Stability-Activity Relationships of a Family of G-tetrad Forming Oligonucleotides as Potent HIV Inhibitors

A BASIS FOR ANTI-HIV DRUG DESIGN*

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Recently, we have demonstrated that T30695, a G-tetrad-forming oligonucleotide, is a potent inhibitor of human immunodeficiency virus, type I (HIV-1) integrase and the K⁺-induced loop folding of T30695 plays a key role in the inhibition of HIV-1 integrase (Jing, N., and Hogan, M. E. (1998) J. Biol. Chem. 273, 34982–34999). Here we have modified T30695 by introducing a hydrophobic bulky group, propynyl dU, or a positively charged group, 5-amino dU, into the bases of T residues of the loops, and by substitution of the T-G loops by T-T loops. Physical measurements have demonstrated that the substitution of propynyl dU or 5-amino dU for T in the T residues of the loops did not alter the structure of T30695, and these derivatives also formed an intramolecular G-quartet structure, which is an essential requirement for anti-HIV activity. Measured IC_{50} and EC_{50} values show that these substitutions did not induce an apparent decrease in the ability to inhibit HIV-1 integrase activity and in the inhibition of HIV-1 replication in cell culture. However, the substitution of T-T loops for T-G loops induced a substantial decrease in both thermal stability and anti-HIV activity. The data analysis of T30695 and the 21 derivatives shows a significant, functional correlation between thermal stability of the G-tetrad structure and the capacity to inhibit HIV-1 integrase activity and between thermal stability of the G-tetrad structure and the capacity to inhibit HIV-1 replication, as assessed with the virus strains HIV-1 RF, IIIB, and MN in cell culture. This relationship between thermostability and activity provides a basis for improving the efficacy of these compounds to inhibit HIV-1 integrase activity and HIV-1 replication in cell culture.

Anti-HIV chemotherapy has been studied intensively for over a decade. To date, most compounds that have been approved for the treatment of HIV infection belong to the class of 2',3'-dideoxynucleoside analogues (1), such as AZT (zidovudine) (2), DDC (zalcitabine), DDI (didanosine) (3, 4), D4T ( stavudine) (5), and 3TC (lamivudine) (6). These 2',3'- dideoxynucleoside analogues act as competitive inhibitors of the reverse transcriptase, thus stopping the viral replication cycle at the reverse transcription step. Although the combination therapy, which uses two or more drugs simultaneously to inhibit HIV activity, can reduce HIV load to undetectable levels in the blood of many HIV-positive patients, the viruses in T cells are still capable of replicating and infecting other cells (7–9). The results from the assay for integrated and total HIV-1 DNA (7) demonstrated that integrated HIV-1 DNA in resting CD4+ T cells from patients receiving combination treatment is not significantly decreased, and resting CD4+ T cells seem to be a stable reservoir for integrated HIV-1 DNA. However, unintegrated HIV-1 DNA seems to be relatively unstable in vitro with a short half-life in vivo. Thus, development of new drugs against the HIV enzyme called integrase could be a major advance in the treatment of HIV infection because it may eliminate HIV-1 from intracellular sites. Integrase is the only enzyme that catalyzes the integration of the HIV-1 proviral DNA into a host chromosome, which is an essential step in HIV-1 viral replication. The recently reported candidates for pharmaceutical inhibition of HIV-1 integrase had IC_{50} values in the micromolar range for inhibition of HIV-1 integrase activity (10).

A family of G-tetrad-forming oligonucleotides was recently developed as potential anti-HIV therapeutic drugs (11–13). These compounds have shown a strong interaction with HIV-1 integrase in vitro, and to inhibit the integration of viral DNA into host DNA. In previous studies (12, 13), the most potent inhibitors of HIV-1 integrase were found to be T30695, 5'-ggtggtgggtgggtgggt-3', T30177, 5'-ggtggtgggtgggtgggt-3', T30177, 5'-ggtggtgggtgggtgggt-3', and T30177, 5'-ggtggtgggtgggtgggt-3'. IC_{50} values of inhibition for HIV-1 integrase 3'-processing and strand transfer, obtained from a gel-based method, were 47 and 24 nM for T30695 and 79 and 49 nM for T30177. Compared with T30177, T30695 forms an even more stable and orderly G-quartet fold. Our NMR and kinetic data demonstrated that in response to K⁺ binding, T30695 folded into a stable and symmetric G-tetrad complex (13, 14). The folding has been shown to be a two-step process, which is dependent on the nature of the alkaline metal ion. The first step of the process involves the coordination of one K⁺ ion, which competes with a Li⁺ ion to bind within the core of two G-quartets. The second step involves the binding of two additional K⁺ ions to the loop domains. NMR and optical analysis have shown that the second binding step is associated with substantial ordering of the oligonucleotide fold. NMR data and molecular modeling have determined (14, 15) that T30695 in the absence of K⁺ (with Li⁺ ions) forms an intramolecular G-quartet structure with the
opened loop structures. Upon coordination with three K⁺ ions, the loop structure is rearranged, and the bases of loops are folded onto the underlying G-quartets. The structure of T30695 in the presence of K⁺ becomes symmetric and compact. The inhibition of HIV-1 integrase activity was found to greatly increase upon K⁺ binding to the loops. Thus, the folding of the loop domains of these oligonucleotides plays an important role in the function of G-tetrad-forming oligonucleotides.

