DNA Aptamers Selected against the HIV-1 RNase H Display in Vitro Antiviral Activity†

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ABSTRACT: The DNA polymerase of the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is a target widely used to inhibit HIV-1 replication. In contrast, very few inhibitors of the RNase H activity associated with RT have been described, despite the crucial role played by this activity in viral proliferation. DNA ligands with a high affinity for the RNase H domain of HIV-1 RT were isolated by systematic evolution of ligands by an exponential enrichment strategy (SELEX), using recombinant RTs with or without the RNase H domain. The selected oligonucleotides (ODNs) were able to inhibit in vitro the HIV-1 RNase H activity, while no effect was observed on cellular RNase H. We focused our interest on two G-rich inhibitory oligonucleotides. Model studies of the secondary structure of these ODNs strongly suggested that they were able to form G-quartets. In addition to the inhibition of HIV-1 RNase H observed in a cell free system, these ODNs were able to strongly diminish the infectivity of HIV-1 in human infected cells. Oligonucleotides described here may serve as leading compounds for the development of specific inhibitors of this key retroviral enzyme activity.

The reverse transcriptase (RT)[1] plays a key role in the life cycle of retroviruses (*1*). This enzyme catalyzes the conversion of retroviral single-stranded genomic RNA to double-stranded DNA following the entry of the virus into the cytoplasm of the host cell. RT is a multifunctional enzyme displaying DNA polymerase activity on both RNA and DNA templates, and a ribonuclease H activity on RNA−DNA hybrids (RNase H). It also plays a role during strand transfer. All these steps are crucial for viral replication. The RT of human immunodeficiency virus type 1 (HIV-1) is a heterodimeric enzyme possessing 66 and 51 kDa subunits (p66 and p51, respectively). Both DNA polymerase and RNase H activities reside in the p66 polypeptide. The RNase H domain is located in the C-terminal part and the DNA polymerase domain in the N-terminal portion of the protein. The p51 subunit lacks the C-terminal region carrying the RNase H activity. In the heterodimeric crystal, p66 forms a cleft where the template−primer duplex can be bound between the polymerase and RNase H active sites (*2*). Actually, in the RT−DNA cocrystal, obtained with a 19-base/18-base dsDNA, the primer−template duplex is bent, and is located between both active sites.

Vaccination against HIV-1 is still a long-range goal. Thus, the main clinical treatment for controling the development of acquired immunodeficiency syndrome (AIDS) today is the use of inhibitors targeted against some virally encoded enzymes such as the HIV-1 protease and reverse transcriptase in the so-called multitherapy approach. Reverse transcriptase is a target widely used for therapeutic intervention after HIV infection. Nucleoside analogues and non-nucleoside RT inhibitors (NNRTIs) are effective at blocking viral replication and slowing the onset and progression of AIDS. Recently, new inhibitors of HIV-1 RT have been identified using the SELEX approach (systematic evolution of ligands by exponential enrichment). The development of SELEX has allowed the isolation of oligonucleotide sequences with the capacity to recognize virtually any class of target molecule with high affinity and specificity. These oligonucleotides, termed "aptamers", are emerging as a class of molecules that rival antibodies in both therapeutic and diagnostic applications (for recent reviews, see refs *3*−*5*). High-affinity nucleic acids ligands have been described that may be useful both as inhibitors of HIV-1 RT and for structural studies of the enzyme. The first results using SELEX allowed the selection of RNA pseudoknots that bind specifically to HIV-1 RT and inhibit the DNA polymerase activity (*6*). Interestingly, Burke et al. (*7*) found several new RNA inhibitors that differ significantly from the pseudoknot previously described. A

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; AMV, avian myeloblastosis virus; M-MLV, Moloney murine leukemia virus; RT, reverse transcriptase; ODN, oligodeoxynucleotide; AZT, 3′-azido-3′-deoxythymidine; ddI, dideoxyinosine; ddC, dideoxycytidine; SELEX, systematic evolution of ligands by exponential enrichment; NNRTI, non-nucleoside reverse transcriptase inhibitor.

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wide variety of single-stranded DNA ligands to HIV RT were also obtained (*8*). All these ODNs selectively inhibit the DNA polymerase activity in the nanomolar range.

