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Anti-gene peptide nucleic acid targeted to proviral HIV-1 DNA inhibits in vitro HIV-1 replication

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Abstract

Highly active antiretroviral therapy (HAART) is unlikely to affect reservoirs of HIV in latently infected cells. Anti-gene compounds, such as peptide nucleic acids (PNAs), which block transcriptional activity via sequence-specific invasion of double-stranded DNA may be an effective strategy to target cells harbouring proviral HIV DNA. Here we show that a PNA oligomer (PNA_{HIV}), 15 bases in length, linked to a nuclear localization signal (NLS), substantially suppressed HIV-1 replication in chronically infected lymphocytes and macrophages and efficiently prevented mitogen-induced HIV-1 reactivation in lymphocytes, as determined by HIV-p24 antigen production in supernatants and FACS analysis for intracellular HIV accumulation. In contrast, a mismatched PNA did not show any effect on HIV expression. Semi-quantitative RT-PCR and quantitative real-time RT-PCR demonstrated a decrease of HIV RNA expression in infected cells treated by PNA_{HIV} indicating that inhibition of HIV-1 replication occurred at the transcription step. In conclusion, the use of anti-gene PNA to target the HIV-1 proviral DNA in the quest for new antiretroviral agents appears quite promising.

Keywords: Peptide nucleic acid (PNA); Proviral DNA; Latent infection; HIV

1. Introduction

In human immunodeficiency virus (HIV)-infected individuals, who are successfully treated with drug combination antiretroviral therapy ("highly active anti-retroviral therapy" or HAART), plasma virus levels may remain below the detection limit for a prolonged period. However, when the antiviral therapy is discontinued, the virus reappears. Therefore, there is a latent reservoir of virus that resists current therapies (Bukrinsky et al., 1991; Chun et al., 1997a,b). There is strong evidence that this latent reservoir is comprised of resting CD4+ T cells or other long-lived infected cells, such as cells of the monocyte-macrophage lineage, harbouring proviral HIV DNA (Bukrinsky et al., 1991; Chun et al., 1995, 1997a,b; Zack et al., 1990, 1992; Sun et al., 1997). It is believed that this population of latently infected cells play a critical role in the pathogenesis of HIV infection (Michie et al., 1992; Fauci, 1993; Chun et al., 1998) and it is assumed that a cure can only be achieved if the latent reservoirs are also eliminated. Thus, novel antiretroviral compounds

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able to affect HIV in latently infected cells are urgently needed.

A possible strategy to attack HIV-1 in latently infected cells may be to use anti-gene compounds able to inhibit RNA synthesis by interacting specifically with proviral HIV-DNA sequences. Peptide nucleic acids (PNAs) are synthetic DNA mimics in which the negatively charged deoxyribose phosphate backbone has been replaced by an uncharged pseudo peptide backbone and only the four natural nucleobases are retained (Kuhn et al., 1999; Nielsen et al., 1994; Nielsen, 1995; Uhlmann et al., 1998). The absence of a negatively charged backbone facilitates PNA invasion of the DNA double helix to form a stable PNA–DNA hybrid with high mismatch discrimination. (Almarsson et al., 1993; Egholm et al., 1993). PNA–DNA interaction is further stabilized in chromatin of live cells. Indeed, it has been demonstrated that in the cellular environment, PNA–DNA hybrids are more stable than their homologous DNA–DNA because they are ionic strength-independent (Tomac et al., 1996) and PNA binding to supercoiled DNA is stronger than to linear DNA (Bentin and Nielsen, 1996). In addition, the PNAs are highly resistant to proteases and nucleases (Demidov et al., 1994).

Previous experiments with isolated nuclei (Boffa et al., 1997) and in vivo (Tyler et al., 1999) have shown that mixed sequence PNAs are highly effective in blocking transcription of the targeted gene without inhibiting RNA synthesis in unrelated genes. Still a major problem in the application of PNAs as anti-gene agents is their restricted ability to penetrate the nucleus of a cell in culture or in vivo (Tyler et al., 1999). However, there are indications that anti-gene PNAs, if artificially allowed to enter the nucleus, can inhibit transcription (Bonham et al., 1995; Boffa et al., 1996, 1997). Recent trials of a few vectors have also shown a successful delivery of the fused PNAs to the nucleus of live cells (Tyler et al., 1999; Scarfi et al., 1997; Aldrian-Herrada et al., 1998; Pooga et al., 1998; Branden et al., 1999). In particular, PKKKRKV, a basic nuclear localization signal (NLS) peptide, has been shown to specifically enhance the nuclear uptake of PNAs without altering their anti-gene effects (Boffa et al., 1997, 2000; Cutrona et al., 2000).

The use of potent gene-intervening reagents and the selection of optimal genetic targets are equally critical elements of a successful anti-gene strategy. The viral DNA targets selected for anti-gene intervention should be conserved regions resistant to mutational changes. The 15 bp sequence that we have chosen as a target in our studies is a region comprising partially (11 out of 16 bases) the HIV poly(purine) tract sequence (PPT) and a 4-base (TTTT) 5′-flanking sequence (designated PPT^{TTTT}). This sequence is present twice in the HIV-1 proviral DNA: (i) in the 3′ of the *pol* gene coding for the integrase viral protein and (ii) in the *nef* gene upstream of the 3′-LTR and is conserved in all HIV-1 strains. Our goal was to block viral transcription elongation by a PNA oligomer able to bind to the template strand of the proviral target sequence.

