Intramolecular G-quartet Motifs Confer Nuclease Resistance to a Potent Anti-HIV Oligonucleotide*

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We have identified a potentially therapeutic anti-human immunodeficiency virus (HIV-1) oligonucleotide composed entirely of deoxyguanosines and thymidines (T30177, also known as AR177: 5’-g*tggtgggtgggtggg*t-3’, where asterisk indicates phosphorothioate linkage). In acute assay systems using human T-cells, T30177 and its total phosphodiester homologue T30175 inhibited HIV-1-induced syncytium production by 50% at 0.15 and 0.3 μM, respectively. Under physiological conditions, the sequence and composition of the 17-mer favors the formation of a compact, intramolecularly folded structure dominated by two stacked guanine quartet motifs that are connected by three loops of TGs. The molecule is stabilized by the coordination of a potassium ion between the two stacked quartets. We now show that these guanine quartet-containing oligonucleotides are highly resistant to serum nucleases, with half-lives of 5 h and >4 days for T30175 and T30177, respectively. Both oligonucleotides were internalized efficiently by cells, with intracellular concentrations reaching 5-10-fold above the extracellular levels after 24 h of incubation. In contrast, single-base mutated variants or random sequence control oligonucleotides that could not form the compactly folded structure had markedly reduced half-lives (t1⁄2 from ~3 to 7 min), low cellular uptake, and no sequence-specific anti-HIV-1 activity. These data suggest that the tertiary structure of an oligonucleotide is a key determinant of its nuclease resistance, cellular uptake kinetics, and biological efficacy.

Guanine-rich nucleic acid strands, under physiological salt and pH conditions, can adopt a higher order, thermodynamically stable conformation containing square-planar arrangement of four guanines that are hydrogen-bonded in the Hoogsteen manner and stabilized by a monovalent cation (1–4). Depending upon the base composition, sequence, and concentration of the nucleic acids, guanine quartet-containing structures (or G-quartets)1 can be generated from DNA or RNA, either by the intramolecular folding of a single G-rich strand, or by the association of multiple strands (1–7). Believed to be ubiquitous in nature, G-quartets are proposed to participate in diverse biological processes including the modulation of telomere activity, dimerization of HIV RNA, and site-specific genetic recombination in immunoglobulin switch regions (5–9). In addition, using a combination of rational drug design and combinatorial screening methods, several biologically active oligonucleotides have been described, each of unique specificity and the potential to form G-quartet motifs (10–15). In particular, we have identified a family of deoxyguanosine- and thymidine-rich (deoxyribo)oligonucleotides that are potent inhibitors of HIV-1 expression and viral p24 synthesis in culture (16-17). One such inhibitor is T30175, a 17-mer oligonucleotide synthesized with a natural phosphodiester backbone (Table I). A more potent version, T30177, has the same sequence, but contains a single phosphorothioate internucleoside linkage at both the 5’- and 3’-termini. Under physiological conditions, the specific sequence of G and T nucleotides and the small size of the oligonucleotide favor the formation of an intramolecular G-quartet-containing structure over an intermolecular four-stranded one (17). This G-quartet motif has been implicated in providing the three-dimensional shape to T30177 that leads to its remarkable antiviral activity (17). Previously we had observed that a single 4-day treatment regimen of T30177 or various modified versions of the oligonucleotide can suppress HIV-1-induced syncytium formation and viral p24 synthesis in vitro for more than 4 weeks (16–18). The long term suppression of HIV-1 growth by T30177 suggested that this oligonucleotide may have a long biological half-life and/or favorable uptake properties. We hypothesized that the intramolecular G-quartet motifs may protect the phosphodiester linkages of T30177 from single strand-specific endonucleases, and the terminal phosphorothioate linkages may confer protection from exonucleases (19, 20). To test this, we utilized quantitative approaches to examine the cellular uptake and susceptibility of oligonucleotides to nucleases in the serum, or within cells. Our studies focused on the nuclease resistance and biological efficacy of T30177 in comparison to its total phosphodiester and single-base mutant versions and a random sequence 17-mer oligonucleotide control.

