Novel bimodular DNA aptamers with guanosine quadruplexes inhibit phylogenetically diverse HIV-1 reverse transcriptases

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ABSTRACT

DNA aptamers RT5, RT6 and RT47 form a group of related sequences that inhibit HIV-1 reverse transcriptase (RT). The essential inhibitory structure is identified here as bimodular, with a 5′ stem–loop module physically connected to a 3′-guanosine quadruplex module. The stem–loop tolerates considerable sequence plasticity. Connections between the guanosine triplets in the quadruplex could be simplified to a single nucleotide or a nonnucleic acid linker, such as hexaethylene glycol. All 12 quadruplex guanosines are required in an aptamer retaining most of the original loop sequence from RT6; only 11 are required for aptamer R1T (single T residue in intra-quadruplex loops). Circular dichroism (CD) spectroscopy gave ellipticity minima and maxima at 240 nm and 264 nm, indicating a parallel arrangement of the quadruplex strands. The simplified aptamers displayed increased overall stability. An aptamer carrying the original intra-quadruplex loops from RT6 inhibited RT in K+ buffers but not in Na+ buffers and displayed significant CD spectral broadening in Na+ buffers, while R1T inhibited RT in both buffers and displayed less broadening in Na+ buffers. The bimodular ssDNA aptamers inhibited RT from diverse primate lentiviruses with low nM IC50 values. These data provide insight into the requirements for broad-spectrum RT inhibition by nucleic acid aptamers.

INTRODUCTION

Antiviral chemotherapy has achieved spectacular results in prolonging the survival of patients infected with HIV-1. Morbidity and mortality related to HIV-1 have dramatically declined in developed countries, converting HIV infection into a treatable chronic disease. However, current antiviral drugs do not eradicate the virus, and prolonged treatment can have serious side effects and select drug-resistant viral strains (1). In addition, millions of new infections occur worldwide each year (2,3). Continued efforts toward the discovery of new antiviral strategies therefore remain imperative.

The reverse transcriptase (RT) of HIV-1 is a primary target for inhibition by current drugs, which include the nucleoside analog RT inhibitors (NRTIs, primarily chain terminators) and the nonnucleoside RT inhibitors (NNRTIs, noncompetitive allosteric inhibitors of polymerization by RT). Nucleic acid aptamers comprise a third class of RT inhibitors. Because many aptamers compete with the template/primer duplex for access to the enzyme (4–6), they have been referred to as TRTIs (template/primer analog RT inhibitors) (7). Aptamers are derived from the combinatorial method of in vitro selection, or SELEX (for Selective Evolution of Ligands by EXponential enrichment). Numerous aptamers have been identified that bind RT with high affinity and that inhibit its enzymatic activity in vitro (4,5,8–15) [reviewed in (16)]. Several of these aptamers have also been demonstrated to interfere with viral replication in cell culture (7,12,15,17,18). Clinical application of RNA aptamers may eventually take the form of gene therapy, wherein genes that direct the expression of the therapeutic aptamer are delivered to target cells (e.g. CD34+ stem cells) for intracellular expression. Direct clinical application of DNA aptamer inhibitors of RT will require further improvements in delivery to the appropriate target cells. However, both RNA and DNA aptamers are valuable research tools for dissecting the molecular mechanisms of viral replication and pathogenesis.

While both RNA and DNA aptamers to RT have been described, DNA aptamers offer several unique advantages and opportunities. (i) They can be synthesized at large
scale cheaply and efficiently using technology that is available worldwide. (ii) DNA aptamers can be stored in desiccated form for years, then be refolded and fully activated upon rehydration, and their shelf-life can be further prolonged by storage in the presence of metal chelators, such as EDTA. (iii) Chemical derivatization can be readily accomplished by existing synthetic methods to adapt a given aptamer to a variety of delivery and diagnostic platforms. (iv) Nucleic acids are generally nonimmunogenic, so their repeated use is unlikely to induce an inflammatory immune response. (v) Several recent studies with RT mutants—including drug-resistant RT (19,20)—and with RT from phylogenetically diverse viruses (14) suggest that the genetic threshold for the development of significant resistance to some ssDNA aptamers may be very high.

Five sets of ssDNA aptamers to HIV-1 RT have been described. The present study builds from the aptamer set selected by Schneider et al. (11), who identified at least seven sequence families from among 30 aptamer isolates. Several aptamers from this set strongly inhibit the DNA-dependent DNA polymerase activity of HIV-1 RT and bind RT with dissociation constant ($K_d$) values near 1 nM (11). We recently demonstrated that a 49-nt version of aptamer RT1, designated RT149, inhibits RT from diverse strains of HIV-1, HIV-2 and SIVcpz (a close relative of HIV-1 that infects chimpanzees) (14). Mutational and footprinting analysis, combined with molecular modeling, support a two-helix structure for RT149 in which one helix occupies the primer/template-binding cleft in the protein and the other helical structural unit is thought to interact with the back side of the fingers or thumb domain (5). Other work has evaluated the influence of specific RT point mutations on binding by RT149 and several other aptamers (19,20). Although some progress has been made in identifying how the RT1 family of ssDNA aptamers interacts with HIV-1 RT, relatively little is known about the other structural families from this set.

