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Inhibition of HIV-1 replication by anti-trans-activation responsive polyamide nucleotide analog

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Abstract

Efficient replication and gene expression of human immunodeficiency virus-1 (HIV-1) involves specific interaction of the viral protein Tat, with its trans-activation responsive element (TAR) which forms a highly stable stem-loop structure. We have earlier shown that a 15-mer polyamide nucleotide analog (PNA) targeted to the loop and bulge region of TAR blocks Tat-mediated transactivation of the HIV-1 LTR both in vitro and in cell culture (Mayhood et al., Biochemistry 39 (2000) 11532). In this communication, we have designed four anti-TAR PNAs of different length such that they either complement the entire loop and bulge region (PNA_{TAR-16} and PNA_{TAR-15}) or are short of few sequences in the loop (PNA_{TAR-13}) or in both the loop and bulge (PNA_{TAR-12}), and examined their functional efficacy in vitro as well as in HIV-1 infected cell cultures. All four anti-TAR PNAs showed strong affinity for TAR RNA, while their ability to block in vitro reverse transcription was influenced by their length. In marked contrast to PNA_{TAR-12} and PNA_{TAR-13}, the two longer PNA_{TARs} were able to efficiently sequester the targeted site on TAR RNA, thereby substantially inhibiting Tat-mediated transactivation of the HIV-1 LTR. Further, a substantial inhibition of virus production was noted with all the four anti-TAR PNA, with PNA_{TAR-16} exhibiting a dramatic reduction of HIV-1 production by nearly 99%. These results point to PNA_{TAR-16} as a potential anti-HIV agent.

Keywords: Tat-mediated transactivation; Polyamide nucleotide analog; Trans-activation response region; Antiviral; Reverse transcription; HIV-1 replication

1. Introduction

Acquired immune deficiency syndrome (AIDS) has reached worldwide epidemic proportions in spite of enormous efforts for the prevention of AIDS. The current anti-AIDS drugs mainly target two key enzymes in the HIV-1 life cycle, reverse transcriptase and protease. The major impediment in successful HIV-1 therapy is the rapid emergence of drug resistant strains harboring mutations in genes encoding these viral enzymes (Larder, 1995). This limitation has necessitated attempts world-wide to explore additional targets and strategies for blocking HIV-1 replication.

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HIV-1 gene expression is regulated through a complex interplay of specific cis-acting DNA elements within its long terminal repeat (LTR) with host cell proteins/factors as well as with its own accessory proteins (Bohan et al., 1992; Farese-DiGiorgio et al., 1999). One of these cis-acting elements called trans-activation response region (TAR) in the 5′-LTR of viral genome is essential for transcriptional activation by the transactivator protein Tat (Isel and Karn, 1999; Jeang et al., 1999; Veschambre et al., 1995). A three nucleotide U-rich bulge located between +23 and +25 has been identified as the site of Tat binding (Long and Crothers, 1999).

Intensive research over the last decade on the transactivation mechanism involving Tat-TAR interaction has yielded significant biological and virological insights (Katz and Skalka, 1994). It is now clear that the primary role of Tat is in regulating productive and processive transcription from the HIV-1 LTR (Cullen, 1993; Jeang et al., 1999; Veschambre et al., 1995). Natural or induced mutations that destabilize TAR by disrupting base pairing in the stem region abolish Tat-stimulated transcription resulting in premature transcription termination at random locations downstream of the viral RNA start site (Jeang et al., 1999; Selby et al., 1989). Given the functional importance of Tat-TAR interaction, both the Tat and TAR element represent attractive targets for drug design. A number of reports have suggested various chemicals, genetic inhibitors, Tat peptide analogs, TAR RNA decoys, TAR circle, TAR ribozyme, extra cellular anti-Tat monoclonal antibody and single-chain anti-Tat antibodies, among others, to sequester Tat's function, thereby reducing transcription and viral load. These agents affect the interaction between Tat and TAR, thereby preventing transcriptional activation of HIV-1 genome either by steric hindrance, sheer displacement mechanism or by deprivation of the functional molecules. Recently, it has also been shown that shielding the bulge-loop region of TAR with PNA and other oligo analogues in an anti-sense fashion inhibits HIV-1 reverse transcription (Boulme et al., 1998).

In a recent report, it has been shown that a 12-mer PNA and its analogues inhibit Tat-dependent transcription in HeLa cell nuclear extract (Arzumanov et al., 2001). These results are in agreement with our own earlier studies, where we demonstrated that a 15-mer polyamide nucleotide analog (PNA), targeted to the stem-loop region of HIV-1 TAR, effectively competes with Tat for TAR and prevents Tat-mediated transactivation in cell culture (Mayhood et al., 2000). We have now extended these studies, in order to identify the appropriate length of anti-TAR PNA for efficient blocking of Tat-mediated transactivation, using four anti-TAR PNAs of varying length ranging from 12- to 16-mer ($PNA_{TAR\text{-}12}$, $PNA_{TAR\text{-}13}$, $PNA_{TAR\text{-}15}$ and $PNA_{TAR\text{-}16}$). Using the luciferase reporter gene constructs, we now demonstrate that a 16-mer PNA complementing both the loop and bulge regions of TAR efficiently inhibits Tat-mediated transactivation of HIV-1 LTR. Further, transfection of this 16-mer PNA in HIV-1-infected CEM cells effectively blocks HIV-1 production, thus suggesting that anti-TAR PNA may be a potentially attractive candidate for antiviral therapy.

2. Materials and methods

2.1. PNA oligomers

The PNA oligomers targeted to TAR regions of HIV-1 genome as well as scrambled PNA were synthesized at Applied Biosystems Inc. (Fig. 1).

2.2. Plasmid constructs

The plasmid pEM-7 encoding the HIV-1 TAR under the control of the T7 promoter was used for transcribing the wild type TAR RNA (Gunnery et al., 1992) for gel shift analysis and primer extension studies. The plasmids, pHIV-1 LTR-Luc, pCMV-Tat (pcDNA3-Tat), pCMV-R.Luc and pcDNA3.1 were used in the transfection experiments to investigate the effect of PNA_{TAR} on HIV-1 LTR. The plasmid pHIV-1 LTR-Luc (a kind gift from Dr. M. B. Mathews) contains the firefly luciferase gene cloned downstream of the HIV-1 LTR. The plasmid pCMV-Tat, encodes for the Tat protein under the control of the CMV promoter

(Fujinaga et al., 1999). The plasmid pCMV-R.Luc (Promega Corp.) encodes for the Renilla Luciferase downstream of the CMV promoter and pcDNA3.1 (Invitrogen Corp.) encodes for the CMV promoter.

2.3. Transcription of HIV-1 TAR RNA template

HIV-1 TAR RNA template was transcribed after initially linearizing the plasmid pEM-7 with *Hin*dIII as described previously (Mayhood et al., 2000). For preparing the unlabeled transcript, in vitro transcription reaction was carried out using T7 RNA polymerase in accordance with the Manufacturer's protocol (Roche Molecular Biochemicals). The internally labeled transcript was similarly prepared except that the rNTP mixture contained 1 mM each of ATP, GTP, CTP and 20μM α-^{32}P UTP (specific activity: 1 μCi/10 pmol; Perkin–Elmer Life Sciences Inc.). Following the transcription reaction, 25 U of DNase I (RNase free) was added and further incubated for 30 min to digest the DNA. The labeled transcript was purified by 10% polyacrylamide-urea gel electrophoresis. The radioactive band was excised from the gel, extracted in 0.5 M ammonium acetate, desalted on a NAP-10 column (Pharmacia Inc), lyophilized, and dissolved in 10 mM Tris–HCl, pH 7.8, 60 mM KCl and 10 mM DTT and stored at −70 °C. The specific radioactivity of the resulting purified transcript was determined by A260 absorbance and Cerenkov counting.