To investigate the structure and activity of these tetrad-forming oligonucleotides and to improve inhibition of HIV-1 integrase activity and/or inhibition of HIV-1 replication in cell culture, we have designed derivatives by adding positively charged or large hydrophobic groups into T30695. The derivatives were designed to replace T residues in the loop domains with 5-amino dU or with 5-propynyl dU or to substitute G in the loop domains with T. The derivatives were monitored for melting temperature (T_m), inhibition of HIV-1 integrase activity (IC₅₀), and the inhibition of HIV-1 replication in cell culture (EC₅₀). Based upon these measurements, we propose a relationship between thermal stability of the G-quartet structure and its ability to inhibit the activity of HIV-1 in cell culture, which could be useful as the basis for improvement of these oligonucleotides as anti-HIV drug candidates.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Oligonucleotides used in this study were synthesized on an Applied Biosystems Inc. DNA synthesizer, model 380D or 394, using standard phosphoramidite chemistry or fast deblocking expedite chemistry on a Milligen synthesizer as described previously (11, 13). Purification was accomplished by preparative anion exchange high pressure liquid chromatography on Q-Sepharose, followed by pressure filtration in H₂O (Amicon) to remove metal ions. Product purity was confirmed by analytical Q-Sepharose chromatography and by denaturing electrophoresis of ³²P-labeled oligomers on a 20% polyacrylamide/bis-acrylamide (19/1), 7 M urea gel matrix. Oligomer folding was monitored by native gel electrophoresis on a 15% acrylamide (19:1) matrix in Tris-borate EDTA. 5-Propynyl dU was obtained from Glenn Research and was used in oligonucleotide synthesis.

Thermal Denaturation—Oligonucleotides at 7 μM in strand equivalents (20 mM Li₃PO₄, pH 7) were heated to 90 °C for 5 min and then incubated for 1 h at 37 °C in the presence of KCl at 0.1, 0.5, and 1.0 mM. Subsequent to the incubation step, thermal denaturation profiles of the oligonucleotides were obtained at a rate of 1.25 °C/min over a range of 20–90 °C. Absorbance was measured at 240 nm by an HP8452A (Hewlett-Packard) diode array spectrophotometer using an HP89090A temperature regulator.

The thermal denaturation curves of the oligonucleotides were analyzed by an intramolecular folding equilibrium (16) as shown in the following equations.

\[ A(T) = (1 - \alpha)A_{w} + \alpha A_{st} \]  
(Eq. 1)

The oligonucleotides with 0.1 mM KCl in 20 mM Li₃PO₄, pH 7

| Oligomer | Sequence | T_m (°C) | ΔH * (kcal/mol) | ΔS * (cal/K mol) | ΔG * (T = 295K) (kcal/mol) | Fitting coefficient |
|----------|----------|----------|----------------|----------------|---------------------------|-------------------|
| T30695   | 5'-g*gggTgggTgggTgggTt-3' | 46.3 | -53.29 | -166.90 | -4.06 | 0.9995 |
| T30925   | 5'-g*gggTgggTgggTgggTt-3' | 51.0 | -64.49 | -199.02 | -5.77 | 0.9995 |
| T30926   | 5'-g*gggTgggTgggTgggTt-3' | 51.1 | -64.17 | -197.98 | -5.76 | 0.9992 |
| T30927   | 5'-g*gggTgggTgggTgggTt-3' | 51.6 | -64.64 | -199.15 | -5.89 | 0.9997 |
| T30928   | 5'-g*gggTgggTgggTgggTt-3' | 53.7 | -70.25 | -215.01 | -6.82 | 0.9991 |
| T30929   | 5'-g*gggTgggTgggTgggTt-3' | 48.6 | -62.79 | -195.24 | -5.19 | 0.9996 |
| T30924   | 5'-g*gggTgggTgggTgggTt-3' | 48.7 | -45.59 | -141.72 | -3.78 | 0.9994 |

* PT linkages; T, propynyl dU.
FIG. 3. A stereo view of the top of the molecular structure of T30695 in the K⁺ form. The structure shows that each of the T methyl groups of the two thymidines (green) is pointed out from the folded surface.

### Table II

The oligonucleotides with 0.5 mm KCl in 20 mm Li₃PO₄, pH 7

| Oligomer | Sequence | Tₘ | ΔH * | ΔS * | ΔG * (T = 295K) | Fitting coefficient |
|----------|----------|----|------|------|-----------------|-------------------|
|          |          | °C | kcal/mol | (cal/K·mol) | kcal/mol        |                   |
| T30924   | 5'-g*gtgtgtgtgtgtgtgtgtgg*+t-3' | 59.7 | -68.88 | -207.07 | -7.80 | 0.9993          |
| T30925   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 61.8 | -76.80 | -229.36 | -9.14 | 0.9997          |
| T30926   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 61.5 | -77.50 | -230.38 | -9.09 | 0.9994          |
| T30927   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 62.2 | -74.55 | -222.39 | -8.94 | 0.9996          |
| T30928   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 63.2 | -89.47 | -266.08 | -10.97 | 0.9919          |
| T30929   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 59.1 | -81.74 | -246.13 | -9.13 | 0.9991          |
| T30930   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 57.6 | -64.3 | -194.50 | -6.92 | 0.9994          |

### Table III

The oligonucleotides with 1.0 mm KCl in 20 mm Li₃PO₄, pH 7

| Oligomer | Sequence | Tₘ | ΔH * | ΔS * | ΔG * (T = 295K) | Fitting coefficient |
|----------|----------|----|------|------|-----------------|-------------------|
|          |          | °C | kcal/mol | (cal/K·mol) | kcal/mol        |                   |
| T30924   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 69.8 | -66.97 | -195.32 | -9.35 | 0.9963          |
| T30925   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 68.8 | -79.90 | -227.92 | -10.66 | 0.9997          |
| T30926   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 69.1 | -68.27 | -199.60 | -9.39 | 0.9991          |
| T30927   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 68.7 | -79.40 | -232.38 | -10.85 | 0.9996          |
| T30928   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 71.1 | -74.95 | -217.83 | -10.69 | 0.9996          |
| T30929   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 66.4 | -80.41 | -236.90 | -10.53 | 0.9994          |
| T30930   | 5'-g*gtgtgtgtgtgtgtgtgtgtgtgg*+t-3' | 65.0 | -77.81 | -230.23 | -9.89 | 0.9984          |