The second set of ssDNA aptamers, isolated by Andreola et al. (12), was targeted to interact with the RNase H domain of RT by utilizing alternate cycles of positive selection for binding to the p66/p51 heterodimer with cycles of negative selection to remove aptamers binding the p51/p51 homodimer. Two species from this selection, designated ODN93 and ODN112, inhibit both DNA polymerization and RNase H activities in vitro with half-maximal inhibitory values ($IC_{50}$) of 500 nM. When added to cell culture simultaneously with virus, these same DNAs interfered with viral infectivity. Truncated version of these anti-RNase H aptamers, designated 93del and 112del, both form guanosine quadruplex structures. Both molecules also cross-react with HIV-1 integrase, blocking both 'end-processing' and strand-insertion activities by integrase, with $IC_{50}$ values in these reactions as low as 10–100 nM (21).

The other three aptamer sets each have distinct features. DeStefano and Cristaforo (13) selected GC-rich duplexes with recessed 3'-ends that resemble the primer/template substrate utilized by the virus during (+)-strand genome synthesis. Somasunderam et al. (15) used combinatorial bead chemistry to identify sulfur-containing thioaptamers that bind RT with $K_d$ values as low as 70 nM and that interfere with viral replication in cell culture. Several of these DNAs are G-rich and have the potential to form quadruplex structures (15). More recently, Bowser and colleagues (22) used capillary electrophoresis to effect the rapid selection of ssDNA aptamers with especially high affinity for HIV-1 RT ($K_d = 0.18$ nM).

The aptamers from the five selections above comprise a highly diverse collection of sequences and secondary structures, including several that form guanosine quadruplex structures. Quadruplexes are a highly stable, multi-layer fold in which four guanosines within a given layer hydrogen bond from the Watson–Crick face of each guanosine to the Hoogsteen face of another guanosine, and successive layers stack together [reviewed in (23)]. Sequences capable of forming quadruplex structures are found in human (24) and *Oxytricha nova* (25) telomeres, and in several promoter sequences (26–32). Recent advances in quadruplex aptamer identification, characterization and delivery have led to increased interest in developing them as tools for modulating biology. For example, ssDNA aptamers to the blood clotting factor thrombin (33) and to insulin (34) both form quadruplexes and have been studied extensively. AS1411 is a quadruplex-forming DNA aptamer that targets nucleolin and that is currently in clinical trials as a treatment for various cancers (35), and several DNA quadruplexes have shown potent inhibition of HIV-1 integrase (36,37).

In the present work, we reinvestigated several molecules from the Schneider set of ssDNA aptamers selected to bind HIV-1 RT (11). Three aptamers demonstrate bimodular structure comprising quadruplex and helical elements. Both elements are required for RT inhibition, and they must be physically connected. We find that there are relatively few sequence constraints within either element or in the connection between them, and the chemical nature of the linkages between the two major structural elements and within the quadruplex played no significant roles in determining inhibition of HIV-1 RT. Importantly, the bimodular aptamer exhibited potent inhibition of RT derived from phylogenetically diverse HIV and SIV strains. Our findings aid in understanding the mechanism of HIV RT inhibition utilizing structured DNA inhibitors and reinforce the potential clinical and research value of quadruplex DNA aptamers.

**MATERIALS AND METHODS**

**RT and DNA oligonucleotides**

RT were expressed in *Escherichia coli* and purified as described in (4). Except where noted, all assays utilized RT from HIV-1 strain HXB2 (group M, subtype B). Aptamer RT6 was synthesized and purified by Operon Biotechnologies, Inc., (Huntsville, AL, USA; www. operon.com). All other aptamer oligonucleotides and primer/template substrates were synthesized and purified by Integrated DNA Technologies, Inc. (www.idtdna.com). The original full-length aptamers were 81 nt in length (11). The 'full-length' aptamers studied here were synthesized as 80-nt molecules by removing the 3'-terminal dG residue. This change was without functional
Inhibition of RT enzymatic activities

Aptamer DNA was denatured by heating to 95°C for 5 min, followed by slow cooling at a rate of 2°C per minute to room temperature and stored in frozen condition. Subsequent dilutions were made from this refolded stock without additional refolding. Measurements of DNA-dependent DNA polymerization (DDDP) were carried out essentially as described (4). For DDDP assays, an 18-nt Cy3-labeled DNA primer was annealed to a 103-nt synthetic DNA template corresponding to the primer-binding sequence and U5 segments of HIV-1 strain HXB2. Reactions were assembled in reaction buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 5 mM MgCl2, 10 mM DTT) with 30 nM primer, 45 nM template and 0.2 mM dNTPs. After annealing primer and template strands, DNA aptamers were added. Reactions were initiated by adding RT to a final concentration of 3 nM active site. For IC50 measurements, final aptamer concentrations were 0 nM, 0.3 nM, 1.0 nM, 3.0 nM, 10 nM, 30 nM, 100 nM and 300 nM. Additional reactions included 1 µM and 3 µM where indicated. After 10 min at 37°C, reactions were quenched by addition of 2 volumes (20 µl) of gel-loading buffer (95% formamide with 0.01% bromophenol blue). For reactions assessing monovalent ion dependence, 75 mM KCl was replaced with 75 mM NaCl where indicated.

