Inhibition of HIV-1 Tat-Dependent *Trans* Activation by Steric Block Chimeric 2′-*O*-Methyl/LNA Oligoribonucleotides†

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ABSTRACT: The HIV-1 *trans*-activation responsive element (TAR) RNA 59-residue stem−loop interacts with the HIV *trans*-activator protein Tat and other cellular factors to stimulate transcriptional elongation from the viral long terminal repeat (LTR). Inhibition of these interactions blocks full-length HIV transcription and hence replication. We have found that three types of 12-residue oligonucleotide analogues, namely, a 2′-*O*-methyl oligoribonucleotide (OMe), a chimeric oligonucleotide containing 7×OMe and 5×5-methyl C locked nucleic acid (LNA) residues, and a peptide nucleic acid (PNA), inhibit Tat-dependent in vitro transcription in HeLa cell nuclear extract equally efficiently (50% inhibition at 100−200 nM) and sequence specifically. The results are correlated with surprisingly similar binding strengths to a model 39-residue TAR under transcription conditions. A 12-mer containing 11 contiguous LNA residues was less effective in both Tat-dependent transcription inhibition and TAR 39 binding. Anti-TAR 3′-carboxyfluorescein- (FAM-) labeled OMe and OMe/LNA chimeric 12-mers were also efficient Tat-dependent in vitro transcription inhibitors as were 3′-FAM-labeled OMe oligonucleotides containing some phosphorothioate (PS) linkages. By use of a HeLa cell line containing stably integrated plasmids expressing firefly luciferase under HIV-LTR/Tat dependence as well as a *Renilla* luciferase constitutive control, we showed submicromolar, selective, dose-dependent, and sequence-dependent intracellular inhibition of Tat-TAR *trans* activation by the anti-TAR 3′-FAM 12-residue 7×OMe/5×LNA oligonucleotide when delivered by cationic lipid. No intracellular activity was observed for the corresponding anti-TAR 3′-FAM OMe 12-mer. An alternating PS-containing 3′-FAM OMe 12-mer oligonucleotide exhibited partial inhibition of *trans*-activation activity, but this was correlated with a similar effect on control gene expression, suggesting nonspecific inhibition.

Oligonucleotides and their analogues have found wide utility as sequence-specific regulators of gene expression in cells (*1*−*3*). A popular route of inhibition of gene function is by complementary binding of oligonucleotides to a region of RNA, usually mRNA or viral RNA, which has been termed the "antisense approach". It had been expected that the effect of such binding would be to sterically block important cellular functions of the RNA such as translation. However, it was found that, for phosphodiester (PO)[1] and phosphorothioate (PS) oligodeoxyribonucleotides, an overriding mechanism of gene inactivation is via the induction of RNase H, especially when the oligonucleotide is targeted to mRNA coding regions, resulting in endonucleolytic cleavage of the RNA strand of the RNA−oligonucleotide hybrid (reviewed in ref *4*).

The steric block mechanism of action of antisense oligonucleotides has received relatively little attention until recently, since oligonucleotide analogues and chimeras targeted to coding regions appeared to have far greater potency when they had the ability to induce RNase H, directed through at least partial regions of PO or PS sequence within the oligonucleotide (*5*). An advantage of a steric block mode of action is that an oligonucleotide mishybridized to an incorrect RNA by virtue of partial complementarity would be less likely to lead to unwanted gene inactivation, whereas a similarly mishybridized RNase H-inducing oligonucleotide could lead to RNA cleavage and therefore less specificity. The effectiveness of steric block oligonucleotides was shown in 1997 for a 20-mer 2′-*O*-(2-methoxyethyl) oligoribonucleotide in inhibition of initiation of translation by targeting of

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; FAM, 6-carboxyfluorescein; FCS, fetal calf serum; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; LNA, locked nucleic acids; LTR, long terminal repeat; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; OMe, 2′-*O*-methyl; PAGE, polyacrylamide gel electrophoresis; PBS, primer binding site; PCR, polymerase chain reaction; PNA, peptide nucleic acids; PO, phosphodiester; PS, phosphorothioate; SDS, sodium dodecyl sulfate; TAK, Tat-associated kinase; Tat, *trans*-activator protein; TAR, *trans*-activation responsive region.

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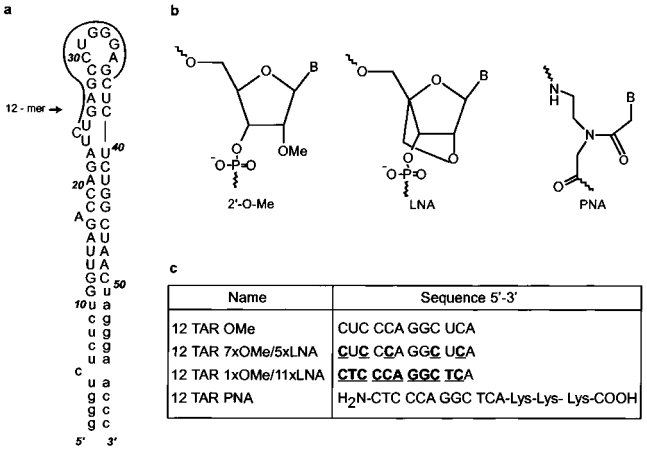


Name	Sequence 5′-3′
12 TAR OMe	CUC CCA GGC UCA
12 TAR 7xOMe/5xLNA	**CUC CCA GGC UCA**
12 TAR 1xOMe/11xLNA	**CTC CCA GGC TCA**
12 TAR PNA	H_2N-CTC CCA GGC TCA-Lys-Lys- Lys-COOH

FIGURE 1: Oligonucleotide analogues and their binding sites on TAR RNA. Panel a: Full-length 59-mer TAR RNA found at the 5′-end of HIV-1 transcripts and TAR 39 model stem−loop RNA used for binding studies (capitals section only). The location of the 12-mer oligonucleotide binding region is marked. Panel b: Chemical structures of the backbones of the three types of oligonucleotide analogues studied. Panel c: Names and sequences of the 12-mer oligonucleotide analogues studied. Underlined and bold residues represent LNA units.

the 5′-cap region of ICAM-1 mRNA in endothelial cells (*6*). Since then, there has been a range of examples of the use of steric block oligonucleotides employing a variety of chemistries to block cellular gene expression, notably via inhibition of elongation of translation (*7*, *8*), altering splicing pathways (*9*−*11*), or redirection of polyadenylation (*12*). All of these examples have used oligonucleotide analogues of 18−30 residues, except for the arrest of translation by a 13-mer PNA (*7*).

Few studies have focused on the use of steric block oligonucleotides to inhibit RNA−protein interactions important to HIV replication. Steric blocking of in vitro HIV reverse transcription was achieved using either 21-mer PNA (*13*) or 16-mer oligonucleotide analogues targeted to the primer binding site (PBS) or the *trans*-activation responsive region TAR (*14*). The 59-residue TAR stem−loop element (Figure 1a) is a particularly good target site for investigation, since it has a very high degree of sequence conservation and occurs at the 5′-end of all HIV-1 RNA transcripts. Further, transcription of HIV is regulated by a virally encoded *trans*-activator protein Tat that interacts with TAR during transcriptional elongation (for recent reviews see refs *15*−*17*). Together with a Tat-associated kinase (TAK), which includes host cellular factors cyclin T1 and the kinase cdk9, Tat forms a ternary complex with TAR RNA. Tat-dependent *trans* activation involves cdk9-mediated hyperphosphorylation of the C-terminal domain of RNA polymerase II and resultant stabilization of the transcription complex leading to a substantial stimulation of full-length transcription. Tat recognition of TAR is localized to a pyrimidine-rich bulge (Figure 1a), whereas cyclin T1 action appears to involve in some way the apical loop. Since *trans* activation is essential for HIV replication, small molecule or peptidomimetic inhibitors that bind TAR and block the action of Tat or its cellular partners are potential antiviral agents (for a review see ref *18*).