**EXPERIMENTAL PROCEDURES**

Synthetic Oligonucleotides—Oligonucleotides used in this study (Table I) were synthesized, purified, and characterized using procedures described previously (16–18). Oligonucleotides RAN1G1 and RAN1G2, containing 2’-deoxy-6-thioguanosine (6-thio-DG) were synthesized using phosphoramidites described by Rao et al. (21).

Table I

| Oligonucleotide | Sequence (5’→3’) | Description |
|-----------------|-----------------|-------------|
| RAN1G1          | 5’-g*tggtgggtgggtggg*t-3’ | 6-thio-DG-containing oligonucleotide |
| RAN1G2          | 5’-ggtgggtgggtgggg*t-3’ | 6-thio-DG-containing oligonucleotide |

Internal Labeling of Oligonucleotides with 32P—The 17-base oligonucleotides were radiolabeled essentially according to Zendegui et al. (22). Briefly, for each oligonucleotide to be radiolabeled (see Table I for labeling position), two short oligonucleotides corresponding roughly to the 5’- (termed “oligo A”) and 3’- (termed “oligo B”) halves of the final product, and a third oligonucleotide complementary to oligos A and B (termed “oligo C,” 15 bases long) were synthesized. Oligo B was labeled with 32P-ATP (Amersham Corp.) and polynucleotide kinase (New England Biolabs, Beverly, MA) using standard methods (23), and the product was purified electrophoretically using 20% polyacrylamide, 7 M urea gels in TBE buffer (80 mM Tris; 90 mM borate, 2 mM EDTA), essentially...
the three 2-base "loops" are protected from interaction with aqueous solute, including enzymes. Previously reported NMR data and energetically favored over multimeric structures (17). The model suggests that the phosphodiester linkages of the three 2-base "loops" are protected from interaction with aqueous solute, including enzymes.

Oligonucleotides unable to form intramolecular G-quartets may still participate in multistranded G-quartet-formation.

According to published procedures (23). The 32P-labeled oligo B was then mixed with equimolar amounts of oligos A and C in 10 mM Tris-Cl, pH 8.0, in a screw-capped microcentrifuge tube, heated to 95 °C, and cooled slowly to room temperature to allow oligos A and B to anneal to oligo C. Oligos A and B, which had hybridized to oligo C, were then ligated by T4 DNA ligase (New England Biolabs) using standard buffer conditions (23). The ligated material, corresponding to the desired dideoxynucleotide, was separated from oligo C and other oligonucleotides using urea-polyacrylamide gels, as described above. Appropriate size standards were run in adjacent lanes to allow for the positive identification of the 17-base 32P-labeled oligonucleotide. The labeled material was eluted from the gel using water, desalted using a SepPak cartridge, concentrated by lyophilization, resuspended in water, and stored at -20 °C until needed. For the uptake and stability studies, the labeled material was premixed with unlabeled oligonucleotides to a final concentration of 1 μM and incubated in screw-capped 1.5-ml polypropylene tubes at 37 °C. At various time points ranging from 0 to 96 h, 150-μl aliquots were removed and oligonucleotides were extracted by the phenol/chloroform method (23). The oligonucleotides were precipitated with ethanol, solubilized in formamide-containing sample buffer, and fractionated by denaturing polyacrylamide gels (23). Known amounts of 32P-oligonucleotides were run in parallel lanes as standards. The gels were fixed in a 10% methanol plus 10% acetic acid solution, dried, and exposed to Kodak XAR-5 film for autoradiography. The radioactivity associated with each band on the gel was quantified using a Fuji Bioimager and Fuji MacBas software.