2.4. Gel retardation assay

The affinity and specificity of the various anti-TAR PNAs for the TAR RNA was evaluated by gel mobility shift analysis. Varying concentrations of anti-TAR PNAs or scrambled PNA were incubated with 6.4 nM ^{32}P-labeled TAR RNA

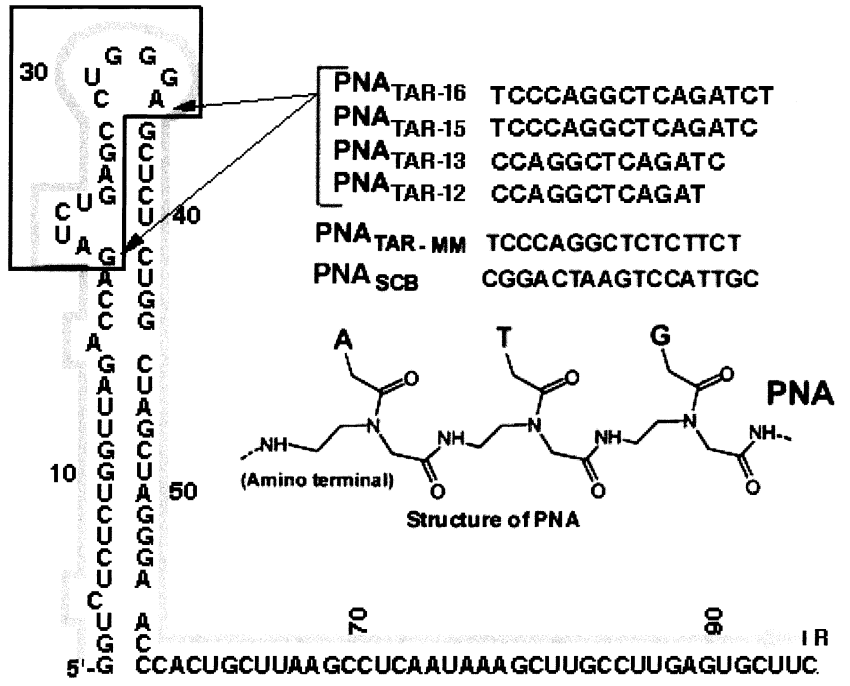

Fig. 1. General structure and sequence of PNA. The general structure of PNA, where bases are linked with peptide bonds, are shown (Nielsen, 1999). The sequence of the individual anti-TAR PNA (PNA_{TAR}) of varying lengths, the mismatched anti-TAR PNA ($PNA_{TAR\text{-}MM}$) as well as the 17-mer scrambled PNA (PNA_{SCB}) used in the experiments, are listed. Secondary structure of the HIV-1 TAR RNA genome corresponding to the RNA stem-loop and bulge, where the complementary anti-TAR PNA binds, as shown in the box.

transcript (5000 Cerenkov cpm) for 1 h at 37 °C in a binding buffer containing 50 mM Tris–HCl, pH 7.8, 60 mM KCl, 5.0 mM $MgCl_2$, 10 mM DTT, 10% glycerol, 0.01% NP-40 and 500 ng of r(I–C). Three microliters of RNA gel loading dye (0.27% bromophenol blue and 30% glycerol) were added to the samples and subjected to electrophoresis on a native 6% polyacrylamide gel in Tris–Borate buffer. The gels were pre-run at 120 V for 30 min at 4 °C in Tris–Borate buffer, pH 8.2. The RNA-PNA complexes were resolved at a constant voltage of 120 V at 4 °C for 3 h and subjected to phosphorImager analysis (Molecular Dynamics).

2.5. Reverse transcription of TAR RNA primed with 17-mer DNA primer

Reverse transcription catalyzed by HIV-1 RT on TAR RNA in the presence or absence of the individual anti-TAR PNA or scrambled PNA was monitored by gel extension analysis. To this end, the 17-mer DNA primer was 5′-labeled using α-^{32}P-ATP and T_4 polynucleotide kinase according to the standard protocol and annealed in a 2:1 molar ratio of RNA template to primer. The individual anti-TAR PNAs at the indicated concentrations were pre-incubated with 10 nM of the annealed template-primer either at 37 °C or at 25 °C for the indicated times in a reaction buffer containing 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 100 μg/ml BSA, 60 mM KCl and 5 mM $MgCl_2$ and used in the extension reaction. Reverse transcription was initiated by the addition of 50 nM of HIV-1 RT and 100 μM each of the 4 dNTP mix. The reactions were performed at 25 °C and terminated by the addition of equal volume of Sanger's gel loading solution (Sanger et al., 1977). The products were resolved on an 8% polyacrylamide–urea gel and visualized on a phosphorImager.

2.6. Tissue culture and transfection

Lymphocyte CEM (12D7) cells were maintained in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in 5% CO_2 containing humidified air. For transfections, the cells were grown to mid-log phase, washed with phosphate-buffered saline (PBS) without Ca^{+2} or Mg^{+2}, resuspended in unsupplemented RPMI-1640 medium (5.0×10^6 cells in 250 μl) and electroporated at 250 V and 900 microfarad capacitance with optimum amounts of the plasmids pHIV-1 LTR-Luc and pCMV-Tat, using a Bio-Rad Gene pulsar II. In order to monitor the efficiency of transfection, the cells were co-transfected with the reporter plasmid, pCMV-R.Luc. The effect of anti-TAR PNA on Tat-mediated transactivation of the HIV-1 LTR was monitored by co-transfecting the individual anti-TAR PNAs at the indicated concentrations. In order to determine the specificity of Tat-TAR interaction, a 17-mer control PNA containing scrambled sequence was co-transfected in an independent experiment. The transfected cells were plated in 10 ml of serum free RPMI-1640 media, allowed to recover from the effects of electroporation at 37 °C for 2 h and then grown in 10 ml of complete RPMI-1640 medium. Eighteen hours post-transfection, the cells were harvested and analyzed for luciferase activity. To monitor the effect of anti-TAR PNA on cell viability, an aliquot of the transfected cell culture was withdrawn prior to harvesting and examined using the calcein AM component from the Live-Dead viability kit (Molecular probes) as per the manufacturer's protocol.

2.7. Production of pseudotyped HIV

Pseudotyped HIV-1 virions were produced in 293T cells by co-transfection of pHIV-$_{1JR\text{-}CSF\text{-}luc}$env(−) (Planelles et al. 1995) with the pVSV-G retroviral vector, encoding the vesicular stomatitis virus protein G under the control of the CMV immediate-early promoter (BD Biosciences Clontech.) using the calcium phosphate transfection system (Life Technologies). Virus stocks were harvested at 24, 48 and 72 h post-transfection, an aliquot was removed for p24 antigen quantitation using the ELISA p24 antigen kit (Abbott Laboratories) and the remaining stock was frozen at −80 °C.

2.8. Infections

The effect of anti-TAR PNA on HIV-1 production was monitored in CEM cells infected with the pseudotyped HIV-1 virions expressing the firefly luciferase reporter. Briefly, pseudotyped HIV-1 virions in the presence of 10 μg of polybrene/ml were added to 5×10^6 CEM cells in a final volume of 1.0 ml to achieve multiplicities of infection (MOI) of 10. The cell cultures were incubated at 37 °C for 1 h, cells were gently spun out, washed with PBS and resuspended in 1.0 ml of complete RPMI medium. The infected cells were further incubated for 1 h in a 37 °C incubator and then transfected with varying amounts of the individual anti-TAR PNA, scrambled PNA or the mismatched TAR PNA as described above. A mock transfection of infected cells was similarly carried out. The cells were grown in 10 ml of complete RPMI medium. Forty-eight hours post-transfection, the cells were harvested and expression of the pseudovirus was analyzed by estimating the firefly luciferase activity.

