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Tricyclo-DNA Containing Oligonucleotides as Steric Block Inhibitors of Human Immunodeficiency Virus Type 1 Tat-Dependent *Trans*-Activation and HIV-1 Infectivity

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ABSTRACT

Replication of human immunodeficiency virus type 1 (HIV-1) is controlled by a variety of viral and host proteins. The viral protein Tat acts in concert with host cellular factors to stimulate transcriptional elongation from the viral long terminal repeat (LTR) through a specific interaction with a 59-residue stem–loop RNA known as the *trans*-activation responsive element (TAR). Inhibitors of Tat-TAR recognition are expected to block transcription and suppress HIV-1 replication. In previous studies, we showed that 2′-*O*-methyl (OMe) oligonucleotide mixmers containing locked nucleic acid (LNA) residues are powerful steric block inhibitors of Tat-dependent *trans*-activation in a HeLa cell reporter system. Here we compare OMe/LNA mixmer oligonucleotides with oligonucleotides containing tricyclo-DNAs and their mixmers with OMe residues in four different assays: (1) binding to the target TAR RNA, (2) Tat-dependent *in vitro* transcription from an HIV-1 DNA template directed by HeLa cell nuclear extract, (3) *trans*-activation inhibition in HeLa cells containing a stably integrated firefly luciferase reporter gene under HIV-1 LTR control, and (4) an anti-HIV β-galactosidase reporter assay of viral infection. Although tricyclo-DNA oligonucleotides bound TAR RNA more weakly, they were as good as OMe/LNA oligonucleotides in suppressing *in vitro* transcription and *trans*-activation in HeLa cells when delivered by cationic lipid. No inhibition of *in vitro* transcription and *trans*-activation in HeLa cells was observed for tricyclo-DNA/OMe mixmers, even though their affinities to TAR RNA were strong and their cell distributions did not differ from oligonucleotides containing all or predominantly tricyclo-DNA residues. Tricyclo-DNA 16-mer showed sequence-specific inhibition of β-galactosidase expression in an anti-HIV HeLa cell reporter assay.

INTRODUCTION

TRANSCRIPTION of the human immunodeficiency virus type 1 (HIV-1) genome is activated by a mechanism that involves the interaction of the viral protein Tat with the *trans*-activation responsive element (TAR) RNA, which is found at the 5′-leader sequence of the HIV-1 mRNA (for a recent review, see Brady and Kashanchi, 2005). Tat promotes the binding of the positive transcriptional elongation factor P-TEFb, which is composed of two proteins, cyclin T1 and the kinase cdk9. The formation of a quaternary complex between Tat, P-TEFb and

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TAR leads to an increase in the stability and processivity of RNA Pol II and thus enhances substantially transcriptional elongation from the HIV-1 long terminal repeat (LTR) promoter. The high degree of conservation of the RNA sequence of the apical region of TAR makes this a promising target for anti-HIV drug development. Over the last several years, a number of peptides, peptidomimetics, aminoglycoside antibiotics, and nonpeptidic small molecules of different types have been suggested as Tat-competitive binders to TAR RNA (for recent reviews, see Krebs et al., 2003; Richter and Palu, 2006). However, none of these compounds has proved suitable for clinical development.

Synthetic oligonucleotides and their analogs that are complementary to the apical region of TAR RNA are obvious alternative inhibitors of Tat-mediated *trans*-activation (for review, see Fabani et al., 2006; Richter and Palu, 2006; Turner et al., 2006). Since the potential of oligodeoxynucleotides to inhibit viral replication in cell culture was discovered (Zamecnik and Stephenson, 1978), numerous approaches for targeting of HIV-1 TAR RNA have emerged following distinct mechanistic pathways. Thus, anti-TAR antisense oligodeoxynucleotides with diester or phosphorothioate linkages were designed to activate RNase H and cleave the RNA moiety of a DNA·RNA heteroduplex (Vickers et al., 1991; Boulmé et al., 1997). Other strategies involving cleavage of RNA use oligonucleotide modifications that either form small ribozymes when bound to the target (Bramlage et al., 1998), or are linked to a ribozyme domain (Puerta-Fernández et al., 2003). Furthermore, the discovery of the phenomenon of RNA interference (RNAi) increased the impact of the oligonucleotide field in antiviral therapy (for a recent review, see Morris and Rossi, 2006). Small interfering (si) RNA targeted against TAR RNA has been shown to successfully suppress HIV-1 replication (Jacque et al., 2002), but more recently TAR was found to be a relatively unfavorable target for siRNA (Yoshinari et al., 2004). Recently, nuclease-resistant modified oligonucleotide aptamers have been selected *in vitro* to bind tightly to the TAR RNA and form a loop–loop kissing complex and block Tat binding and transcriptional elongation (Darfeuille et al., 2002a,b; Kolb et al., 2005).

Steric block oligonucleotides that are unable to induce RNase H cleavage have been used to inhibit viral replication (recently reviewed in Ivanova et al., 2006). We showed previously that 12-mer oligonucleotides containing all 2′-*O*-methyl (OMe) nucleotides, or a mixture of OMe and locked nucleic acid (LNA) monomers (Koshkin et al., 1998; Wahlestedt et al., 2000), or 12-mer peptide nucleic acid (PNA) prevented HIV-1 Tat binding to TAR RNA and inhibited Tat-dependent *in vitro* transcription (Arzumanov and Gait, 1999; Arzumanov et al., 2001a). Furthermore, we found that 12-mer or 16-mer steric-block OMe/LNA mixmer oligonucleotides complementary to TAR effectively inhibited Tat-dependent *trans*-activation when delivered by cationic lipids in a HeLa cell reporter assay (Arzumanov et al., 2001b, 2003). The 16-mer OMe/LNA mixmer targeted to TAR was found to inhibit dose-dependently and sequence-specifically syncitia formation induced by HIV-1 infection when delivered by cationic lipids into HeLa T4 LTR cells (Brown et al., 2006). PNA complementary to TAR and their peptide conjugates are also promising analogs that are capable of blocking reverse transcription and Tat-dependent *trans*-activation in reporter assays and also have shown anti-HIV activity (Kaushik et al., 2002a,b; Tripathi et al., 2005; Turner et al., 2005b).