Cellular Uptake and Stability Assays—HeLa cells were seeded at a density of 1 × 10^5 cells/well in 12-well plates, in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin-streptomycin, and grown overnight at 37 °C. Beginning the next day, at various time points (0–48 h), the medium was replaced with 0.35 ml of fresh medium containing a mixture of radiolabeled (3 × 10^6 cpm) and unlabeled (1 μM) oligonucleotide. The starting period of oligonucleotide addition was staggered so that all incubations were completed at the 48-h time point. At the end of incubations, the extracellular medium was removed from each well and a small aliquot was used for determining the amount of radioactivity remaining in the medium. The remaining portion was transferred to microcentrifuge tubes containing 350 μl of phenol/chloroform/isoamyl alcohol (25:24:1), mixed by vortexing, and kept on ice until further processing. The cells in corresponding wells were washed four times with Dulbecco's phosphate-buffered saline (from Life Technologies) containing 0.1% NaN3 and detached from the plate using trypsin-EDTA (Life Technologies). The cells were then resuspended in 350 μl of Dulbecco's phosphate-buffered saline plus azide, and a small aliquot was used for determining

![Predicted three-dimensional structure of T30175 or T30177 under physiological conditions.](image)

**Fig. 1.** Predicted three-dimensional structure of T30175 or T30177 under physiological conditions. The primary sequence of the oligonucleotide is shown at left. The guanosines participating in quartet-formation are connected by lines. In the wire-frame rendering (center) and space-filling model (right), the phosphate oxygens have been marked in yellow, the quartet-forming guanosines are shown in gray, and other bases are represented in blue. The two stacked G-quartets are stabilized by potassium cation (red). The compact, monomeric form is consistent with previously reported NMR data and energetically favored over multimeric structures (17). The model suggests that the phosphodiester linkages of the three 2-base "loops" are protected from interaction with aqueous solute, including enzymes.
the amount of cell-associated radioactivity. Cell numbers were obtained by counting the cells recovered from nonradioactive, but otherwise identically treated set of wells. The remaining material was transferred to new tubes containing 350 μl of phenol/chloroform followed by vortex mixing. The oligonucleotides were extracted from each sample and analyzed by gel electrophoresis and phosphorimaging, as described above. Correlation was made for loss of material during incubation and extraction steps by processing a known amount of radiolabeled oligonucleotide by exactly the same procedure at the 0 time point. The volume of HeLa cells used in calculating cellular concentrations was estimated to be 2.3 × 10^6 μl/cell, as determined by the Coulter procedure described by McShan et al. (28).

For subcellular localization studies, HeLa cells grown on coverslips were incubated with 0.1–5 μM 5′-fluorescein-conjugated version of T30177 for varying time periods (0–48 h). Cells were washed, fixed, mounted on glass slides, and examined with a Nikon Axiophot microscope equipped for fluorescence imaging.

Anti-HIV-1 Assays—Acute HIV-1 infection assays were carried out essentially as described (16, 17), and the anti-HIV effect was measured as reduction in virus-induced syncytium production or decrease in viral p24 levels. Briefly, human SUP T1 cells cultured in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (2 × 10^5 cells/200 μl/well in 96-well plate) were infected with HIV (at 0.1 multiplicity of infection) for 1 h at 37 °C, washed with medium, and resuspended in medium containing increasing concentrations of test oligonucleotides. After 4 days, the number of syncytia per well was calculated and expressed as percentage of inhibition compared to the untreated control cells. In addition, supernatant from wells was analyzed for the presence of the HIV-1 antigen p24 using the Coulter p24 antigen capture kit.

RESULTS

Structural Model of the Anti-HIV-1 Oligonucleotide—Structural analysis by NMR (17) has shown that in the presence of one equivalent of potassium ion, T30175 or T30177 can adopt a hydrogen-bonded, thermodynamically stable conformation containing two stacked G-quartet motifs. As shown in Fig. 1, the wire-frame and space-filling structures obtained by molecular modeling suggest a compact cube-shaped structure for the oligonucleotide. Thermal denaturation analysis was used to confirm the presence of stable tertiary structure in T30175 and T30177 and to show the inability of mutated variants of T30175 and T30177 to form intramolecular quartets.