2.9. Luciferase assays

Luciferase assays were performed by using the Promega Dual Luciferase assay kit. Briefly, the transfected cells were harvested, washed once with PBS without Ca^{+2} or Mg^{+2} and resuspended in 50 μl of the reporter lysis buffer (Promega). Cell lysis was carried out by incubating the samples at room temperature for 15 min on a rocking shaker. The lysate was centrifuged at 15,000 rpm for 10 min and the cell extracts were assayed for firefly and Renilla luciferase activity in a 96-well fluorotrac plate using a Packard Top Count Luminescence Counter. The results of at least three separate transfections were analyzed for each experiment.

3. Results

3.1. Binding specificity of PNA_{TAR} to TAR RNA

Labeled TAR RNA corresponding to nucleotides +1 to +82 of the HIV-1 LTR was transcribed in vitro using T7 RNA polymerase and used for determining the binding specificity of the individual PNA_{TAR}. The sequence of the HIV-1 TAR, PNA_{TAR}, mismatched PNA_{TAR} and scrambled PNA are shown in Fig. 1. Four anti-TAR PNAs of different length that either complement the entire loop and bulge region of TAR ($PNA_{TAR\text{-}16}$ and $PNA_{TAR\text{-}15}$) or are short of a few sequences either in the loop ($PNA_{TAR\text{-}13}$) or in both the loop and bulge ($PNA_{TAR\text{-}12}$) were used in this study. In order to determine the relative ability of the individual anti-TAR PNAs to bind with TAR RNA, we performed gel mobility shift assays (Fig. 2). As seen in Fig. 2, a distinct shift in the mobility of TAR RNA was observed due to the formation of specific [PNA_{TAR} -TAR RNA] complex (panels A, B, C and D; lanes 2–8). This mobility shift was concentration-dependent, as is evident from an incomplete shift seen at lower concentrations of PNA_{TAR} to TAR RNA (lanes 2 and 3) and a complete shift seen at higher concentrations (lanes 4–8). Although all the anti-TAR PNAs displayed strong affinity for TAR, the extent of gel retardation at lower concentrations varied with the length of the individual PNA; higher binding was noted with $PNA_{TAR\text{-}15}$ and $PNA_{TAR\text{-}16}$, as compared to $PNA_{TAR\text{-}12}$ and $PNA_{TAR\text{-}13}$ (lanes 2 and 3). The slower moving complex was not seen with scrambled PNA (panel E), thus suggesting the specificity of this interaction.

3.2. Inhibition of reverse transcription of TAR RNA in the presence of anti-TAR PNA

Since PNA–RNA or PNA–DNA duplexes exhibit higher Tm values than the corresponding RNA–DNA or DNA–DNA duplexes (Lee et al., 1998), it was interesting to examine if the individual anti-TAR PNA was able to block reverse transcription of HIV-1 TAR. Ability to block reverse transcription would have multiple effects on viral replication besides influencing Tat-mediated transactivation. For this purpose, TAR RNA primed with the labeled 17-mer DNA primer was incubated in the absence or presence of the individual anti-TAR PNA or scrambled PNA at 37 °C followed by initiation of reverse transcrip-

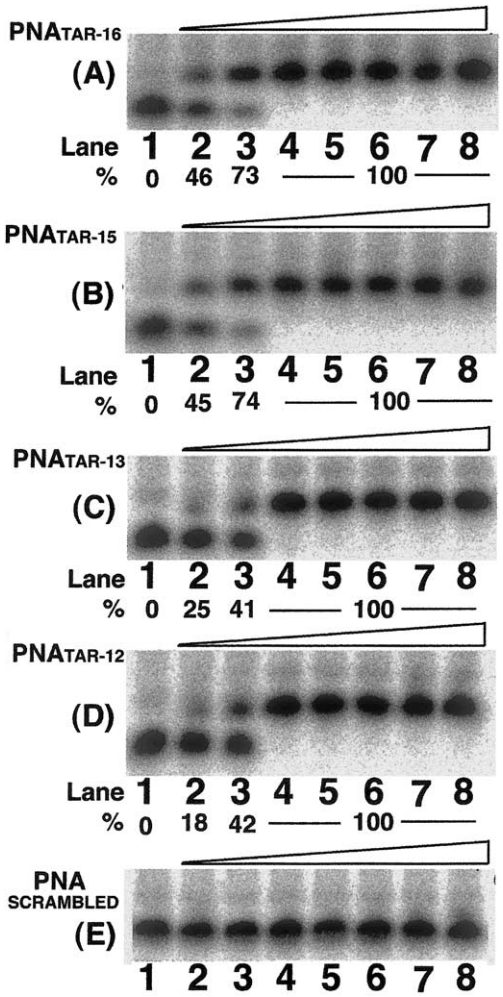

Fig. 2. Specificity of interaction of PNA_{TAR} to its target sequence on the HIV-1 genomic RNA. The binding affinity of the individual anti-TAR PNA and scrambled PNA to the TAR RNA was assessed by gel mobility shift analysis. Anti-TAR PNAs or scrambled PNA at indicated concentrations, were incubated with 6.4 nM of the labeled TAR RNA transcript for 1 h at 37 °C in binding buffer and subjected to native polyacrylamide gel electrophoresis. The RNA-PNA complexes were resolved at a constant voltage of 120 V at 4 °C and visualized by phosphorImaging. The extent of gel shift was determined by quantifying the probe RNA band on the phosphorImager using Image-Quant software (Molecular Dynamics). Panels A, B, C, D and E represent gel-shift of labeled TAR RNA in the presence of PNA_{TAR-16}, PNA_{TAR-15}, PNA_{TAR-13}, PNA_{TAR-12} and PNA_{SCB}, respectively. Lanes 1–8 indicate gel-shift carried out at the following concentrations of the individual anti-TAR PNA or scrambled PNA: 0, 2.5, 5.1, 9, 16, 32, 48 and 64 nM, respectively. The percent of labeled TAR RNA retarded due to PNA binding is as indicated.

tion by HIV-1 RT. The results are presented in Fig. 3. PNA_{TAR-16} and PNA_{TAR-15} caused a prominent pause in reverse transcription at the 42-mer position prior to the loop site targeted by these two PNAs. Likewise, the other two anti-TAR PNAs, PNA_{TAR-13} and PNA_{TAR-12} also exhibited a prominent pause at nucleotide position 44, prior to the targeted site. In addition, another minor pause at nucleotide 43 was also seen, but appeared to be a natural pause on this template as noted from the control set carried out in the absence of PNA. These results suggest that the individual anti-TAR PNA bind to their target site on TAR and block reverse transcription, probably by inhibiting the strand displacement activity of HIV-1 RT. Interestingly, while complete blockage at nucleotide position 42 was seen in case of PNA_{TAR-16} and PNA_{TAR-15}, further extension of some of the accumulated products beyond position 44 was observed with PNA_{TAR-13} and PNA_{TAR-12} upon prolonged reaction. Reverse transcription of HIV-1 TAR in the presence of scrambled PNA was similar to the control, indicating the specificity of the interaction of the individual anti-TAR PNA with its target sequence.

It is possible that incubation of PNA_{TAR} and RNA template at 37 °C may have facilitated their interaction by destabilizing the secondary structure of TAR. In order to evaluate whether PNA_{TAR} is able to invade the stem-loop of TAR and block reverse transcription at ambient temperature, we incubated varying concentration of the individual PNA_{TAR} with the pre-primed HIV-1 TAR at 25 °C. The pattern of reverse transcription products seen in Fig. 4 indicates a distinct difference in the ability of the various PNA_{TAR} to block reverse transcription of TAR. PNA_{TAR-16}, PNA_{TAR-15} and PNA_{TAR-13} were able to invade and block reverse transcription in a concentration dependent manner at ambient temperature and the pattern was similar to that observed at 37 °C. The highest inhibition was observed with PNA_{TAR-16}. In contrast, the ability of PNA_{TAR-12} to block reverse transcription was greatly diminished at ambient temperature. The higher efficiency of PNA_{TAR-16} in sequestering TAR and blocking reverse transcription suggests that targeting the entire stem-loop and bulge region-spanning nu-

cleotides 19–34 in the HIV-1 LTR is essential for maximal efficiency.