Most of the antiviral compounds inhibiting HIV replication in cell culture, such as AZT, ddI, ddC, nevirapine, etc., act primarily by inhibiting the DNA polymerase function of the RT, either as chain terminators or by directly interacting with the enzyme. However, it is important to recall that point mutations in the RNase H domain of reverse transcriptase, located at the carboxy end of the enzyme, provoke a marked decrease in the level of virus proliferation, an indication of the crucial role of RNase H during the retroviral cycle (*9*). A functional RNase H activity is essential for retrovirus replication. Unfortunately, very few inhibitors of this enzyme have been described, and no clear specificity for the retroviral RNase H emerges from those experiments. The lack of specificity may be explained because the structure and properties of the retrovirally associated RNase H are very similar to those of other cellular RNases H. Several molecules are able to inhibit retroviral RNase H in vitro, although at high concentrations, including DNA (*10*), sulfated polyanions (*11*), and suramin (*12*). The inhibition of HIV-1 RNase H by azidothymidylate (AZT-MP) with an IC_{50} of 50 μM has been reported (*13*, *14*). HIV RNase H and RT activities have been shown to be inhibited by *N*-ethylmaleimide at millimolar concentrations (*12*, *15*, *16*), pyridoxal phosphate (*12*), and vanadyl ribonucleoside complexes (*17*). Allen et al. (*18*) have reported the inhibition of HIV-1 RNase H activity by nucleotide dimers and monomers with IC_{50} values of $\sim$10−15 μM. A promising compound [the *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone] inhibiting the DNA polymerase activity (probably by binding to the NNRTI binding pocket) was also able to inhibit the RNase H activity of HIV-1 RT in the micromolar range by binding to a second site located in this domain (*19*). Illimaquinone, a natural marine product, inhibits preferentially the HIV-1 RNase H activity but also inhibits the RNase H activity of HIV-2 RT, MLV RT, and *Escherichia coli* RNase H (*20*). Phosphorothioate oligonucleotides are also inhibitors of the DNA polymerases and RNase H activities (*21*, *22*). Recently, a product isolated from constituents of *Juglans mandshurica* inhibited the DNA polymerase activity of HIV-1 with an IC_{50} of 40 nM, while the RNase H was inhibited with an IC_{50} of 39 μM (*23*). The specific high-affinity RNA ligands to AMV and M-MLV reverse transcriptases identified by SELEX (*24*) inhibited the DNA polymerase activity with IC_{50} values of 25 and 9 nM for AMV and M-MLV RTs, respectively, and 60 and 75 nM for the RNase H activities of AMV and M-MLV, respectively.

In this study, we used SELEX to isolate high-affinity ligands that are able to inhibit specifically the RNase H activity associated with the HIV-1 RT. We previously expressed and purified two forms of recombinant HIV-1 RT, the p51−p51 form which contains only the DNA polymerase domain (*25*) and the complete heterodimeric p66−p51 enzyme (*26*). The rationale of the work developed here was to use differential screening by alternatively using the p51−p51 RT homodimer (RNase H−) and the p66−p51 heterodimer (RNase H+) in selection and counterselection steps, respectively. This strategy led us to select oligonucleotides that are able to bind specifically to the RNase H domain of HIV-1 RT. We describe here the isolation of these molecules, the characterization of their secondary structure, and their inhibitory properties.

EXPERIMENTAL PROCEDURES

Materials

Recombinant p51−p51 HIV-1 RT expressed in the yeast *Saccharomyces cerevisiae* was purified as previously described (*25*). Recombinant heterodimeric p66−p51 RT was obtained as described previously (*26*). PCR was performed using the Goldstar Red DNA polymerase from Eurogentec.

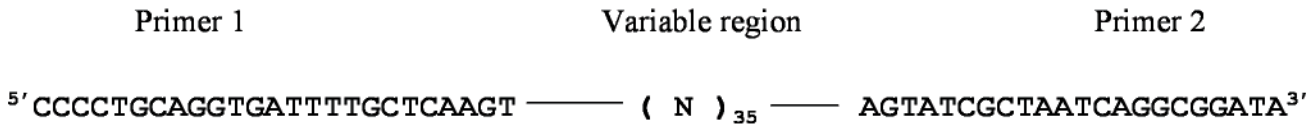
Methods

Polymerase Chain Reaction. A library of synthetic DNAs (Figure 1A) containing 35 random nucleotides flanked by invariant primer annealing sites (primers 1 and 2) was amplified by the polymerase chain reaction. In the first four rounds, primer 2 was biotinylated to allow the regeneration of single-stranded ODNs (*8*). Briefly, the PCR products were incubated with 100 μL of streptavidin−agarose beads (Sigma) for 30 min at 20 °C. After five washing steps with buffer A [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 50 mM NaCl], the matrix-bound dsDNA was denatured in 0.15 M NaOH. After centrifugation, free DNA present in the supernatant was precipitated with ethanol. Single-stranded DNA (50−200 pmol) was obtained and used for further selection steps. After four rounds of selection, running a second PCR using only primer 1 allowed the regeneration of single-stranded DNA.