Nuclease Resistance of Oligonucleotides—To test the influence of G-quartet motifs on the nuclease resistance of T30177 and its variants, 33P-labeled oligonucleotides were incubated with aliquots of bovine serum for varying time periods, and any intact material was extracted and analyzed by denaturing gel electrophoresis followed by quantitative phosphorimaging. Because the 33P atom was introduced at an internal site on the sugar-phosphate backbone (Table I), the label was not accessible to the abundant terminal phosphatases inside and outside the cell (22). As shown in Fig. 1, the wire-frame and space-filling structures obtained by molecular modeling suggest a compact cube-shaped structure for the oligonucleotide. Thermal denaturation analysis was used to confirm the presence of stable tertiary structure in T30175 and T30177 and to show the inability of mutated variants of T30175 and T30177 to form intramolecular quartets.

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![Fig. 2. Stability of T30177 and its variants in serum. Oligonucleotides were internally labeled with \( ^{33}P \) and incubated (1 μM, 2 Ci/mmol) with aliquots of fetal bovine serum (not heat-treated) at 37 °C for the indicated time periods. At each time point, extractable oligonucleotides were analyzed using urea-polyacrylamide gel electrophoresis followed by autoradiography (A) and quantitative phosphorimaging (B). A, size markers, in bases, are indicated at right. The major band at 0 time point corresponds to the intact material (17-mer). The single-base mutant T30526 and random sequence oligonucleotides T30523 and T30527 were digested shortly after exposure to serum. The degradation products appeared even at the 0 time point because of the few seconds of exposure to serum nucleases during sample processing. B, about 75% of the T30177 was recoverable intact after 4 days of exposure to serum. The \( t_{1/2} \) values of T30175, T30526, T30527, and T30523 were 5, 7, 3, and 2.8 min, respectively. The data are an average of two representative experiments carried out using the same batch of serum. The \( t_{1/2} \) values of oligonucleotides varied slightly (−20%) depending on the batch of serum used, but their relative stability remained unchanged.

Followed by autoradiography (A) and quantitative phosphorimaging (B). A, size markers, in bases, are indicated at right. The major band at 0 time point corresponds to the intact material (17-mer). The single-base mutant T30526 and random sequence oligonucleotides T30523 and T30527 were digested shortly after exposure to serum. The degradation products appeared even at the 0 time point because of the few seconds of exposure to serum nucleases during sample processing. B, about 75% of the T30177 was recoverable intact after 4 days of exposure to serum. The \( t_{1/2} \) values of T30175, T30526, T30527, and T30523 were 5, 7, 3, and 2.8 min, respectively. The data are an average of two representative experiments carried out using the same batch of serum. The \( t_{1/2} \) values of oligonucleotides varied slightly (−20%) depending on the batch of serum used, but their relative stability remained unchanged. 
corresponding to the oligonucleotide lacking the 3' terminal T, since 3'-exonucleases are known to occur in serum (22). Breakdown products shorter than the observed 16-mer were not as abundant, indicating their greater susceptibility to nucleases and suggesting that if the folded oligonucleotide is destabilized, it is quickly digested by nucleases. The time lag between the appearance of the n \(-\) 1 form of T30177 in comparison to T30175 reflects the greater nuclease resistance of phosphorothioate versus phosphodiester linkages (19, 20). In contrast, a random sequence oligonucleotide of the same length (T30523), with a phosphodiester backbone, had a \(t_{1/2}\) of less than 3 min in serum, with multiple breakdown products evident at the earliest time points. The \(t_{1/2}\) of an end-protected (terminal phosphorothioates) version of the same oligonucleotide (T30527), was only \(-7\) min. Based on these observations, we hypothesized that the G-quartet motifs were responsible for the unexpectedly long half-life of T30177 and T30175 in serum. To confirm this, we tested the nuclease resistance of a mutated version of T30175 containing a single G \(\rightarrow\) A base change in the fourth position from the 5'-end (T30526), a substitution that should prevent stable intramolecular quartet formation (30). The results showed that T30526 had a \(t_{1/2}\) of only 3 min in serum (Fig. 2), slightly greater than the \(t_{1/2}\) of the randomly selected oligonucleotide T30523, but much lower than the \(-5\) h half-life of T30175. Together, these observations strengthened the argument that the G-quartet motifs of T30175 and T30177 were the primary determinants of oligonucleotide half-life in serum.