3.3. Anti-TAR PNA blocks tat-mediated transactivation of the HIV-1 LTR in cell culture

Results of the above in vitro experiment demonstrated that the four different anti-TAR PNAs of varying length and sequence differed in their ability to block reverse transcription on HIV-1 TAR. Since HIV-1 Tat enhances transcription elongation via interacting with TAR, it was of interest to probe which of the four anti-TAR PNAs could block the function of Tat in cell culture. To this end, we used a reporter plasmid construct expressing the firefly luciferase under the control of the HIV-1 LTR. CEM cells were transfected, with the reporter plasmids, in the absence or presence of the pCMV-Tat along with varying amounts of the individual anti-TAR PNA or nonspecific scrambled PNA. The expression of luciferase was then estimated by the Dual Luciferase Assay kit. Expression of Renilla luciferase driven by the CMV promoter did not significantly change in the absence or presence of the Tat expression clone or in the presence or absence of anti-TAR PNA, and was therefore used as a control to normalize for transfection efficiency (data not shown). On the other hand, expression of the firefly luciferase driven by the HIV-1 LTR directly correlated with the concentrations as well as the lengths of the individual PNA_{TAR}, thus pointing to the promoter specificity of anti-TAR PNA. These results are presented in Fig. 5, as the percent inhibition of Tat-mediated transactivation of the HIV-1 LTR at the indicated concentrations

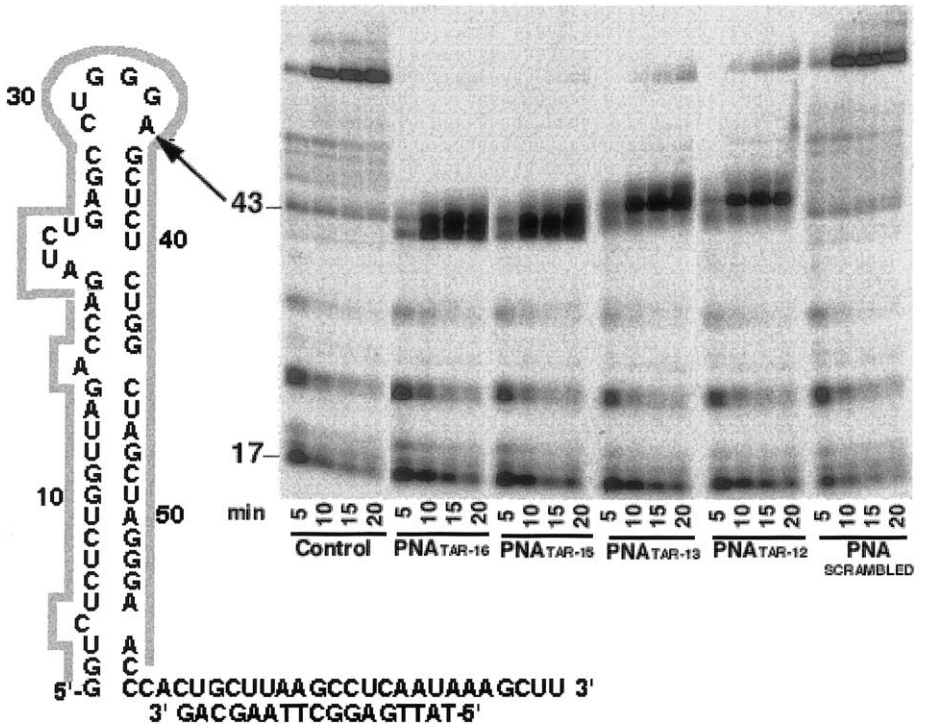

Fig. 3. Effect of anti-TAR PNA on reverse transcription of HIV-1 TAR RNA. The individual anti-TAR PNA or scrambled PNA (1 μM) were pre-incubated at 37 °C with the TAR RNA template primed with the 5′-^{32}P labeled 17-mer DNA primer. Reverse transcription reactions were initiated by the addition of enzyme and dNTP mix and aliquots were withdrawn at 5, 10, 15 and 20 min of incubation at 25 °C and quenched with the Sanger's stop dye. Control set represents the reactions carried out in the absence of anti-TAR PNAs or scrambled PNA. The position of the 17-mer primer is indicated. The position marked as 43 corresponds to the beginning of the loop region on TAR RNA targeted by the anti-TAR PNA.

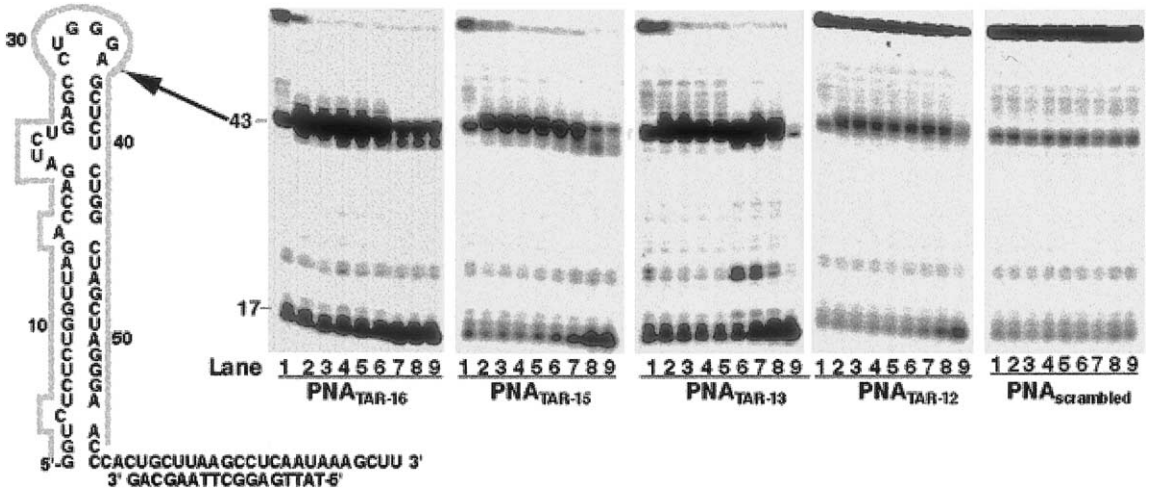

Fig. 4. Inhibition of reverse transcription of TAR RNA as a function of individual anti-TAR PNA concentration. The TAR RNA template primed with the 5′-^{32}P labeled 17-mer DNA primer was incubated in the absence or presence of increasing concentrations of PNA_{TAR} or scrambled PNA at 25 °C for 2 h. Reverse transcription was initiated by the addition of the four dNTP mix and HIV-1 RT as described in Section 2. The reaction products were analyzed on a denaturing 8% polyacrylamide-urea gel and subjected to phosphorImager analysis. Lane 1 in each set represents the control reaction carried out in the absence of PNA. Lanes 2–9 represent extension of the 17-mer primer in the presence of the indicated PNA at 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 μM concentration, respectively. The position of the 17-mer primer is indicated on the left. The position marked as 43 corresponds to the beginning of the loop region on TAR RNA targeted by the anti-TAR PNA.

of the individual PNA_{TAR} or scrambled PNA calculated from the respective ratios of the firefly and Renilla luciferase activities.

As seen from the Figure, the individual anti-TAR PNA significantly inhibited the Tat-mediated transactivation of the HIV-1 LTR. The extent of inhibition varied with the individual PNA_{TAR} as well as its concentration. Of the four anti-TAR PNAs, PNA_{TAR-16} was most effective in inhibiting Tat-mediated transactivation. Co-transfection of as low as 1 μg PNA_{TAR-16} resulted in 75% inhibition of Tat- mediated transactivation, which subsequently increased to 88% and 97% inhibition at 2.5 and 5.0 μg, respectively. The percent inhibition with PNA_{TAR-15} at these concentrations ranged from 40 to 87%. The extent of inhibition in case of PNA_{TAR-13} and PNA_{TAR-12}, was significantly lower with a maximum of 40–50% inhibition seen at the highest concentrations tested. Furthermore, we noted that transfection of anti-TAR PNA at the indicated concentrations had no adverse effect on cell viability (data not shown), thus indicating that these molecules may not be toxic to the cells.