Ecker et al. showed some years ago that oligonucleotides can strand invade the apical stem−loop of TAR RNA and inhibit Tat binding in vitro (*19*). Strand invasion was poor for short phosphodiester or phosphorothioate oligonucleotides, but longer phosphorothioates of 26−28 residues were able to induce a partially sequence-specific antisense effect in a *trans*-activation reporter assay when transfected into cultured cells (*20*). By contrast, short oligoribonucleotides and steric block 2′-*O*-methyl (OMe) oligoribonucleotides were found to bind much more strongly to the TAR loop, allowing the formation of a half-pseudo-knot structure, but no cellular data were reported (*19*).

More recently, we showed that OMe oligoribonucleotides of 12−16 residues targeted to the apical stem−loop bound TAR RNA in vitro with affinities in the 20−100 nM range and blocked Tat binding very efficiently (*21*, *22*). We have further shown that such OMe oligoribonucleotides block sequence specifically Tat-dependent transcription in vitro directed by HeLa cell nuclear extract on a DNA template containing the HIV-1 LTR (*23*, *24*). This ability to inhibit Tat-dependent transcription extends also to chimeric 12-mer OMe oligoribonucleotides containing 5-propynyl-2′-*O*-methyl C or by 5-methyl C locked nucleic acid (LNA) monomeric units and to a PNA 12-mer (*24*). An independent study showed recently that a 15-mer PNA targeted to the same apical region of TAR blocked Tat-dependent in vitro transcription and, when electroporated into CEM cells, could inhibit HIV LTR-driven chloramphenicol acetyltransferase (CAT) expression in a transient plasmid reporter system (*25*).

We now compare the effectiveness of a variety of steric block oligonucleotides to bind to TAR RNA and also to inhibit Tat-dependent in vitro transcription. We show that, under the ionic conditions of in vitro transcription, OMe, chimeric OMe/LNA, and PNA oligonucleotides of the same length (12 residues) bind to TAR equally strongly and inhibit Tat-dependent transcription equally well. Further, when delivered by cationic lipid into HeLa cells, a 3′-carboxyfluorescein-labeled 12-mer OMe/LNA chimeric oligonucleotide was able to inhibit sequence specifically and with dose dependence Tat-dependent HIV-1 LTR *trans* activation in a stably integrated plasmid system involving double luciferase reporters. This 12-mer is the shortest steric block oligonucleotide analogue reported to date to show intracellular antisense activity as an inhibitor of transcription/translation.

MATERIALS AND METHODS

Oligonucleotides. 2′-*O*-Methyl oligoribonucleotides and TAR 39 RNA were synthesized as described previously (*21*, *22*). Phosphorothioate linkages were introduced using the Beaucage Reagent (Glen Research). LNA and mixed 2′-*O*-methyl/LNA oligoribonucleotides were prepared by standard phosphoramidite solid-phase synthesis as described previously using LNA monomers of T, G, A, and 5-methyl C (*26*, *27*). PNA−triLys conjugates were synthesized by standard Fmoc chemistry manually on a Tentagel resin preloaded with one Fmoc-Lys(Boc) residue (5 μmol) as described previously (*28*). 3′-FAM 2′-*O*-methyl oligonucleotides and 2′-*O*-methyl/LNA chimeric oligonucleotides were synthesized on 1 μmol scale using a FAM-functionalized controlled pore glass support described previously (*29*).

Oligonucleotide−TAR Apparent Dissociation Constants. The 20 μL binding reactions included varying concentrations of the oligonucleotide analogue and 4−5 nM 5′-^{32}P-labeled

TAR 39 RNA (4.5×10^4 cpm pmol^{-1}) (*21*) incubated for 20 min at 30 °C in one of three buffer conditions: (1) 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 5 mM DTT, 0.01% Triton X-100, and 0.08 unit μL^{-1} RNasin (Promega), (2) the same as (1) but with 80 mM KCl, and (3) transcription buffer: 20 mM HEPES (pH 7.9), 2 mM DTT, 10 μM $ZnSO_4$, 10 mM creatine phosphate (Boehringer Mannheim), 80 mM KCl, and 3 mM $MgCl_2$. The 10 μL loading buffer was added to each sample to give 0.025% bromophenol blue and 13% sucrose, and electrophoresis was carried out on an 8% native polyacrylamide gel and run in 20 mM Tris−acetate (pH 8.3), 1 mM EDTA, and 0.2% glycerol at room temperature for 1 h. The gel was dried and visualized by autoradiography. The dried gels were also exposed to a phosphor storage screen (Molecular Dynamics) and scanned by a Model 425S PhosphorImager (Molecular Dynamics). The resulting digitized images were analyzed by Geltrak (*30*) on a DEC/Alpha 2100 (Digital Equipment Corp., Maynard, MA) through an X-terminal. Apparent dissociation constants (K_d) were estimated from

$$[\mathrm{RNA}] = (A \pm (A^2 - 4[\mathrm{RNA}]_0[\mathrm{Oligo}]_0)^{1/2})/2[\mathrm{RNA}]_0 \quad (1)$$

where $A = [\mathrm{RNA}]_0 + [\mathrm{Oligo}]_0 + K_d$, $[\mathrm{RNA}]_0$ and $[\mathrm{Oligo}]_0$ are initial concentrations of TAR 39 RNA and oligonucleotide analogues, and [RNA] is the fraction of bound radiolabeled probe. The experimental data were fitted to eq 1 using the nonlinear least-squares method with KaleidaGraph software (Abelbeck Software). For calculation of K_d the average values of three experiments were used.

Association and Dissociation Kinetics. To find the association (k_{on}) and dissociation (k_{off}) rate constants, 400 μL binding reactions were prepared containing 25 nM 5′-^{32}P-labeled TAR 39 RNA (2×10^4 cpm pmol^{-1}) in buffer conditions 2 or 3 described above and oligonucleotide analogues at two concentrations (approximately 5−10-fold over K_d). Reactions were incubated at 30 °C for prescribed intervals and quenched by addition of 10 μL of loading buffer (40% sucrose, 0.075% bromophenol blue) to 20 μL reaction samples and snap-freezing on dry ice/2-propanol. These quenched reactions were individually thawed, loaded, and electrophoresed at 5 °C on a native polyacrylamide gel as described above. Dried gels were analyzed as before. Association and dissociation rate constants for a bimolecular reaction were estimated using the Levenberg−Marquardt algorithm to refine the constants, with numerical integration (at 0.25 s intervals) to calculate the binding reaction at each value of the constants. The data fit was not perfect for a single-step bimolecular binding isotherm, and there was some evidence for a slightly faster initial binding phase, but deviations were small and appeared unlikely to affect the numerical values of overall rate constants significantly.