**Fig. 3.** Cellular uptake of T30177 and its variants. \(^{32}\)P-Labeled oligonucleotides (1 \(\mu\)M; 4 Ci/mmol) was added to the growth medium of HeLa cells and incubation continued for various time periods. After the treatment, medium was separated from the cells and oligonucleotides extractable from each fraction were analyzed by denaturing gel electrophoresis. Autoradiographic analysis (A) showed that the G-quartet-containing oligonucleotides T30177 and T30175 remained intact in the medium for up to 48 h and accumulated inside cells, whereas only trace levels of intact T30526 (G \(\rightarrow\) A mutant version of T30175) and T30527 (random sequence control with terminal phosphorothioate linkages) could be detected. B, quantitative analysis of radioactivity in bands, by phosphorimaging, showed that cellular concentration (closed squares) of intact T30177 or T30175 was about 6-fold higher than in the extracellular medium (open squares) after 48 h of incubation. Data are an average of two representative experiments. In parallel experiments, cells were also treated with radiolabeled T30523, but the unmodified random sequence oligonucleotide was very nuclease-sensitive and could not be detected after \(-15\) min of incubation.
Cellular Uptake of Oligonucleotides—The relative nuclease resistance of each oligonucleotide was also tested in cellular uptake assays, in which labeled oligonucleotides were added to the growth medium supporting HeLa cells in culture for varying time periods, and any intact material remaining inside or outside the cells was extracted and analyzed by denaturing gel electrophoresis. About 80% of T30177 and 40% of T30175 remained either intact or in the n − 1 form after 24 h of incubation (Fig. 3). In contrast, very low cellular levels of the mutant or control oligonucleotides were detected, since nearly all of the oligonucleotides were digested during the first hour of exposure to the extracellular medium. Quantitative analysis of cellular uptake indicated that the intracellular levels of intact T30177 increased with time, to about 5–10-fold above the extracellular levels (Fig. 3B). The cellular concentration of intact T30175 also increased above the extracellular levels, but to a lesser extent than T30177, reflecting the greater susceptibility of the terminal phosphodiester linkages in T30175 to exonucleases. Nevertheless, the uptake and stability of T30175 was comparable to or better than that reported for oligonucleotides with nuclease-resistant backbones (31–33). Subcellular localization studies, by microscopic analysis of cells treated with a fluorescein-conjugated homologue of T30177, revealed the punctate cytoplasmic distribution pattern typical for oligonucleotides with nuclease-resistant backbones (34, 35). Cellular uptake was evident 20 min after treatment and accumulation continued for the 48 h duration of the study. There was also some hazy fluorescence associated with the nucleus (figure not shown).

Anti-HIV-1 Activity of Oligonucleotides—To correlate the uptake and stability data with antiviral activity, T30177 and its variants were assessed for anti-HIV effect in a series of acute HIV-1 infection assays using the inhibition of virus-induced syncytium formation or viral p24 antigen levels as assay end points (16, 17). The concentration of T30177 required to inhibit viral activity by 50% (EC50) was 0.15 μM, while the EC50 for the total phosphodiester version (T30175) or a 3'-protected variant (T09100) were determined to be 0.3 and 0.5 μM, respectively (Fig. 4). We then tested the single-base mutated variants of T30175 or T09100, in which certain G nucleotides participating in quartet formation were substituted either with A (T30526), or with 6-thio-dG (RAN1G1, RAN1G2), a synthetic guanosine analog that has been designed to prevent the formation of hydrogen bonds necessary for quartet formation (21). In each case, the substituted oligonucleotides had markedly reduced anti-HIV activity, with EC50 values in the 10 μM range (Fig. 4). These data supported the idea that the G-quartet motifs are more essential than end modifications for maintaining the anti-HIV oligonucleotides in a functional conformation.