3.4. Inhibition of HIV-1 production in CEM cells by anti-TAR PNA

Since a concentration-dependent gradient of inhibition of Tat-mediated transactivation of the HIV-1 LTR was noted with the anti-TAR PNA of varying lengths, we investigated their efficacy in HIV-1-infected cell cultures. We therefore transfected the pseudotyped HIV-1 virion-infected lymphocyte CEM $CD4^+$ cells, with varying concentrations of the individual anti-TAR PNA, scrambled PNA or mismatched anti-TAR PNA. The effect of anti-TAR PNA on HIV-1 production in CEM cells was monitored by analyzing the expression of the firefly luciferase reporter cloned in place of nef in the $HIV\text{-}_{1JR\text{-}CSF}env(-)$ cassette (Planelles et al., 1995). The firefly luciferase activity was normalized to the total protein in the cell extract. Luciferase expression obtained in

the absence of PNA in the mock-transfected HIV-1 infected CEM controls was taken to be hundred percent and that obtained in the presence of PNA was calculated relative to this value. These results are presented in Fig. 6, as the percent luciferase activity obtained relative to the control at indicated concentrations of the anti-TAR PNA, mismatched TAR PNA or scrambled PNA.

A substantial inhibition of virus production was seen with all four anti-TAR PNAs. The extent of inhibition was concentration-dependent and varied depending on the length of the individual PNA_{TAR}. Of the four anti-TAR PNAs, PNA-

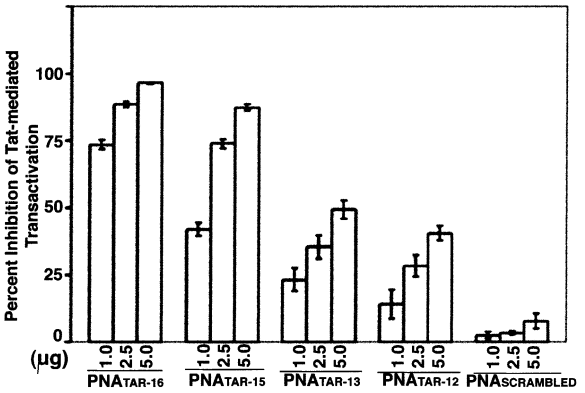

Fig. 5. Effect of anti-TAR PNA on Tat-mediated transactivation in CEM cells. CEM cells (5.0×10^6 cells in 250 μl) were transfected with the reporter plasmids pHIV-1 LTR-Luc (2 μg) and pCMV-R.Luc (0.6 μg) in the absence or presence of Tat expression vector, pCMV-Tat (1.0 μg). The individual PNA_{TAR} or scrambled PNA (PNA_{SCB}) were cotransfected at 1.0, 2.5 and 5.0 μg concentrations. The transfected cells were harvested 18 h post-transfection and analyzed for the individual luciferase activities. Expression of Renilla luciferase driven by the CMV promoter did not significantly change either in the absence or presence of the Tat expression clone or in the presence or absence of anti-TAR PNA, and was, therefore, used as a control to normalize for transfection efficiency. Expression of the firefly luciferase driven by the HIV-1 LTR correlated with the concentrations as well as the lengths of the individual PNA_{TAR}. These results are presented as the percent inhibition of Tat-mediated transactivation of the HIV-1 LTR at the indicated concentrations of the individual PNA_{TAR} or scrambled PNA calculated from their respective ratios of the firefly and Renilla luciferase activities. The results are expressed as mean values along with standard deviations of three independent experiments.

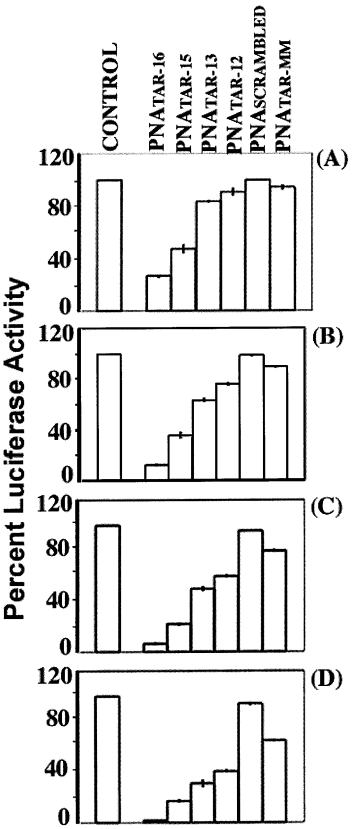

Fig. 6. Effect of anti-TAR PNA on HIV-1 production in CEM cells. CEM cells were transiently infected with the pseudotyped HIV-1 virus expressing the firefly luciferase reporter and then transfected with indicated amounts of the individual anti-TAR PNA (PNA_{TAR}), mismatched anti-TAR PNA ($PNA_{TAR\text{-}MM}$), or nonspecific scrambled PNA (PNA_{SCB}). The effect of anti-TAR PNA on HIV-1 production was monitored by analyzing the expression of the firefly luciferase in the cell extracts at 48 h post-transfection. Luciferase activity was monitored in a 96-well fluorotrac plate using a Packard Top Count Luminescence Counter. The firefly luciferase activity was normalized to the total protein in the cell extract. Luciferase expression obtained in the absence of PNA in the mock-transfected HIV-1-infected CEM controls was taken to be hundred percent and the extent of luciferase expression in the presence of the PNA was calculated relative to this value. These results are presented as the percent luciferase activity obtained relative to the control at the indicated concentrations of individual PNAs. Panels A, B, C, D represent transfections carried out at 1.0, 2.5, 5.0 and 10.0 μg of the individual PNAs. The values shown are the average of three sets of experiments. The bars represent the standard deviation.

TAR-16 complementing the entire loop and bulge region of the TAR as well as a single nucleotide in the stem, was most effective in suppressing virus production exhibiting a dramatic abolishment at the highest concentration (10 μg). In contrast, $PNA_{TAR\text{-}15}$ having the same sequence as $PNA_{TAR\text{-}16}$ except for a single nucleotide complementing the stem, was significantly less effective than $PNA_{TAR\text{-}16}$ in suppressing virus production, exhibiting an inhibition ranging from 50 to 85%. Further decrease in the length of the anti-TAR PNA such that they are short of few sequences in the loop ($PNA_{TAR\text{-}13}$) or in both the loop and bulge ($PNA_{TAR\text{-}12}$) resulted in a further decrease in their efficacy to inhibit HIV-1 production. Thus, $PNA_{TAR\text{-}13}$ and $PNA_{TAR\text{-}12}$ exhibited inhibition ranging from 10-70%. Scrambled PNA exhibited no inhibition of virus production at similar concentrations, indicating the specificity of anti-TAR PNA. A mismatched 16-mer anti-TAR PNA ($PNA_{TAR\text{-}MM}$) having three nucleotide mismatches in the bulge region of the TAR, exhibited substantially reduced antiviral efficacy (6–40% inhibition of virus production). These results clearly demonstrate the importance of targeting the bulge region of the TAR.

4. Discussion

Currently, clinical treatment of AIDS patients with highly active antiretroviral therapy (HAART) involving a combination of drugs targeting the two key HIV-1 enzymes, namely the reverse transcriptase and protease, has been successful in reducing the viral load (Aalen et al., 1999; Centers for Disease Control and Prevention, 1998). However, the two major impediments to successful HIV-1 therapy are the rapid emergence of drug-resistant viral strains (Condra et al., 1995; Ho et al., 1994; Husson et al., 1993; Kavlick et al.,, 1998; Kojima et al., 1995; Larder et al., 1989; Markowitz et al., 1995; Partaledis et al., 1995; Otto et al., 1993; Patick et al., 1995; Shirasaka et al., 1995) and the persistence of integrated provirus in host cells (Chun et al., 1998; Selby et al., 1989; Wong et al., 1997). In recent years, it has become apparent that identification of novel non-mutable viral targets and the selection of potent gene-intervening reagents are clearly needed to empower anti-HIV-1 therapeutic strategies.