Inhibition of Tat-Dependent Transcription. The −346 to +524 fragment (*Eco*RV−Xba I) of plasmid p10SLT (*31*) carrying the wild-type HIV-1 LTR (strain NL4-3) was amplified by PCR with 200 pmol of the primers LTR PRI, 5′-ATC GAT CCA CTG ACC TTT GGA TGG TGC TTC-3′, and XBA PRI, 5′-CTA GAG TCG CTG CTG CTT GCT GTG CCT TTT-3′, and a *Taq* DNA polymerase kit (Promega) according to the manufacturer's specifications. The reaction product was purified by use of an UltraClean PCR Cleanup DNA purification kit (MO BIO Laboratories, Inc., Solana Beach, CA). Cell-free transcription reactions were carried out essentially as described (*31*, *32*) with minor differences. Thus the transcription reaction mixture (40 μL) contained 15 μL of HeLa cell nuclear extract (*33*), 10 nM template DNA, 80 mM KCl, 2−4 mM $MgCl_2$ (depending on the HeLa nuclear extract), 20 mM HEPES (pH 7.9), 2 mM DTT, 10 μM $ZnSO_4$, 10 mM creatine phosphate (Boehringer Mannheim), 100 μg mL^{-1} creatine kinase (Boehringer Mannheim), 1 μg of poly[d(I-C)] (Boehringer Mannheim), 50 μM ATP, GTP, and CTP, 5 μM UTP, [α-^{32}P]-UTP (10 μCi), 1 unit μL^{-1} RNasin (Promega), 200 ng of recombinant Tat protein (*33*), and increasing concentrations of inhibitor oligonucleotide. The reactions were incubated at 30 °C for 20 min and then stopped by addition of 50 μL of 150 mM sodium acetate solution, 0.5% SDS, 10 mM EDTA, and 20 μg mL^{-1} tRNA, extracted with an equal volume of phenol/chloroform followed by precipitation with 2 volumes of ethanol. The reaction products were analyzed by 6% PAGE containing 7 M urea followed by autoradiography. The autoradiographs were scanned densitometrically using a Personal Densitometer SI (Molecular Dynamics, Inc.), and the resulting digitized images were analyzed by Geltrak software (*30*).

Cell Line HeLa Tet-Off/Tat/luc-f/luc-R. HeLa Tet-Off cells (Clontech), maintained with neomycin and doxycyclin, were transformed sequentially with three plasmids: (1) pTRE Tat (hygromycin selection) constructed from pREV-TRE (Clontech) and a synthetic HIV-1 Tat gene (*34*), (2) pRT 5 (blasticidin selection) constructed from pGL3-Basic (Promega) and an HIV-1 LTR-firefly luciferase gene insert from pD5 (3.3) (*35*), and (3) pRT 215 (zeocin selection) constructed from SV40-zeo2 (Invitrogen) with a CMV-*Renilla* luciferase (pRL-CMV, Promega) insert.

Inhibition of Tat Trans Activation in Cells. In each experiment two identical plates were prepared, one for the luciferase assay and the other for the cytotoxicity assay. HeLa Tet-Off/Tat/luc-f/luc-R cells were plated out in 96-well plates at 7500 cells per well in DMEM medium (Life Technologies)/10% Tet System Approved FCS (Clontech) in the absence of doxycyclin and incubated overnight at 37 °C. Oligonucleotides were prepared at a concentration of 1 μM in Opti-MEM serum-free medium (Life Technologies), and the cationic lipid Effectin 12 (Cambio) was added to a concentration of 4.5 μg mL^{-1} from a 0.9 mg mL^{-1} stock. Complexes were allowed to form at room temperature for 30 min. Subsequent dilutions were prepared from this oligonucleotide/Effectin 12 mixture. Medium was removed from the cells, which were briefly washed with phosphate buffered saline before addition of 100 μL of oligonucleotide/Effectin 12 dilution. After 3 h in the presence of the oligonucleotide/Effectin 12 mixture, the medium was removed and replaced with 100 μL of DMEM/10% FCS. Cells were analyzed 18 h after the removal of the oligonucleotide.

(*a*) *Luciferase Assay*. Cell lysates were prepared and analyzed using the Dual Luciferase Reporter Assay System (Promega) and relative light units for both firefly and *Renilla* luciferase read sequentially using a Berthold Detection Systems Orion Microplate luminometer. Each data point was averaged over four replicates.

(*b*) *Toxicity Assay*. The extent of toxicity was determined by measurement of the proportion of live cells colorimetrically using CellTiter 96 AQ_{ueous} One Solution Assay (Prome-