Selection Procedure. The random ssDNA library was incubated with p51−p51 RT for 30 min at 37 °C in a selection buffer containing 50 mM Tris-HCl (pH 8.0), 6 mM Mg^{2+}, and 10 mM dithiothreitol. After filtration on HAWP filters (Millipore), the flow-through was incubated with p66−p51 RT. KCl (200 mM final concentration) was added, and incubation was performed for 30 min at 37 °C. After filtration, the ODNs retained on the filters were eluted by incubating them for 20 min at 37 °C in 200 μL of 7 M urea and 200 μL of phenol. ODNs were precipitated and amplified by PCR as described above. For the first round of selection, 5 μM ODNs and 0.5 μM RT were used in 300 μL. The concentrations were progressively decreased to reach the following levels in the last round (round 8): 5 nM RT and 200 nM ODNs. The same concentration of the p51−p51 and p66−p51 dimers was used at each round. Following round 8 of selection, ODNs were amplified using primers 1 and 2 and ligated in the pGEM-T vector. After transformation of *E. coli* DH5α, isolates were sequenced using the Thermosequenase sequencing kit (Pharmacia). In all the subsequent experiments, we used ODNs synthesized without primers, except in Figure 5 where ODNs 102 and 112 contained the primers.

HIV-1 RNase H Assay. The substrate for RNase H activity was prepared as previously described (*12*). The template for *E. coli* RNA polymerase was single-stranded calf thymus DNA. HIV-1 RT (4.5 pmol) was incubated with the appropriate ODN (concentrations as indicated in the legends of each figure) in the presence of selection buffer for 30 min at 37 °C. The components of the incubation mixture were added to produce final concentrations of 50 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 6 mM $MgCl_2$, 80 mM

A: Library



B: Variable region of selected ODNs

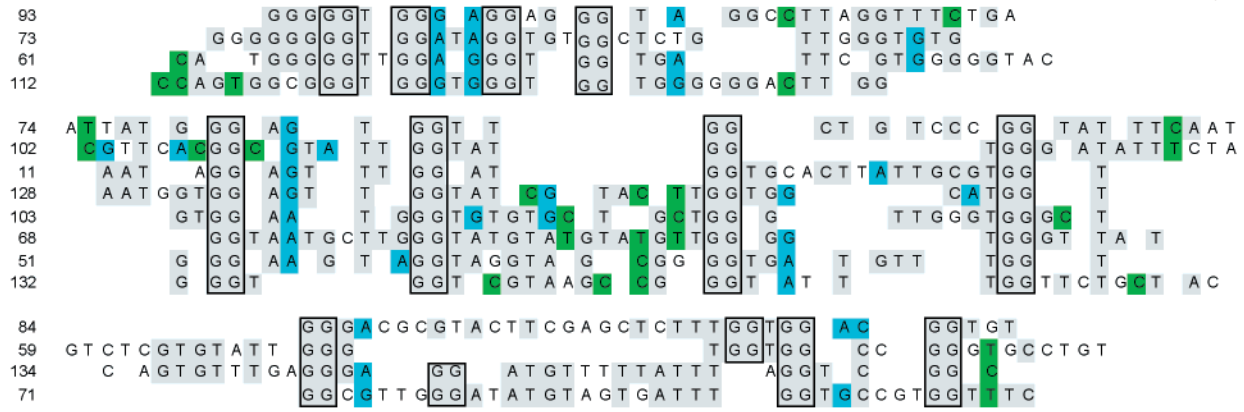


FIGURE 1: Classification of ODNs obtained from the enriched library. (A) Library of synthetic ODNs. (B) Variable region of selected ODNs. The clone numbers are indicated on the left. The G-residues chosen for allowing the best sequence alignment giving the shortest sequences between G-doublets are boxed. These boxed residues might contribute to G-quartets. In gray are shown the identical nucleotides, in blue the purines, and in green the pyrimidine positions.

KCl, and the labeled nucleic acid duplex (20 000 cpm) in a final volume of 50 μL. After incubation for 10 min at 37 °C, the reaction was stopped by addition of 1 mL of cold 10% TCA containing 20 mM pyrophosphate. Samples were filtered on nitrocellulose filters and washed, and the amount of radioactivity was determined.

DNA Polymerase Assay. The DNA polymerase activity was measured as described previously (*27*). As for the RNase H assay, the enzyme was first incubated with ODNs in a volume of 20 μL; then the reaction mixture was added, and the incubation proceeded for 10 min at 37 °C.

Gel Retardation Assay. A 5′-end-labeled ODN (2−4 nmol) was incubated with increasing concentrations of reverse transcriptase, in 10 μL under the conditions used for in vitro selection. The capacity of candidates to form complexes with the target was evaluated by electrophoresis on a 6% acrylamide gel in TBE buffer [89 mM Tris-HCl (pH 8), 90 mM borate, and 90 mM EDTA]. Gels were run at room temperature.

UV Cross-Linking Experiments. Reverse transcriptase was incubated with [^{32}P]ODNs in 50 mM Tris (pH 7.9) and 6 mM Mg^{2+} for 10 min at 37 °C. Samples were then irradiated with a UV transilluminator (254 nm, 2.4 mW/cm^2) at room temperature for 10 min and analyzed on a 12% SDS−polyacrylamide gel.