Reaction products were separated using denaturing (8 M urea) 10% PAGE and scanned for fluorescence using a Fujifilm FLA5000 imaging system. RT activity data were collected using Fujifilm Multi Gauge V2.3 image analysis software. Competitive inhibition by aptamers is due to a decrease in the concentration of available enzyme, and is described by the two-state model for RT-aptamer binding [Equation (1)]:

\[ Y = \frac{100[\text{aptamer}]}{[\text{IC50} + [\text{aptamer}]]} \]

where \( Y \) is the measured percent activity at a given inhibitor concentration. This equation can be rearranged to a convenient form [Equation (2)] for curve-fitting with GraphPad Prism software to obtain IC50 values for aptamer inhibition:

\[ Y = \frac{100}{[1 + 10^{\log(\text{IC50} - X)}]} \]

where \( X \) is the log of the inhibitor concentration. Error terms for reported IC50 values are the standard deviations among triplicate assays.

Circular dichroism spectroscopy

DNA aptamer samples (SN, RIT and RT6-B) were prepared essentially as for inhibition assays, by heating to 90°C and renaturing at 25°C for 20 min. The concentrations 4 µM or 2 µM were adjusted in the corresponding buffers (K-buffer = 50 mM Tris–HCl pH 8.3, 75 mM KCl, 5 mM MgCl2, 10 mM DTT; Na-buffer = 50 mM Tris–HCl pH 8.3, 75 mM NaCl, 5 mM MgCl2, 10 mM DTT). Near-UV circular dichroism (CD) spectra were acquired at 25°C using an Aviv 62DS spectrometer (Lakewood, NJ) at 1.0 nm intervals between 350 nm and 210 nm in a 1.0 mm quartz cuvette, averaging each data point for 10 s.

RESULTS

Identification of a novel inhibitory DNA structure within ssDNA aptamers RT6, RT5 and RT47

Aptamers RT5, RT6 and RT47 form a subgroup of closely related sequences within Family VI of the ssDNA aptamers identified by Schneider et al. (11). Consistent with previous results, we obtained strong inhibition of DNA polymerization by RT from a subtype B strain of HIV-1 by 80-nt versions of each of these three aptamers. The concentration required for half-maximal inhibition (IC50) by RT6 was 36 ± 3 nM and was similar for the other two (data not shown). All three aptamers share the same sequence in the first 5–6 nt of the initial 35 N random segment (Figure 1A), and this shared segment is complementary to nucleotides within the 5′-primer-binding constant region. The remainder of each molecule includes four or more stretches of guanosine tri-nucleotide repeats. Five such repeats are evident in RT6. Aptamer RT6-A was generated by removing the 25 nt on the 3′-side of the fourth repeat of RT6 and modifying 5 nt between the third and fourth repeat. Aptamer RT6-A was more than 2-fold more potent for RT inhibition relative to the original RT6 aptamer (IC50 = 16 ± 3 nM). Similar 3′ deletions from RT5 and RT47 inactivated these aptamers for RT inhibition unless three guanosines were appended onto the 3′-ends of the truncated species to restore their original termini (data not shown). Removing both the 3′-terminal 25 nt and the 5′-terminal three unpaired nucleotides (ACG) of RT6 generated a 53-nt species designated RT6-B that retained potent RT inhibition (IC50 = 5.7 ± 0.8 nM). Finally, the 41-nt aptamer variant R1T (IC50 = 14 ± 2 nM, Figure 1C) was generated in which the intra-quadruplex loop sequences connecting the guanosine triplets in RT6-B were replaced with single thymidine nucleotides. These data suggest a structural model in which each of these aptamers forms a 5′-helical domain connected to a 3′-guanosine quadruplex domain (Figure 1B).

Mutational support for quadruplex formation

To establish the quadruplex positions at which guanosine is required, each of the nucleotides that are proposed to define the G-quadruplex structures within RT6-A was individually changed to adenosine. All 12 G-to-A substitutions abolished RT inhibition (data not shown). These data provide strong evidence that RT inhibition by RT6-A (and by the original RT6) requires a three-tiered quadruplex. When similar mutations were generated in R1T, 11 of the 12 G-to-A mutations were similarly disruptive. However, deleting the first guanosine of the second triplet or changing it to A, C or T only reduced RT inhibition by two- to threefold (TQK4, Figure 2A, and data not shown). These data suggest that only two tiers
### Table 1. Oligonucleotides used in this study

| Oligos for activity assays |  |
|---------------------------|--|
| Cy3Primer | Cy3-GTCCTGTGTCGCGCGTCCCA |
| 103Template | AAGTTGTGTTGCTGGTAGCTGTTGACTGTTACTTAGAGATCCCTCAGACCTTTTT |
| AGTGTGTTGCTGGTAGCTGTTGACTGTTACTTAGAGATCCCTCAGACCTTTTT |