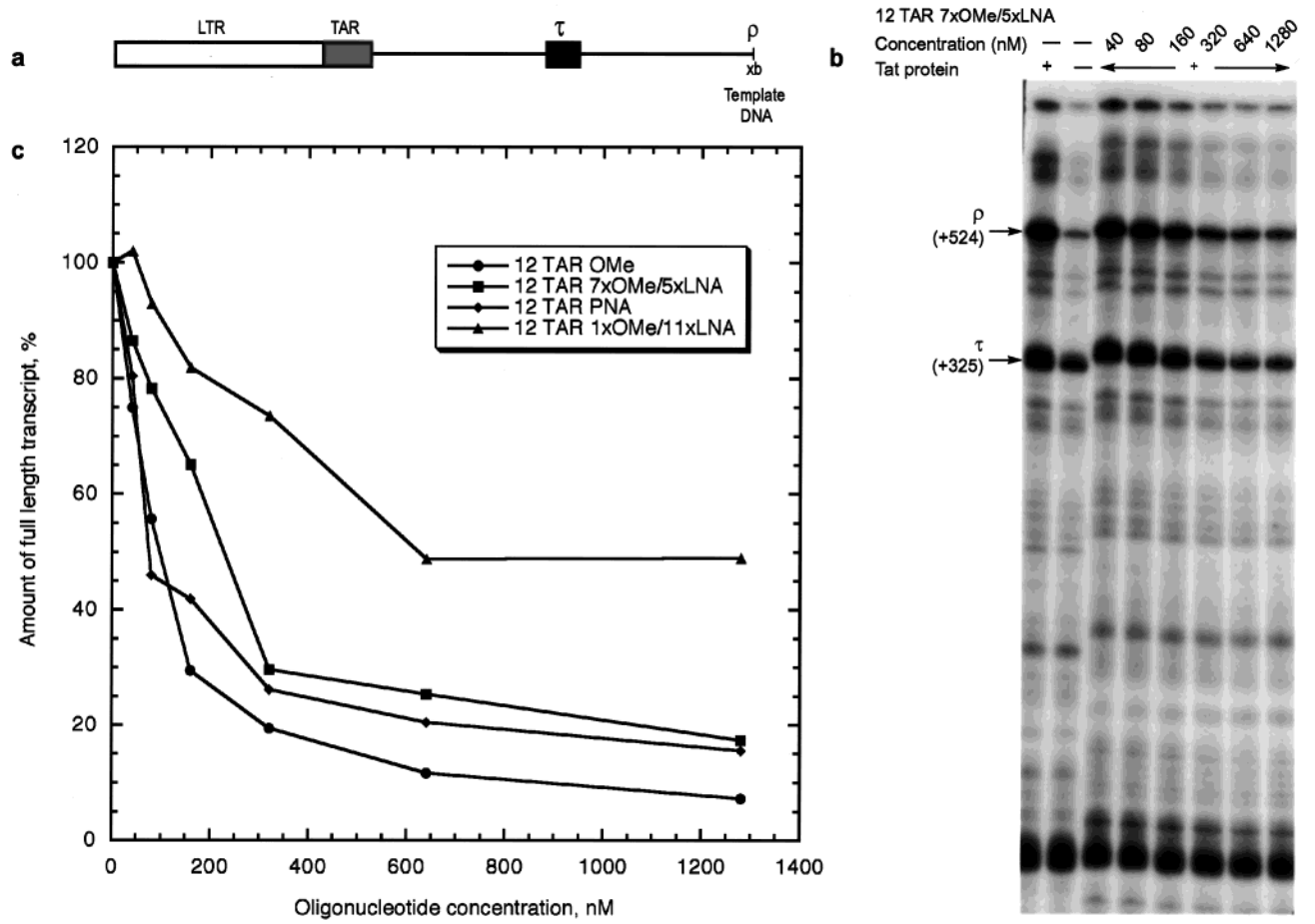


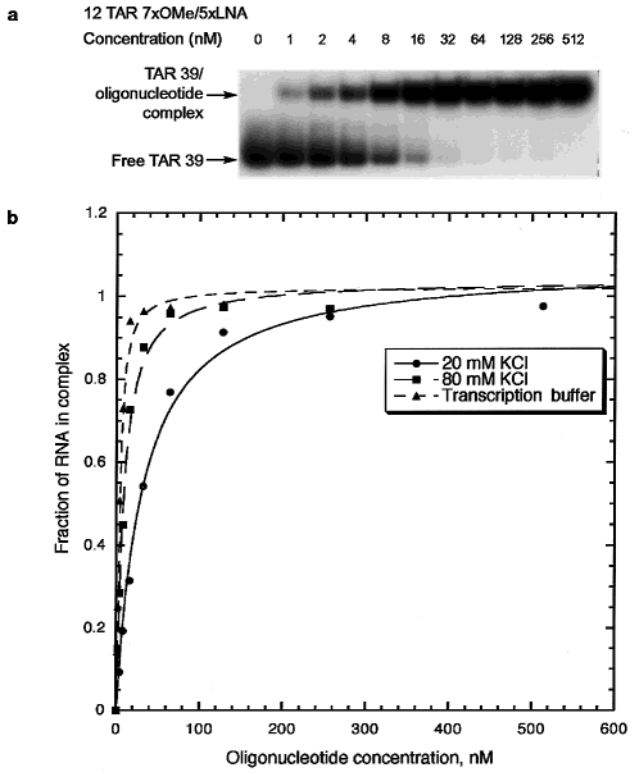
FIGURE 2: Oligonucleotide analogue inhibition of Tat-dependent in vitro transcription in HeLa cell nuclear extract. Panel a: HIV-1 template containing LTR and TAR region obtained from plasmid p10SLT (*31*). xb = *Xba*I restriction site. Panel b: Typical autoradiograph of transcription products produced from the HIV template in the presence (lane 1) or absence (lane 2) of Tat and increasing concentrations of 12 TAR 7×OMe/5×LNA (remaining lanes). The runoff (ρ) and internal artificially terminated (τ) products are marked. Panel c: Graph of amounts of full-length transcripts as a function of oligomer concentration (see inset for key).

ga). The absorbance at 490 nm was read using a Molecular Devices Emax Microplate Reader. Each data point was averaged over four replicates.

RESULTS

Inhibition of Tat-Dependent Transcription by Steric Block 12-mer Oligonucleotides. We reported recently that an OMe oligonucleotide of 16 residues complementary to the apical loop and stem of TAR RNA inhibited sequence specifically Tat-dependent transcription of a DNA template containing the HIV-1 LTR in the presence of HeLa cell nuclear extract (*23*, *24*). The inhibition was dose dependent with 50% inhibition at about 100 nM, whereas neither scrambled nor mismatched oligonucleotides showed inhibition at 1 μM. Inhibition was also seen for shorter 14-mer, 12-mer, and even 10-mer OMe oligonucleotides. In a preliminary study, three other 12-mer steric block oligomers of the same sequence, selected for the reported properties of the component nucleotide analogues to enhance RNA binding, showed similar (but not enhanced) abilities to inhibit in vitro transcription (*24*). These were an OMe oligonucleotide where all 6 cytosines were replaced by 5-propynyl C (*36*), a chimeric OMe/LNA oligonucleotide where 5 cytosines were replaced by 5-methyl C LNA units (*26*), and a PNA oligomer (*28*, *37*).

Oligonucleotides containing contiguous LNA monomers (Figure 1b) have among the highest melting temperatures (T_m) recorded for an analogue, and a sequence containing 11 contiguous LNA residues would be expected to have a T_m of 90 °C or more under medium salt conditions [100 mM NaCl, 10 mM Na_2HPO_4 (pH 7.0)] (*26*). We therefore compared a 12-mer oligonucleotide containing 11 contiguous LNA units (12 TAR 1×OMe/11×LNA) with the same sequence where 5 cytosine residues were substituted by 5-methyl C LNA units, the remaining being OMe residues (12 TAR 7×OMe/5×LNA), as well as the corresponding fully OMe oligonucleotide (12 TAR OMe), and a PNA oligomer (12 TAR PNA) (Figure 1b,c). We found that in vitro transcription of a DNA template containing the HIV-1 LTR in the presence of HeLa cell nuclear extract and HIV-1 Tat protein was inhibited with very similar dose dependence for 12 TAR OMe, 12 TAR 7×OMe/5×LNA, and 12 TAR PNA (Figure 2a,b). The full-length transcript (ρ) was 50% reduced at 100−200 nM as previously observed (*24*). Unexpectedly, 12 TAR 1×OMe/11×LNA was a much poorer inhibitor and showed a 50% reduction in full-length transcript at only about 600 nM. Note that the DNA template happens also to contain an artificial terminator (τ) (*31*) and concomitant reductions in transcription termination are also observed at this position (Figure 2b).

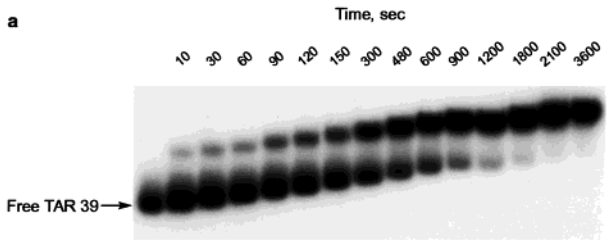


c

Oligonucleotide analogues	50 mM Tris HCl, pH 7.4, 20 mM KCl	50 mM Tris HCl, pH 7.4, 80 mM KCl	Transcription buffer, pH 7.9
12 TAR OMe	60.7±7.5	12±1.5	3.2±0.7
12 TAR 7xOMe/5xLNA	25.4±5.8	6.6±0.4	2.0±0.2
12 TAR 1xOMe/11xLNA	89±12.3	35.7±2.6	11.6±2.5
12 TAR PNA	1.2±0.3	3.3±0.5	2.5±0.6

FIGURE 3: Oligonucleotide analogue binding to TAR 39 RNA. Panel a: Autoradiograph of a PAGE mobility shift experiment showing 5′-^{32}P-labeled TAR 39 and complex formation as a function of increasing concentrations of 12 TAR 7×OMe/5×LNA. Panel b: Graph showing the fraction of TAR 39 RNA complex formation as a function of oligonucleotide concentration (12 TAR 7×OMe/5×LNA) for three buffer conditions (see inset for key). Panel c: Apparent dissociation constants (K_d, nM) for oligomers under three buffer conditions.

