomer **1707** is somewhat more inhibitory than **1676**. Thus **1707** inhibits CAT expression approximately 50% at a concentration of 32 nM, whereas 62 nM **1676** is required to give the same level of inhibition. Although this result is not unexpected, given the relative binding affinities of the two oligomers for TAR, it is likely that other factors, such as the relative uptake efficiency of the two oligomers, could also play a role in determining the overall efficacy of the antisense oligomers in cell culture.

The results of our experiments are consistent with those of others who have shown that anti-TAR oligonucleotides can inhibit TAT-mediated transcription in vitro[25–27] and that anti-TAR peptide nucleic acids have antiviral activity in cell culture.[19,20] In combination these results suggest that TAR may provide a good target for disrupting HIV activity by antisense oligonucleotides.

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