Design of tetraplex specific ligands: cyclic naphthalene diimide

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Yugo Esaki, a Md. Monirul Islam, a Satoshi Fujii, c Shinobu Sato, a, b and Shigeori Takenaka* a, b

Cyclic naphthalene diimide 1 bound to hybrid-type tetraplex DNA from 5'-AGGG(TTAGGG)3-3' (K=8.6 × 10^6 M^-1) with 260-fold greater affinity than binding to double stranded oligonucleotide consisting of 5'-GGG AGG TTT CGC-3' and 3'-CCC TCC AAA GCG-5' (nK=3.3 × 10^4 M^-1) with 0.5 μM of IC50 for telomerase activity.

Telomerase is a ribonucleoprotein that adds DNA sequence repeats to eukaryotic chromosome termini. Constitutive telomerase activity can lead to cell immortalization and cancer, so drugs that target the activity of this enzyme are garnering great interest. Telomere DNA is known to form tetraplex structures through guanine (G) -quartet formation,1 and because this structure inhibits telomerase access, ligands that strongly bind these structures and stabilize these complexes are expected to be highly specific anticancer agents.2,3 Until recently, these tetraplex-specific ligands have been exclusively designed to stack with the plane formed by G-quartet,2 or to arrange their four substituents in the four grooves of tetraplex DNA.3

In this study, we designed and synthesized two cyclic naphthalene diimide compounds (1 and 2) by linking two substituents of different lengths with 2,2'-Cyclohexane-1,1-diyldiacetic acid. Human telomere DNA is known to have several tetraplex structure conformations, including hybrid, basket, chair, or propeller type.4 All of these tetraplex structures retain spaces at both termini of three stacked G-quartets to permit stacking with ligand.5 Cyclic naphthalene diimides as shown in Fig. 1A are expected to bind these sites through a stacking interaction (Fig. 1B). These compounds are also expected to have reduced binding for double stranded DNA (dsDNA) because of their linker substituents (Fig. 1B&S1), so cyclic naphthalene diimides should have higher affinity for tetraplex DNA compared to dsDNA. This interaction has already been reported as classical intercalator.6 Connecting substituents from the amido parts of naphthalene diimides might be more effective for reducing dsDNA affinity, due to blocking of one face in the intercalator plane with the aliphatic chain, so intercalation into duplex DNA be prevented, while end stacking onto G-tetrads can utilize a single face of the naphthalene diimide. To evaluate the selectivity of these derivatives for hybrid-type tetraplex DNA and dsDNA, we evaluated the interactions of 1 and 2 with 5'-AGGG(TTAGGG)3-3' (a-core) and with double stranded oligonucleotide consisting of 5'-GGGAGGTTTCGCG-3' and 3'-CCCCTCACCAGCG-5' (dsOligo). The a-core is known to form either hybrid or basket type tetraplexes in the presence of K+ or Na+, respectively,5 so the presence of these ions was used to test selective binding to these conformations. The different conformations were confirmed by circular dichroism spectra of a-core in the presence of 0.10 M KCl or NaCl (Fig. S10A). The naphthalene diimide derivatives (1-3) had absorption maxima at 384 nm and showed hypochromic effects upon the addition of a-core or dsOligo. A hypochromic effect of 60% was observed for the interaction between non-cyclic naphthalene diimide 3 with dsOligo. A binding constant of 6.0 × 10^5 M^-1 was obtained by Scatchard analysis of absorption change upon the addition of varied amounts of dsOligo (Table 1), which was in agreement with a similar report using calf thymus DNA.7 The ratio of ligand per dsOligo used for binding was n=3, a reasonable result considering that a typical intercalator covers two base pairs upon binding to dsDNA, in addition to the expected relative difficulty of binding at terminal sites. The binding constant and number of ligand with DNA was estimated using the absorption change upon the addition of DNA, and was subsequently fitted with the Scatchard equation [r/L=K(n-r)]; the DNA concentration used was defined as per a-core
or dsDNA molecule). However, the product of the binding constant and binding stoichiometry ($aK$) was obtained using Benesi-Hildebrand analysis, with changing absorption using an excess amount of DNA. Fig. 2 shows binding the data of derivative 1 with a-core or dsOligo in the presence of KCl, and Table 1 shows the binding analysis of 1 with a-core or dsOligo estimated by Scatchard (C) or Benesi-Hildebrand (D) plot, respectively.