To try to understand the behavior of the various oligomers in the in vitro transcription inhibition assay, we compared their abilities to strand invade and bind to a 39-residue synthetic model TAR RNA (TAR 39) under various salt conditions using a PAGE mobility shift assay (Figure 3a). The fraction of RNA bound by the oligomer was measured as a function of increasing oligomer concentration (Figure 3b). Under standard buffer conditions (50 mM Tris-HCl, pH 7.4, 20 mM KCl) used previously for assessing Tat-TAR and oligonucleotide-TAR binding (*21*, *22*, *38*), there were significant differences between the four 12-mers, with strongest binding for 12 TAR PNA ($K_d = 1.2 \pm 0.3$ nM), much weaker binding for the chimeric 12 TAR 7×OMe/5×LNA (25.4 ± 5.8 nM) and 12 TAR OMe (60.7 ± 7.5 nM), and poorest for 12 TAR 1×OMe/11×LNA (89 ± 12.3 nM) (Figure 3c). When the KCl content of the buffer was raised to 80 mM, the three phosphodiester-containing oligonucleotides were more tightly bound to TAR 39. The greatest gain in binding strength (5-fold) was achieved for 12 TAR OMe, less for the chimeric OMe/LNA (4-fold), and least for the 12-mer containing 11 LNA units (2.5-fold). By contrast, 12 TAR PNA was 3-fold poorer in binding than under lower salt conditions.



b

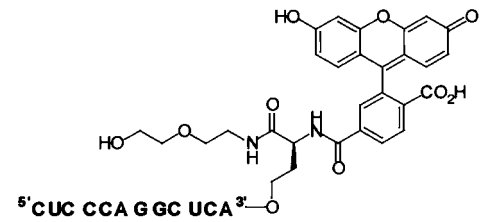
Analogue	k_{on}, M^{-1} sec^{-1}		k_{off}, sec^{-1}	
	50 mM Tris HCl, pH 7.4, 80 mM KCl	Transcription buffer, pH 7.9	50 mM Tris HCl, pH 7.4, 80 mM KCl	Transcription buffer, pH 7.9
12 TAR OMe	$(3.9\pm0.6)\times10^4$	$(2\pm1)\times10^6$	$(3.1\pm0.1)\times10^{-4}$	$(4.3\pm0.9)\times10^{-3}$
12 TAR 7xOMe/5xLNA	$(1.1\pm0.1)\times10^5$	$(2.2\pm0.6)\times10^6$	$(8\pm2)\times10^{-4}$	$(2.1\pm0.2)\times10^{-3}$
12 TAR PNA	$(6.1\pm4.6)\times10^5$	$(4.9\pm1.9)\times10^5$	$(3.3\pm1.6)\times10^{-3}$	$(1.5\pm1.1)\times10^{-3}$

FIGURE 4: Rates of association and dissociation of oligonucleotide analogues with TAR 39 RNA. Panel a: Autoradiograph of PAGE mobility shift experiment showing 5′-^{32}P-labeled TAR 39 and complex formation with 12 TAR 7×OMe/5×LNA as a function of time of incubation. Panel b: Kinetic constants for association (k_{on}) and dissociation (k_{off}) for oligonucleotide analogues under two buffer conditions.

Further gains in binding strength were apparent for the three phosphodiester-containing oligonucleotides under transcription buffer conditions [80 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES (pH 7.9), 2 mM DTT, 10 μM $ZnSO_4$, 10 mM creatine phosphate] whereas 12 TAR PNA binding strength was essentially unaltered. Therefore, under transcription buffer conditions, three of the four oligomers (12 TAR OMe, 12 TAR 7×OMe/5×LNA, and 12 TAR PNA) all had very similar binding strengths (2−3 nM) (Figure 3c). By contrast, the oligomer containing 11 contiguous LNA residues was 5−6-fold less strongly bound by TAR 39 RNA than the chimeric OMe/LNA oligonucleotide. Therefore, the TAR binding strengths under transcription buffer conditions closely parallel the levels of inhibition of Tat-dependent in vitro transcription. This may help to explain why the 12 TAR 1×OMe/11×LNA is less able to inhibit transcription while the other three oligomers inhibit transcription to a similar extent (Figure 2c).

We also measured the association (k_{on}) and dissociation (k_{off}) rates of the three most strongly inhibitory oligomers using a PAGE mobility shift assay (Figure 4). Whereas for 12 TAR PNA the association rate hardly altered between medium salt conditions (80 mM KCl) and transcription buffer conditions, the OMe and OMe/LNA oligonucleotides were 50-fold and 5-fold faster binding, respectively. Similarly, k_{off} for 12 TAR PNA was almost unaffected by the change in buffer conditions, whereas under transcription conditions 12 TAR OMe dissociates 14-fold faster and 12 TAR 7×OMe/5×LNA 3-fold faster. Thus under transcription buffer conditions, the 12-mer OMe and the OMe/LNA chimeric oligonucleotides exhibit identical association rates and are 4-fold faster than the PNA 12-mer. Note that this result holds despite the PNA having three lysine residues attached to improve solubility and to aid RNA binding (*28*). Very few

a



b

Name	Sequence 5′-3′
12 TAR OMe FAM	CUC CCA GGC UCA-FAM
12 TAR OMe FAM 5′-Thio	Cp(S)Up(S)Cp(S) CCA GGC UCA-FAM
12 TAR OMe FAM 3′-Thio	CUC CCA GGCp(S)Up(S)Cp(S)A-FAM
12 TAR OMe FAM Alt-Thio	CUp(S)C Cp(S)CAp(S) GGp(S)C Up(S)CA-FAM
12 TAR 7xOMe/5xLNA FAM	**C**U**C** C**C**A GG**C** U**C**A-FAM
12 TAR OMe FAM mism	CUC C***GC A***GC UCA-FAM
12 TAR 7xOMe/5xLNA FAM mism	**C**U**C** C***GC A***G**C** U**C**A-FAM
12 TAR 7xOMe/5xLNA FAM scr	AU**C** GC**C** UG**C** **C**A**C**-FAM

FIGURE 5: 3′-FAM-labeled oligonucleotide analogues. Panel a: Chemical structure of the 6-carboxyfluorescein (FAM) linked to the 3′-end of 12 TAR OMe. Panel b: Names and sequences of the 3′-FAM oligomers used in in vitro transcription and cellular studies. mism = mismatched; scr = scrambled. Underlined and bold residues are 5-methyl C LNA; underlined and italic residues denote mismatched residues.

differences in dissociation rates are observed between the three 12-mers under these conditions.

Binding and Transcription Inhibition Properties of 3′-Carboxyfluorescein-Functionalized Anti-TAR Oligonucleotides. We now wished to determine the binding and transcription inhibition ability of OMe and OMe/LNA chimeric 12-mers that carry a 3′-carboxyfluorescein label. Our aim was to obtain oligomers capable of being tracked within cells in culture as well as able to inhibit intracellularly Tat-dependent *trans* activation. For this purpose, we recently designed a new and convenient method for synthesis of 3′-conjugates of oligonucleotides in high yield (*29*). In this case, the oligonucleotides are synthesized on a prefunctionalized solid support such that release from the support and deprotection under standard aqueous ammonia conditions produce oligonucleotides joined at the 3′-end to a carboxyfluorescein (or another) moiety through an isomerically pure L-homoserine linker (Figure 5a).