2. Materials and methods

2.1. Compounds

The NLS-PNAs were obtained from Oswel DNA service, Southampton, GB. The sequence of anti-HIV PNA (PNA_{HIV}) is TTTTAAAAGAAAAGG-PKKKRKV. The sequence of control is a mismatched PNA (PNA_{ctr}) TTTTGAACGATAAGG-PKKKRKV. U75875, a synthetic peptidomimetic (Upjohn Laboratories, Kalamazoo, MI), is an inhibitor of HIV-1 protease. U75875 is for laboratory use only and is not to be administered to humans or food-producing animals or plants.

2.2. Viruses

A laboratory monocyte-tropic strain of HIV-1 (HTLV-III-Ba-L, also called $HIV\text{-}1_{Ba\text{-}L}$) was used to infect macrophages. A laboratory lymphocyte-tropic strain of HIV-1 (HTLV-IIIB, also called HIV-IIIB) and three primary isolates (HIV-106, HIV-112 and HIV-AB1) were used to infect lymphocytes. Both the laboratory strains are available through the AIDS Research and Reference Reagent Program (NIH, Bethesda, MD). The primary isolates of HIV-1 were obtained from 3 HIV-1-Ab seropositive individuals who had experienced a virologic failure (plasma HIV RNA levels >500 copies/ml on at least two consecutive measurements) during HAART including at least one protease inhibitor. Isolation of these strains from the plasma of infected individuals was performed in peripheral blood mononuclear cells (PBMC) cultures; the supernatants of these cultures were used as the source of the virus. Titration to determine the infectivity of laboratory strains and primary isolates was performed in human lymphocytes (Andreoni et al., 1992; Bergamini et al., 1994) The titer of the virus stocks, expressed as 50% cell culture infectious dose ($CCID_{50}$), was determined as previously described (Karber, 1931).

2.3. Viral detection

HIV-p24 antigen production in supernatants was assessed by a sandwich ELISA (Abbott, Pomezia, Italy).

2.4. Cells

Peripheral blood obtained from HIV-negative donors was enriched for PBMCs by centrifugation over Ficoll Hypaque. PBMCs were then further enriched for lymphocytes or monocytes by elutriation (Placido et al., 1997). Cells obtained by this method are >90% pure as determined by cytofluorimetric (FACS) analysis. H9/IIIB (H9) is a CD4+ T-cell line persistently infected with HIV-1.

2.5. Assay of antiviral activity

To evaluate anti-HIV efficacy of PNA_{HIV} in chronically infected lymphocytes, elutriated lymphocytes were washed

twice with phosphate-buffered saline (PBS), counted, and incubated for 72 h into 15 ml polyethylene tubes at a concentration of 6×10^6 cells/ml in Roswell Park Memorial Institute (RPMI)-1640 medium with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, (complete medium) supplemented with 5 μg/ml phytohemoagglutinin (PHA) (Sigma Chimica, Milano, Italy). At the end of the incubation period the cells were washed twice and incubated with 300 $CCID_{50}$ of the HIV-1 isolate IIIB in complete medium supplemented with 10 U/ml of recombinant interleukin-2 (IL-2) (Collaborative Research Incorporated, Bedford, MA). After 24 h of incubation the cells were washed twice in PBS to eliminate excess virus and dispensed in each well of a 48-well plate at a concentration of 10^6 cells/ml in complete medium supplemented with 10 U/ml of IL-2. The cells were washed and fed every 3 days. At day 10 after infection the cells were washed and re-fed with complete medium supplemented or not with different concentrations of PNA_{HIV} or PNA_{ctr} After 48 h (day 12) the cells were washed, re-fed and cultured for further 48 h in the presence of the same concentrations of PNAs as before. At day 14 samples of supernatants were collected for HIV-p24 testing.

To evaluate anti-HIV efficacy of PNA_{HIV} in chronically infected macrophages, elutriated monocytes were re-suspended in complete medium at a concentration of 2×10^5 cells/ml and dispensed in each well of a 48-well plate. Monocytes were allowed to mature into macrophages for 7 days and then infected with 300 $CCID_{50}$ of HIV-$1_{Ba\text{-}L}$. Two hours after infection, the cultures were extensively washed to remove excess virus and then cultured under the same conditions as before. Cells were washed and fed every 3–4 days. At day 11 after infection the cells were washed and re-fed with complete medium supplemented or not with different concentrations of PNA_{HIV} or PNA_{ctr}. After 48 h (day 13) the cells were washed, re-fed and cultured for further 48 h in the presence of the same concentrations of PNAs as before. At day 15 samples of supernatants were collected for HIV-p24 testing. The ability of PNA_{HIV} to prevent mitogen-induced expression of HIV-1 in lymphocytes was analyzed as follows. Just after elutriation, lymphocytes were distributed into 15 ml polyethylene tubes at a concentration of 6×10^6 cells/ml in complete medium and incubated with 300 $CCID_{50}$ of the different HIV-1 isolates. After 24 h the cells were washed twice in PBS to eliminate excess virus and inoculated in each well of a 48-well plates at a concentration of 10^6 cells/ml in complete medium supplemented with 5 μg/ml PHA and 10 U/ml IL-2 in the presence or in the absence of 10 μM PNA_{HIV} or 10 μM PNA_{ctr} At day 3 after infection the cells were washed to remove PHA and re-fed with complete medium supplemented with IL-2 and the same concentrations of PNAs as before. The cells were then re-fed every 3–4 days. At given time points (see Section 3) samples of supernatants were collected for HIV p24 testing.