where

\[
α = (0.5 + 0.5 (K_{eq} - 1)/(1 - K_{eq})^2 + 4αK_{eq})^{1/2} \quad (\text{Eq. 2})
\]

\[
K_{eq} = \exp \left( -\frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \right) \quad (\text{Eq. 3})
\]

where \(K_{eq}\) is the constant for the random coils to folded oligonucleotide equilibrium, \(α\) is the fraction of folded strands, \(1 - α\) is the fraction of random coils, \(A(T)\) is the absorbance at temperature \(T\), \(A_m\) is the absorbance when all strands are random coils, \(A_s\) is the absorbance when all strands are folded, and \(σ\) is the cooperativity of the melting transition, which is referred to the helix interruption constant and \(σ = \exp(\Delta S/R)\) where \(ΔS\) is in units/mol of interruption. In our analysis study, the values of \(σ\) are in the range of 0.9–0.999, determined by an optimized fitting program. Values for \(T_m\) and \(ΔG\) were obtained on the basis of the fitting procedure, which inputs the values of \(ΔH^*, ΔS^*, A_m\), and \(A_s\) estimated from the experimental measurements and then uses an optimized fitting program to search for the best fit.

**Assays for Inhibition of HIV-1 Integrase Activity in Vitro and for Inhibition of HIV-1 Replication in Cell Culture—**Anti-HIV integrase activity was determined utilizing a 96-well scintillation proximity assay (SPA) according to the manufacturer’s protocol (Amersham Pharmacia Biotech). Briefly, each test reaction contained 1) tritiated oligonucleotide substrate in assay buffer supplemented with 50 mM MnCl₂ (pH was adjusted according to the experimental protocol), 2) diluted test material, and 3) diluted integrase enzyme (final concentration, ∼50 mM) in a total of 100 μl; the total mixture contained a final concentration of 20 mM HEPES, pH 7.5, 10 mM dithiothreitol, 0.05% (w/v) Nonidet P-40, and 0.05% (w/v) sodium azide. Following incubation for 1 h at 31–33 °C, the reaction was stopped with 50 mM EDTA, pH 8, and 110 μl of SPA columns. The oligonucleotide solution was heated 90 °C for 5 min and cooled at 4 °C for 30 min. 20% polyacrylamide gels (with 10 × Trisborate EDTA, 10% ammonium persulfate, and 30 μl of N,N,N',N'-tetramethylthelylenediamine) in 1× TBA buffer was precooled in a 4 °C cold room for an hour. Then the gels with loaded samples were run for 4–6 h in the 4 °C cold room.
bead/denaturing reagent was added to each well and mixed gently. Plates were sealed and incubated at room temperature for 30 min; all compounds were tested in duplicate. The degree of integrase-catalyzed oligonucleotide strand transfer was then quantified with a Wallac Microbeta scintillation counter, and the resulting data were utilized to calculate the IC_{50} value for each test compound.

Virus culture assays for inhibition of the HIV-1 cytopathicity induced by the three HIV-1 virus strains, IIIB, MN, and RF, in MT-4 cells were carried out as described elsewhere (17).

RESULTS

Molecular Structure and Thermal Stability—T30695, 5'-ggggggggggggggt-3', forms an extremely stable intramolecular G-quartet structure in the presence of K^+ via a two-step process that involves the binding of one K^+ ion to a central pair of G-quartets and two additional K^+ ions to loops (Fig. 1, A and B). NMR data and molecular modeling (14, 15) have demonstrated that T30695 in the presence of Li^+ forms an asymmetric, less stable G-quartet structure with twisted G-quartet plates and opened loops, referred to as the Li^+ form structure (Fig. 2). Upon coordination with three K^+ ion equivalents, the structure of T30695 becomes symmetric and compact (15 Å width and 15 Å length), referred to as the K^+ form structure (Fig. 2). This coordination greatly increases the thermal stability of the molecular structure of T30695 and its activity on HIV-1 integrase inhibition. As seen in Tables I—III, the T_m of T30695 increased as a function of K^+ concentration, e.g. 46.3 °C at 0.1 mM KCl, 59.7 °C at 0.5 mM KCl and 69.8 °C at 1.0 mM KCl. The free energy, ΔG°, of the molecular structure of T30695 decreased from −4.06 kcal/mol at 0.1 mM KCl to −9.35 kcal/mol at 1.0 mM KCl.

The NMR and molecular modeling observations (14, 15) suggest that several features of the structure of T30695 should be considered when attempting to rationalize the inhibition of HIV-1 integrase, even though the molecular structure of the integrase-T30695 complex is not available yet. The K^+ form structure of T30695 lacks a groove, and its two ends are nearly planar. This cylindrical symmetry and large surface area may increase the probability of T30695 binding to HIV-1 integrase. The base planes of all G and T residues of T30695 are coordinated with K^+ ions, and the coordination greatly increases the thermal stability of the structure while greatly decreasing its thermal capacity for dimers or higher aggregate formation. The higher K^+ concentration results in higher stability of the structure while greatly decreasing its capacity for dimers or higher aggregate formation. The higher stability and lowered capacity aggregation for G-tetrad may enhance the ability to resist nuclease degradation and for its efficient delivery into cells. Three unhydrated K^+ ions are bound to T30695, creating a cylinder with positive charges inside and negative charges on the surface, which also could be a factor for enhanced interaction between T30695 and HIV-1 integrase.