By use of fluorescently labeled OMe/LNA oligonucleotides, we concluded that about 50% LNA units was needed to obtain both good cellular and nuclear uptake when packaged by cationic lipids (Arzumanov et al., 2003). Because the replacement of LNA units by isomeric α-LNA, which has completely different stereochemistry, did not reduce the *trans*-activation inhibitory activity, it seemed that the hydrophobicity of LNA monomers may help in cationic lipid packaging as well as directing the oligonucleotide to the nucleus. Recently, we showed that oligonucleotide analogs containing certain amino modifications: N3′-P5′-phosphoamidates, N3′-P5′-phosphothioamidates, OMe/2′-amino-LNA or OMe/2′-amino-LNA/2′-glycylamino-LNA mixmers targeted to HIV-1 TAR region could also inhibit *trans*-activation in our cell reporter system (Ivanova et al., 2005).

Another type of conformationally restricted oligonucleotide analog, tricyclo-DNA, has a rather hydrophobic sugar unit, elicits strong RNA-binding ability, and has good biostability (Steffens et al., 1999; Renneberg and Leumann, 2002). Lipofectamine-mediated delivery into HeLa cells of nanomolar concentrations of a 17-mer tricyclo-DNA complementary to the 3′-cryptic splice site of β-globin mRNA was shown to result in correction of aberrant splicing with up to 100-fold enhanced efficiency relative to the corresponding OMe-phosphorothioate RNA (Renneberg et al., 2002). Furthermore, in analysis by fluorescence microscopy, a stronger nuclear localization of 15-mer tricyclo-DNA compared with the corresponding all LNA oligonucleotide was seen following Lipofectamine-mediated transfection, which was suggested as a possible reason for the four to five times higher observed antisense efficacy of tricyclo-DNA (Ittig et al., 2004). However, mixmers of OMe/LNA were not studied in these assays, nor were mixmers of tricyclo-DNA with OMe units.

Here we show that 16-mer tricyclo-DNA oligonucleotides are able to inhibit Tat-dependent *trans*-activation in HeLa cells as effectively as OMe/LNA mixmers when delivered by Lipofectamine 2000, but tricyclo-DNA mixmers with OMe are ineffective. Neither TAR RNA-binding ability nor nuclear delivery ability, as

judged by confocal microscopy, was correlated with effectiveness at Tat-dependent *trans*-activation inhibition in HeLa cells. However there was good correlation with their ability to inhibit Tat-dependent transcription *in vitro*. A 16-mer tricyclo-DNA oligonucleotide showed sequence-specific inhibition of β-galactosidase expression in an anti-HIV reporter assay, and only a little less effectively than a 16-mer OMe/LNA mixmer.

MATERIALS AND METHODS

RNA, tricyclo-DNA, OMe/tricyclo-DNA, and OMe/LNA oligonucleotides

39-mer model TAR RNA (see Fig. 1A, below) and OMe/LNA mixmers containing a 3′-FAM label (Ic, IIc, see Table 1) were chemically synthesized as described previously (Mestre et al., 1999; Arzumanov et al., 2001a,b). The 3′-label was 3′-(6-fluorescein) CPG (Glen Research).

Tricyclo-DNA and OMe/tricyclo-DNA oligonucleotides (Ia, Ib, IIa, IIm, and IIb) were prepared on a 1.3 μmol scale on a Pharmacia Gene Assembler Special™ or a PerSeptive Biosystems Expedite DNA Synthesizer using the modified phosphoramidite chemistry procedure as reported previously (Renneberg and Leumann, 2002; Renneberg et al., 2002; Ittig et al., 2004). OMe phosphoramidites were obtained from Glen Research and LNA phosphoramidites were from Exiqon. All oligomers were synthesized with a 3′-fluorescein label using LC fluorescein CPG (Roche Diagnostics). Deprotection and cleavage from the solid support were carried out under standard conditions (conc. NH_3, 16 hours, 55°C). Crude oligonucleotides were purified by reversed phase high-performance liquid chromatography (HPLC). The molecular masses of all oligonucleotides were confirmed by ESI mass spectrometry.

Oligonucleotide-TAR RNA-binding assay

The binding affinities of the oligonucleotides to ^{32}P-labeled 39-mer TAR RNA in two different buffers were evaluated by a polyacrylamide gel mobility-shift assay as described previously (Arzumanov et al., 2001a,b). Apparent dissociation constants (K_D) shown (Table 2) are calculated as the average value from a minimum of three experiments.

Tat-dependent in vitro *transcription*

Tat-dependent *in vitro* transcription was carried out in the presence of oligonucleotide inhibitors using a DNA template, which is a fragment (−346 to +524) of plasmid p10SLT and carries the wild-type HIV-1 LTR (strain NL4-3) as described previously (Arzumanov et al., 2001b). The oligonucleotides were incubated with 10 nM of the template at 30°C for 20 minutes in a 40-μL reaction mixture containing 15 μL HeLa cell nuclear extract, 80 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES pH 7.9, 2 mM DTT, 10 μM $ZnSO_4$, 10 mM creatine phosphate, 100 $\mu g \cdot ml^{-1}$ creatine kinase, 1 μg poly[d(I-C)] (Roche, Nutley, NJ), 50 μM ATP, GTP, and CTP, 5 μM UTP (including [α-^{32}P]UTP, 10 μCi), 1 U $\cdot$ μl^{-1} RNasin (Promega, Madison, WI), and 200 ng recombinant Tat protein. The reaction products were resolved by 6% polyacrylamide gel electrophoresis (PAGE) with 7 M urea and detected by phosphoimaging.

Tet-off/Tat/luc-f/luc-R HeLa cell line

HeLa cells containing the stably integrated HIV-1 Tat gene under Tet-off promoter, firefly luciferase gene under the HIV-1 LTR, and the *Renilla* luciferase gene under cytomegalovirus (CMV) promoter, described previously (Arzumanov et al., 2001b), were grown in Dulbecco's-modified Eagle medium (DMEM) medium supplemented with 10% Tet System Approved fetal bovine serum (FBS) (Clontech, Palo Alto, CA), penicillin, and streptomycin at 37°C under 5% CO_2/95% air.