2.6. Cytofluorimetric (FACS) analysis

FACS analysis of intracellular HIV-p24 antigen was performed as previously described (Bergamini et al., 1999). In brief, at day 10 after infection lymphocytes were treated with 10 μM PNA_{HIV} or 10 μM PNA_{ctr}. After 48 h the cells were washed, re-fed and cultured for a further 18 h in the presence of 1 μg/ml of the protein transport inhibitor brefeldin A. Then, the cells were collected, washed twice in PBS and fixed in 4% paraformaldehyde at 4 °C for 15 min. After two further washes, the cells were re-suspended for 30 min at room temperature in 30 μl PBS containing 0.1% saponin, 1% BSA and 0.5 μg/million cells of phycoerythrin-conjugated (PE) mouse anti-HIV p24 antigen (Immunotech, Marseille, France). As a last step, the cells were washed twice in PBS containing 0.01% saponin. Paired isotype-specific control Abs (PharMingen) were run with each sample. FACS analysis was performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Five thousand cells were computed in list mode and analysed using the FACScan research software (Becton Dickinson). Lymphocytes were differentiated from macrophages and dead cells on the basis of forward angle and 90° scatter. Five thousand cells were analyzed for each sample.

2.7. RT-PCR and sequencing

Total RNA was isolated using SV Total RNA Isolation System (Promega Corporation, Madison, WI), inclusive of DNase I digestion, and then ethanol precipitated. RT-PCR was performed using Access RT-PCR System (Promega) that allows to carry out reverse transcription and amplification in one reaction tube containing 10 μl AMV/*Tfl* 5× reaction buffer, 0.2 mM each deoxynucleotide, 1 μM each primer, 1 mM $MgSO_4$, 5 U of AMV reverse transcriptase and 5 U of *Tfl* DNA polymerase in a total volume of 50 μl. Amounts of RNA in reaction were derived from serial dilutions (12–300 ng). The reaction was performed on DNA Thermal Cycler 480 (Perkin-Elmer). The following HIV-1 primers were used in this study (numbering of nucleotide positions corresponds to that for the HIV-1 individual isolate TH4-7-5, strain IIIB RNA sequence (Neumann et al., 1995); GenBank accession number: L31963): NEF/R (product 531 bp in length containing PNA_{HIV} *nef* target sequence), sense primer, in *nef* gene, (5′-GCAGCTNTAGATCTTAGCCACTT-3′; nucleotides 9080–9102), anti-sense primer, in R sequence, (5′-TCCCAGGCTCAGATCTGGTCTAAC-3′; nucleotides 9628–9651); U3/U3 (product 254 bp in length downstream of PNA_{HIV} *nef* target sequence), sense primer, in U3 sequence, (5′-CAGATATCCACTGACCTTTGG-3′; nucleotides 9232–9252), anti-sense primer, in U3 sequence, (5′-CAGCGGAAAGTCCCTTGTAG-3′; nucleotides 9466–9485). β-actin was the housekeeping gene selected as internal standard and the primers used were: sense primer (5′-CGTACCACTGGCATCGTGAT-

3′; nucleotides 506–525) and anti-sense primer (5′-GTGTTGGCGTACAGGTGTTTG-3′; nucleotides 937–957) (Thellin et al., 1999; Castello et al., 2002). The length of the amplification product was 452 bp. The thermal cycling protocol consisted of 45 min incubation at 48 °C followed by heating to 94 °C for 2 min, then 33 or 21 cycles, respectively for HIV-1 or β-actin, of 94 °C for 60 s, 58 °C for 30 s, and 68 °C for 60 s, with a final extension at 68 °C for 7 min. 10 μl of the amplification product from each PCR were separated on 1.8% agarose gel, stained with ethidium bromide and visualized by UV irradiation. The absence of DNA contamination in the RNA preparation was monitored performing parallel reactions with the exclusion of the AMV-RT in the reaction mix. NEF/R PCR product was purified with GeneDia Seq-Prep Kit and was sequenced in both directions using CEQ 2000 automated sequencer (Beckman Coulter).