Antiviral Assays. The infectivity was assayed on P4 cells expressing CD4 receptors and the *lacZ* gene under the control of the HIV-1 LTR (*28*). P4 cells were plated using 100 μL of DMEM in addition to 10% fetal calf serum, gentamicin (45 μg/mL), and Geneticin (200 μg/mL) in 96 multiwells plates, at a density of 12 500 cells/well. After overnight incubation at 37 °C, the supernatant was discarded and 200 μL of fresh medium containing HIV-1 in the presence or absence of oligonucleotides was added. Twenty-four hours later, the supernatant was discarded and the wells were washed three times with 9‰ NaCl. Each well was refilled with 200 μL of a reaction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM β-mercaptoethanol, 0.05% Triton X-100, and 5 mM 4-methylumbelliferyl-β-D-galactoside (4-MUG). After 3 h at 37 °C, the reaction products were assessed in a fluorescence microplate reader (Cytofluor II) at 405 nm.

For the cellular viability experiments, the vybrant MTT cell proliferation assay kit (Molecular Probes) was used according to the manufacturer's indications.

RESULTS

Selected ODN Sequences. To isolate molecules that are able to inhibit HIV-1 RNase H activity, we used the SELEX approach. The starting library, containing approximately 10^{14} unique DNA species, was randomized over a 35-nucleotide stretch flanked by a 25-nucleotide fixed region at the 5′-end and a 21-nucleotide fixed region at the 3′-end. Selected aptamers were obtained by first incubating the recombinant p51−p51 RT lacking the RNase H domain of HIV-1 RT with ODNs in a buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM Mg^{2+}, and 10 mM dithiothreitol. The p51−p51·ODN complexes were filtered, and the flow-through was incubated with the heterodimeric p66−p51 HIV-1 RT. To increase the stringency of the selection, KCl was added at a final concentration of 200 mM. After a second filtration step involving the ODN·p66−p51 complex, ODNs were eluted from the filters and amplified by PCR prior to the next selection round. This two-step procedure was aimed at selectively enriching the DNA pool in candidates exhibiting affinity for the RNase H domain at the expense of sequences that would bind to the RT molecule outside of the targeted RNase H polypeptide. After eight rounds of selection and amplification, the PCR-derived molecules were cloned and sequenced. No consensus can be identified by comparison of either the primary sequences or the secondary structures predicted by a folding algorithm (*29*). Many folded candidates exhibited stem−loop structures derived from base pairing between nucleotides from the primers and the central variable region. For instance, several ODNs possess a G-rich sequence in the central 35-nucleotide cassette, which is able to base pair with the invariant CCCC 5′-end. However, this double-stranded motif did not appear in every selected candidate and is therefore not likely a characteristic of anti-RNase H sequences.

As shown in Figure 1B, the selected sequences exhibited a very high proportion of G- and T-residues in the variable region. On the basis of primary sequence similarities, we ranked the selected candidates in three classes (Figure 1B). ODNs 93 and 74 are the heads of the first two groups, whereas sequences in the last group (84, 59, 134, and 71) exhibit lower degrees of homology. Such G-rich oligomers are prone to forming G-quartet structures. The sequences of the selected ODNs were tentatively aligned on the putative G-residues involved in G-quartet interactions.

The first group comprising ODNs 93, 73, 61, and 112 presented a G-cluster at the 5′-end. The G-residues that are potentially able to form G-quartets are separated by very short loops (three residus at most). The second group of ODNs (74, 102, 11, 128, etc.) is characterized by much longer sequences between the G-boxes compared to the first one.

It should be mentioned that many other selected ODNs were found, showing G-rich sequences but no possibility of forming G-quartet structures.

Effect of Selected ODNs on RNase H and DNA Polymerase Activity. The ability of the isolated ODNs to inhibit the RNase H activity of the HIV-1 RT was determined by measuring the decrease of TCA-precipitable counts as described in the RNase H assay (see Experimental Procedures). As shown in Figure 2, ODN 93 was a potent inhibitor with an IC_{50} of 500 nM. ODNs 73, 112, and 103 exhibited the same IC_{50} (data not shown). Other ODNs (61, 74, and 134) were weaker inhibitors with IC_{50} values of ~5−6 μM. ODNs 102 and 84 did not show any inhibition.

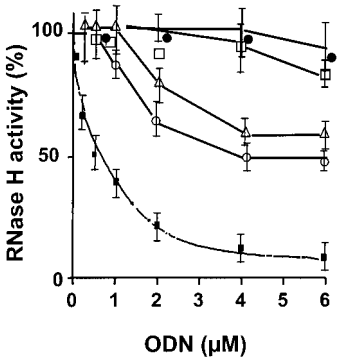


FIGURE 2: Effect of selected ODNs on RNase H activity. The HIV-1 RT p66−p51 heterodimer was incubated with increasing concentrations of ODNs, in the selection buffer. The reaction mixture was then added and incubation carried out for 10 min at 37 °C. The RNase H substrate used in the assay was prepared by synthesizing a labeled RNA in the presence of [^{3}H]UTP and *E. coli* RNA polymerase using single-stranded calf thymus DNA as a template. Effect of ODN 93 (■), ODN 61 (○), ODN b33 (●), and the library (△) on HIV-1 RNase H and (□) effect of ODN 93 on *E. coli* RNase H activity. The b33 sequence is GCTGGTCTCT-GCGGGTTGTTGCGCCGCGGCACCCTTGGCA.