Naphthalene diimide 3 showed similar binding affinities with dsOligo in the presence of KCl and NaCl. The binding constant of 3 with a-core in the presence of K$^+$ was $K=1.6 \times 10^6 \text{ M}^{-1}$ with a binding number of ca. 2. On the other hand, absorption of 3 showed a 20% hypochromic effect upon the addition of a-core in the presence of Na$^+$, and $nK=1.1 \times 10^5 \text{ M}^{-1}$ by Benesi-Hildebrand for this interaction due to unsaturation even at high concentrations of a-core. Binding constants of 3 for a-core in K$^+$ are 3-16 times higher than a-core in Na$^-$ or dsOligo in the presence of K$^+$ or Na$^+$.

The absorption maximum of cyclic naphthalene diimide 2 at 384 nm showed a 50% hypochromic effect upon the addition of a-core in the presence of K$^+$, with a binding constant of $K=1.5 \times 10^6 \text{ M}^{-1}$ and a binding amount of $n=2$ based on absorption changes using various amounts of a-core. The binding constant of 2 to a-core was similar to that of 3 in K$^+$ and Na$^+$, but then $nK=3.0 \times 10^6 \text{ M}^{-1}$ obtained for 2 and dsOligo revealed a 47-times higher preference of 2 for a-core than dsOligo. The introduction of a cyclic linker substitute in naphthalene diimide apparently retains the binding affinity for a-core while diminishing the affinity for dsDNA.

An unwinding experiment of plasmid DNA with Topoisomerase I using large amounts of 2 resulted in the unwinding of super helicity (Fig. S13B and S14D), suggesting that 2 can weakly bind dsDNA with partial stacking between base pairs in the DNA duplex.

We also synthesized a cyclic naphthalene diimide derivative with a shorter linker chain (1) in an attempt to reduce intercalation in dsDNA, then evaluated its interaction with a-core and dsOligo. Results show a binding constant of $K=8.6 \times 10^5 \text{ M}^{-1}$ with $n=2$ for 1 with a-core, with a diminished affinity relative to 3 of $nK=3.3 \times 10^5 \text{ M}^{-1}$ for dsOligo. Cyclic naphthalene diimide 1 did not show any unwinding of super coiled plasmid DNA, even using excess amounts of 1 in the Topoisomerase I assay (Fig. S13A and S14B&c). The data indicates that 1 has a 260-times preference for a-core over dsOligo. Although the binding affinity of 1 for a-core in the presence of Na$^+$ was similar to that of 2 and 3, it is clear that 1 has a preference for hybrid tetraplex DNA compared to the basket conformation. This might be due to the fact that basket type a-core crosses its oligonucleotide chain over the G-quartet diagonally and disrupts access of 1 to the G-quartet plane.

Stabilization of DNA structure for 1-3 was tested using DNA melting temperature measurements (Fig. S11&S12), and results are summarized in Table 2. Similar stabilization effects for the use of 2 were observed in both a-core and dsOligo, which was expected due to the partial binding of this compound to dsDNA. Derivative 1 imparted little stabilization to dsOligo, highlighting the selective stabilization of hybrid-type a-core with 1. Circular dichroism spectra of a-core showed little change upon the addition of 1 (Fig. S10B), indicating that binding of 1 apparently does not disturb the structure of a-core. Isothermal titration calorimeter (ITC) measurements were also carried out for the binding of 1-3 with a-core. A typical experimental result is shown in Fig. S15 and results are summarized in Table 3. The order of binding affinities of 1-3 observed using ITC was in agreement with results obtained from changes in absorption spectra using varied concentrations of 1-3. The non-cyclic 3 showed

**Table 1 Binding parameters of 1-3 with a-core or dsOligo in the presence of K$^+$ or Na$^+$**

<table>
<thead>
<tr>
<th>DNA</th>
<th>$10^5K/M^1 (\alpha)$</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-core (K$^+$)</td>
<td>86 (1.9)</td>
<td>15 (1.6)</td>
<td>16 (1.5)</td>
<td></td>
</tr>
<tr>
<td>a-core (Na$^+$)</td>
<td>2.2*</td>
<td>1.1*</td>
<td>1.1*</td>
<td></td>
</tr>
<tr>
<td>dsOligo (K$^+$)</td>
<td>0.33*</td>
<td>0.30*</td>
<td>6.0 (2.8)</td>
<td></td>
</tr>
<tr>
<td>dsOligo (Na$^+$)</td>
<td>-</td>
<td>-</td>
<td>6.0 (3.0)</td>
<td></td>
</tr>
</tbody>
</table>

$^*$50 mM Tris-HCl (pH 7.4) and 100 mM NaCl or KCl.

$^*$Benesi-Hildebrand analysis, $aK$.