**DISCUSSION**

We had previously discovered that the oligonucleotide T30177 is a specific and long-term suppressor of HIV-1 growth in standard cell culture-based assays (16–18). Composed entirely of (deoxy)guanosines and thymidines, the energetically favored and biologically active conformation of the 17-base anti-HIV oligonucleotide is a compact, intramolecular structure of two stacked G-quartets connected by three loops of GT and stabilized by one potassium ion (Fig. 1). Data presented in this report support the idea that the stacked G-quartet motifs of T30177 are the primary determinants of its remarkable nuclease resistance, superior cellular uptake kinetics, and long-term biological efficacy.

The vast difference between the serum and cellular half-lives of T30177, T30175, and their mutated variants (Table I) confirm that subtle changes in sequence or composition that interfere with G-quartet formation and, in turn, influence the three-dimensional shape of the oligonucleotides can markedly reduce the stability of oligonucleotides. According to the T30177 structure model (Fig. 1), the intramolecular folds provide significant occlusion of phosphodiester linkages in the three loops and prevent single-strand exonucleases from accessing their cleavage sites, leading to very long oligonucleotide half-life in serum and inside cells. Biological assays suggest that T30177 inhibits a preintegration step in the HIV-1 infection cycle, possibly by interfering with the activity of the integrase enzyme found in the nucleus and cytosol of infected cells (18), by a mechanism distinct from that of antisense, triplex-forming, and other oligonucleotide inhibitors of HIV expression (28, 36–38). The mechanism by which T30177 may be transported across the plasma or endosomal membrane to its site of action inside cells is an enigma. In the case of antisense oligonucleotides with total phosphorothioate backbones, biological activity can be improved considerably by coadministration with membrane perturbants such as cationic lipids or fusogenic liposomes (34, 35). However, since T30177 (or T30175) is efficacious without the need for uptake enhancers, the G-quartet-mediated folding may contribute to efficient cellular internalization. This enhancement could be due to the compact oligonucleotide size, or to the additional neutralization of the phosphate charges in the oligonucleotide backbone by “cation

[Fig. 4. Anti-HIV activity of T30177 and its variants, measured as reduction in viral-induced syncytium production. A, variants with different end-modifications but unaltered sequences had potent and specific antiviral activity (EC50: T30177, 0.15 μM; T30175, 0.3 μM; T09100, 0.5 μM). In comparison, a single-base mutant version (T30526), not expected to form the G-quartets (26), had no specific antiviral activity. B, in related experiments, the replacement of specific quartet-forming G nucleotides in T09100 with 6-thio-dGs (RAN1G1 and RAN1G2; see Table I) resulted in the loss of specific antiviral activity. The 6-thio-dG-modified oligonucleotides lack the ability to form hydrogen bonds crucial for quartet formation (21). Similar anti-HIV-1 data were obtained when viral p24 levels were measured (not shown). Data points represent the mean ± S.E. for three separate experiments.]
condensation” (39), resulting from increased cation binding to the phosphates brought into close proximity by G-quartet formation and folding. Charge neutralization may improve the permeability of an otherwise negatively charged oligonucleotide across the membrane lipid barrier.

While the existence of short endogenous G-quartet-forming oligonucleotides in cells has not yet been demonstrated, substantial evidence exists for the quartet fold as a recognition element in the telomere (1–4). More generally, compact, multistranded nucleic acid arrays are thought to play a role in diverse biological functions, such as recombination and retroviral dimerization (5–9). Although the structure model for T30177 (Fig. 1) is based upon the formation of two stacked G-quartets, in cross-section the motif is reminiscent of multistranded nucleic acid configurations occurring inside cells (1–4). Thus the stacked G-quartets of T30177 may be described as an example of a stable intramolecular, multistranded fold. Given the remarkable stability and favorable cellular uptake characteristics of T30177, and its general characteristics as a multistranded nucleic acid array, it is interesting to consider the possibility that molecules of this kind maybe used for purposes other than HIV-1 treatment, by competing for macromolecular targets that bind to multistranded nucleic acid structures. Thus, T30177 motif may serve as a prototype for the derivation of a broader class of potentially therapeutic oligonucleotide-based inhibitors that may interfere with the activity of proteins that interact with nucleic acid folds. The tertiary structure of the oligonucleotides may be selectively manipulated to improve their nuclease resistance, uptake properties, and biological specificity.

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