2.8. Real-time RT-PCR

Total RNA was reverse-transcribed to cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA): 50 μl of 2× RT Master Mix (2× RT buffer, 2× dNTP mixture, 2× random primers, 5 U of MultiScribe RT) was added into each tube containing 50 μl of RNA sample (500–1500 ng). Incubation conditions were 10 min at 25° C and 2 h at 37 °C. We used real-time RT-PCR assays and optimized them on a TaqMAN ABI 7000 (Applied Biosystems) in a reaction volume of 25 μl containing 900 nM of each primer, 200 nM of each probe, 12.5 μl of TaqMan universal Master Mix (Applied Biosystems) and 3 μl of cDNA. Incubation conditions were: 2 min at 50 °C (for optimal AmpErase UNG activity), 10 min at 95 °C (for deactivation of AmpErase UNG activity and activation of AmpliTaq Gold) then 40 cycles of 15 s at 95 °C and 1 min at 60 °C (for probe/primer hybridization and DNA synthesis). The sequences of primer set and TaqMan probe were designed in U3 region of the HIV sequence deduced via sequence analysis of NEF/R PCR product using the Primer Express 2.0 software (Applied Biosystems). Primers and probe sequences were homologous to both the reference sequence used in this study (GenBank accession number: L31963) and NEF/R PCR product sequence. The sequences were the following: sense primer 5′-CACCAGGACCAGGGATCAGA-3′, nucleotides 9216–9235; anti-sense primer 5′-GCTCAACTGGTACTAGCTTGTAGCA-3′, nucleotides 9257–9281; probe 5′-CCACTGACCTTTGGATG-3′, nucleotides 9239–9255. Commercially available pre-developed TaqMan endogenous reference β2 microglobulin gene was used in order to normalize the amount of cDNA added per sample. A comparative C_T (threshold cycle) method was used to determine relative quantification of gene expression. All PCR reactions were performed in triplicate.

2.9. Assessment of PNAs toxicity

Cell viability was measured as propidium iodide (PI) staining and flow cytometric. Briefly, the cells were collected by low speed centrifugation, washed twice in PBS and re-suspended in 0.5 ml of PBS containing 2 μg/ml PI (Sigma). PI fluorescence was measured using a FACScan flow cytometer (Becton Dickinson). The forward and side scatter of particles were simultaneously measured. Cell debris were excluded from analysis by appropriately raising the forward scatter threshold. At least 10^4 cells of each sample were analyzed.

The effect of PNA_{HIV} on [^{3}H]-thymidine incorporation in lymphocytes was assessed as follow. Lymphocytes were grown in the presence or absence of different concentrations of PNA_{HIV} or PNA_{ctr} and supplemented with 1 μCi of [*methyl-*^{3}*H*]thymidine/ml (86 Ci/mmol) (Amersham, International, Buckingham-Shire, England). After 48 h the cells were harvested, washed, counted and re-suspended in 200 μl of lysis buffer containing 1% NP-40 in PBS. The nucleic acids were precipitated by adding cold 10% trichloroacetic acid (TCA). Precipitates were collected on GF/C glass fiber filters (Whatman Inc., Maidstone, Kent, England) and washed extensively with ice-cold 10% TCA and once in 70% ethanol. Filtres were dried and placed in scintillation vials, and radioactivity was counted in the scintillation counter. Protein content in each lysate was estimated by the Bio-Rad protein assay. Results were expressed as counts per minute per milligram of protein. After cell lysis, the radioactivity incorporated into DNA was determined in a β-counter.

To measure the effect of PNA_{HIV} or PNA_{ctr} on protein synthesis in macrophages, these cells were cultured for 5 days with or without PNAs in methionine-free medium supplemented with 1 μCi [^{35}S]-methionine (Amersham International, Buckingham-Shire, England). After cell lysis, the radioactivity incorporated into acid-insoluble material was determined. Results were expressed as counts per minute per milligram of protein.

2.10. Statistical analysis

Non-parametric statistical analysis was performed using the Mann–Whitney *U* test. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Anti-HIV activity of PNA_{HIV} in chronically infected cells

We first assessed the efficacy of PNA_{HIV} to inhibit HIV-1 production in cells (lymphocytes and macrophages) with chronic productive infection. As shown in Fig. 1, a dose-dependent inhibition of viral replication was observed in

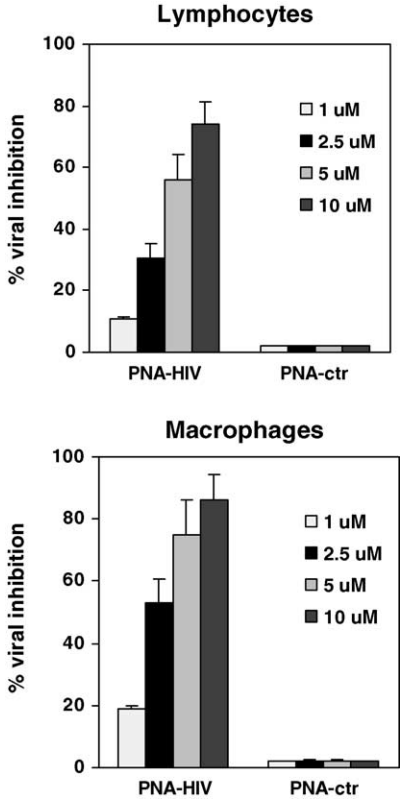

Fig. 1. Anti-HIV activity of PNA_{HIV} and PNA_{ctr} in chronically infected lymphocytes and macrophages. The antiviral activity of PNA_{HIV} was assessed by measuring the amount of HIV p24 antigen in the supernatants with respect to untreated cells. Average HIV p24 production in untreated controls was 356 ng/ml in lymphocytes and 119 ng/ml in macrophages. Results are average values for three experiments each run in triplicate; differences among triplicate were <20%. The error bars represent the standard errors.