**Full-length aptamers and terminal deletions**

| -35 | RTS | ATCCCTGATTAGGATCTCAAGGCTTgggAAggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| -35 | RT47 | ATCCCTGATTAGGATCTCAAGGCTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 35±3 | RTS | ATCCCTGATTAGGATCTCAAGGCTTgggAAggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 35±3 | RTS-A | ATCCCTGATTAGGATCTCAAGGCTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 5.7±0.8 | R2-B | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggCCGCTAAAAACGGg |

**R1T family**

| 14±2 | R1T | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 13±2 | R2T | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| -35 | R1T | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 20±3 | R1T | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 18±7 | R1A | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | R2A | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | R4A | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | R1C | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | R2C | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 3.5±0.1 | SN | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |

**Modifications to helical domain**

| 59±2 | S1 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 110±5 | S2 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 32±1 | S4 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 27±6 | S5 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 10.0±1.5 | ZAM1 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| 26±7 | Srevarstem | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |

**Mutations establishing a three-tiered quadruplex**

| >300 | TQK1 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK2 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK3 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK4 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK5 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK6 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK7 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK8 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK9 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK10 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK11 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | TQK12 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | ML2 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |
| >300 | ML4 | CCGCCGTTGAGCATCTCAAGGCGTTgggAAaggAAATACAGGATCTCAGGCAAAATACCTGCGggg |

**Topological variants**

| >1000 | Stem | CCGCGTATTGAGCAGATCTCAGGCG |
| >1000 | QL6 | CCGCGTATTGAGCAGATCTCAGGCG |
| -30 | Dy1 | CCGCGTATTGAGCAGATCTCAGGCG |
| -30 | Dy12 | CCGCGTATTGAGCAGATCTCAGGCG |
| -30 | Dy13 | CCGCGTATTGAGCAGATCTCAGGCG |
| 21.0±4 | Dy15 | CCGCGTATTGAGCAGATCTCAGGCG |
| 22±4 | Acut | CCGCGTATTGAGCAGATCTCAGGCG |
| 33±2 | Acutrev | CCGCGTATTGAGCAGATCTCAGGCG |
| 48±1 | Bcut | CCGCGTATTGAGCAGATCTCAGGCG |
| 99±2 | Ecut | CCGCGTATTGAGCAGATCTCAGGCG |
| >300 | 91del | CCGCGTATTGAGCAGATCTCAGGCG |
| >300 | Stem-91del | CCGCGTATTGAGCAGATCTCAGGCG |

**Intermodule linker variants**

| 13±2 | L3 | CCGCGTATTGAGCAGATCTCAGGCG |
| 28±2 | L4 | CCGCGTATTGAGCAGATCTCAGGCG |
| 13±1 | L6 | CCGCGTATTGAGCAGATCTCAGGCG |
| 120±6 | L10 | CCGCGTATTGAGCAGATCTCAGGCG |
| 29±1 | HBG | CCGCGTATTGAGCAGATCTCAGGCG |

Underlined nucleotides form the base-paired helical element; bold nucleotides comprise the guanosine triplet repeats.
of the quadruplex may be absolutely required within R1T. However, inserting or removing one guanosine from each (TGGG) repeat in the original R1T quadruplex to yield (TGGGG)₄ in ML4 and (TGG)₄ in ML2 prevents aptamer-mediated RT inhibition (data not shown). The potential significance of these findings is discussed below.

Sequence plasticity of quadruplex loops and stem

To define further the sequence requirements within intra-quadruplex loops, the single Ts in each loop of R1T were changed to other sizes and sequences. Replacement with single adenosines (R1A, IC₅₀ = 18±7 nM, Figure 2B) or cytidines (R1C, IC₅₀ = 26±9 nM) was well tolerated,
while replacement with single guanosines was not (R1G, IC₅₀ ≈ 250 nM). Expanding the loops with runs of two (R2T, IC₅₀ = 13 ± 2 nM), four (R4T, IC₅₀ ≈ 35 nM) or eight (R8T, IC₅₀ = 20 ± 3 nM) thymines was also well-tolerated. In contrast, expansion of the loops to two (R2A), four (R4A) or eight (R8A) adenosines or with two (R2C) or four (R4C) cytidines yielded aptamers with greatly reduced capacity for inhibiting RT (Figure 2B). To eliminate the possibility that RT-loop nucleotide contacts are essential for aptamer recognition, the intra-quadruplex loops were replaced with the relatively inert, nonnucleosidic hexaethylene glycol (HEG) to yield the SN aptamer. Interestingly, inhibition by the SN aptamer is more than 10 times more potent (IC₅₀ = 3.5 ± 0.1 nM, Figure 2C) than the original RT6, and more than fourfold more potent than R1T. We conclude that specific loop nucleotides within the quadruplex region are not required for inhibition or for stability, and that the loops are only minimally constrained as to sequence. Additional variants of R1T were generated to identify sequence constraints within the 7-bp stem comprising the 5'-structural domain. Deletion of 2 bp (S1, IC₅₀ = 59 ± 2 nM) or 3 bp (S2, IC₅₀ = 110 ± 5 nM) from the stem progressively compromised RT inhibition (data not shown), while lengthening the stem to 22 bp slightly improved inhibition (ZAM1, IC₅₀ = 10.0 ± 1.5 nM). Reversing the sequence orientation within this domain (Srevstem, IC₅₀ = 26 ± 7 nM) had minimal impact on IC₅₀ (Figure 2D). Replacing the 10 nt at the end of the R1T stem with a CCCT tetraloop (S4, IC₅₀ = 32 ± 1 nM, Figure 2E) had only a twofold effect on RT inhibition. These results establish that the 5'-domain requires a generic double-helical fragment stabilized by seven or more base pairs of essentially any sequence.