ODN 93 displays an important improvement concerning the inhibition properties with respect to the oligonucleotides present in the original library, for which 50% inhibition was not reached even at concentrations as high as 10 μM. Importantly, b33, a DNA aptamer directed against the human RNase H type II, did not inhibit the HIV-1 RNase H. This oligomer was identified through an independent in vitro selection within a library of DNA sequences containing a window of 40 randomized nucleotides (F. Pileur et al., unpublished observations). As the anti-HIV-1 RNase H, ODN b33 (GCTGGTCTCTGCGGGTTGTTGCGCCGCG-GCACCCTTGGCA) also contained a high proportion of Gs (37%) and could also be folded to generate four-stranded structures.

To ascertain the specificity of the inhibition, we compared the effect of ODN 93 on RNase H enzymes with different origins. In contrast to HIV-1 RNase H, no inhibition was observed either with the *E. coli* RNase H (Figure 2) or with the human RNase H type II activity (not shown), showing an important specific effect toward the retroviral RNase H.

Oligonucleotides, selected according to the previously described procedure, were also assayed for their capacity to inhibit the DNA polymerase of HIV-1 RT. All the tested ODNs were able to inhibit this activity with an IC_{50} of ~500 nM, i.e., significantly lower than the pool of the starting library (Figure 3A). This might correspond to a specific effect, as ODN 93 did not inhibit AMV DNA polymerase activity under conditions where the HIV-1 RT was inhibited (Figure 3B).

All together, these results demonstrate that ODN 93 and related structures (ODNs 112, 73, and 61) are indeed aptamers targeted to the HIV-1 RNase H domain of the RT.

Electrophoretic Mobility Retardation Analysis of the Interaction between Selected DNA Ligands and HIV-1 RT. To check whether oligonucleotides isolated by SELEX were able to interact with the HIV-1 RT, gel mobility retardation assays were carried out. Native gel electrophoresis performed

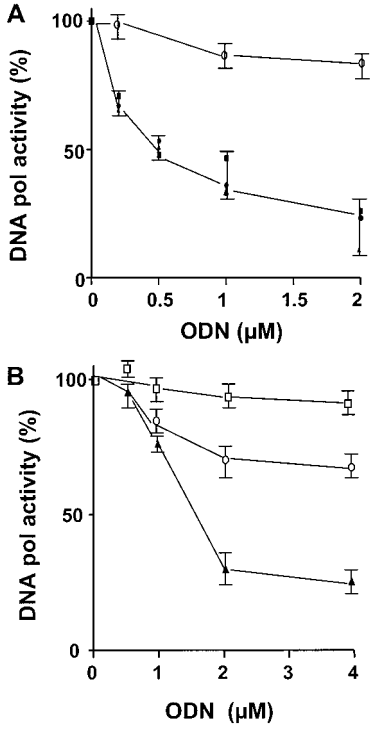


FIGURE 3: Effect of selected ODNs on retroviral DNA polymerase activities. (A) Oligonucleotides were tested on the DNA polymerase activity of HIV-1 RT: library (○), ODN 73 (■), ODN 61 (▲), and ODN 68 (●). (B) ODN 93 was tested on the DNA polymerase activities of AMV (□) and HIV-1 (▲) reverse transcriptases. The ODN pool of the original library was tested on AMV DNA polymerase (○).

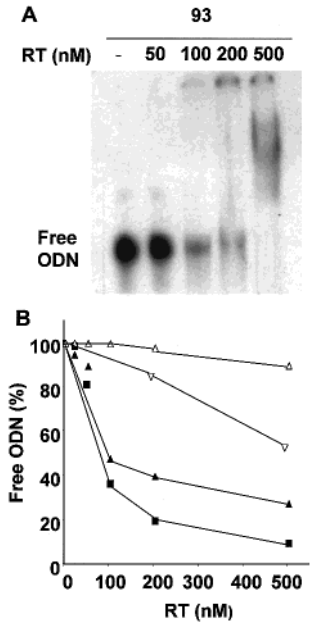


FIGURE 4: Gel retardation assay. (A) The 5′-end-labeled oligonucleotide 93 was incubated with increasing concentrations of the p66−p51 reverse transcriptase under selection conditions. The products were analyzed by electrophoresis on a 6% acrylamide gel in TBE buffer as described in Methods. (B) Scanning profile of the region corresponding to the migration of free ODN: ODN 93 (■), ODN 112 (▲), ODN 93A (▽), and b33 (△). Results are representative of two to four independent experiments. Standard deviations are within 5−10%.