cells treated with PNA_{HIV}, as determined by HIV-p24 antigen production. In particular, the 50% effective concentration (EC_{50}) of PNA_{HIV} was 5.8 μM and 3 μM in lymphocytes and macrophages, respectively. The anti-viral activity of PNA_{HIV} did not increase with concentrations up to 30 μM in both cell types. In contrast, PNA_{ctr} had no effect on HIV-1 replication, suggesting that the anti-HIV effect of PNA_{HIV} is sequence-specific and is not a merely unspecific effect of the NLS conjugate. These findings were confirmed and extended by FACS analysis for intracellular HIV p24 production. Indeed, as shown in Figs. 2 and 3, PNA_{HIV} treatment reduced the percentage of HIV p24 positive cells with respect to untreated samples and PNA_{ctr}-treated cells ($85 \pm 16\%$ in untreated samples, 79 ± 12 in PNA_{ctr}-treated cultures and $20 \pm 9\%$ in cultures exposed to PNA_{HIV}, $p < 0.05$). These results suggest that the reduction of HIV p24 production observed in PNA_{HIV}-treated cultures is due to the ability of this compound to completely inhibit viral replication in a percentage of infected cells rather than to a partially inhibitory effect exerted on the totality of infected cells.

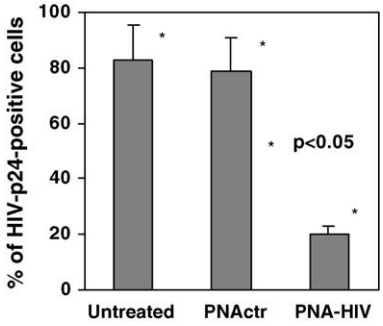

Fig. 2. PNA_{HIV} treatment reduces the number of HIV-1 productively infected cells. The percentages of HIV p24-positive cells were obtained by FACS. Percent of p24-positive cells in the PNA_{HIV}-treated group were compared to untreated or PNA_{ctr}-treated cells. Results are average values for three experiments. The error bars represent the standard errors.

3.2. PN_{AHIV} blocks the activation of HIV-1 expression in lymphocytes

Experiments were carried out to evaluate the ability of PNA_{HIV} to block the activation of HIV-1 expression in lymphocytes. To prevent cell activation before infection, lymphocytes were sorted freshly from blood with minimal manipulation. After infection, resting lymphocytes were treated with PNA_{HIV} or PNA_{ctr} and then stimulated by PHA and IL-2. As shown in Fig. 4, PNA_{HIV}, but not PNA_{ctr}, was able to prevent mitogen-induced replication of the laboratory strain and of the three different clinical isolates resistant to HAART.

3.3. Sustained inhibition of HIV replication by PNA_{HIV} in chronically infected macrophages

In view of the potential use of PNA_{HIV} in patients (usually treated with antiviral drugs for prolonged periods), we next assessed whether a breakthrough of viral replication could occur when HIV-infected macrophages were maintained in culture for a long period after viral infection, in the continuous presence of PNA_{HIV}. As shown in Fig. 5, 10 μM PNA_{HIV} inhibited HIV-p24 production for 11 days, without any detectable loss of efficacy. In addition, no decrease of antiviral activity was detected when infected macrophages were cultured for 14 additional days after the removal of PNA_{HIV}. This suggests that the binding of PNA_{HIV} to the target site on HIV-1 proviral DNA is very stable. In contrast, the inhibition of viral replication by U75875, an inhibitor of HIV-1 protease (Bergamini et al., 1999), was completely lost when this compound was removed from infected cultures.

3.4. PNA_{HIV} inhibits HIV-1 transcription

Semi quantitative RT-PCR and quantitative real time RT-PCR were performed on total RNA extracted from chronically infected H9 cells cultured in the presence or in the absence of 10 μM PNA_{HIV} or 10 μM $PNA_{ctr.}$ The HIV primers