Tat-dependent trans*-activation in HeLa cells*

Tat-dependent *trans*-activation experiments in the presence of oligonucleotide inhibitors were carried out as

TABLE 1. OLIGONUCLEOTIDES USED IN THIS STUDY[a]

No.	*Oligonucleotide analog*	*Sequence 5′–3′*
Ia	12 TAR 1xOMe/11xTricyclo-DNA FAM	ctc cca ggc tcA-FAM
Ib	12 TAR 7xOMe/5xTricyclo-DNA FAM	cUc CcA GGc UcA-FAM
Ic	12 TAR 7xOMe/5xLNA FAM	CUC CCA GGC UCA-FAM
IIa	16 TAR Tricyclo-DNA FAM	ctc cca ggc tca gat c-FAM
IIm	16 TAR Tricyclo-DNA-mism FAM	ctc cca ccc tca cat c-FAM
IIb	16 TAR 10xOMe/6xTricyclo-DNA FAM	cUc CcA GGc UcA GAt C-FAM
IIc	16 TAR 10xOMe/6xLNA FAM	CUC CCA GGC UCA GATC-FAM

[a]Tricyclo-DNA residues are shown in lower case. Capitals show 2′-*O*-Me nucleotides. Underlined monomers are LNA nucleotides (5-methyl C or T).

TABLE 2. OLIGONUCLEOTIDE BINDING TO 39-MER TAR RNA

No.	*Oligonucleotide analog*	*Binding K_d (nM) TK-80*[a]	*Binding K_d (nM) transcription buffer*[b]
Ia	12 TAR 1xOMe/11xTricyclo-DNA FAM	131.7 ± 15.5	9.9 ± 3.5
Ib	12 TAR 7xOMe/5xTricyclo-DNA FAM	176.2 ± 33.9	23.2 ± 5.1
IIa	16 TAR Tricyclo-DNA FAM	65.8 ± 7.5	5.5 ± 0.6
IIm	16 TAR Tricyclo-DNA-mism FAM	n.b.[c]	n.b.[c]
IIb	16 TAR 10xOMe/6xTricyclo-DNA FAM	98.1 ± 4.9	4.5 ± 0.1
Ic	12 TAR 7xOMe/5xLNA FAM	5.7 ± 0.7[d]	1.1 ± 0.3[d]
IIc	16 TAR 10xOMe/6xLNA FAM	9.3 ± 0.4[d]	3.3 ± 0.8[d]

[a]TK-80: 50 mM Tris.HCl pH 7.4; 80 mM KCl.

[b]Transcription buffer: 20 mM HEPES, pH 7.9; 10 μM $ZnSO_4$; 2 mM DTT; 80 mM KCl; 3 mM $MgCl_2$; 10 mM creatine phosphate.

[c]No binding up to 10 μM concentration.

[d]Data published before (Arzumanov et al., 2003) and shown here for comparison.

described previously (Arzumanov et al., 2003). Cells were aliquoted in two identical 96-well plates at concentration of 10^4 cells per well and incubated at 37°C for 18 hours. One of the plates was used in the luciferase assay and the other in the cytotoxicity assay. Oligonucleotides were prepared at a concentration of 1 μM in serum-free Opti-MEM (Invitrogen, San Diego, CA). To each solution was added an equal volume of cationic lipid Lipofectamine 2000 in Opti-MEM (10 μl lipid per 1 mL medium). Complexes were formed at room temperature for 20 minutes. Subsequent dilutions were made from this oligonucleotide/lipid mixture and added to HeLa cells (100 μL of oligonucleotide/lipid dilution per well), which had been briefly washed previously with phosphate-buffered saline solution (PBS). After 3 hours of transfection, the medium was replaced with 100 μL of DMEM/10% fetal bovine serum (FBS) and cells were incubated for 18 hours at 37°C, after which the luciferase and cell toxicity levels were measured.

Luciferase assay

Cells were lysed and the lysates were analyzed using a Dual Luciferase Reporter Assay System (Promega). The relative light units for both firefly and *Renilla* luciferase were read on a Berthold Detection Systems Orion Microplate luminometer. Each data point was averaged over two replicates of at least three experiments.

Toxicity assay

The proportion of live cells was determined by absorbance measurement at 490 nm using a Molecular Devices Emax microplate Reader (Menlo Park, CA) after treatment with Cell Titer 96 AQueous One Solution Assay (Promega). Each data point was averaged over two replicates of at least three experiments.

Confocal microscopy

Oligonucleotide cell distribution was detected using confocal microscopy, as described previously (Arzumanov et al., 2003). Hela cells were plated on eight-well Lab-Tek chambered coverglasses (Fisher Scientific, Pittsburgh, PA) in DMEM/10% FBS at 2×10^4 cells per well and incubated at 37°C overnight. After washing with PBS, cells were incubated with 200 μL of oligonucleotide/Lipofectamine 2000 complex in Opti-MEM for 3 hours and with 50 μL of 20 μg mL^{-1} hydroethidine in Opti-MEM for 30 minutes. The cell images were taken in Opti-MEM (without Phenol Red) medium with a Radiance 2100 confocal system using a Nikon Eclipse TE300 inverted microscope, a 40× Planapo objective, NA 1.3.A 60× Planapo, NA 1.4.A, and a 488-nm argon laserline to excite the fluorescein (FAM) label. An HQ 515/30 emission filter was used for observation of the green emission. Hydroethidine was excited with a 543-nm (green) HeNe laser and detected using a HQ 570LP (orange) emission filter. A dual fluorescence method was used with a differential interference contrast (DIC) transmission channel. The images in the three channels were viewed sequentially at approximately one frame per second with a scanning resolution of 512 × 512 pixels, and a Kalman average of 10 frames.

Inhibition of HIV infectivity in HeLa P4 cells

Infectivity was assayed on HeLa P4 cells expressing receptors CD4 and CXCR4 and carrying the stably integrated *lacZ* gene under the control of the HIV-1 LTR

(gift of J.L. Darlix, INSERM U412 ENS, Lyon) by a modification of a previously published method (Ventura et al., 1999). P4 cells were plated using 100 μL of DMEM plus 10% (vol/vol) FBS, 2 mM glutamine, 500 μg ml^{-1} of antibiotic G418 (Gibco-BRL) in two identical 96-well plates at 10,000 cells per well. One of the plates was used for the β-galactosidase activity and the other for the cytotoxicity assay. After incubation overnight at 37°C under 5% CO_2, the supernatant was discarded and the cells were washed with PBS.