In the present study, we demonstrate the potential of PNA complementary to the HIV-1 TAR apical-stem loop and bulge to inhibit virus replication in cell culture. We identified the TAR RNA sequences that may be critical for Tat-mediated transactivation of the HIV-1 LTR (Fig. 6). The significance of Tat-TAR interaction in regulating gene expression is well documented (Jeang et al., 1999; Karn, 1999). Activation of transcriptional elongation occurs following the recruitment of Tat to the transcription machinery via a specific interaction with an RNA regulatory element called TAR, a 59-residue RNA leader sequence in the long terminal repeat (LTR) (Karn, 1999). The main advantage of targeting the TAR element is that it is conserved and folds into a stable stem-loop structure. Any mutational changes in TAR that destabilize this structure also abolish Tat-TAR interaction.

We have earlier used PNA to target two critical sites on the 5′ (U5) nontranslated region of the HIV-1 genome. Our investigations using PNA targeted to the PBS region of the HIV-1 genome demonstrated the effective inhibition of the initial priming process by $tRNA_3^{Lys}$ of HIV-1 cDNA synthesis (Lee et al., 1998). In another study, we demonstrated that a 15-mer PNA targeted to the stem-loop region of HIV-1 TAR binds to TAR effectively and prevents Tat-TAR interaction, thereby blocking Tat-mediated transactivation in cell culture (Mayhood et al., 2000). As an extension of this study, we designed four anti-TAR PNAs of varying length, ranging from 12 to 16-mer ($PNA_{TAR\text{-}12}$, $PNA_{TAR\text{-}13}$, $PNA_{TAR\text{-}15}$ and $PNA_{TAR\text{-}16}$), and examined their ability to sequester the TAR element and prevent Tat-mediated transactivation as well as their efficacy in inhibiting viral replication. The sequence of these anti-TAR PNAs and the region of TAR RNA that they target are shown in Fig. 1. The rationale for designing these anti-TAR PNAs was to examine whether partial or complete blockage of the loop and bulge region of TAR by varying their length was sufficient to block Tat-mediated transactivation. In vitro, Tat binds to bulge region of TAR

RNA but does not recognize sequences in the loop of the TAR hairpin that are essential for transactivation (Roy et al., 1990). The interaction of Tat with TAR region requires cellular factors such as cyclin T/ CDK9 that bind to the terminal loop region of TAR (Wei et al., 1998). Tat is involved in recruiting cellular kinases that phosphorylate C-terminal domain of RNA pol II, resulting in a more processive RNA Pol II complex (Bieniasz et al., 1999; Chen et al., 1999; Fujinaga et al., 1998; Isel and Karn, 1999; Ivanov et al., 1999; O'Keeffe et al., 2000; Ramanathan et al., 1999; Romano et al., 1999; Napolitano et al., 1999; Wei et al., 1998). These reports underscore the importance of the bulge and loop region of TAR in viral gene regulation.

All the four anti-TAR PNAs used in this study were able to bind to TAR, although subtle differences in their ability to gel-shift TAR RNA were noted at lower molar ratio of PNA_{TAR} to TAR RNA (Fig. 2, panels A, B, C, and D). This difference in their binding ability correlated with their lengths (Fig. 2, panels A, B, C, and D; lanes 2 and 3). The binding specificity of the anti-TAR PNAs is supported by our observation that scrambled PNA did not influence the mobility of TAR RNA (Fig. 2, panel 5).

Earlier we have shown that PNA-bound viral RNA blocks both reverse transcription and RNase H cleavage (Lee et al., 1998). Our findings with the four anti-TAR PNA used in this study, exhibiting pronounced blockage of reverse transcription on TAR RNA are consistent with this observation (Fig. 3). It may be pointed out, however, that the extent of blockage varied with the temperature of incubation of the anti-TAR PNA as well as the PNA_{TAR} itself (Figs. 3 and 4). While $PNA_{TAR\text{-}16}$ and $PNA_{TAR\text{-}15}$ exhibited near complete blockage of reverse transcription, some extension of the product beyond the targeted site was noted in the case of $PNA_{TAR\text{-}13}$ and $PNA_{TAR\text{-}12}$ (Fig. 3). Our observation that inhibition of reverse transcription on TAR RNA was markedly reduced when $PNA_{TAR\text{-}12}$ was incubated at ambient temperature with the pre-annealed template-primer suggests that the TAR stem-loop structure may be more stable at ambient temperature and smaller PNA_{TAR} are relatively less efficient in sequestering this region.

Transcription activation by Tat occurs through TAR and requires the proper folding of the TAR RNA hairpin structure (Cullen, 1993; Jones and Peterlin, 1994). It has been demonstrated that both an intact loop sequence and an intact Tat-binding site are critical structural motifs of the TAR element, and there is no complementation in cis between TAR element carrying mutations in loop or in the Tat-binding site (Churcher et al., 1995). A potential barrier in this interaction would result in down regulation of transcription. This contention is supported by our findings in CEM cells co-transfected with the pHIV-1 LTR-Luc and pCMV-Tat reporter gene constructs in the presence and absence of the four different anti-TAR PNA and nonspecific scrambled PNA (Fig. 5). A substantial increase in the luciferase activity upon co-transfection with pCMV-Tat indicated a significant stimulation of the basal level of transcription of the HIV-1 LTR. All the four anti-TAR PNA were able to sequester the targeted site on the reporter gene construct in cell culture. However, it may be noted that decrease in the length of the anti-TAR PNA by shifting the target by a few nucleotides upstream or downstream resulted in a significant decrease in Tat-mediated transactivation. These results are not surprising since the TAR domain is probably minimally required for Tat response either directly or via its interaction with other transcription factors including the Tat binding pyrimidine bulge, the TAR RNA upper stem, and the loop sequences (Harrich et al., 1994.). Characterization of the RNA protein contact sites using modification interference experiments have mapped the TAR RNA contact site to the trinucleotide bulge region and adjacent base pairs (Weeks et al., 1990). An NMR model of the TAR-argininamide complex, which mimics the RNA-peptide interaction, suggests that the critical arginine residue of Tat hydrogen bonds to G26 and contacts two important phosphates (Puglisi et al., 1992). Furthermore, our data clearly demonstrate that $PNA_{TAR\text{-}16}$ can inhibit HIV-1 replication in cell culture (Fig. 6). This is not surprising since disruption of the Tat-TAR interaction by this PNA is expected to inhibit virus production.

A challenging aspect in the development of antisense strategy is the selection of appropriate targets. In the case of various clades of HIV-1, targeting the numerous regulatory genes on its RNA genome has not always proved efficient (Agrawal et al., 1989; Kim et al., 1995; Kinchington et al., 1992; Lisziewicz et al., 1995, Matsukura et al., 1989). A promising aspect of the present study is that the TAR gene sequence appeared to be accessible to the PNA oligomer. This sequence, therefore, can be considered a vulnerable region of the HIV-1 genome that can be exploited to develop a specific antiviral intervention. Independent studies from a number of labs pertaining to growth characteristics of viral mutants with alterations in the TAR stem, the pyrimidine bulge or the loop sequence have shown deleterious effect on viral gene expression and replication (Harrich et al., 1994; Rounseville et al., 1996, Das et al., 1998).