We therefore prepared both 12 TAR OMe and 12 TAR 7×OMe/5×LNA as 3′-(6-carboxyfluorescein) (FAM) derivatives (Figure 5b). In addition, three 12 TAR OMe FAM derivatives were synthesized with a number of phosphorothioate (PS) linkages, namely, three at the 5′-end (12 TAR OMe FAM 5′-thio), three at the 3′-end (12 TAR OMe FAM 3′-thio), or alternating (12 TAR OMe FAM Alt-thio). Alternating PS linkages have been suggested to significantly improve the nuclear activity of OMe oligonucleotides in modification of splicing pathways (*10*). We first determined how the FAM and PS modifications affected the ability of the oligonucleotides to bind to TAR and to interfere with transcription. At 20 mM KCl concentration, 12 TAR OMe FAM was 2−3-fold weaker in TAR 39 binding strength (Figure 6a) than for the same oligomer that lacked the FAM attachment (Figure 3c). The addition of three PS linkages at either end resulted in a further ca. 2-fold loss in binding strength whereas the alternating PS OMe oligomer was 3-fold destabilized (K_d 436 ± 46 nM). 12 TAR 7×OMe/5×LNA

a

12 TAR	50 mM Tris HCl, pH 7.4, 20 mM KCl	50 mM Tris HCl, pH7.4, 80 mM KCl	Transcription buffer, pH7.9
OMe FAM	146±26	45.4±4.2	9.1±1.5
OMe FAM 5′-Thio	243±27	70.1±4.1	9.2±2.4
OMe FAM 3′-Thio	251±52	46.7±5.3	6.6±1.5
OMe FAM Alt-Thio	436±46	110±9.7	23.2±2.3
7xOMe/5xLNA FAM	36.2±3.9	6.2±0.7	2.1±0.4

b

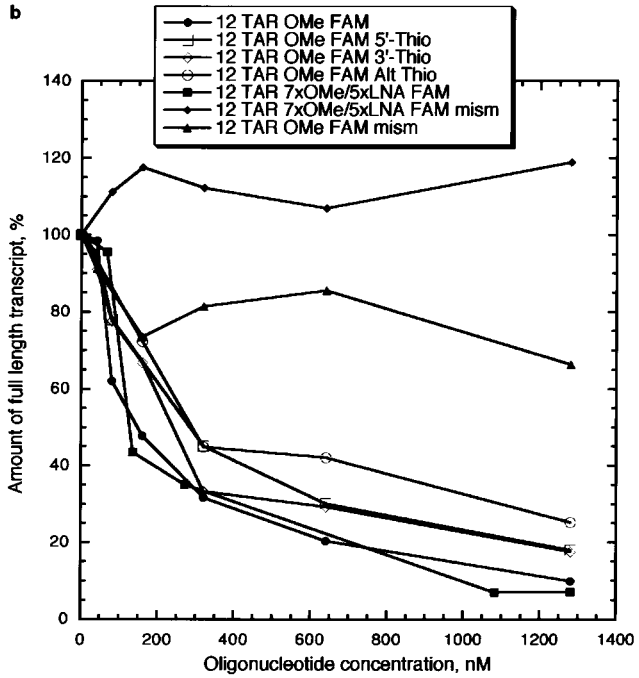


FIGURE 6: Binding and in vitro transcription data for 3′-FAM oligonucleotides. Panel a: Apparent dissociation constants (K_d, nM) for 3′-FAM oligomers under three buffer conditions. Panel b: Graph of the amount of full-length transcripts as a function of oligomer concentration (see inset for key).

FAM was only marginally poorer in binding TAR than its non-FAM counterpart. As the salt concentration was increased, the gain in binding strength was significant in all cases (Figure 6a) and varied in the 3−6-fold range. Further substantial gains in binding strength were apparent under transcription buffer conditions. 12 TAR OMe FAM and OMe oligonucleotides containing three PS linkages were similar in TAR binding strength (6.6−9.2 nM range) but 12 TAR OMe FAM Alt-thio was a little weaker (23.2 ± 2.3 nM). 12 TAR 7×OMe/5×LNA FAM was particularly strongly bound to TAR 39 under transcription buffer conditions (2.1 ± 0.4 nM). The results show that anti-TAR oligomers carrying 3′-FAM and/or three PS linkages maintain strong TAR binding under transcription buffer conditions.

We next measured the ability of the 3′-FAM oligomers to inhibit Tat-dependent in vitro transcription (Figure 6b). All five anti-TAR 3′-FAM oligomers were powerful inhibitors of in vitro transcription and showed a 50% inhibition of full-length transcript at 100−200 nM. Direct comparison of oligomers with and without 3′-FAM substitution showed that there was perhaps a very marginal reduction in effectiveness of the 3′-FAM oligomers, but this difference was not larger than the experimental inaccuracies (data not shown). We also assessed the sequence specificity of the inhibition. Thus 12 TAR OMe FAM and 12 TAR 7×OMe/5×LNA FAM oligomers containing a three-base central mismatch were synthesized (Figure 5b) and showed little or no inhibition

in the in vitro transcription assay (Figure 6b). This shows that the 3′-FAM oligomers retain sequence specificity in transcription inhibition. Further, no inhibition of 12 TAR OMe FAM or 12 TAR 7×OMe/5×LNA FAM was seen during in vitro transcription with a plasmid containing the control CMV promoter (data not shown).

Cellular Activity of Anti-TAR 12-mer Oligonucleotides. To assess the ability of anti-TAR oligonucleotides to inhibit Tat-dependent *trans* activation within cells, we utilized a HeLa cell line (HeLa Tet-Off/Tat/luc-f/luc-R) into which had been stably integrated three plasmids. The first contains the HIV-1 LTR (including the TAR region) fused to the gene for firefly luciferase (*Photinus pyralis*), the second the constitutive CMV promoter fused to the *Renilla* luciferase (*Renilla reniformis*), and the third contains the HIV-1 Tat gene under the control of the Tet-Off promoter. In these cells, Tat is expressed and is able to *trans*-activate the HIV-LTR leading to expression of firefly luciferase. The addition of the tetracycline analogue doxycyclin results in suppression of Tat production and concomitant reduction in firefly (but not *Renilla*) luciferase production. Similarly, inhibition of Tat-dependent *trans* activation would be expected to result in a reduction of firefly (but not *Renilla*) luciferase production. Since the HeLa cell line is a stable cointegrant, the *Renilla* luciferase level provides a control for nonspecific inhibition of transcription. The advantage of a cell line containing stably integrated plasmids is that inhibitors can be tested directly on cells without the need for plasmid cotransfection.

12 TAR OMe FAM oligonucleotide delivered by cationic lipid (Effectin 12) failed to show inhibition of firefly luciferase expression up to 1 μM concentration (Figure 7a). By contrast, 12 TAR 7×OMe/5×LNA FAM showed dose-dependent reduction in firefly luciferase expression, reaching about 75−80% suppression at 1 μM. The suppression achieved is similar to that seen when the HeLa cells are treated with doxycyclin and cultured for a further 48 h (data not shown). Mismatched and scrambled OMe/LNA chimeric oligonucleotides did not show inhibition of firefly luciferase expression (Figure 7a). No inhibitory trends could be observed for any of the oligonucleotide derivatives over the same concentration range in the level of expression of the control *Renilla* luciferase (Figure 7b), although the uncertainties in the measurement of *Renilla* luciferase levels are a little higher. Nor were toxic effects seen over this concentration range of oligonucleotide or cationic lipid using a standard cell toxicity assay (Figure 7c). It should be noted that each assay concentration shows data averaged over four replicates, and the complete experiment has been reproduced at least three times with identical results (data not shown).