For delivery by cationic lipid mixture, 1 μM oligonucleotide and 10 μg mL^{-1} Lipofectamine 2000 in DMEM medium was prepared according to the manufacturer's manual (Invitrogen). After 20 minutes at room temperature, subsequent dilutions were prepared from the oligonucleotide/Lipofectamine complex. HeLa P4 cells were incubated with this complex for 3 hours, washed with PBS, and incubated in DMEM/10% FBS. After incubation for 18 hours at 37°C under 5% CO_2, the supernatant was discarded and 200 μL of fresh medium containing HIV-1_{LAI} was added. Then 24 hours later, the supernatant was discarded and the wells were washed three times with 200 μL of 9% NaCl solution. Each well was refilled with 200 μL of a reaction buffer containing 50 mM Tris-HCl pH 8.0, 100 mM β-mercaptoethanol, 0.05% (vol/vol) Triton X-100, and 5 mM 4-methylumbelliferyl-β-D-galactosidase (4-MUG). After 3 hours at 37°C, the reaction products were measured in a fluorescence microplate reader (Cytofluor II) at 405 nm.

For the toxicity assay, CellTiter 96 AQ_{ueous} One Solution Assay (Promega) was used. The absorbance at 490 nm was read using a Dynex MRX model 96 Well Microplate Reader (Dynex Technologies). Each data point was averaged over two replicates of three separate experiments. The β-galactosidase activities results were normalized to the absorbance data from the toxicity assay, which reflects the amount of live cells.

RESULTS

Binding of Tricyclo-DNA containing oligonucleotides to 39-mer TAR RNA

To prevent the Tat-TAR interaction and have the potential for *trans*-activation inhibition, steric block oligonucleotides containing nucleotide analogues (Fig. 1B–D) must be able to bind specifically and strongly to the target TAR RNA. We chemically synthesized a 12-mer containing 11 tricyclo-DNA units and a single OMe at the 3′-end (Ia, Table 1) and a 16-mer with all tricyclo-DNA units (IIa), as well as a 12-mer mixmer with 7× OMe and 5× tricyclo-DNA units (Ib) and a 16-mer with 6× tricyclo-DNA units and 10× OMe units (IIb), complementary to the apical region of a 39-mer model TAR RNA (Fig. 1A; Mestre et al., 1999). All oligonucleotides contained a 3′-FAM label for cell uptake studies. We measured the ability of these oligonucleotides to bind *in vitro* to a 39-mer model TAR RNA at 37°C in either of two buffers, one with a moderate salt concentration and the second with much higher salt concentration and other additives used *in vitro* transcription assays.

The results (Table 2) show that the apparent dissociation constants of oligonucleotides containing tricyclo-DNA are higher than for the corresponding 12-mer-FAM and 16-

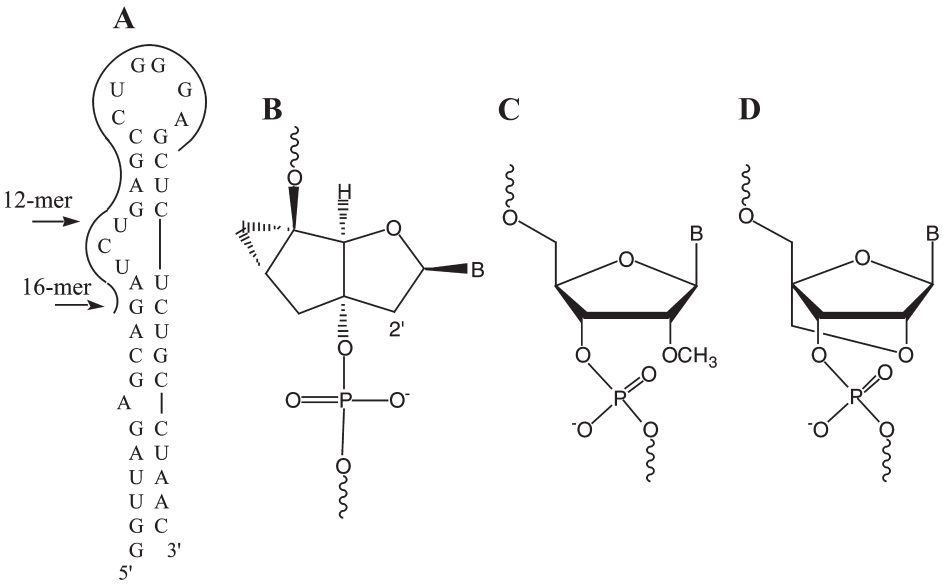

FIG. 1. (**A**) HIV-1 39-mer model TAR RNA stem–loop structure and the binding sites for steric block oligonucleotides with different length containing nucleosides of three types: tricyclo-DNA (**B**), 2′-*O*-Me (**C**), and LNA (**D**).

mer OMe/LNA mixmers from our previous work (Arzumanov et al., 2003). 16-mer tricyclo-DNA containing oligomers had stronger binding affinity to the target than similar 12-mers in both buffers, whereas with OMe/LNA mixmers this is reversed (Table 2 and Arzumanov et al., 2003). The binding is sequence-specific, since mismatched tricyclo-DNA 16-mer IIm did not bind to the TAR RNA in concentrations up to 10 μM. A scrambled tricyclo-DNA 16-mer (ATCGCTCGCACCATGC-FAM) also failed to bind TAR RNA (data not shown).

The 12-mer mixmer Ib showed generally weaker binding than corresponding oligonucleotide containing mostly tricyclo-DNA residues Ia, but the 16-mer mixmer IIb in transcription buffer had similar K_D value to the all tricyclo-DNA 16-mer IIa. The binding to TAR RNA is stronger for all oligonucleotides tested in transcription buffer than for TK buffer, as was observed previously for OMe/LNA mixmers (Arzumanov et al., 2001b, 2003). Under these conditions, all of the exactly matched tricyclo-DNA containing oligonucleotides have sufficiently strong binding (K_D 4.5 to 23.2 nM) for them to be suitable for consideration as steric block agents.