NMR data also showed that upon binding K^+ ions, the bases of T-G-T-G loop domains of T30695 folded into an approximate plane aligned with the G-quartets. The formation of the hydrogen bonds in the loop structure greatly increased the thermal stability of G-quartet structure of T30695. When the bases of T-G-T-G loop domains fold in an approximate plane, each of the

![Fig. 4. A, one-dimensional Ising model employed to fit the melting curve of T30927 in 1.0 mM KCl in 20 mM Li3PO4 buffer (pH 7), obtained by UV absorption at the wavelength of 240 nm. The function used to fit the data is that of Longfellow et al. (16): A(T) = (1 - a1A_a + a2A_s); α = 0.5 + 0.5(K_m - 1)/(1 - K_m)^2 + 4πK_m/3; and K_m = exp(−ΔH + TΔS/RT) (see text for details). B, the calculated ΔG° values of T30924, T30695, T30929, and T30925 were plotted versus Log10[KCl]. These data were fitted to a straight line yielding a slope (ΔΔG°/ΔLog10[KCl]) of 5.8, 5.3, 5.3, and 4.9 for T30924, T30695, T30929, and T30925, respectively. According to the simple model of the transition between the folded and unfolded state for intramolecular tetrad (13), the values of the released K^+ equivalents, ΔS = −ΔG°/2.3RTάLog10(KCl), of T30924, T30695, T30929, and T30925 are about 4.2, 3.8, 3.8, and 3.5, respectively, which correspond to the range of releasing three K^+ ion equivalents.](http://www.jbc.org/)

### Table IV

| Oligomer | Sequence | T<sub>m</sub> (°C) | ΔH° (kcal/mol) | ΔS° (cal/K·mol) | ΔG° (kcal/mol) | Fitting coefficient |
|----------|----------|----------------|----------------|----------------|----------------|-------------------|
| T40101   | 5'-gggIggtgggtgggtggtggt-3' | 69.2 | -73.37 | -214.43 | -10.12 | 0.993 |
| T40102   | 5'-gggIgggggtgggtgggtggt-3' | 69.5 | -64.11 | -187.18 | -8.89 | 0.998 |
| T40103   | 5'-gggIgggggtgggtgggtggt-3' | 69.3 | -59.33 | -173.33 | -8.20 | 0.996 |
| T40104   | 5'-gggIgggggtgggtgggtggt-3' | 69.6 | -71.74 | -209.42 | -9.86 | 0.997 |
| T40105   | 5'-gggIgggggtgggtgggtggt-3' | 67.2 | -82.68 | -243.04 | -10.99 | 0.998 |
| T40106   | 5'-gggIgggggtgggtgggtggt-3' | 65.9 | -50.80 | -149.89 | -6.58 | 0.995 |
| T40107   | 5'-gggIgggggtgggtgggtggt-3' | 69.2 | -49.30 | -144.88 | -6.69 | 0.993 |

The oligonucleotides with 1.0 mM KCl in 20 mM Li3PO4 buffer (pH 7)
T methyl groups (CH₃) is pointed out of the folded plane and faces the solution (Fig. 3). That special orientation for the eight T methyl groups of T30695 provides potential sites for the substitution of other chemical groups, such as bulky groups or charged groups, without disruption of loop structure.

T30924–T30929—In this study, a set of T30695 derivatives were synthesized to investigate the relationship between sequence and thermal stability and the relation between the thermal stability of the oligomers and their anti-HIV activity. One oligonucleotide set (T30925–T30928) was derived from T30695 by replacing one T of the loop domains with a propynyl dU, whereas T30929 and T30924 were derived by the substituting two and three propynyl dUs (Tables I–III and Fig. 1D). Thermal denaturation of these oligonucleotides was measured optically at three K⁺ ion concentrations (0.1, 0.5, and 1.0 mM KCl), and the resulting absorbance versus temperature curves were analyzed by curve fitting, using a two-step formalism for the folding equilibrium proposed by Longfellow et al. (16). Fig. 4A shows a representative analysis of Tₘ for T30927 in 1.0 mM KCl. The data points are UV absorbance values at 240 nm, and the solid line is a curve fitting to the data points with a fitting coefficient of 0.9996, based upon the relationships derived by Longfellow et al. (16). Coefficients of the other members of the set were also in the 0.99 or higher range. Free energy of T30927 folding in 1.0 mM KCl at 295 K, ΔG° = −10.85 (kcal/mol), was obtained from this analysis (Table III), as were equivalent values for the other oligonucleotides of this family.

As seen from these data, the substitution of propynyl dU in the loops does not induce a substantial change in the thermal stability of the G-quartet based folding when compared with T30695 (Tables I–III). The average Tₘ and ΔG of these oligonucleotides were 50.1 ± 3.8 °C and −5.3 ± 1.54 kcal/mol in 0.1 mM KCl (Table I), 60.9 ± 3.3 °C and −8.86 ± 2.11 kcal/mol in 0.5 mM KCl (Table II), and 67.9 ± 3.2 °C and −10.24 ± 0.89 kcal/mol in 1.0 mM KCl (Table III). The slope of a linear regression of ΔG° versus log[K⁺] for T30695 and its derivatives was 5.3 ± 0.5 (independent of propynyl substitution) with fitting coefficients of 0.96 to 1.0 (Fig. 4B). The Δn (=ΔΔG°/2.3RTA log[K⁺(K⁺)]) values of T30695, T30924, T30925, and T30929 (3.8, 4.2, 3.5, and 3.8, respectively) demonstrate that three or more K⁺ equivalents are released from the folded structures upon the melting of T30695 and each of the propynyl dU derivatives. The K⁺-induced loop folding of T30695 by binding three K⁺ ions was previously confirmed by NMR titration study (13), and the loop folding of T30695 plays a key role in structure stability and in inhibition of HIV integrase activity (15). The plots in Fig. 4B show that both thermal stability and K⁺-induced loop folding are not significantly affected by the propynyl substitution. Interestingly, Tₘ values of T30928 suggest that propynyl dU at the 3'-end may lead to a higher thermal stability (Tables I–III).