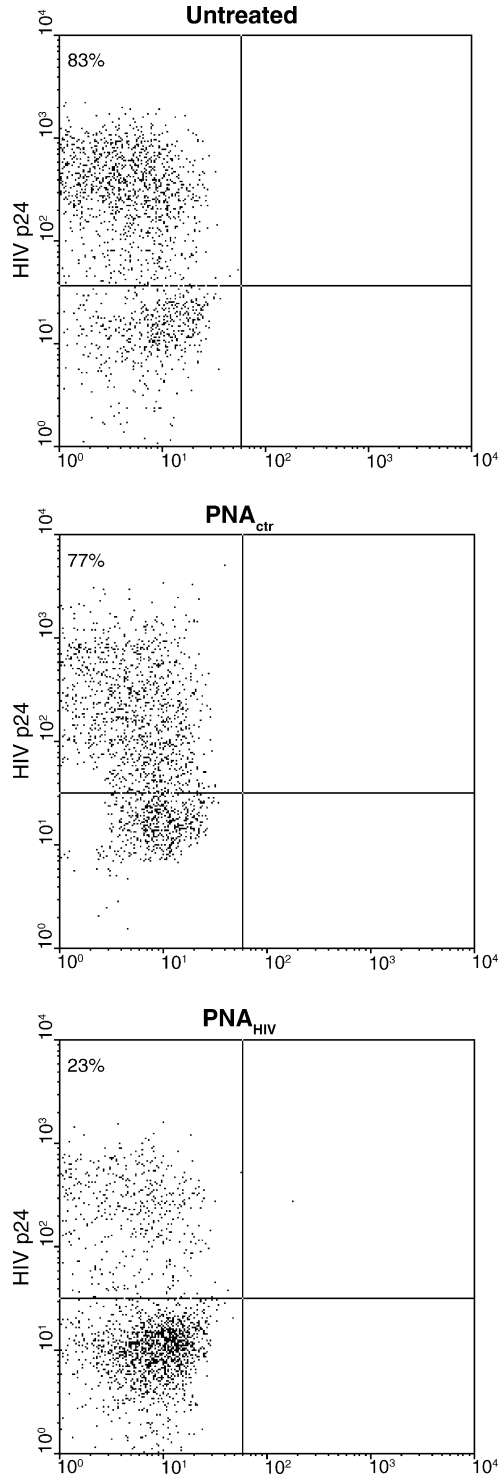

Fig. 3. FACS analysis of a typical experiment out of three summarized in Fig. 2. The data are displayed as dot plots. The quadrants were set according to the negative controls (less than 1% of the isotype control cells appeared positive). Low left quadrants: unstained cells; upper left quadrants: cells stained with PE-conjugated antibody (anti-p24). Five thousand cells were analysed for each sample.

Table 1
Effect of PNA_{HIV} on the incorporation of [^{3}H]-thymidine in lymphocytes and [^{35}S]-methionine in macrophages

CPM ± S.D. at a PNA concentration (μM) of:			
0	5	10	20
Lymphocytes			
1241 ± 317	1145 ± 107	1309 ± 333	1111 ± 71
Macrophages			
8207 ± 471	8051 ± 945	8255 ± 891	8051 ± 839

Lymphocytes and macrophages were grown in the presence of the indicated concentrations of PNA_{HIV} for 48 h. The total amount of radioactivity was determined by scintillation counting and the results are represented as counts per minute incorporated per milligram of protein. Results represent the average ± S.D. of two experiments each carried out in duplicate.

pair NEF/R used in RT-PCR reaction amplifies a sequence encompassing the PNA_{HIV} target sequence located in the *nef* gene. This sequence represents the second site along the HIV-1 genome, where PNA_{HIV} was expected to block RNA polymerase. Since PNA oligomers are able to inhibit RT-PCR, the use of primers such as NEF/R that amplifies a sequence encompassing the PNA_{HIV} target sequence may result in inhibition of product synthesis. To solve this problem an RT-PCR was performed in U3 sequence (primers pair U3/U3) downstream of the PNA_{HIV} *nef* target sequence and identical results were obtained (data not shown). Therefore, also the real-time RT-PCR was located downstream of the PNA_{HIV} *nef* target sequence. As shown in Fig. 6A, RT-PCR demonstrated a substantial decrease in HIV-RNA expression in PNA_{HIV}-treated samples with respect to cultures exposed to PNA_{ctr}. Fig. 6 B also shows that by real time PCR a 75% decrease of HIV RNA expression was found in cells treated with PNA_{HIV} but not in those treated with PNA_{ctr}. Failure of PNA_{ctr} to prevent HIV RNA synthesis confirmed the specificity of the effect of PNA_{HIV}. These results indicate that block of viral expression by PNA_{HIV} occurred at the transcription step.

3.5. Toxicity

As shown in Fig. 7, PNA_{HIV} did not affect cell viability at the concentrations that inhibited HIV replication in both lymphocytes and macrophages. The 50% cytotoxic concentration (CC_{50}) of PNA_{HIV} was 66.6 μM and 103.6 μM in lymphocytes and macrophages, respectively. Similarly, both [^{3}H]thymidine and [^{35}S]methionine incorporation were not impaired with respect to untreated cells at the PNA_{HIV} concentrations that were effective against HIV replication (Table 1).

4. Discussion

A large number of studies have focused on the ability of anti-gene compounds to inhibit transcription initiation by preventing the binding of transcription factors (Grigoriev et al., 1992, 1993a,b). In contrast, only a few studies have focused