Tat-dependent in vitro *transcription*

A good steric block oligonucleotide inhibitor must be able to compete effectively for the target TAR RNA during active transcription. This is a more rigorous dynamic test than equilibrium binding to TAR. Thus, we tested the ability of tricyclo-DNA containing oligonucleotides to inhibit Tat-dependent transcription *in vitro* from a HIV-1 DNA template containing the TAR sequence as directed by HeLa cell nuclear extract (Fig. 2). A representative PAGE analysis (Fig. 2A) shows the levels of ^{32}P-labeled transcription run-off products. In the control lanes 1 and 2, the Tat plus and Tat minus band intensities are seen, and this shows the transcription enhancement due to the presence of Tat. When 16 TAR tricyclo-DNA IIa was added in the presence of Tat, a transcription reduction was seen as the oligonucleotide concentration was increased (Fig. 2A, lanes 3–7). Furthermore, the inhibition is sequence specific, since the mismatched 16 TAR tricyclo-DNA IIm showed no inhibitory activity (Fig. 2A, lanes 8–12). Surprisingly, among the rest of oligonucleotides tested (Table 1), only 12 TAR tricyclo-DNA Ia showed inhibitory activity (Fig. 2B), and there was no effect of either of the OMe mixmers containing tricyclo-DNA (Ib or IIb). The inhibition levels observed for both active sequences Ia and IIa are quite similar. They are slightly higher than those we obtained and previously published for 16-mer OMe/LNA IIc and slightly lower than those for 12-mer OMe/LNA Ic (Arzumanov et al., 2003).

Tat-dependent trans*-activation in HeLa cells*

Because HIV-1 Tat-dependent *trans*-activation occurs in the cell nucleus, the steric block oligonucleotide must

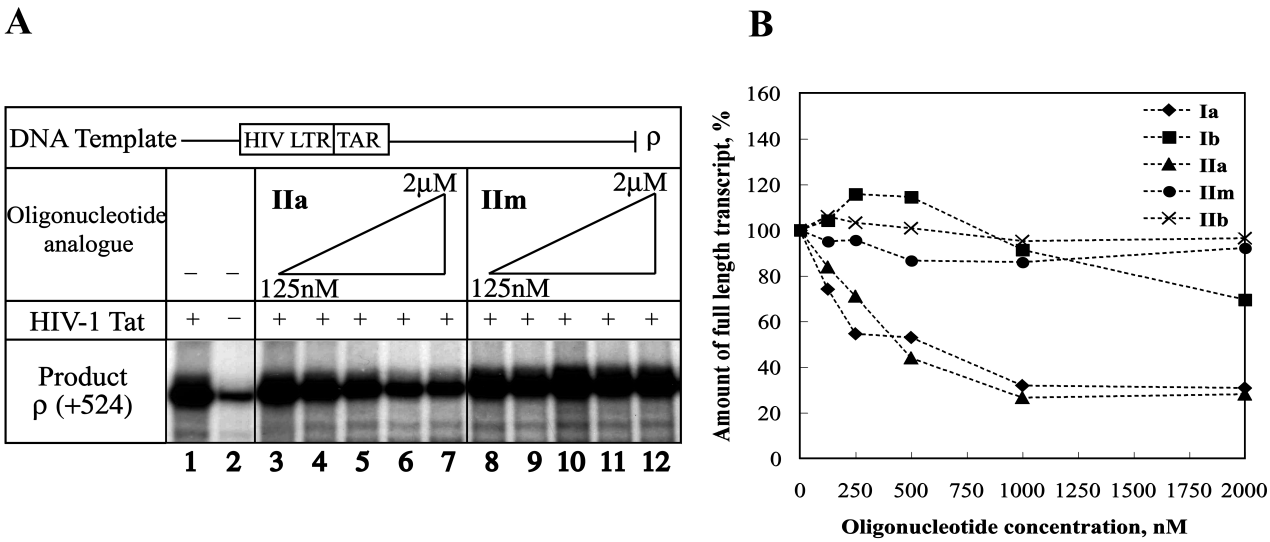

FIG. 2. Inhibition of *in vitro* transcription from an HIV-1 DNA template, which contains the TAR sequence, by steric block oligonucleotides. (**A**) A typical autoradiograph showing the transcription run-off product in the presence (lane 1) or absence (lane 2) of HIV-1 Tat protein, and the effect of increasing concentration (0.125, 0.25, 0.5, 1, and 2 μM) of oligonucleotides in the presence of Tat (IIa, lanes 3–7 or IIm, lanes 8–12). (**B**). The amount of transcript product is shown as a plot of the intensity of the corresponding band in the autoradiograph (**A**) against oligonucleotide concentration. Data presented were averaged over at least three experiments, but error bars are not shown for clarity. The standard deviation for each data point was less than 30% of the averaged value.

be able to enter the nucleus and bind to the TAR RNA to prevent docking of Tat and host cellular factors that trigger transcriptional elongation. The nuclear inhibition activity of the oligonucleotides was tested in a double-luciferase HeLa cell reporter system (Arzumanov et al., 2001b, 2003). The HeLa cells contain three stably integrated plasmids, one expressing the HIV-1 Tat under Tet-off promoter control, the second expressing firefly luciferase under HIV-1 LTR control, and the third expressing *Renilla* luciferase under CMV promoter control. Specific knockdown of firefly luciferase expression is obtained only if the oligonucleotide is taken up by a large majority of cells and is able to inhibit the Tat-dependent transcription from the HIV-1 LTR by steric blocking of the TAR RNA target during the assay period. There should be no effect on the *Renilla* luciferase expression, which is a control for nonspecific inhibition of protein synthesis.

We tested all oligonucleotides (Table 1) up to 500 nM concentration in this cell assay with cationic lipid–mediated delivery for 3 hours, followed by cell growth for 18 hours in the absence of oligonucleotide challenge. Two alternative cationic lipids, Oligofectamine or Lipofectamine 2000, were used. Under these conditions, all oligonucleotides showed >80% cell viability. Dose-dependent knockdown of firefly luciferase expression was observed with both cationic lipids for the sequences containing mostly tricyclo-DNA monomers Ia and IIa and the levels of inhibition were similar to these for the OMe/LNA controls with the same length Ic and IIc (Fig. 3A, Lipofectamine 2000 data shown). Activity levels when oligonucleotides were delivered with Oligofectamine were a little lower (data not shown). Neither of the mixmers Ib or IIb was active with either of the cationic lipids. The mismatched 16–mer IIm did not inhibit Tat-dependent *trans*-activation.