T40101–T40107—This set of T30695 derivatives was designed to investigate the inhibition of HIV-1 integrase activity by a G-tetrad structure with added positive charge in the loop domains, using the substitution of 5-amino dU for T 5-methyl (CH₃). T40101–T40103 possess one such substitution, so that the loop with a 5-amino dU will carry one positive charge. T40104 and T40105 have two such substitutions in two T-G-T-G loop domains (top and bottom). T40106 has two positive charges in a single T-G-T-G loop domain (Fig. 1E). T40107 was designed by substituting three T methyls with three 5-amino dUs. The thermal denaturation measurements in Table IV show that in 1.0 mM KCl, Tₘ values of T40101–T40107 are in the range of 66–70 °C, the same as Tₘ of T30695. The ΔG° values of T40101–T40105 are also in the same range of ΔG° of T30695. T40106 and T40107 have a slight lower ΔG°, which could result from the two substitutions of 5-amino dU in a single T-G-T-G loop domain.

To confirm the obtained Tₘ values of T30695 and its derivatives, we determined the influence on Tₘ by employing a different heating rate in the melting studies. We found that upon alteration of heating rate from 0.5 to 2.5 °C/min, the Tₘ values of these G-quartet structures shifted less than 1 °C when Tₘ was in the range of 60–80 °C (data not shown). Our results suggest that the Tₘ values of these G-tetrad-forming oligonucleotides are independent of the heating rate in the range of 0.5–2.5 °C/min. The previous kinetic studies of T30695 and TBA demonstrated that in the presence of K⁺, the first folding step, forming a self-associated G-quartet structure, is stabilized by a G-tetrad structure with added positive charge in the loop domains.

### Table V

| Oligomer | Sequence | Tₘ (1 mM KCl) | ΔG° (T = 295K) | EC₅₀ | HIV-1 RF | HIV-1 HIB | HIV-1 MN |
|----------|----------|---------------|----------------|------|--------|--------|--------|
| T30177   | 5'-ggtggtgggtgggtggtggt-3' | 54.0 | −6.19 | 82 ± 10 | 170 ± 50 | 480 ± 100 |
| T30986   | 5'-ggtggtgggtgggtggtggt-3' | 69.8 | −9.55 | 8.2 ± 0.6 | 14 ± 3 | 55 ± 20 |
| T30916   | 5'-ggggggtggtgggtggtggtggtg-3' | 45.4 | −3.56 | 280 ± 100 | 400 ± 280 | 340 ± 120 |
| T30917   | 5'-ggggggtggtgggtggtggtggtg-3' | 42.6 | −2.90 | 550 ± 320 | 450 ± 50 | 1100 ± 300 |
| T30918   | 5'-ggggggtggtgggtggtggtggtg-3' | 47.2 | −3.09 | 450 ± 280 | 400 ± 220 | 530 ± 50 |
| T30919   | 5'-ggggggtggtgggtggtggtggtg-3' | 47.3 | −2.26 | 720 ± 120 | 350 ± 130 | 740 ± 130 |
| T30920   | 5'-ggggggtggtgggtggtggtggtg-3' | 46.4 | −2.77 | 420 ± 40 | 180 ± 80 | 680 ± 20 |
| T30921   | 5'-ggggggtggtgggtggtggtggtg-3' | 47.3 | −1.83 | 450 ± 50 | 250 ± 100 | 540 ± 90 |
| T30922   | 5'-ggggggtggtgggtggtggtggtg-3' | 65.1 | −11.08 | 37 ± 14 | 18 ± 3 | 150 ± 50 |
| T30923   | 5'-ggggggtggtgggtggtggtggtg-3' | 65.2 | −9.68 | 68 ± 28 | 26 ± 5 | 212 ± 45 |

**Fig. 5.** Electrophoresis of T30695, TBA, and derivatives in the presence of 10.0 mM KCl in nondenaturating gels (see text for details). A, the oligos used are T30928, T30929, T30924, TBA, and T30695 from left to right. B, the oligos used are T40105, T40102, T40103, TBA, T30695, T40104, and T40106 from left to right.
shown that K

T30920, and T30921. Previous studies (13, 15) have shown that T30695 is a potent HIV-1 inhibitor and has suggested that the intramolecular G-tetrad fold might be a requirement for the inhibition of HIV integrase activity. To confirm this hypothesis, we monitored the inhibitory capacity of the entire set of 22 T30695 derivatives in this study. Because the members of this set are very similar in overall sequence structure but show systematic variation in the free energy of folding, we have used this set to verify whether the capacity to inhibit integrase is a direct function of the stability of the intramolecular G-tetrad fold. To acquire quantitative data for this analysis, the measurements of the IC50 values of T30695 and its derivatives were carried out in a 96-well-based HIV-1 SPA.

The data in Table VI were obtained with gel-based methods (12, 13) or by the SPA assay (Fig. 6A) and revealed a relatively small error for all IC50 values. T30695 and T30177 show strong inhibition of HIV-1 integrase. However, the derivatives of T30695 or T30177 with the substitutions of T-T or T-G loops for T-G loops, such as the TBA and T30676–T30679, gave poorer IC50 values. As seen in Fig. 6B for 3° processing of group B and Fig. 7C for strand transfer of group B, the rate of migration of an oligonucleotide in nondenaturating gels depends on the size of its molecular structure. The same migrational rates indicate that these oligonucleotides have the same structural size. These results show clearly that T30924–T30929 and T40101–T40107 form an intramolecular G-quartet structure the same as that of T30695. Further evidence to support the suggestion was provided by running nondenaturating gels at 4°C. T30695 and TBA were used as controls because the structures of T30695 and TBA have been determined to form an intramolecular G-quartets with two G-quartets in central by NMR (15, 20, 21). In the presence of K+ (T30917, T30919, and T30920, and T30922 and T30923, had similar Tm values and ΔG0 values. However, Tm values of T30916–T30921 were about 46.0 ± 3.4°C to nearly 20°C lower than that of T30695. In each of those instances, the observed decrease in thermal stability of the G-quartet structures was caused by the conversion of a single T-G loop to form a T-T loop, in general agreement with previous studies (14). The studies have proven that loop folding is crucial to overall stability of T30695 derivatives.