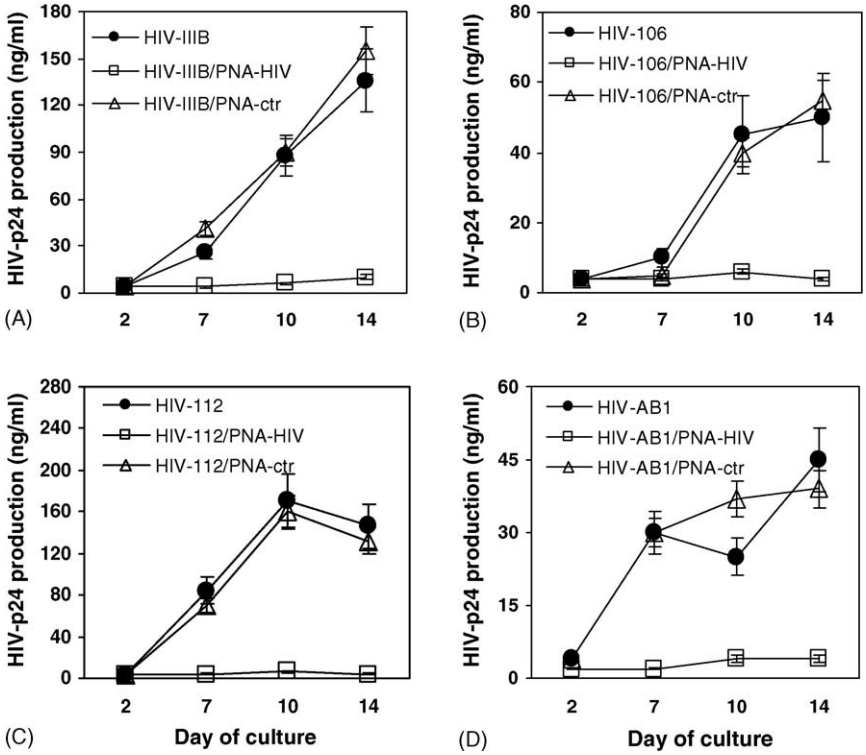

Fig. 4. PNA_{HIV} prevents mitogen-induced expression of HIV-1. Freshly sorted lymphocytes were infected with a laboratory strain (HIV-IIIB) and three different HIV-1 isolates (HIV-106, HIV-112, HIV-AB1). Results are average values for three experiments each run in triplicate. The error bars represent the standard errors.

on anti-gene compounds targeted far downstream of the transcription initiation site, so that they are presumed to interfere with transcription elongation by acting as a roadblock to RNA polymerase along the DNA. Here, we have analyzed the ability of a PNA targeted to a sequence located in a coding region

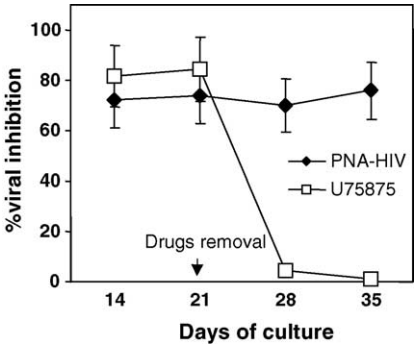

Fig. 5. Sustained inhibition of HIV replication by PNA_{HIV} in chronically infected macrophages. At day 10 after infection the cultures were washed and re-fed with fresh medium containing or not 10 μM PNA_{HIV} or 10 μM U75875. At day 12 the cultures were washed and re-fed with fresh medium containing the same concentrations of drugs as before. At day 21 the cultures were extensively washed to remove antiviral compounds and then run in the absence of these. Results (representative of 2 experiments with two different donors, each carried out in triplicate) are % inhibition of HIV p24 production in supernatants of drug-treated infected macrophages, compared with untreated cells. Average HIV p24 production in infected controls was 135, 111, 87, and 62 ng/ml, at days 14, 21, 28 and 35, respectively. The error bars represent the standard errors.

far downstream of the start site of the HIV-1 genome to inhibit HIV-1 replication by arresting transcription elongation. PNA_{HIV} is complementary and anti-parallel to the template strand of the proviral DNA target sequence; it is supposed to invade the double helix and to replace the coding strand by forming a high-energy hybrid by Watson–Crick bonds.

As we have shown here, PNA_{HIV} inhibited HIV-1 replication in both lymphocytes and macrophages. PCR results suggest that PNA_{HIV} prevented viral RNA synthesis, by arresting elongation of transcription. However, complete inhibition of viral replication could not be obtained in either cell types. The inability of PNA_{HIV} to completely inhibit HIV-1 replication could be due to the ability of RNA polymerase to bypass the PNA/DNA hybrid with read through transcription beyond the PNA block (Giordano et al., 1989; Dubendorff and Studier, 1991). PNAs targeted to multiple sites on the viral genome may be expected to provide an additive effect and could be effectively used to ensure complete blockage of the process of viral RNA transcription.

The importance of latent reservoirs for HIV-1 is highlighted by studies on potent antiretroviral drugs that block new rounds of infection and produce a dramatic drop in plasma virus in two weeks. The rapid decline shows that virus production results largely from continuous rounds of virus infection and replication in host cells with rapid turnover of free virus and virus-producing cells (Ho et al., 1995; Wei et al., 1995; Perelson et al., 1996; Coffin, 1995). Subsequently, however, plasma virus declines at a slower rate, presumably