**Loop sequences affect sensitivity to ionic environment**

Potassium ions strongly favor quadruplex formation, while sodium ions are usually less stabilizing and lithium ions are destabilizing (40,41). We observed equivalent DNA polymerization activity by HIV-1 RT in buffers containing either K⁺ or Na⁺. RT inhibition by aptamers SN, R1T and RT6-B was therefore monitored in the original buffer containing K⁺ and also in a buffer in which K⁺ was replaced with Na⁺. The same RT was nearly inactive in buffers containing Li⁺, preventing analysis of inhibition in the presence of Li⁺. All three aptamers strongly inhibited RT in the presence of K⁺. Aptamers SN and R1T were also strongly inhibitory in the Na⁺-containing buffer, while aptamer RT6-B lost all inhibition under these conditions (Figure 3). Because these three aptamers differ only in the loop sequences connecting the individual guanosine triplets of the quadruplex, we conclude that the loop sequences in RT6-B render that aptamer sensitive to ionic destabilization, while the simplified structures of R1T and SN stabilize those aptamers to ionic substitution.

Circular dichroism (CD) spectroscopy is a powerful analytical tool for identifying quadruplexes DNA structures. For quadruplexes in which all four strands are in the same orientation (parallel), CD minima and maxima are typically near 240 nm and 264 nm, respectively. For quadruplexes in which strands orientation alternates (antiparallel), the corresponding values are typically near 265 nm and 295 nm, respectively. The CD spectra for RT6-B and R1T both show maxima at 262 nm and minima at 242 nm at room temperature in the presence of K⁺ (Figure 4). In Na⁺ buffer the ellipticity is strongly flattened for RT6-B, especially at low DNA concentration, indicating destabilization of the quadruplex structure. Slight flattening is also evident in R1T, although to a lesser extent. Quadruplexes containing HEG loops, such as the one in aptamer SN, have previously been shown to be more stable than quadruplexes with single T loops in both K⁺ and Na⁺ buffers (42); thus, SN is also expected to retain its quadruplex in the Na⁺ buffer. These data provide independent confirmation of guanosine quadruplex formation within the RT6 family of aptamers, and they establish that the structural integrity of the simple quadruplex within R1T is less sensitive to ionic conditions, consistent with the inhibition data above.

**A generic, physical connection is required between the duplex and quadruplex modules**

RT inhibition was measured for several variants of R1T to determine the importance of the physical connection
between the two structural modules (Figure 5A). No inhibition was observed for a 28-nt oligo containing only the 5' stem–loop motif (stem), nor for a 16-nt oligo containing the (TGGG)₄ quadruplex motif (Q16). Similarly, no inhibition was observed when both oligos were added simultaneously at a ratio of 1:1, even when their individual concentrations were increased to 3000 nM (Figure 5B). A physical (covalent) connection between the DNA duplex and G-quadruplex is therefore required to effect inhibition.

The stem–loop and G-quadruplex elements within aptamers RT6, RT5 and RT47 are joined by single-stranded segments with the sequences TTA, CTT and CC, respectively, while in R1T the connector is TT. To determine the effect of varying this connector sequence, RT inhibition was measured for variants of R1T in which the connector was changed to several other sequences. Changing those two thymidines to two adenosines (A₂ connector in L6), or expanding it to four (T₄ in L3) or eight (T₈ in L4) thymidines had minimal effect on IC₅₀ values (13 ± 1, 13 ± 2 and 28 ± 2 nM, respectively) (data not shown). To determine whether the negative charges or other nucleic acid-related features of these connector sequences are required for RT inhibition, the duplex and quadruplex modules were joined via HEG (Figure 5C). The IC₅₀ value of the HEG-linked molecule (29 ± 3 nM, HEG) is similar to that of the T₈-linked molecule. Thus, the primary role of the connector is to provide a physical connection between the two domains, rather than to provide a specific structural or functional role. However, there is an optimal range of lengths for the connector, as increasing it to T₁₆ significantly weakened inhibition (L₁₀, IC₅₀ = 120 ± 6 nM).