Development of novel therapies with an appropriate delivery system targeting several stages of the viral life cycle and not vulnerable to the genetic flexibility of the virus is critical towards a successful drug intervention. The TAR motif seems to be an attractive candidate for anti-retroviral therapy given that it is independent of the genetic flexibility of the virus and can be attributed with a pleiotropy of functions. In addition, other critical domains in the 5′ non-translated region of the HIV-1 genome comprising of the primer-binding site, the A-loop region located upstream of the PBS, and the bulge region located down stream of the PBS, also warrant investigation as targets for drug intervention. The current use of PNAs by a number of laboratories holds tremendous promise as a successful antisense strategy. Recently, it has been shown that PNAs complementary to the template region of the RNA domain of human telomerase inhibit cellular telomerase and cause telomeres to be shortened in cells (Herbert et al., 1999; Shammas et al., 1999). Specifically designed PNAs have been successfully used for in vivo inhibition of delta-opoid receptor gene function (Fraser et al., 2000). It may however be pointed out that the therapeutic potential of the present unmodified form of PNA is vastly limited due to its inefficient delivery across cell membranes. In this context, the recent advances made for the delivery of exogenous proteins into living cells with the help of membrane-permeable carrier peptides such as HIV-1 Tat-(48–60), Antennapedia-(43–58), the arginine-rich peptides and transportan among others holds tremendous promise (Derossi et al., 1996; Fawell et al., 1994; Futaki et al., 2001). Using this strategy, Good et al. (2001) have recently shown that a 10-mer peptide-PNA conjugate targeted against ribosomal RNA and against messenger RNA of Acp protein of bacteria can effectively cure the HeLa cells infected with E. coli K12 strains, thereby raising the possibility for anti-infective drug development (Good et al., 2001). Studies in our laboratory are underway to analyze the effect of PNA-transportan conjugates targeted against the critical regions of the HIV-1 genome in the quest for novel inhibitors of HIV-1.

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References

Aalen, O.O., Farewell, V.T., De Angelis, D., Day, N.E., Gill, O.N., 1999. New therapy explains the fall in AIDS incidence with a substantial rise in number of persons on treatment expected. AIDS 13, 103–108.

Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P.S., Konopka, A., Maizel, J., Zamecnik, P.C., 1989. Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues. Proc. Natl. Acad. Sci. USA 86, 7790–7794.

Arzumanov, A., Walsh, A.P., Rajwanshi, V.K., Kumar, R., Wengel, J., Gait, M.J., 2001. Inhibition of HIV-1 Tat-dependent trans activation by steric block chimeric 2′-*O*-methyl/LNA oligoribonucleotides. Biochemistry 40, 14645–14654.

Bieniasz, P.D., Grdina, T.A., Bogerd, H.P., Cullen, B.R., 1999. Recruitment of cyclin T1/P-TEFb to an HIV type 1 long terminal repeat promoter proximal RNA target is both

necessary and sufficient for full activation of transcription. Proc. Natl. Acad. Sci. USA 96, 7791–7796.

Bohan, C.A., Kashanchi, F., Ensoli, B., Buonaguro, L., Boris-Lawrie, K.A., Brady, J.N., 1992. Analysis of Tat transactivation of human immunodeficiency virus transcription in vitro. Gene Expr. 2, 391–407.

Boulme, F., Freund, F., Moreau, S., Nielsen, P.E., Gryaznov, S., Toulme, J.J., Litvak, S., 1998. Modified (PNA, 2′-*O*-methyl and phosphoramidate) anti-TAR antisense oligonucleotides as strong and specific inhibitors of in vitro HIV-1 reverse transcription. Nucleic Acids Res. 26, 5492–5500.

Centers for Disease Control and Prevention, 1998. HIV/AIDS surveillance report, midyear ed. vol. 10. Centers for Disease Control and Prevention, Public Health Service, Department of Health and Human Services, Atlanta, GA.

Chen, D., Fong, Y., Zhou, Q., 1999. Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription. Proc. Natl. Acad. Sci. USA 96, 2728–2733.

Chun, R.F., Semmes, O.J., Neuveut, C., Jeang, K.T., 1998. Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. J. Virol. 72, 2615–2629.

Churcher, M.J., Lowe, A.D., Gait, M.J., Karn, J., 1995. The RNA element encoded by the trans-activation-responsive region of human immunodeficiency virus type 1 is functional when displaced downstream of the start of transcription. Proc. Natl. Acad. Sci. USA 92, 2408–2412.

Condra, J.H., Schleif, W.A., Blahy, O.M., Gabryelski, L.J., Graham, D.J., Quintero, J.C., Rhodes, A., Robbins, H.L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppler, H., Squires, K.E., Deutsch, P.J., Emini, E.A., 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 374, 569–571.

Cullen, B.R., 1993. Does HIV-1 Tat induce a change in viral initiation rights? Cell 73, 417–420.

Das, A.T., Klaver, B., Berkhout, B., 1998. The 5′ and 3′ TAR elements of human immunodeficiency virus exert effects at several points in the virus life cycle. J. Virol. 72, 9217–9223.

Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G., Prochiantz, A., 1996. Cell internalization of the third helix of the *Antennapedia homeodomain* is receptor-independent. J. Biol. Chem. 271, 18188–18193.

Farese-DiGiorgio, A., Pairot, S., Patino, N., Condom, R., DiGiorgio, C., Aumelas, A., Aubertin, A.M., Guedj, R., 1999. Synthesis of a new class of HIV-1 inhibitors. Nucleosides Nucleotides 18, 263–275.

Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L.L., Pepinsky, B., Barsoum, J., 1994. Tat-mediated delivery of heterologous proteins into cells. Proc. Natl. Acad. Sci. USA 91, 664–668.

Fraser, G.L., Holmgren, J., Clarke, P.B., Wahlestedt, C., 2000. Antisense inhibition of delta-opioid receptor gene function in vivo by peptide nucleic acids. Mol. Pharmacol. 57, 725–731.

Fujinaga, K., Cujec, T.P., Peng, J., Garriga, J., Price, D.H., Grana, X., Peterlin, B.M., 1998. The ability of positive transcription elongation factor B to transactivate human immunodeficiency virus transcription depends on a functional kinase domain, cyclin T1, and Tat. J. Virol. 72, 7154–7159.

Fujinaga, K., Taube, R., Wimmer, J., Cujec, T.P., Peterlin, B.M., 1999. Interactions between human cyclin T, Tat, and the transactivation response element (TAR) are disrupted by a cysteine to tyrosine substitution found in mouse cyclin T. Proc. Natl. Acad. Sci. USA 96, 1285–1290.

Futaki, H., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., Sugiura, Y., 2001. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J. Biol. Chem. 276, 5836–5840.

Good, L., Awasthi, S.K., Dryselius, R., Larsson, O., Nielsen, P.E., 2001. Bactericidal antisense effects of peptide–PNA conjugates. Nat. Biotechnol. 19, 360–364.

Gunnery, S., Green, S.R., Matthew, M.B., 1992. Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis in vivo and in vitro: relationship between structure and function. Proc.Natl. Acad. Sci. USA 89, 11557–11561.

Harrich, D., Hsu, C., Race, E., Gaynor, R.B., 1994. Differential growth kinetics are exhibited by human immunodeficiency virus type 1 TAR mutants. J. Virol. 68, 5899–5910.

Herbert, B.-S., Pitts, A.E., Baker, S.I., Hamilton, S.E., Wright, W.E., Shay, J.W., Corey, D.R., 1999. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc. Natl. Acad. Sci. USA 96, 14276–14281.

Ho, D.D., Toyoshima, T., Mo, H., Kempf, D.J., Norbeck, D., Chen, C.M., Wideburg, N.E., Burt, S.K., Erickson, J.W., Singh, M.K., 1994. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. J. Virol. 68, 2016–2020.

Husson, R.N., Shirasaka, T., Butler, K.M., Pizzo, P.A., Mitsuya, H., 1993. High-level resistance to zidovudine but not to zalcitabine or didanosine in human immunodeficiency virus from children receiving antiretroviral therapy. J. Pediatr. 123, 9–16.

Isel, C., Karn, J., 1999. Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation. J. Mol. Biol. 290, 929–941.

Ivanov, D., Kwak, Y.T., Nee, E., Guo, J., Garcia-Martinez, L.F., Gaynor, R.B., 1999. Cyclin T1 domains involved in complex formation with Tat and TAR RNA are critical for tat-activation. J. Mol. Biol. 288, 41–56.