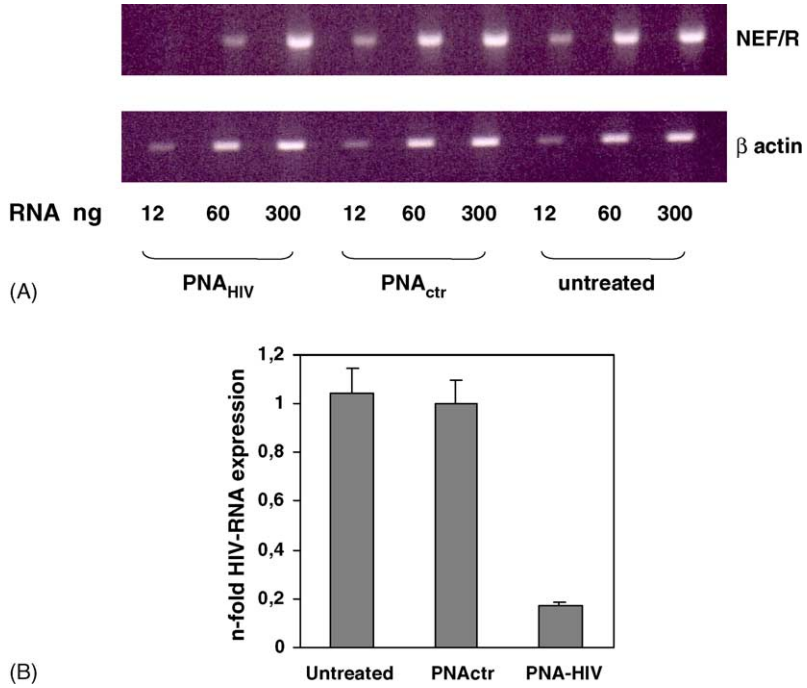

Fig. 6. PCR and real-time PCR analysis of HIV-RNA expression in H9 cells treated with PNA$_{HIV}$. 10^6 chronically infected H9 cells were cultured in 15 ml polyethylene tubes for 48 h in 500 μl of complete medium, at 37 °C in a humidified atmosphere of 5% CO_2 in air, in the presence or in the absence of different concentrations of PNA$_{HIV}$ or PNA$_{ctr}$. (**A**) Representative profile of HIV-RNA expression by limiting dilution RT-PCR, using NEF/R primers. β-actin RNA expression was determined in parallel as a control. Paired mock infected samples were used as negative controls and assayed undiluted. (**B**) Analysis of HIV-RNA expression by real time PCR. Results are expressed as the ratio between the expression of HIV RNA and the expression of the β2 microglobulin gene, using uninfected controls as calibrator, and represent the mean ± S.D. of two experiments. HIV p24 antigen production was assessed in the supernatants from the culture used for HIV-RNA testing. Results were as follows: untreated controls: 574 ng/ml; 10 μM PNA$_{HIV}$: 94 pg/ml (84% reduction); 10 μM PNA$_{ctr}$: 560 pg/ml. Trypan blue dye exclusion test carried out just before RNA extraction did not show significant differences among the different cell samples (data not shown).

reflecting the loss of long-lived productively infected cells (Perelson et al., 1997). Despite their extremely low frequency, (<10^7 per individual), loss of latently infected cells with replication competent proviral genomes takes place on a very slow time scale, with a half-life of 6 months or greater (Chun et al., 1997b; Michie et al., 1992; Ramratnam et al., 2000), and

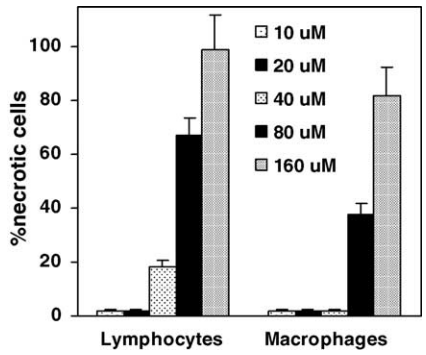

Fig. 7. Effect of PNA$_{HIV}$ on the viability of lymphocytes and macrophages. Elutriated lymphocytes and macrophages were grown in the presence of the indicated concentrations of PNA$_{HIV}$ for 48 h. The percentage of necrotic cells (calculated with respect to untreated samples) was determined by propidium iodide staining and cytofluorimetric analysis. Results are average values for two independent experiments. The toxicity profile of PNA$_{ctr}$ was superimposable to that of PNA$_{HIV}$ (data not shown).

the viral reservoir in these cells is unlikely to be affected by HAART because of low cell turnover and/or low level of virus expression (Zack et al., 1990, 1992; Sun et al., 1997). Moreover, immune activation associated with HIV infection itself, as well as with infection with opportunistic pathogens, may induce viral replication in latently infected cells (Fauci, 1993; Feinberg, 1996; Pantaleo and Fauci, 1995; Stanley et al., 1996; Staprans et al., 1995). It has been established that HIV infection can progress even though only a small fraction of the susceptible cell population is infected (Fauci, 1993) and it is assumed that a cure can only be achieved if the latent reservoirs of replication competent provirus are also eliminated. We wish to stress that any specific clinical or therapeutic inference from our data should be made only with great caution at this time. However, as we show here, anti-gene PNA both downregulated active HIV expression in chronically infected cells and inhibited HIV reactivation in mitogen-stimulated cells. For these reasons, anti-gene PNAs represent a promising strategy aimed at eradicating those minor populations of infected cells that serve as reservoirs of inducible and replication-competent HIV.

Another impediment to controlling HIV-1 infection is the emergence of drug resistant viral strains which have been directly correlated with mutations in the genes encoding the viral reverse transcriptase and/or protease, the two key viral

enzymes in HIV-1 life cycle that are targeted by the currently available antiretroviral compounds (Martinez-Picado et al., 2000; Brown et al., 2000; Buhler et al., 2001). Along this line, is important to note that anti-gene PNA may overcome the barrier of virus mutability, and consequent drug resistance, since they can be easily targeted to conserved regions of the HIV-1 genome. In particular, we show here that PNA_{HIV} inhibited with the same efficiency the mitogen-induced reactivation of three clinical HIV-1 variants resistant to reverse transcriptase and/or protease inhibitors. This is consistent with the fact that the PPT sequence is conserved in all HIV-1 strains.

In conclusion, our approach using anti-gene PNA to target the HIV-1 proviral DNA in the quest for new antiretroviral agents appears quite promising. In addition, anti-gene PNA are potential effective antiretroviral compounds that may be useful in combination with reverse transcriptase and/or protease inhibitors to increase the efficacy of the antiviral treatments and to prevent the emergence of drug-resistant HIV variants.

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