**Topological malleability of the connectivity between the structural elements**

In all of the aptamer variants above, the two structural elements are connected via one flexible linker between the 3'-end of the helical element and the 5'-end of the quadruplex element. The 5’- and 3’-ends of the molecule are located, respectively, at the termini of the helical and quadruplex elements. Several variants of R1T were evaluated to determine whether alternative topologies are allowed. To accommodate relocating the 5’- and 3’-termini to other positions, the helical fragment was joined to the quadruplex by separate thymidine dinucleotides at each end of the quadruplex (Figure 5A). For variants Dyl1, Dyl2, Dyl3 and Dyl5, the 5’- and 3’-termini lie within the original loop at the distal end of the helical element. The helical portions of these molecules contain a total of 15 bp, 19 bp, 22 bp or 35 bp, including an internal mismatch derived from the original stem in RT6. All four variants strongly inhibit HIV-1 RT (e.g. IC₅₀ = 21.0 ± 3.5 nM for Dyl5) (Figure 5D). The 5’- and 3’-termini were next relocated to internal positions within the quadruplex (Figure 5A). Placing the 3'-end after the second guanosine triplet (Acut, IC₅₀ = 22 ± 4 nM, Figure 5D) yielded inhibition within a factor of two of the potency.
of R1T. Reversing the sequence within the central loop at the distal end of the helical element (Acutrev, IC50 = 33/C6 2 nM) had only a slight effect on IC50. Inhibitory potency was further reduced when the 3'-end was placed after the first guanosine triplet (Bcut, IC50 = 48/C6 1 nM) or after the third guanosine triplet (Ecut, IC50 = 99/C6 2 nM), likely reflecting a destabilization of the quadruplex module. Potent inhibitors based on R1T can therefore be generated by joining the helical fragment and quadruplex by either a single or a double linker between the helical and quadruplex elements.

Cross-clade inhibition of divergent primate lentiviral RT's

The ssDNA aptamers studied here were originally selected to bind RT from HIV-1 strain BH10 (group M, subtype B). The inhibition studies presented above all utilize RT from the closely related HIV-1 strain HXB2 (six amino acid differences in 560 positions, preserving 98.9% identity). To define the phylogenetic breadth of inhibition by these bimodular ssDNA aptamers, DNA-dependent DNA polymerization was monitored for a panel of five RT variants from diverse primate lentiviral strains from within HIV-1 group M (average 92% identity with RT from BH10) and three from outside this group (10). Aptamer SN was chosen for this analysis, as it was the most potent inhibitor among those studied here. All eight lentiviral RT’s were inhibited. With the exception of the HIV-2 isolate, all IC50 values were (within error) within a factor of two of being identical to IC50 for inhibition of the subtype B enzyme (Table 2). The SN aptamer (and related forms) are therefore highly promising reagents for developing broad-spectrum anti-HIV agents.

DISCUSSION

‘Universalist’, bimodular, single-stranded DNA aptamers with stem-plus-quadruplex domains

This study demonstrates a novel structural paradigm for bimodular DNA inhibitors of HIV-1 RT, in which one module is a helical stem and the other is a guanosine quadruplex. We identified this architecture within aptamers RT5, RT6 and RT47—isolated previously (11)—and defined the sequence components required to achieve

Table 2. Inhibition of phylogenetically diverse primate lentiviral reverse transcriptases

| Strain       | Phylogenetic groupa | Percent identityb | IC50 (nM)   |
|--------------|---------------------|-------------------|-------------|
| HXB2         | HIV-1 (M:B)         | 98.9              | 3.5 ± 0.9   |
| 92UG021      | HIV-1 (M:A/D)c      | 92.9              | 3.0 ± 0.3   |
| 98CN009      | HIV-1 (M:C)         | 92.9              | 3.4 ± 1.1   |
| 94CY017      | HIV-1 (M:A)         | 90.4              | 7.2 ± 0.7   |
| 93TH253      | HIV-1 (M:A/E)c      | 90.5              | 6.1 ± 0.6   |
| MVP5180      | HIV-1 (O)           | 78.2              | 4.9 ± 0.4   |
| TAN1         | SIVcpzP.t.s.        | 72.7              | 2.1 ± 0.3   |
| EHO-287      | HIV-2 (B)           | 60.5              | 12.0 ± 0.5  |

aHIV-1 phylogenetic groupings are designated by ‘(group:subtype)’.
bPercent amino acid identity in complete RT sequences in comparison with HIV-1 strain BH10.
cStrains 93TH243 and 92UG021 are intersubtype recombinants. RT from the former groups with subtype A; RT from the latter groups with subtype D (10).
the functional structures by monitoring RT inhibition for a collection of 60 variants of RT6. Structural interpretations were further confirmed by CD spectroscopy. The most potent variant, designated SN, strongly inhibited not only RT from HIV-1 subtype B, which was the subtype originally used in generating this family of aptamers, but also several other HIV-1, SIVcpz and HIV-2 RTs ranging from approximately 60% to 92% amino acid identity with RT from subtype B. Inhibition of these RT and additional members of the RT diversity panel was previously monitored for four RNA pseudoknot aptamers (10) and for three ssDNA aptamers (14). ssDNA aptamer RT8 was a ‘specialist’, exhibiting strict specificity for RT from subtype B. In contrast, ssDNA aptamers RT1t49 and RT1t49(-5) were ‘universalists’ for primate lentiviral RTs, inhibiting every member of the panel. The four RNA aptamers displayed intermediate behavior, inhibiting two or more members of the panel while leaving more than half of the panel uninhibited. Thus, the ‘universalist’ inhibitory behavior of the bimodular aptamers studied here (especially SN) is similar to that of RT1t49 and RT1t49(-5).