The *Renilla* luciferase expression level was affected only in the case of 12–mer Ia (Fig. 3B). However 16 TAR tricyclo-DNA IIa did not show any effect on the *Renilla* luciferase levels, which confirms the sequence-specific cellular activity. Both 12–mer Ia and 16–mer IIa were tested in the cell assay with unassisted delivery for 24 hours, but neither showed any firefly luciferase inhibitory activity up to 10 μM concentration (data not shown), which is consistent with previous observations in other assay systems (Ittig et al., 2004).

Note that some increases in luciferase fluorescence for particular constructs are occasionally observed as concentration of oligonucleotide/cationic lipid is increased (Fig. 3), reflecting the sensitivity of the assay to additives in cell growth conditions, as previously observed (Arzumanov et al., 2001b, 2003; Turner et al., 2005a,b). A significant reduction in *Renilla* luciferase expression is expected if there is any nonspecific transcription/translation suppressive effect, which is only seen in the case of oligonucleotide Ia. Thus, in the case of Ia, the firefly luciferase reduction level may be partly due to a nonspecific effect. We do not have an explanation for the nonspecific effect seen here, which in our experience is rare in this assay for oligonucleotides not containing phosphorothioate linkages (Arzumanov et al., 2001b, 2003).

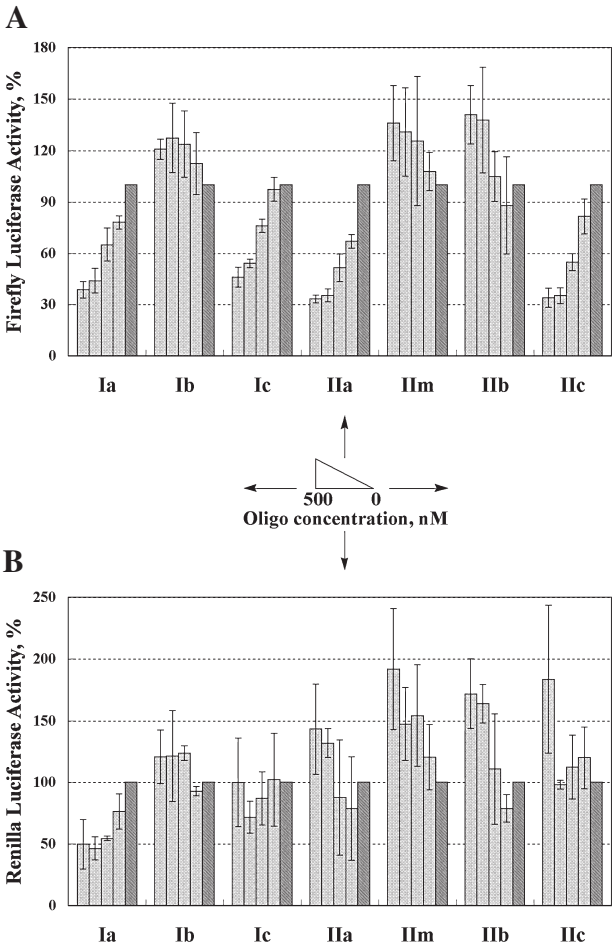

FIG. 3. Inhibitory effect of steric block oligonucleotides delivered by cationic lipid Lipofectamine 2000 in the HeLa reporter assay. (**A**) Luminescence levels measured due to firefly luciferase activity (normalized to cell viability count) after cell incubation with oligonucleotides Ia, Ib, Ic, IIa, IIm, IIb, or IIc, shown as percentage of firefly luciferase luminescence of untreated cells (the last bar in each series). *Left to right:* oligonucleotide concentrations 500, 250, 125, 62.5, and 0 nM, respectively. (**B**) Luminescence levels measured due to *Renilla* luciferase activity. Note that in this assay the batch of control oligonucleotide IIc did not contain a 3′-fluorescein label, but this has already been shown to make no difference to the cellular activity of such oligonucleotides (Arzumanov et al., 2003).

Oligonucleotide uptake into HeLa cells

Because the steric block oligonucleotides were 3′-fluorescein-labeled, we were able to observe their cell uptake

by confocal microscopy. All newly synthesized oligonucleotides Ia–IIb (Table 1) were taken up well by most of the HeLa cells treated when delivered with Lipofectamine 2000 for 3 hours (Fig. 4), but none of them was taken into the cells when incubated in the absence of cationic lipids, even after 24 hours (data not shown). The confocal microscopy images of the cells transfected with 16-mers tricyclo-DNA IIa, OMe/tricyclo-DNA mixmer IIb, and OMe/LNA mixmer IIc in the presence of cationic lipid are shown in Fig. 4. Both tricyclo-DNA containing oligonucleotides IIa and IIb (Fig. 4A,B) showed strong nuclear uptake with some remaining fluorescence in cytosolic compartments, even though the mixmer IIb did not have any inhibitory activity in the HeLa cell reporter system (Fig. 3). This shows that the inactivity of mixmer IIb is not due to poor nuclear penetration.

Although previous studies showed a stronger nuclear uptake for 15–mer all-tricyclo-DNA compared with a 15–mer all LNA oligonucleotide (Ittig et al., 2004), no significant difference in distribution within the cells was found for the tricyclo-DNA containing oligonucleotide IIa and tricyclo-DNA/OMe mixmer IIb compared to the OMe/LNA mixmer IIc (Fig. 4C). These results show that both tricyclo-DNA and LNA units are capable of directing excellent cell uptake and nuclear entry in the presence of cationic lipids. Note that we have previously shown that a FAM-labeled all OMe 16-mer oligonucleotide was poorly taken up by HeLa cells under similar conditions and failed to show inhibition of Tat-dependent *trans*-activation (Arzumanov et al., 2003).