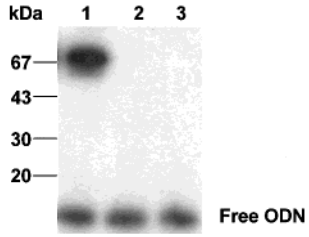


FIGURE 5: Cross-link of ODN 93 with both forms of RT. ^{32}P-labeled ODN 93 was incubated with RT. The samples were then UV-irradiated for 10 min at room temperature and analyzed on 12% SDS−polyacrylamide gels: lane 1, ODN 93 and p66−p51 RT; lane 2, ODN 93 and p51−p51 RT; and lane 3, ODN 93 alone.

with oligonucleotide 93 showed that the magnitude of the band corresponding to the labeled free oligonucleotide decreased with increasing RT concentration (Figure 4A); a decrease of ∼50% for an HIV-1 RT concentration of 70−80 nM was determined by scanning the autoradiograph (Figure 4B). Substituting the five G-residues at the 5′-end of the 93 aptamer sequence, thus generating the control sequence 93A, strongly weakened the binding to HIV-1 RT. RT concentrations higher than 500 nM were needed to obtain 50% binding (Figure 4B). A similar binding pattern was obtained for aptamer 112. In contrast, ODN b33, the G-rich 40mer selected against the human RNase H, did not bind to the HIV-1 RT under the conditions used in our assay.

To locate the aptamer binding site on the RT dimer, UV cross-linking experiments were performed between the ^{32}P-labeled ODN 93 and either the RT or the p51−p51 homodimer. While the entire RT gave a strong cross-link, the p51−p51 dimer (without RNase H) was not able to cross-link the ODN, suggesting that the RNase H domain mediates the binding to the RT (Figure 5, compare lanes 1 and 2). These data were confirmed by gel retardation experiments performed with the p51−p51 enzyme. In contrast to the p66−p51 heterodimer, no interaction was observed between the labeled ODN and the p51−p51 enzyme at concentrations up to 500 μM (not shown).

Are Aptamers Forming G-Tetrad Structures? The G-rich oligonucleotides may generate four-stranded structures through G-quartet formation (*30*−*32*). It was therefore of interest to determine whether the G-rich aptamers selected against the RNase H domain of HIV-1 RT did actually fold into tetraplexes and, subsequently, whether the structure accounted for the specific inhibitory properties displayed by these ODNs. To address this question, two sets of experiments were carried out. First, we monitored the behavior of the oligomers under salt conditions known to have an impact on G-tetrad stability. Second, we evaluated the properties of mutated sequences at G-positions putatively involved in G-quartet formation.

The G-rich oligonucleotides can be very polymorphic, since the structures that are adopted may depend on the presence of different monovalent cations. Cations are known to stabilize G-quartets in the following order: $K^+ > Ca^{2+} > Na^{2+} > Mg^{2+} > Li^{2+}$ (*33*). To determine the influence of different salts on the structure of our anti-RNase H aptamers, the molecules were denatured by heating for 2 min at 100 °C in the presence of either 200 mM LiCl, NaCl, or KCl. After renaturation by slow cooling at room temperature, the ODNs were analyzed by electrophoresis on native polyacrylamide gels (Figure 6). Aptamers 93 or 112 exhibited one band in the presence of Li^+ (Figure 6A, lanes 1 and 4)

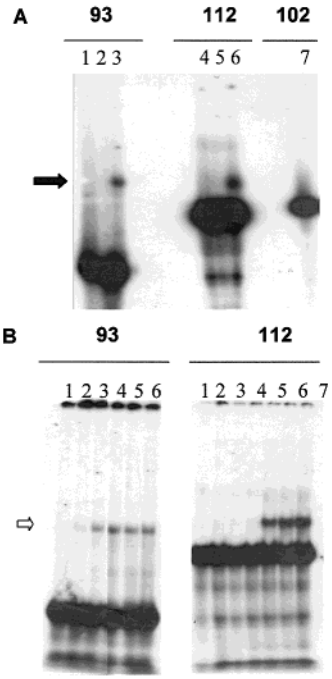


FIGURE 6: Effect of salts on oligonucleotide structures. (A) The 5′-end-labeled ODNs 93, 112, and 102 were heated at 100 °C for 2 min followed by slow cooling at room temperature in a buffer containing 50 mM Tris-HCl (pH 8), 6 mM Mg^{2+}, and either 200 mM LiCl (lanes 1 and 4), NaCl (lanes 2 and 5), or KCl (lanes 3, 6, and 7). Samples were loaded on 6% acrylamide gels in TBE buffer and submitted to electrophoresis. (B) The ODNs were heated in the presence of salts at 100 °C for 2 min and slowly cooled at room temperature before loading on a 6% acrylamide gel. With ODN 112, the buffer conditions were 0, 10, 30, 50, 100, 150, and 200 mM KCl and 200, 190, 170, 150, 100, 50, and 0 mM NaCl (lanes 1−7, respectively). For ODN 93, the conditions were the same as for ODN 112 except that 50 mM KCl and 150 mM NaCl were ommitted. Arrows denote the low-mobility species. ODNs 112 and 102 are longer ODNs containing the constant regions: 25 nucleotides at the 5′-end and 21 nucleotides at the 3′-end.