Mutational analysis revealed a simple core structure in these bimodular aptamers. The helical module can be of any sequence. Internal bulges or mismatches are not required; converting them into fully base paired sites had no consequence on inhibition. Variants with seven or more base pairs in the stem gave similar RT inhibition, while variants with shorter stems were less effective. The distal end of this stem can either be a closed loop of any sequence, or it can contain the 5' and 3' termini. Mutations within the quadruplex module retained RT inhibition, so long as the altered sequence retained the capacity to form the quadruplex structure. Aptamer RT1T (IC\textsubscript{50} = 14 ± 2 nM), which may assume a two-layer quadruplex (see below), yielded slightly weaker inhibition than the three-layer quadruplex aptamers RT6-B (IC\textsubscript{50} = 5.7 ± 0.8 nM) and SN (IC\textsubscript{50} = 3.5 ± 0.1 nM). Replacing the T's with HEG (SN) gave the strongest inhibition among all variants tested here. We conclude that the duplex and quadruplex structural modules, linked by a short tether, are sufficient for potent inhibition of HIV-1 RT, and that sequence-specific recognition plays little or no role in specifying the protein–ssDNA interaction.

**Interaction models for the helical element**

RT contains a strongly electropositive trough running between the DNA polymerase and RNase H active sites (43–45), which binds helical nucleic acids in co-crystal structures and in modeled complexes (6,13,44,46–48). The binding interactions of a dual-stem aptamer, denoted RT1t49, have been modeled based on Fe(II)-mediated DNA cleavage mapping (5). Differences in the hydroxyl radical cleavage patterns in the presence of wild-type RT and active site mutants suggested that the 5'-end of RT1t49 is near the polymerase active site, and that the long stem of this aptamer extends towards the RNase H active site (5). This model places the second structural domain—a short duplex stem—on the back side of the fingers or thumb domain, perhaps close to the normal trajectory of the 5' single-strand extension in the template strand (49). A similar mode of interaction by the helical domain of these bimodular aptamers would place the quadruplex module on the back side of the fingers or thumb domain of RT.

**Structure of the quadruplex element**

Our mutational and spectroscopic data support a quadruplex with parallel strands of repeated guanosines arranged into at least two stacked layers. All 12 guanosines were immutable within RT6-A, implying a three-layer stack in this context. By extension, three stacked layers are proposed for the original aptamers RT6-B, RT6, RT5 and RT47. Adding or removing a guanosine from each of the four repeats to shorten or extend the stack within RT1T abolished inhibition, again implying a three-layer stack. In contrast, only 11 of the 12 guanosines were strictly required in RT1T. RT inhibition was reduced but not abolished upon deletion or mutating the first guanosine of the second repeat within RT1T to A, C or T, potentially indicating that the nucleotide in this position is translocated to the loop, and that only two stacked layers are absolutely required in this context. An alternative possibility is that the three remaining guanosines in this tier of the quadruplex may provide sufficient stabilization to allow the fourth nucleotide to stack onto the quadruplex so as to retain overall structure. Our results are also in agreement with other authors' findings that quadruplex stability is affected by the sequence of the loops (42,50).

In particular, the RT6-B aptamer inhibits RT only in buffer that contains K\textsuperscript{+} ions and shows considerable broadening of its CD spectrum in buffer that contains Na\textsuperscript{+}, while the more compact RT1T inhibits in both K\textsuperscript{+} and Na\textsuperscript{+}, and shows less line broadening.

Some of the quadruplex modules included within these bimodular aptamers are analogous to quadruplexes that have been studied previously. A systematic survey of quadruplexes with differing numbers of repeated G's or with differing loop sequences identified (G\textsubscript{3}T\textsubscript{4}) as being especially stable (51,52). The (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} quadruplex is very similar to the (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} quadruplex within RT1T. CD spectroscopy of (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} indicated parallel strands within the stack (51), similar to the results obtained here for RT1T and RT6-B. In analogy with aptamer SN, the sequence (G\textsubscript{3}T\textsubscript{4})\textsubscript{4}G\textsubscript{3} was previously shown to form a threelayered quadruplex and displayed greater thermal stability than (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} (42). The (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} quadruplex—also referred to as T30923 and as T30695—has been studied for inhibition of HIV-1 integrase (53,54), in addition to structural studies (36,51–53). Importantly, an NMR structure of (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} shows only two layers of stacked guanosine tetrads with partially ordered ‘GT’ loops (36,53). These observations are consistent with the suggestion above that aptamer RT1T may only require two layers of stacked quadruplex. However, the (G\textsubscript{3}T\textsubscript{4})\textsubscript{4} quadruplex studied here (denoted Q16 when used in isolation) was unable to inhibit RT without a physical connection to the helical domain, even when used at 3000 nM concentration. The well-studied aptamer ‘TBA’ (GGTTGGTGGTGATTGG) forms a two-layer quadruplex that binds and inhibits
thrombin in the low nanometer range (33). However, in preliminary assays, we observe no inhibition of thrombin-mediated cleavage of a model, chromogenic peptide even in the presence of 3000 nM aptamer SN (data not shown), suggesting that TBA may be a poor analog of SN. In sum, most of the quadruplex modules within these aptamers are best modeled as three-layered stacks of parallel strands, in some cases with single-nucleotide or nonnucleic acid loops, and we cannot rule out that two layers may be sufficient for RT.