Jeang, K.T., Xiao, H., Rich, E.A., 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. J. Biol. Chem. 274, 28837–28840.

Jones, K.A., Peterlin, B.M., 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. 63, 717–743.

Karn, J., 1999. Tackling Tat. J. Mol. Biol. 293, 235–254.

Katz, R.A., Skalka, A.M., 1994. The retroviral enzymes. Annu. Rev. Biochem. 63, 133–173.

Kavlick, M.F., Wyvill, K., Yarchoan, R., Mitsuya, H., 1998. Emergence of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 variants, viral sequence variation, and disease progression in patients receiving antiretroviral chemotherapy. J. Infect. Dis. 177, 1506–1513.

Kim, S.G., Hatta, T., Tsukahara, S., Nakashima, H., Yamamoto, N., Shoji, Y., Takai, K., Takaku, H., 1995. Antiviral effect of phosphorothioate oligodeoxyribonucleotides complementary to human immunodeficiency virus. Bioorg. Med. Chem. 3, 49–54.

Kinchington, D., Galpin, S., Jaroszewski, J.W., Ghosh, K., Subasinghe, C., Cohen, J.S., 1992. A comparison of gag, pol and rev antisense oligodeoxynucleotides as inhibitors of HIV-1. Antiviral Res. 17, 53–62.

Kojima, E., Shirasaka, T., Anderson, B.D., Chokekijchai, S., Steinberg, S.M., Broder, S., Yarchoan, R., Mitsuya, H., 1995. Human immunodeficiency virus type 1 (HIV-1) viremia changes and development of drug-related mutations in patients with symptomatic HIV-1 infection receiving alternating or simultaneous zidovudine and didanosine therapy. J. Infect. Dis. 171, 1152–1158.

Larder, B.A., 1995. Viral resistance and the selection of antiretroviral combinations. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 10 (Suppl. 1), S28–S33.

Larder, B.A., Darby, G., Richman, D.D., 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Lee, R., Kaushik, N., Modak, M.J., Vinayak, R., Pandey, V.N., 1998. Polyamide nucleic acid targeted to the primer binding site of the HIV-1 RNA genome blocks in vitro HIV-1 reverse transcription. Biochemistry 37, 900–910.

Lisziewicz, J., Sun, D., Lisziewicz, A., Gallo, R.C., 1995. Anti-Tat gene therapy: a candidate for late-stage AIDS patients. Gene Ther. 2, 218–222.

Long, K.S., Crothers, D.M., 1999. Characterization of the solution conformations of unbound and Tat peptide-bound forms of HIV-1 TAR RNA. Biochemistry 38, 10059–10069.

Markowitz, M., Mo, H., Kempf, D.J., Norbeck, D.W., Bhat, T.N., Erickson, J.W., Ho, D.D., 1995. Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. J. Virol. 69, 701–706.

Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C.A., Mitsuya, H., Wong-Staal, F., Cohen, J.S., Broder, S., 1989. Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate oligodeoxynucleotide against rev (art/trs) in chronically infected cells. Proc. Natl. Acad. Sci. USA 86, 4244–4248.

Mayhood, T., Kaushik, N., Pandey, P.K., Kashanchi, F., Deng, L., Pandey, V.N., 2000. Inhibition of Tat-mediated transactivation of HIV-1 LTR transcription by polyamide nucleic acid targeted to TAR hairpin element. Biochemistry 39, 11532–11539.

Napolitano, G., Licciardo, P., Gallo, P., Majello, B., Giordano, A., Lania, L., 1999. The CDK9-associated cyclins T1 and T2 exert opposite effects on HIV-1 Tat activity. AIDS 13, 1453–1459.

Nielsen, P.E., 1999. Peptide nucleic acids as therapeutic agents. Curr. Opin. Struct. Biol. 9, 353–357.

O'Keeffe, B., Fong, Y., Chen, D., Zhou, S., Zhou, Q., 2000. Requirement for a kinase-specific chaperone pathway in the production of a Cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated tat stimulation of HIV-1 transcription. J. Biol. Chem. 275, 279–287.

Otto, M.J., Garber, S., Winslow, D.L., Reid, C.D., Aldrich, P., Jadhav, P.K., Patterson, C.E., Hodge, C.N., Cheng, Y.S., 1993. In vitro isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease. Proc. Natl. Acad. Sci. USA 90, 7543–7547.

Partaledis, J.A., Yamaguchi, K., Tisdale, M., Blair, E.E., Falcione, C., Maschera, B., Myers, R.E., Pazhanisamy, S., Futer, O., Cullinan, A.B., Stuver, C.M., Byrn, R.A., Livingston, D.J., 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease. J. Virol. 69, 5228–5235.

Patick, A.K., Rose, R., Greytok, J., Bechtold, C.M., Hermsmeier, M.A., Chen, P.T., Barrish, J.C., Zahler, R., Colonno, R.J., Lin, P.F., 1995. Characterization of a human immunodeficiency virus type 1 variant with reduced sensitivity to an aminodiol protease inhibitor. J. Virol. 69, 2148–2152.

Planelles, V., Bachelerie, F., Jowett, J.B., Haislip, A., Xie, Y., Banooni, P., Masuda, T., Chen, I.S., 1995. Fate of the human immunodeficiency virus type 1 provirus in infected cells: a role for vpr. J. Virol. 69, 5883–5889.

Puglisi, J.D., Tan, R., Calnan, B.J., Frankel, A.D., Williamson, J.R., 1992. Conformation of the TAR RNA-arginine complex by NMR spectroscopy. Science 257, 76–80.

Ramanathan, Y., Reza, S.M., Young, T.M., Mathews, M.B., Pe'ery, T., 1999. Human and rodent transcription elongation factor P-TEFb: interactions with human immunodeficiency virus type 1 tat and carboxy-terminal domain substrate. J. Virol. 7, 5448–5458.

Romano, G., Kasten, M., De Falco, G., Micheli, P., Khalili, K., Giordano, A., 1999. Regulatory functions of Cdk9 and of cyclin T1 in HIV tat transactivation pathway gene expression. J. Cell. Biochem. 75, 357–368.

Rounseville, M.P., Lin, H.S., Agbottah, E., Shukla, R.M., Rabson, A.B., Kumar, A., 1996. Inhibition of HIV-1 replication in viral mutants with altered TAR RNA stem structures. Virology 216, 411–417.

Roy, S., Delling, U., Chen, C.H., Rosen, C.A., Sonenberg, N., 1990. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated transactivation. Genes Dev. 4, 1365–1374.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Selby, M.J., Bain, E.S., Luciw, P.A., Peterlin, B.M., 1989. Structure, sequence, and position of the stem-loop in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat. Genes Dev. 3, 547–558.

Shammas, M.A., Simmons, C.G., Corey, D.R., Shmookler Reis, R.J., 1999. Telomerase inhibition by peptide nucleic acids reverses 'immortality' of transformed human cells. Oncogene 18, 6191–6200.

Shirasaka, T., Kavlick, M.F., Ueno, T., Gao, W.Y., Kojima, E., Alcaide, M.L., Chokekijchai, S., Roy, B.M., Arnold, E., Yarchoan, R., Mitsuya, H., 1995. Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. Proc. Natl. Acad. Sci. USA 92, 2398–2402.

Veschambre, P., Simard, P., Jalinot, P., 1995. Evidence for functional interaction between the HIV-1 Tat transactivator and the TATA box binding protein in vivo. Mol. Biol. 250, 169–180.

Weeks, K.M., Ampe, C., Schultz, S.C., Steitz, T.A., Crothers, D.M., 1990. Fragments of the HIV-1 Tat protein specifically bind TAR RNA. Science 249, 1281–1285.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., Jones, K.A., 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell 92, 451–462.

Wong, J.K., Hezareh, M., Günthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A., Richman, D.D., 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278, 1291–1295.