OMe oligonucleotides containing PS linkages were also assayed in the double luciferase reporter assay. Neither 12 TAR OMe FAM 5′-thio nor 12 TAR OMe FAM 3′-thio showed dose-dependent inhibition of firefly luciferase expression (Figure 8a). Similarly, no significant inhibition was seen in the expression levels of the control *Renilla* luciferase (Figure 8b), neither did the oligonucleotides show cell toxicity at these concentrations (up to 1 μM) (Figure 8c). For 12 TAR OMe FAM Alt-thio, a partial reduction (30−40%) of firefly luciferase expression was seen (Figure 8a), but this was paralleled by a similar drop in expression of the control *Renilla* luciferase (Figure 8b) but without any effect on cell toxicity (Figure 8c). This suggests that this

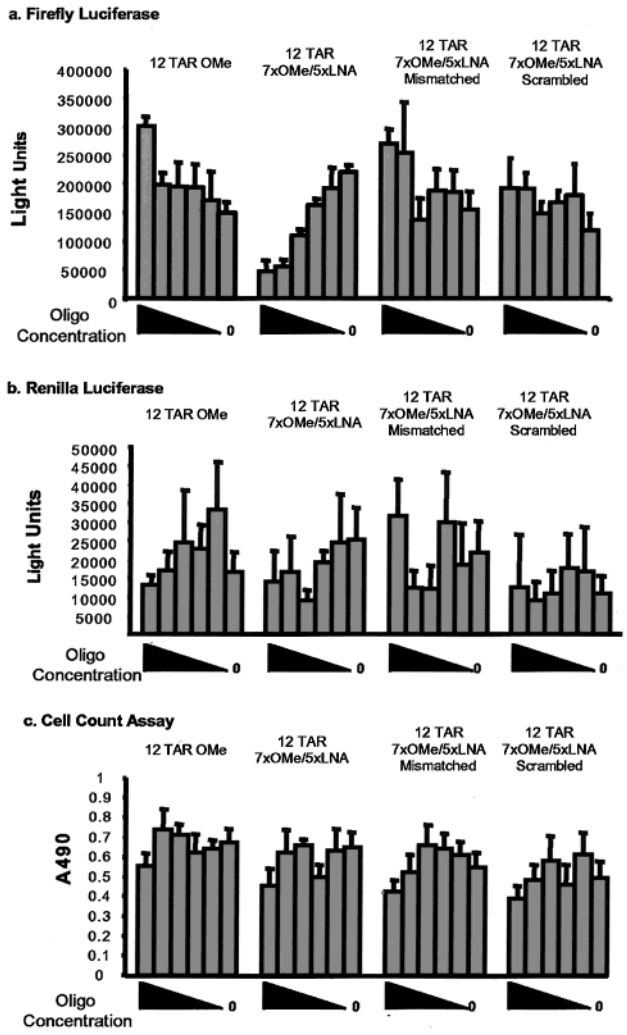


FIGURE 7: Effect of 3′-FAM oligonucleotide analogues delivered by the cationic lipid Effectin 12 on HeLa Tet-Off/Tat/luc-f/luc-R cells. 12 TAR OMe FAM, 12 TAR 7×OMe/5×LNA FAM antisense, mismatched, and scrambled oligonucleotide concentrations are shown as a black wedge from left to right: 1000, 500, 250, 125, 62.5, and 0 nM. Panel a: Levels of firefly luciferase. Panel b: Levels of *Renilla* luciferase. Panel c: Cell count.

activity was the result of a nonspecific effect on transcription or translation rather than specific Tat-dependent transcription inhibition.

DISCUSSION

The HIV-1 TAR RNA apical stem−loop is a particularly attractive target for drug design. First, it is the RNA partner within a complex involving HIV-1 Tat protein and host cellular factors involved in the essential mechanism of *trans* activation required for efficient full-length viral RNA production and hence viral replication (*15*−*17*). Second, there is a very high degree of sequence conservation of this region of HIV RNA. For example, mutations that disrupt the structure of the apical loop, stem, or bulge of TAR and interfere with either Tat binding or cellular factor action do not support *trans* activation (*35*, *39*). The TAR stem−loop is therefore ideal for exploration of the steric-block antisense approach to inhibition of virus-controlled gene expression.

In previous studies we verified the sequence specificity of strand invasion of TAR and showed efficient blockage of Tat binding in vitro by 2′-*O*-methyl oligoribonucleotides

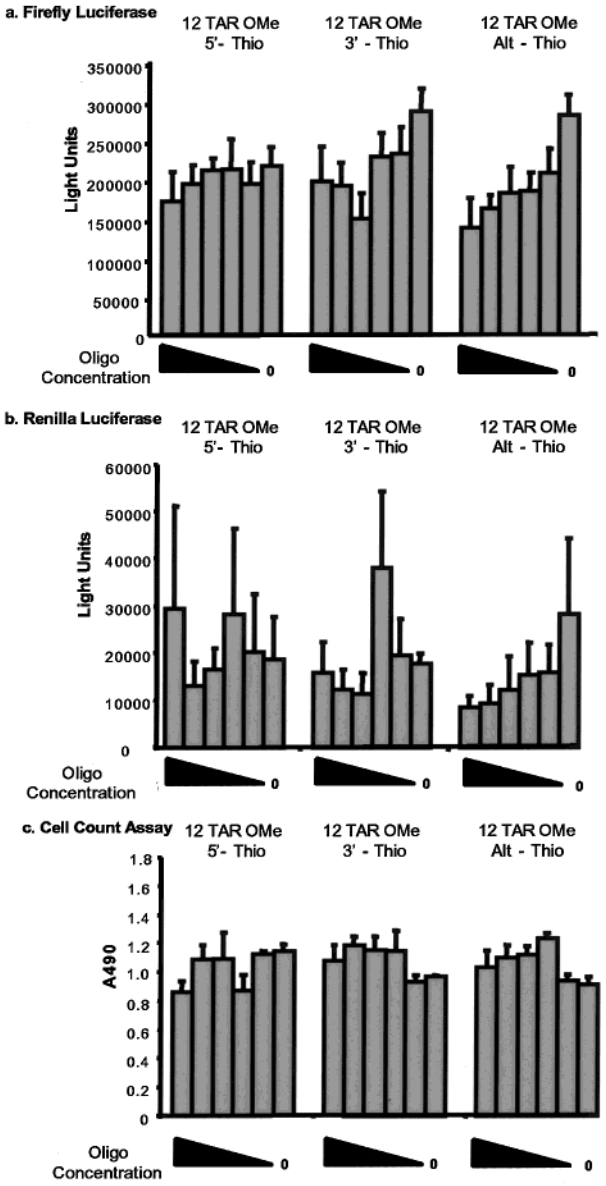


FIGURE 8: Effect of 3′-FAM PS oligonucleotide analogues delivered by the cationic lipid Effectin 12 on HeLa Tet-Off/Tat/luc-f/luc-R cells. 12 TAR OMe FAM 5′-thio, 12 TAR OMe FAM 3′-thio, and 12 TAR OMe FAM Alt-thio concentrations are shown as a black wedge from left to right: 1000, 500, 250, 125, 62.5, and 0 nM. Panel a: Levels of firefly luciferase. Panel b: Levels of *Renilla* luciferase. Panel c: Cell count.

(OMe) antisense to the apical stem−loop (*21*). More recently, we showed that OMe oligonucleotides are able to inhibit very effectively and sequence specifically Tat-dependent in vitro transcription directed by HeLa cell nuclear extract (*23*, *24*). Our current study has demonstrated that 12-residue oligonucleotide analogues of several types (OMe, OMe/LNA chimeras, and PNA) are equally efficient in inhibition of Tat-dependent in vitro transcription and that this inhibition is correlated with similar binding strengths under transcription buffer conditions. The results with 12 TAR PNA are in agreement with a previous study where 100 nM 15-mer anti-TAR PNA was found to inhibit Tat-dependent in vitro transcription, although the full concentration dependence was not shown (*25*). Our results show that antisense PNA is not unique, or more suited than other oligonucleotide analogues such as OMe or OMe/LNA chimeras, to sterically block transcriptional activation at the TAR RNA target, as was suggested previously (*25*).