Inhibition of HIV infectivity in HeLa P4 cells

The most promising steric block oligonucleotide candidate 16 TAR tricyclo-DNA IIa and the corresponding OMe/LNA mixmer IIc were tested for inhibition of HIV-1 infectivity in a HeLa P4 cell line expressing receptors CD4 and CXCR4 and carrying the stably integrated *lacZ* gene under the control of the HIV-1 LTR (Ventura et al., 1999; Brown et al., 2006). The transfections were carried out with Lipofectamine 2000 assistance for 3 hours. At 18 hours later HIV-1_{LAI} was added and the cells were incubated for a further 24 hours. Dose-dependent knockdown of HIV-1-induced β-galactosidase expression was observed for 16–mer tricyclo-DNA IIa (Fig. 5) for concentrations up to 1 μM (25% inhibition), whereas no HIV-1 inhibitory activity was seen for the mismatched control IIm. A little higher inhibitory activity was measured for 16–mer OMe/LNA mixmer IIc (40% at 1 μM),

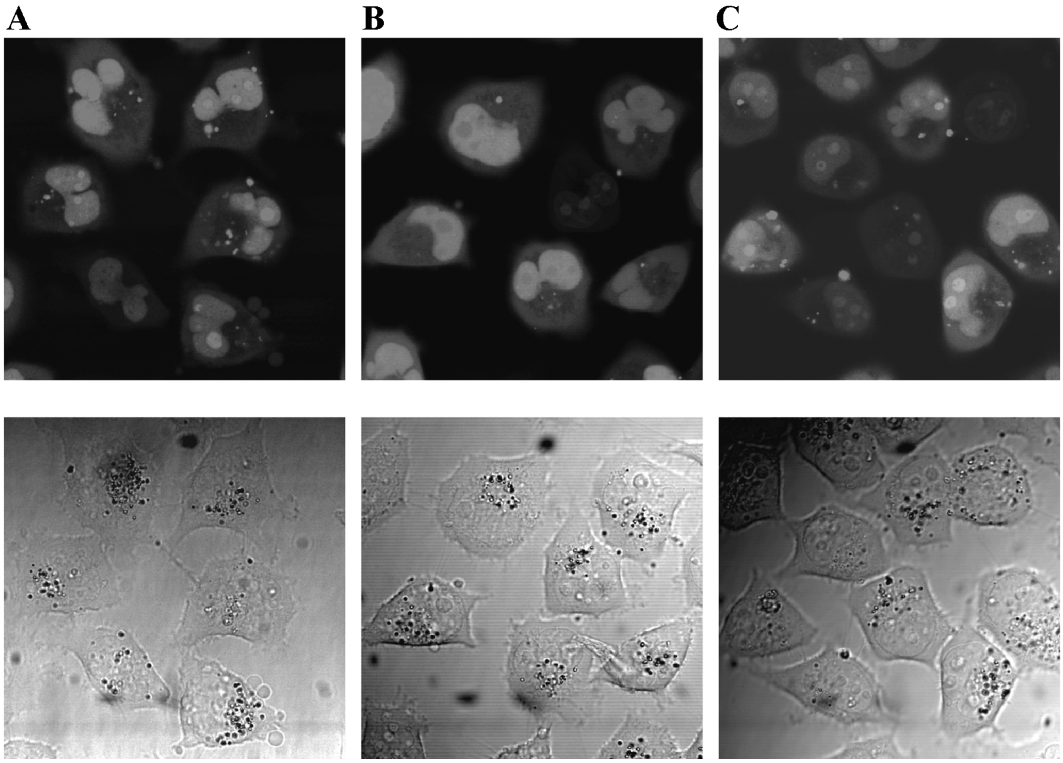

FIG. 4. Live HeLa cell uptake of 16 TAR tricyclo-DNA FAM IIa (**A**), 16 TAR 10xOMe/6xTricyclo-DNA FAM IIb (**B**), and 16 TAR 10xOMe/6xLNA FAM IIc (**C**) when delivered by Lipofectamine 2000. The cell nucleus is stained red (hydroethidine; see Materials and Methods). *Top panels:* The FAM-labeled oligonucleotides show a green fluorescence, seen here as white patches. *Bottom panels:* Differential interference contrast (DIC) transmission channel showing cell outlines.

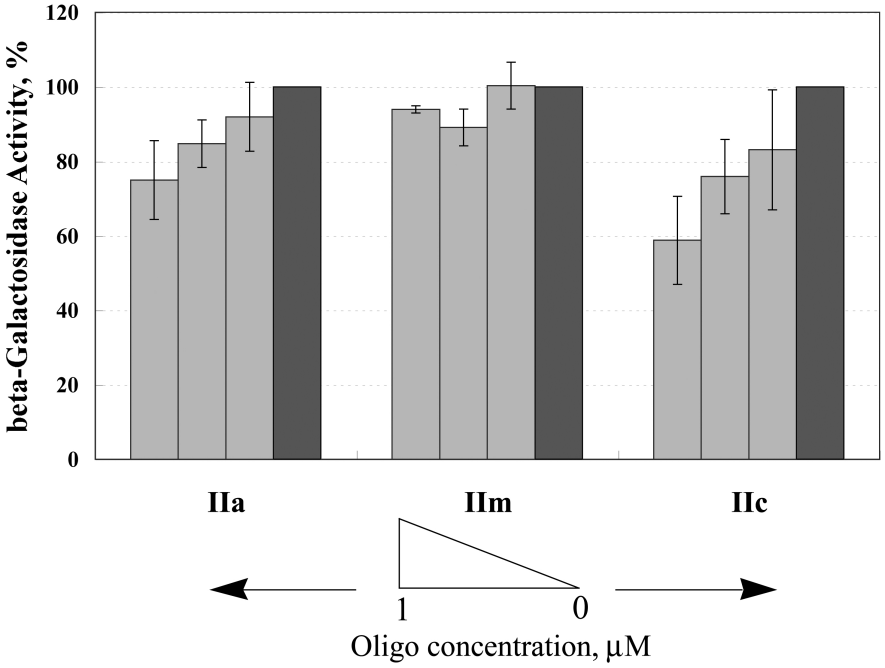

FIG. 5. Inhibition of HIV infectivity in HeLa P4 cells by 16 TAR tricyclo-DNA IIa, mismatched control IIm, and 16 TAR OMe/LNA IIc (without 3′-FAM) delivered with Lipofectamine 2000. Bars left to right represent β-galactosidase activity levels, normalized to cell viability, for oligonucleotide concentrations 1, 0.5, 0.25, and 0 μM, respectively

which is very similar to that obtained in an analogous anti-HIV-1 syncitia reduction assay for the same oligonucleotide from another recent study (Brown et al., 2006). No inhibitory effect was seen for a scrambled 16-mer OMe/LNA oligonucleotide (AUCGCUCGCACCATGC, LNA underlined, data not shown). These results show that tricyclo-DNA oligonucleotides can exhibit sequence-dependent antiviral activity in a similar way to OMe/LNA mixmers.