Inhibition of HIV-1 Integrase Activity and Inhibition of HIV-1 Replication in Culture—Previous studies (13, 15) have shown that T30695 is a potent HIV-1 inhibitor and has suggested that the intramolecular G-tetrad fold might be a requirement for the inhibition of HIV integrase activity. To confirm this hypothesis, we monitored the inhibitory capacity of the entire set of 22 T30695 derivatives in this study. Because the members of this set are very similar in overall sequence structure but show systematic variation in the free energy of folding, we have used this set to verify whether the capacity to inhibit integrase is a direct function of the stability of the intramolecular G-tetrad fold. To acquire quantitative data for this analysis, the measurements of the IC50 values of T30695 and its derivatives were carried out in a 96-well-based HIV-1 SPA.

The data in Table VI were obtained with gel-based methods (12, 13) or by the SPA assay (Fig. 6A) and revealed a relatively small error for all IC50 values. T30695 and T30177 show strong inhibition of HIV-1 integrase. However, the derivatives of T30695 or T30177 with the substitutions of T-T or T-G loops for T-G loops, such as the TBA and T30676–T30679, gave poorer IC50 values. As seen in Fig. 7, the plots of Log (IC50 vs. Tm) revealed an apparent linear correlation between the inhibition of HIV-1 integrase activity and structural stability in both assay systems. The correlation coefficients for a linear fitting of the data points in Fig. 7A for group A, Fig. 7B for 3° processing of group B and Fig. 7C for strand transfer of group B are 0.73, 0.55, and 0.74, respectively. Thus, the data support a correlation between activity as an integrase inhibitor and the stability of the intramolecular tetrad fold within members of the T30695 family.

Previous work has also demonstrated (15) that T30695 and T30177 can inhibit HIV-1 integrase in the K+ form structure but not in the Li+ form structure. TBA, which cannot form orderly loop structures, has high IC50 values in the presence and absence of K+ ions. Compared with the matched pairs of
T30695 derivatives (Table V), elimination of the T residue at the 3'-end had no apparent influence on both \( T_m \) and EC\(_{50} \) values. However, the substitution of a T-G loop by a T-T loop in the sequence caused a marked decrease in both the thermal stability and the inhibition of HIV-1 replication in cell culture (Table V). The decrease in the thermal stability of the G-quartet structure substituted with a T-T loop has been shown to be due to loss of a K\(^+\) binding site between the loops and G-quartets (15). The corresponding decrease in IC\(_{50}\) is ascribed to the requirement that T30695 loops must be folded to inhibit the HIV-1 integrase activity (15).

IC\(_{50}\) and EC\(_{50}\) Values of T30924–T30929—As seen in Table VII and Fig. 6B, IC\(_{50}\) values of the derivatives with propynyl T, T30924–T30929, were in the range of 50–200 nM. As mentioned in last section, the substitution of the T methyl by a propynyl dU in the loop residues did not disrupt the K\(^+\) form structure of T30695. T30929 with two propynyl dUs in a single T-G-T-G loop domain had an IC\(_{50}\) close to that of T30695. The IC\(_{50}\) values of T30924 and T30926 seem to show that the substitution of the T methyl group by a hydrophobic bulky group in residue 8 of the sequence only causes a minor decrease in the inhibition of HIV-1 integrase activity. The residue, T8,
folds into a T-G-T-G loop plane with a pseudo T-G loop when binding a K⁺ ion with a G-quartet (Fig. 1). As seen in Table VIII, T30924–T30929 have the same EC₅₀ as T30695, apparently showing that the substitution of a hydrophobic bulky group in the loop structure has no an apparent effect on the inhibition of HIV-1 replication in cell culture.

The IC₅₀ and EC₅₀ values of T30924–T30929, for the inhibition of HIV-1 integrase activity and of HIV-1 replication in cell culture respectively, were about the same as those of T30695 (Tables VII and VIII). Thus, the conclusion to be drawn from these derivatives is that a substitution of a hydrophobic bulky group for a T methyl group does not alter the structure and thermostability of the T30695 and also does not disrupt the interaction between T30695 and HIV-1 integrase, keeping IC₅₀ and EC₅₀ values unchanged.

IC₅₀ Values of T40101–T40107—Although T40101–T40107 form a same G-quartet structure with T30695, the IC₅₀ values of T40101–T40107 were decreased to 6–9-fold compared with that of T30695 (Table IX and Fig. 6C). We tentatively postulate that the substitution of a positively charged group for a T methyl group weakens the interaction between T30695 and HIV-1 integrase, whereas the substitution of a hydrophobic bulky group does not. Based upon a computed model of the T30695-integrate complex,² T30695 appears to be bound into the binding site of HIV-1 integrase, nearby many residues with positively charged side chains, such as Lys₁₁₀, Lys₁₁₃, and Lys₁₆₀. Thus, the decrease in the inhibition of HIV integrase activity for T40101–T40107 may be caused by the charge-charge interaction between the positively charged loops of T30695 derivatives and the positive charges of the lysine residues in the binding site of HIV-1 integrase.

Therapeutic Index—The therapeutic index of T30695 and its derivatives, shown as a ratio of CC₅₀ (50% cytotoxic concentration) to EC₅₀, were obtained from the measurements of cytotoxicity and anti-HIV activity with three virus strains: HIV-1 RF, IIIB, and MN (Table X). The method of the measurements was described elsewhere (17). T30695 is seen to have a therapeutic index in the range of 200 for the virus strain HIV-1 RF and MN and in the range of 50 for HIV-1 IIIB. Similar values were observed for T30177. Compared with T30695, the therapeutic index of T30923 was markedly decreased for all three virus strains. The substitution of phosphodiester linkages for PT linkages at G₁ and G₁₅ seems to have a strong influence on the therapeutic indexes for HIV-1 IIIB and MN. From the ratios of CC₅₀ to EC₅₀ of T30924–T30929, it seems that there is no influence on the therapeutic index following substituting hydrophobic bulky groups in the T residues.