Interaction models for the quadruplex element

Aptamers with diverse quadruplex modules can accomplish RT inhibition, arguing for a promiscuous interaction with the quadruplex domain. In particular, the intraquadruplex loops tolerate considerable plasticity. The loops in aptamers RT5, RT47 and RT6 are unrelated to each other. Inhibition was improved by simplifying the loops to single A (R1A), C (R1C) or T residues (R1T) and was further improved by replacing the loops with HEG (SN). For each of these four alterations, inhibition correlated with previously measured increases in the stabilities of isolated quadruplex DNAs (42,51,52). Loop substitutions with multi-homonucleotide sequences other than T (R2C, R2A, etc.) did not inhibit RT. Furthermore, aptamers with the phosphodiester chain termini located within the quadruplex retained inhibition. Thus, the loops are more likely to serve simply to stabilize the folded quadruplex rather than to interact directly with the protein.

The interaction model noted above for the helical domain based on RT11t49 (5) places the quadruplex module on the back side of the fingers or thumb domain of RT. An alternative interaction mode is suggested by studies of two quadruplexes, 93del and (G3T)4, which bind HIV-1 integrase. Integrase is in the same structural superfamily as RNase H, with the greatest similarity in the central catalytic core (IN residues 60–160) (55,56). In this alternative model, the quadruplex domains of the bimodular aptamers bind the RNase H domain of RT, effectively reversing the orientation of the helical domain relative to the model suggested above. Quadruplex 93del (GGGGTG GGAGGAGGGT) was originally selected as a longer ODN93 for its ability to bind mature heterodimer RT and not to bind a p51/p51 homodimer lacking the RNase H domain (21). An NMR structure of 93del shows an interlocking dimer of two three-layer quadruplexes, arranged in predominately parallel strands. Those authors modeled the 93del dimer as docking into the channel formed by HIV-1 IN, and they suggest potential interactions of the quadruplex loops with the protein (55). Quadruplex (G3T)4 also binds and inhibits HIV-1 IN (36,53,57,58). Shortening or extending the run of G’s [(G3T)4 and (G4T)4, respectively] did not diminish IN inhibition, a result that was interpreted as indicating DNA–IN interaction through loops on the ends of the quadruplex, rather than a side-on interaction (53). Computational modeling of that interaction places the (G3T)4 quadruplex in the catalytic domain of HIV-1 IN. Interestingly, this NMR structure shows the guanosine triplets arranged into antiparallel strands, even though CD analysis shows an ellipticity maximum near 264 nm, indicative of a parallel strand arrangement. Computational docking models again suggest essential interactions between quadruplex loops and the integrase catalytic core, particularly Asp64, Asp116 and Glu152 (53).

The data and models from studies of these IN aptamers are at odds with our RT inhibition studies with the bimodular aptamers. Specifically, we observe similar degrees of RT inhibition for a multitude of loop analogs, and RT inhibition is disrupted by shortening [ML2, (TG2)4] or extending [ML4, (TG4)4] the run of G’s, in contrast with the effects of similar alterations within IN-binding quadruplexes (55). Furthermore, a hybrid molecule carrying the stem and connector elements and 93del in place of the (TG4)4 quadruplex (stem-93del) did not inhibit RT in pilot reactions. Thus, RT and the members of the RT6 family of ssDNA aptamers are structurally and functionally distinct from previously described quadruplex aptamers. While, it is possible that the quadruplex domains of the bimodular aptamers interact with the RNase H domain of RT, we conclude that the IN-binding quadruplex models are not appropriate models for defining this interaction. New experimental data are needed to address this question directly.

CONCLUSION

The bimodular RT-binding aptamers presented here represent a novel architecture for RT antagonists. We demonstrate that a helical fragment connected to diverse quadruplex domains is able to specify the correct localization and the strength of binding to RT protein, yielding competition with primer/template for access to the protein. The interactions appear distinct from those observed previously for quadruplex–integrase, quadruplex–thrombin and quadruplex–RNase H complexes. It is likely that the helical and quadruplex modules contact the protein directly, and that their physical connection can facilitate cooperativity of binding, with both modules interacting simultaneously. Further, it is possible that simultaneous binding at two sites, facilitated by a flexible linker, aids in the cross-clade recognition exhibited both by the stem-plus-quadruplex bimodular aptamers studied here and by the dual-stem aptamers studied previously (5,11,14). Current work seeks to determine whether the bimodular RT-binding aptamers described here confer antiviral bioactivity in cell culture and whether they interact with other cellular components. The results of those studies, and those presented here, will aid the design of RT antagonists for use in drug discovery and studies of HIV-1 replication.

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