DISCUSSION

Prerequisites for therapeutic applications of antisense oligonucleotides are high binding affinity to the RNA target and good resistance to degradation by cellular nucleases. Several analog types fulfil these criteria. Especially attractive are analogs where the sugar moiety is conformationally restricted, such that preorganization of the structure of the oligonucleotide single strand entropically favors high stability upon duplex formation. Among the best such analogs are LNA (Koshkin et al., 1998), hexitol nucleic acids (HNA) (Van Aerschot et al., 1995), cyclohexene nucleic acids (CeNA) (Wang et al., 2000), bicyclo-DNA (Tarköy et al., 1994), and tricyclo-DNA (Steffens and Leumann, 1999). Tricyclo-DNA showed a very high affinity for RNA and an increased biostability compared with many other analog types.

The only previous comparative study of tricyclo-DNA was with all-LNA oligonucleotides (Ittig et al., 2004). Although LNAs were superior in binding RNA, tricyclo-DNA oligonucleotide 9-15 mers targeted against 3′ and 5′ splice sites of exon 4 of cyclophilin A pre-mRNA showed at least four to five times stronger nuclear antisense effects than corresponding all LNA oligonucleotides upon Lipofectamine-mediated transfection into HeLa cells. It was shown that tricyclo-DNA was more strongly taken up into the cell nucleus, perhaps due to a more hydrophobic backbone (Ittig et al., 2004). Previously, we reported that LNA mixmers with OMe monomers are highly effective in inhibition of HIV-1 Tat-dependent *trans*-activation in a HeLa cell double-luciferase reporter assay with cationic lipid delivery (Arzumanov et al., 2001b, 2003) and may have advantages over all-LNA oligonucleotides. Therefore, it was important to compare tricyclo-DNA with OMe/LNA mixmers and also to determine to what extent tricyclo-DNA could be mixed with OMe residues to perhaps enhance activity further.

Our results show that tricyclo-DNA 12-mer Ia with 11 tricyclo units and the all tricyclo-DNA16–mer IIa behave very similarly to the corresponding OMe/LNA mixmers Ic and IIc, respectively in: (1) ability to inhibit Tat-dependent *in vitro* transcription (Fig. 2), (2) ability to inhibit Tat-dependent *trans*-activation and thus firefly luciferase gene expression in our HeLa cell reporter system

with cationic lipid–mediated delivery (Fig. 3), and (3) ability to enter the cell nucleus (Fig. 4). These similarities occurred despite the fact that 12-mer tricyclo-DNA Ia showed weaker binding affinity to TAR RNA compared to 12-mer OMe/LNA oligomer Ic (Table 2), although the difference in K_D was less apparent for 16-mers IIa and IIc in transcription buffer.

By contrast, tricyclo-DNA mixmers with OMe units Ib and IIb had binding constants to TAR RNA in the same range as tricyclo-DNAs Ia and IIa (Table 1) and were taken into the cell nucleus (Fig. 4), but did not inhibit either Tat-dependent *in vitro* transcription (Fig. 2) or Tat-dependent *trans*-activation in the HeLa cell assay (Fig. 3). Thus, tricyclo-DNA mixmers with OMe must have very different properties to OMe/LNA mixmers within cells and are clearly unable to compete sufficiently for the TAR target in the presence of the active Tat-dependent transcription system, even though the equilibrium RNA binding strengths would have appeared to be perfectly sufficient. We also found that a mixmer containing tricyclo-DNA and LNA units was inactive in inhibition of Tat-dependent *in vitro* transcription and in the HeLa cell assay (data not shown). The results show that RNA binding strength and the ability to enter the cell nucleus when delivered by cationic lipid are not reliable enough predictors alone of nuclear steric block activity. In the case of the TAR target, the ability to inhibit Tat-dependent *in vitro* transcription is an additional requirement and a better predictor of cell activity.

For the Tat-TAR model, the ultimate goal is to select candidate steric block oligonucleotide types as potential antivirals. In this respect, the TAR 16-mer tricyclo-DNA appeared a little less active than 16-mer OMe/LNA oligonucleotide in the HeLa P4 cell β-galactosidase HIV reporter assay when transfected with cationic lipid (Fig. 5). The activity level of the 16-mer OMe/LNA oligonucleotide was very similar to that obtained in a syncitia reduction assay previously reported with the same lipofection agent (Brown et al., 2006). Recent studies of the anti-HIV activity of PNA-peptides targeted to TAR (Tripathi et al., 2005) suggest that inhibition of HIV infection may reflect more the ability to block reverse transcription of the HIV viral RNA at the TAR site within cytosolic compartments rather than inhibition of Tat-dependent gene expression from an integrated HIV provirus within the cell nucleus. The different levels of anti-HIV activity of tricyclo-DNA and OMe/LNA 16-mers we observed is consistent with this interpretation. We are currently extending our own studies of PNA–peptide conjugates targeted to TAR (Turner et al., 2005b) to address these issues and it will thus be of importance to compare tricyclo-DNA, OMe/LNA, and PNA in their abilities to block reverse transcription *in vitro*, in infected cells and in HIV virions, as well as other parameters such as cell delivery, to adequately assess their antiviral potential.

In summary, we have shown that a 16-mer all tricyclo-DNA oligonucleotide targeted to HIV-1 TAR region acts sequence specifically as an effective steric block inhibitor of Tat-dependent *trans*-activation *in vitro* and in Hela cells as well as of HIV infectivity when delivered with cationic lipid Lipofectamine 2000. Thus, our study confirms that tricyclo-DNA oligonucleotides are promising antisense agents with a potential for therapeutic applications.

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