Relationship between Thermal Stability and Anti-HIV-1 Activity—Our previous results have shown that the Tₘ of T30695 homologues is correlated with the IC₅₀ for integrase inhibitors (13) and have suggested that the structure of the intramolecular G-quartet might be required for anti-HIV integrase activity. Here we analyzed results obtained from many derivatives to confirm whether the capacity to inhibit HIV-1 integrase is direct function of the stability of the intramolecular G-tetrad fold. As seen in Fig. 7, the three plots of log(IC₅₀) versus Tₘ of T30695 were obtained based on the data from Table VI. The correlation coefficients for fitting the data points in plots A, B, and C were 0.73, 0.55, and 0.74. The slopes of linear regression for plots A, B, and C were −0.022, −0.022, and −0.028, respectively. Fig. 8 shows three related plots of log(EC₅₀) versus Tₘ of T30695 derivatives for the inhibition of the three HIV-1 viral strains RF, IIIB, and MN, respectively. These data were obtained from the data in Tables V and VIII. The mean square coefficients of plots in Fig. 8 are 0.90 (A), 0.93 (B), and 0.86 (C). The Pearson correlation coefficients (P values) of the plots between A and B, between A and C, and between B and C were 0.88, 0.91, and 0.92, respectively, as obtained from the SAS computational system.

A few important concepts can be drawn from these analyses. The high correlation coefficients for the data demonstrate a significant, functional relationship between the thermal stability and anti-HIV activity of the folded G-quartet structures, which further confirms our previous observations (13). The relationships between Tₘ and IC₅₀ and between Tₘ and EC₅₀ appear to be independent of the methods used to test anti-HIV activity because the data were obtained from several different assays. The plots for Figs. 7 and 8 demonstrate that the relationship between Tₘ and IC₅₀ is surprisingly similar to that between Tₘ and EC₅₀ which suggests that the inhibition of HIV-1 integrase and the inhibition of HIV-1 replication in cell culture may be depending on closely related structural features of the compounds. Additional observations in cell culture assays have demonstrated that compounds such as T30695 also interfere with virus adsorption and entry into the cell (19), and thus it would be worth further exploring the relationship between Tₘ and EC₅₀ for inhibition of virus adsorption. The relationship between the thermal stability and anti-HIV activity for the tetrad-forming oligonucleotides provides critical information for improving the ability of these compounds to inhibit HIV-1 integrase activity, virus-cell binding, and HIV-1 replication. This should greatly help in the design of anti-HIV therapeutic drugs.

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² R. Mitra, S. Lee, M. E. Hogan, and M. Pettitt, submitted for publication.

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### Table VIII

| Oligomer | Sequence | Tₘ (1 mm KCl) °C | ΔG (T = 295) kcal/mol | HIV-1 RF IC₅₀ nM | HIV-1 IIIB IC₅₀ nM | HIV-1 MN IC₅₀ nM |
|----------|----------|-----------------|-----------------------|-----------------|------------------|-----------------|
| T30924   | 5'-g*ggTggttgggTggg't-3' | 65.0         | -8.89                | 10.5 ± 4        | 14 ± 2           | 54 ± 3          |
| T30925   | 5'-g*ggTggttgggTgggt-3' | 68.8         | -10.66               | 10.0 ± 2        | 15 ± 7           | 85 ± 35         |
| T30926   | 5'-g*gtgtggttgggTgggt-3' | 69.1         | -9.39                | 12.0 ± 2        | 29 ± 9           | 62 ± 21         |
| T30927   | 5'-g*gtgtggtggtggtTgggt-3' | 68.7         | -10.85               | 6.5 ± 2         | 18 ± 9           | 45 ± 25         |
| T30928   | 5'-g*gtgtggtggtggtTgggt-3' | 71.1         | -10.69               | 14.0 ± 6        | 20 ± 8           | 76 ± 34         |
| T30929   | 5'-g*gtgtggtggtggtTgggt-3' | 66.4         | -10.53               | 10.0 ± 2        | 14 ± 2           | 70 ± 30         |

### Table IX

| Oligomer | Sequence | Tₘ (1 mm KCl) °C | IC₅₀ nM |
|----------|----------|-----------------|--------|
| T40101   | 5'-gggIgggtggtggtggtggtTxxx-3' | 69.2 | 264 |
| T40102   | 5'-gggIgggtggtggtggtggtTxxx-3' | 69.5 | 326 |
| T40103   | 5'-gggIgggtggtggtggtggtTxxx-3' | 69.3 | 243 |
| T40104   | 5'-gggIgggtggtggtggtggtTxxx-3' | 69.6 | 278 |
| T40105   | 5'-gggIgggtggtggtggtggtTxxx-3' | 67.2 | 227 |
| T40106   | 5'-gggIgggtggtggtggtggtTxxx-3' | 65.9 | 191 |
| T40107   | 5'-gggIgggtggtggtggtggtTxxx-3' | 66.2 | 216 |
The correlation coefficients (5), 0.93 (5), and 0.86 (5). The Pearson