The binding strengths of OMe, OMe/LNA, and PNA 12-residue oligomers (K_d in the low nanomolar range) would be expected to be sufficient in principle to be competitive with that of the Tat−TAR interaction and with other RNA binding proteins. 3′-FAM attachment and/or substitution with phosphorothioate linkages in OMe oligonucleotides were found to reduce binding strength a little (Figure 6a) but insufficiently to affect transcriptional inhibition (Figure 6b). Interestingly, a 12-mer containing 11 contiguous LNA residues was less effective both in binding TAR and in inhibition of Tat-dependent transcription than the 7×OMe/5×LNA chimera. This is surprising since an all LNA 9-mer was found recently to have a 10 °C higher T_m than a 6×OMe/3×LNA chimera when bound to complementary RNA in medium salt buffer (*40*). More detailed comparative studies of LNA and OMe/LNA chimeras will be needed in order to understand the RNA binding and strand invasion properties of such LNA-containing oligonucleotides, but the more flexible nature of the 7×OMe/5×LNA chimera compared to the 1×OMe/11×LNA may contribute to the more efficient TAR RNA binding of the former.

From the association and dissociation kinetics of OMe, OMe/LNA, and PNA 12-mers, it is clear that PNA is a little slower to associate under transcription buffer conditions. However, at 200 nM concentration, the association rates are all in the millisecond range while dissociation rates are of the order of hundreds of seconds. Such kinetics would be thought to be favorable in principle for competition with transcription, and indeed dose-dependent inhibition of transcription was observed in our assay system. It should be noted that the levels of inhibition (50% at 100−200 nM) seen for the three types of oligonucleotide analogue, and also for 3′-FAM and PS derivatives, are less than might be expected from their apparent binding constants under the same conditions (a few nanomolar). This may reflect limitations due to additional binding competition with proteins present in HeLa cell nuclear extract or to the possibility of more rapid dissociation through displacement of the oligomer by the transcription machinery. Such possibilities will require further more detailed evaluation.

3′-FAM and phosphorothioate substitution of OMe oligonucleotides affected binding constants to TAR 39 RNA a little, but binding was relatively strong (<10 nM) under transcription conditions. 3′-FAM OMe, OMe phosphorothioate, and OMe/LNA chimeric oligonucleotides were all effective inhibitors of Tat-dependent in vitro transcription at levels very similar to the parent oligonucleotide derivatives. This is particularly valuable information since modifications that are in principle destabilizing to duplex formation (such as phosphorothioate) are shown to be tolerated readily under the buffer conditions of in vitro transcription, ionic conditions which may not be too dissimilar to those found within cells.

For cellular studies, we chose a reporter assay for Tat-dependent *trans* activation that is very powerful and informative. First, the HeLa cell line is a clonal cointegrant that does not require transformation by plasmids. All cells therefore would be expected to express the same levels of both firefly luciferase (driven by the HIV-1 LTR) as well as *Renilla* luciferase (driven by the constitutive CMV promoter).

Measurements of the levels of each luciferase are carried out by simple sequential luminescence assay on the same sample. Inhibition of Tat-dependent *trans* activation is reflected in lower levels of firefly luciferase, whereas the level of *Renilla* luciferase acts as an effective internal control for inhibition of general transcription. Such a system is more reproducible and suffers less cell-to-cell variation of expression levels than one involving transient cotransfection of reporter and control plasmids. It is therefore ideally suited to testing of *trans*-activation inhibitors and in distinguishing nonspecific transcription inhibition effects.

One of the 12-mer oligonucleotides tested (12 TAR 7×OMe/5×LNA FAM) showed substantial, dose-dependent and selective inhibition of firefly luciferase in the concentration range investigated (0−1000 nM) when delivered to the HeLa reporter cell line by cationic lipid. This is significant in that, to our knowledge, this is the shortest steric block oligonucleotide analogue to show inhibition of mRNA transcription/translation within cells. No activity was observed for 12 TAR OMe FAM in this concentration range. Preliminary fluorescence studies by fluorescence microscopy suggest that the 12 TAR 7×OMe/5×LNA FAM oligomer was more strongly taken up by HeLa cells with enhanced fluorescence in both nucleus and cytosol compared to 12 TAR OMe FAM (data not shown). A detailed confocal microscopic evaluation, beyond the scope of this current work, will be necessary to study the uptake and compartmentalization of these oligomers.

Only one study of oligonucleotides containing LNA (in this case a 15-mer LNA/DNA hybrid) with activity within cells has been published to date (*27*). Our study is therefore the first report of a LNA/OMe hybrid with cellular activity. It should be noted that neither OMe nor OMe/LNA chimeric oligomer, in the Effectin 12 cationic lipid formulation, showed cell toxicity at these concentrations (Figure 7c). Further, we found that both were entirely stable for 24 h when incubated under transcription conditions that included HeLa cell nuclear extract (data not shown).

It is also interesting that addition of three PS linkages at either end of the OMe 12-mer failed to enhance activity but that addition of five alternating PS linkages did result in some inhibition of both luciferase activities, indicating the likelihood of a nonspecific effect on inhibition of transcription. This could perhaps be due to binding to proteins involved in transcription.

A 13-mer OMe oligonucleotide containing PS end caps has been reported to show significant cellular activity against telomerase RNA when delivered by cationic lipid (*41*). Similarly, an 18-mer OMe oligonucleotide with PS end caps has recently shown antisense activity in human colon cancer cells against the 5′-upstream regulatory element of the gene for thymidylate synthase, again delivered by cationic lipid (*42*). Further, cationic lipid-delivered 20-mer PS OMe oligomers showed some activity at >50 nM concentrations against the 5′-UTR of ICAM-1 mRNA in HUVEC cells, but 2′-*O*-methoxyethyl PO and PS oligomers were considerably more active (*6*). Alternating PO/PS OMe oligonucleotides of 18 residues were also effective in HeLa cells in modification of splicing pathways when delivered either by cationic lipid or by scrape loading (*10*). Thus OMe oligonucleotides as partial or full PS derivatives are in principle perfectly capable of nuclear activity within cells. Whether the lack of selective action of 12 TAR OMe FAM PS-containing oligonucleotides is due to the much shorter 12-mer length or due to cell uptake and compartmentalization problems remains to be defined.

We have not as yet tested 12-mer PNA oligomers in our system. PNA cannot be delivered by cationic lipid unless hybridized to a complementary section of DNA (*43*). Electroporation of a 15-mer anti-TAR PNA into CEM cells transiently cotransfected with a plasmid containing CAT under control of the HIV-1 LTR was shown to cause inhibition of CAT production (*25*). The levels of activity cannot be compared directly with our results because of the different assay type, different cell line, transient versus stable transformant, and different delivery method, but we note that PNA concentrations used during the electroporation step appeared to be in the micromolar range (*25*).

In our case, we are extremely encouraged by the activity of 12 TAR 7×OMe/5×LNA FAM, which contains no PS linkages, in showing at the submicromolar concentration range selective inhibition of Tat-dependent *trans* activation, and hence inhibition of gene expression, as well as the absence of toxic effects. Such OMe/LNA chimeric oligonucleotides deserve further evaluation as sequence-specific gene regulation agents that operate within cells through a steric block mechanism.

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