or Na^+ (Figure 6A, lanes 2 and 5), while a new band with a lower electrophoretic mobility (indicated with an arrow) was present when the ODN was renatured in the presence of KCl (lanes 3 and 6). In contrast, oligonucleotide 102, which weakly inhibited the RNase H activity, presented only one band, even in the presence of KCl (lane 7).

In a second experiment (Figure 6B), the ODNs were renatured in the presence of decreasing NaCl concentrations with concomitantly increasing KCl concentrations. A new band appeared in the presence of 100 mM KCl with ODN 112 (lane 5) and at lower concentrations (10−30 mM KCl, lanes 2 and 3) for ODN 93. These results confirm the presence of two different species in the presence of KCl.

We monitored the inhibitory properties of oligomer 93A derived from aptamer 93; changing the five Gs into five As in the 5′-part of the sequence completely abolished the inhibition of the RNase H activity of the HIV-1 RT. However, changing G-12 into A did not affect the inhibition. Similarly, we screened two mutants of ODN 112. Changing the G at position 18 into an A did not affect the RNase H inhibition. Substituting the six Gs at positions 24−29 with six As did not abolish the inhibition of the retroviral RNase H activity.

Effect of RNase H Inhibitors on Cellular HIV-1 Infectivity. The effect of anti-RNase H aptamers on HIV-1 infectivity

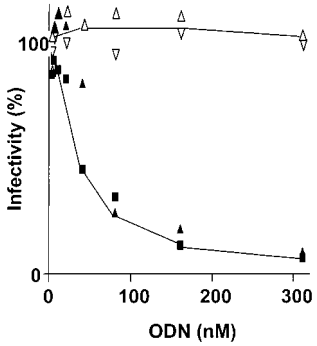


FIGURE 7: Inhibition of viral infectivity. HeLa P4 cells were grown for 24 h in DMEM as described in Methods. Oligonucleotides were added simultaneously with HIV-1_{LAI} particles. The β-gal activity was measured 24 h after infection. Results are expressed as a percentage of infectivity and are representative of three independent experiments, each one in duplicate. The concentration of ODN giving half-maximal infectivity is the IC_{50}. Different concentrations of ODN were used as indicated: ODN 93 (■), ODN 112 (▲), ODN 93A (▽), and ODN b33 (△).

was investigated using human cells infected with HIV-1 as described in Methods. This bioassay is based on the ability of the Tat protein, produced after the genomic integration, to transactivate the HIV-1 LTR driving the expression of the β-galactosidase reporter gene in a HeLa-$CD4^+$ cell line (*34*). The determination of the β-galactosidase activity allows detection of the first cycle of infection. This assay has been used to determine the dose response of drugs and the production of infectious particles after virus infection (*28*, *35*).

ODN 93 or 112 was added to the cell culture at the same time as HIV-1 particles, and inhibited the viral infectivity with an IC_{50} of ∼30 nM (Figure 7). Oligomer 93A, which did not inhibit "in vitro" the HIV-1 RNase H activity, had no effect on HIV-1 infectivity in cell culture. Also, ODN b33, which did not inhibit in vitro the retroviral RNase H activity, had no effect on the infectivity. Oligonucleotides 93 and 112 containing the constant regions (81 nucleotides long) were able to inhibit with an IC_{50} of 10 nM (not shown). In contrast, a mixture of ODNs from the starting library showed 50% inhibition for concentrations higher than 300 nM. ODN 102, which did not inhibit in vitro the RNase H activity, showed inhibition in the same range as the starting library, suggesting that this corresponds to a nonspecific effect. No cytotoxic effect was observed either with ODN 93 or with ODN 112 using the MTT assay (not shown).

DISCUSSION

Specific DNA inhibitors of the RNase H activity associated with the HIV-1 RT were identified by SELEX. No obvious consensus sequences or secondary motif were observed among these ODNs, either full-length or restricted to the variable region. The selected ODNs were characterized by a very high G-content. These aptamers were shown by band-shift assay to bind to the p66−p51 heterodimer of the RT but not to the p51−p51 homodimer devoid of the RNase H domain. This property demonstrates that the counterselection step introduced in the SELEX procedure was efficient and that the RNase H domain was required for aptamer binding. In addition, the isolated ODNs were able to inhibit the RNase H activity to different degrees. The two most powerful

inhibitors, ODNs 93 and 112, were highly specific for the HIV-1 RNase H. Even though the *E. coli* RNase H has been reported to have very important structure homologies with the HIV-1 RNase H domain (*36*), the bacterial enzyme was not inhibited by these ODNs. Therefore, the sequences isolated by SELEX do correspond to aptamers against the RNase H domain of the HIV-1 RT.