therapeutic index (CC50/EC50) of these

Table X

| Oligomer | Sequence       | HIV-1 RF | HIV-1 IIIB | HIV-1 MN |
|----------|---------------|----------|------------|----------|
| T30925   | 5'-g*ggTgggTgggTggg*t-3' | 152 ± 32 | 44 ± 17   | 247 ± 20 |
| T30923   | 5'-g*ggTgggTgggTggg*t-3' | 153 ± 100| 5 ± 1     | 18 ± 7   |
| T30924   | 5'-g*ggTgggTgggTggg*t-3' | 195 ± 80 | 56 ± 15   | 308 ± 30 |
| T30925   | 5'-g*ggTgggTgggTggg*t-3' | 108 ± 35 | 19 ± 1    | 117 ± 50 |
| T30926   | 5'-g*ggTgggTgggTggg*t-3' | 126 ± 35 | 26 ± 12   | 201 ± 23 |
| T30927   | 5'-g*ggTgggTgggTggg*t-3' | 156 ± 50 | 18 ± 6    | 85 ± 10  |
| T30928   | 5'-g*ggTgggTgggTggg*t-3' | 173 ± 80 | 125 ± 30  | 340 ± 80 |
| T30929   | 5'-g*ggTgggTgggTggg*t-3' | 115 ± 60 | 33 ± 14   | 185 ± 80 |
|          |               | 64 ± 5   | 18 ± 7    | 107 ± 30 |

DISCUSSION

Integration of viral DNA into host cell chromosome is an essential step for HIV-1 replication. Based upon recent studies mentioned in the Introduction (7–9), HIV-1 IN as a target for anti-HIV therapy has attracted more attention. The major steps for the integration involved by HIV-1 IN are 1) processing: nicking of 3'-ends of viral DNA adjacent to highly conserved CA dinucleotides and 2) joining: insertion of the pre-cleaved viral DNA 3'-ends into both strands of host DNA (22–24). HIV-1 IN is composed of three functional and structural domains: N-terminal, central core, and C-terminal. Although all three domains are required for 3' processing and strand transfer (25), the central domain is directly involved in catalysis for the strand transfer reaction, demonstrated by a disintegration assay (26). However, no crystal structure has been determined for a full integrase, although structures of all three domains of HIV-1 IN have been identified individually (27–30). The precise functions of the N- and C-terminal domains in the overall integrase are not clear yet. Therefore, it is difficult to design a highly effective anti-HIV IN inhibitor based upon the known structure-activity information of HIV-1 IN.

A recent review reported that a large number of HIV-1 IN inhibitors have been identified to date (18). Most of the inhibitors have IC50 values in the range of 5–100 μM, and very few inhibitors have IC50 values in the nanomole range. Also more than 50% of the reported inhibitors have no antiviral activity in cell culture. A G-quartet oligonucleotide, T30177 (T30695 homologue), as an HIV-1 IN inhibitor was also reported in the review with IC50 in the range of 50 nM and with antiviral activity in cell culture. The inhibition of HIV-1 IN in vitro by the G-quartet oligonucleotides was identified with IC50 in the nanomole range based upon disintegration reaction (10). The results demonstrated that T30695 homologues require a coordination of the enzyme zinc finger region in the N-terminal domain for inhibitory activity and suggested that the zinc finger assists to stabilize the binding interaction between the G-quartet inhibitor and the catalytic domain of HIV-1 IN. The inhibition of HIV-1 virus activity in cell culture by the G-quartet oligonucleotides was also observed previously (19). Based upon DNA sequence analysis, the G-quartet inhibitor was proposed to target the envelope glycoprotein gp120 in cell culture. In the reported HIV-1 IN inhibitors (18), the strong ability to inhibit HIV-1 activity in vitro and in cell culture leads to the G-quartet oligonucleotides to be a useful tool to understand the enzymology of HIV-1 IN and to develop a highly effective anti-HIV therapeutic drugs.

The structure-activity of the G-quartet inhibitors has been studied (13, 15). We have found that the ability to inhibit HIV-1 IN activity in vitro strongly depends on the thermostability and conformation of the G-quartet oligonucleotides. Here we further investigated the relationship between structural stability and anti-HIV ability in vitro and in cells, using 22 T30695 derivatives. The results show clearly that the inhibition of
HIV-1 IN activity in vitro largely decreases when a modification in loop domains of T30695 induces a decrease in thermostability of the G-quartet, in agreement with the previous observation in which the conformation of loop domains of T30695 plays a key role in inhibition of HIV-1 IN activity (15). We also have obtained a linear correlation between thermostability of the G-quartet oligonucleotides and their anti-HIV replication in cell culture, identified with three viral strains, and using 22 T30695 derivatives. Whether the inhibition of HIV-1 replication by the T30695 derivatives in cell culture is due to inhibition of integrase and/or virus adsorption, the high correlation between the $T_m$ and EC$_{50}$ values demonstrates that the structural stability of the G-quartet oligonucleotides is a strong determinant for inhibition of HIV-1 replication in cell culture. This stability-activity correlation provides critical information for new drug design, so that the search for a highly thermostable structure for the G-quartet oligonucleotides will be next priority. Moreover, the correlations between $T_m$ and IC$_{50}$ values and between $T_m$ and EC$_{50}$ values also can be used for a rapid screen of newly designed candidates. Based upon the $T_m$ value of a new candidate, we can quickly make a judgement of whether it is worthwhile to put the candidate through the anti-HIV assay in vitro or virus replication in cells.

Here we also report the EC$_{50}$ values for inhibition of HIV-1 replication for a large number of G-quartet inhibitors in cell culture, using three virus stains: RF, IIIB, and MN. The greater inhibitory potency of T30695 and its derivatives suggests that the G-tetrad-forming oligonucleotides could be novel anti-HIV therapeutic drugs, in accord with previous suggestions (10). To the best of our knowledge, no single integrase inhibitor has so far been shown to owe its anti-HIV activity in vitro to the close relations between $T_m$ and EC$_{50}$ values (11). The conformation of loop domains of T30695 determines in structure-activity studies on T30695, a G-tetrad-forming oligonucleotide.

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Stability-Activity Relationships of a Family of G-tetrad Forming Oligonucleotides as Potent HIV Inhibitors: A BASIS FOR ANTI-HIV DRUG DESIGN

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