The G-richness of aptamers 93 and 112 raised the question of whether they could fold into the characteristic four-stranded structure in which their binding and inhibitory properties might originate. However, experiments performed with aptamer variants and different monovalent cations did not led to clear-cut conclusions. ODN 93 possesses a G-cluster in the 5′-part of the variable region. These Gs are crucial for the aptamer properties since their change into A-residues led to the loss of RNase H inhibition and of the effect on HIV-1-infected cells. Electrophoretic analysis on native gels showed that ODN 93 adopted different structures in the presence of KCl. This was not observed in the presence of LiCl or NaCl. As G-quartets are stabilized by K^+ but not by Li^+ ions, this suggests that ODN 93 may adopt a G-quartet structure.

ODN 112 also shows a region containing six adjacent G-residues. However, in contrast to ODN 93, the change of Gs into As did not affect the inhibition of the RNase H activity. But the A-substituted ODN 112 sequence still retains four G-boxes, which might be significant for maintaining a four-stranded structure.

The molecules selected in this work do not share features with the DNA aptamers previously isolated against the intact HIV-1 RT (*8*). In this latter work, the aptamers showed complementarities between a CCCC sequence in the variable region and the GGGG domain in the conserved 3′-region. They were predicted to form pseudoknots, but no G-quartet structures were proposed.

Our selected DNA ligands share little or no structural similarity with RNA aptamers to HIV-1, AMV, M-MLV, and FIV RTs (*24*, *37*). This is not unexpected since generally the geometry adopted by DNA is different from the one generated by RNA chains of the same sequence.

The DNA polymerase activity, an intrinsic property of reverse transcriptase, was also inhibited by the aptamers inhibiting the retroviral RNase H. It is known that the DNA polymerase and the RNase H sites are overlapping, an unusual property of HIV-1 RT compared to other retroviral enzymes in which these domains are absolutely independent (*38*, *39*). The crystal structure of the HIV-1 RT shows that both the polymerization and RNase H activities are related to the template−primer binding region, spanning ∼18 bp (*40*). Our results may be explained by the possibility that some ligands may occupy different positions in the RNase H and DNA polymerase active sites of HIV-1 RT.

The three-dimensional structure of the RNase H domain associated with HIV-1 RT has been described previously (*36*), and was latter demonstrated to be very similar to that of the catalytic domain of the HIV-1 integrase (*41*). An in vitro selection of RNA ligands has been performed with the integrase as a target (*42*). Not unexpectedly, due to structural differences induced by the different chemistry, the RNA ligands do not display features common to the ODNs obtained in our work. It is interesting to point out that the HIV-1 integrase is inhibited by G-tetrad-forming oligonucleotides T30695 (5′-GGGTGGGTGGGTGGGT) and T30177 (5′-GTGGTGGGTGGGTGGGT) (*43*−*45*). These ODNs are similar to the RNase H aptamer ODN 112 (5′-GGGTGGGT-GGGTGGT), described here, while a GT sequence is found in ODN 112 instead of the TG sequence observed in ODNs T30695 and T30177.

Our results clearly indicate that ODNs 93 and 112 are also powerful inhibitors of HIV-1 replication in infected cells. Changing the G-clusters to As in ODN 93 abolished the effect in HIV-1-infected cell cultures, underlining a correlation between the RT binding capacity, the RNase H inhibitory properties, and the in vitro antiviral effect. The starting library of random oligonucleotides and ODN 102 (which has no effect on the RNase H activity in vitro) did not show a significant antiviral effect when added to infected cells. However, further investigations are necessary to understand the precise mechanism of action of these antiviral agents, as several G- and T-rich oligonucleotides have been described to inhibit HIV-1 replication (*45*, *46*). These ODNs possess phosphodiester bonds that are quickly degraded by cellular nucleases. However, it has been described that the intramolecular G-quartet motif confers nuclease resistance to anti-HIV oligonucleotides (*43*). The largest contribution to the antiviral effect of G-quartet-containing oligonucleotides seems to be related to a blockage of the adsorption and of the penetration of the virus into the cell. The G-quartet structure and a phosphorothioate backbone of the molecule were both essential for preventing cell-to-cell and virus-to-cell infection (*46*). However, in our hands, b33, a G-rich oligonucleotide selected against the human RNase H II, did not exhibit antiviral properties.

Our work clearly indicates the specific inhibitory activity of ODNs 93 and 112 on viral infectivity in infected cells as well as on RNase H activity in vitro. This observation encourages us to perform further experiments with these ligands to investigate the precise antiviral mechanism.

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