**Table 2 Stability of a-core or dsOligo by 1-3 in the presence of K$^+$ or Na$^+$**

<table>
<thead>
<tr>
<th>DNA</th>
<th>$T_m/\circ C$</th>
<th>$\Delta T_m/\circ C$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>a-core (K$^+$)</td>
<td>68</td>
<td>3.0</td>
</tr>
<tr>
<td>a-core (Na$^+$)</td>
<td>56</td>
<td>1.3</td>
</tr>
<tr>
<td>dsOligo (K$^+$)</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>dsOligo (Na$^+$)</td>
<td>61</td>
<td>0</td>
</tr>
</tbody>
</table>

$^*[ligand] = [DNA] = 1 : 1. - 50 \text{ mM Tris-HCl (pH 7.4)} and 100 \text{ mM NaCl or KCl}.$

**Table 3 Thermodynamic data for the binding of 1-3 to a-core in the presence of K$^+$ or Na$^+$**

<table>
<thead>
<tr>
<th>K$^+$</th>
<th>$10^5K/M^1$</th>
<th>$n$</th>
<th>$\Delta H/kcal \text{ mol}^{-1}$</th>
<th>$\Delta S/\text{cal mol}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na$^+$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

50 mM potassium or sodium phosphate buffer (pH7.4).
shows that TS-primer extends the length to form tetraplex structure. Both of the naphthalene diimide planes of 1 and 2 effectively stack with the a-core G-quartet one in these models, and the linker chain of 1 fits within the confines of the cavity created by the hybrid type tetraplex DNA. This model appears to show that the cyclohexane moiety of 1 is more effectively hidden within the hybrid type a-core cavity compared with 2. 

In this study, we successfully designed and synthesized ligands specific for hybrid type tetraplex DNA using cyclization of two linker chains of naphthalene diimide. Results suggest that optimization of the ring structure and/or length of linker chain might allow the development of highly specific telomere inhibitors that can work as highly effective anti-cancer agents.

I am grateful for helping ITC measurement using Nano ITC LV to Dr. Yasuo Asami of TA Instruments. Japan. This work was supported in part by Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan.

Notes and references

† Electronic Supplementary Information (ESI) available: synthetic procedures of 1 and 2, experimental procedures (Topoisomerase I assay, UV-Vis measurement, circular dichroism spectral measurement, melting curve measurement, AFM, ITC, electrochemical telomerase assay, and computer modelling), and Fig. S1-S17. See DOI: 10.1039/c000000x/

Design of tetraplex specific ligand: cyclic naphthalene diimide

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Figure S1. A cartoon for the complex of a cyclic naphthalene diimide with double stranded DNA. (A) The cyclohexyl part prevents the cyclic naphthalene diimide from threading intercalation into double stranded DNA. (B) The linker chains of the cyclic naphthalene diimide also prevent partial intercalation into double stranded DNA. According to the molecular modeling for (C) and (D), 1 is expected to prevent either of the binding modes, whereas 2 is expected to have a “bare” naphthalene diimide plane to allow partial interaction.

Synthesis of cyclic naphthalene diimide 1 and 2
As precursors of 1 and 2, N,N-bis[3-(3-Aminopropyl)methylaminopropyl]-naphthalene-1,4,5,8-tetracarboxylic acid diimide (4) and N,N'-bis[[3-(3-Aminopropyl)piperazin-1-yl]propyl]-naphthalene-1,4,5,8-tetracarboxylic acid diimide (5) were synthesized according to the procedure reported previously [1]. 3 was synthesized according to the procedure reported previously [2].

Synthesis of 1

A suspension of 4 (0.305 g, 0.46 mmol), 1,1-cyclohexanediacid acid (0.175 g, 0.46 mmol), 1-Hydroxybenzotriazole Monohydrate (0.268 g, 1.37 mmol) and (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (0.910 g, 1.37 mol) was dissolved in mixture of chloroform (1.0 l) and triethylamine (30 ml) and the mixture was stirred at room temperature for 120 h. The reaction mixture concentrated to around 100 ml was washed with 50 ml of saturated sodium hydrogen carbonate, and dried over MgSO4. The solvent was removed and the residue was chromatographed on silica gel with chloroform/diethylamine = 1/0.02 as eluent. The fraction showing Rf of 0.31 on TLC with the same solvent was collected and the solvent was removed under reduced pressure. 1 was purified as a collection with a peak at retention time of 16 min by reversed phase HPLC on Inertsil ODS-3 (inner diameter 5mm, size 4.6mm×250mm, GL Science Inc., Tokyo, Japan) in a gradient mode at a flow rate of 1.0 mLmin⁻¹, where the concentration of acetonitrile was changed linearly to 90% from 10% in water containing 0.1% trifluoroacetic acid over 30 min at 40 °C. Elution was monitored by absorption at 250–400 nm. 1 was utilized in all of the experiment after reversed phase HPLC. Yield: 0.06 g (0.02%), MALDI-TOFMS (positive mode, α-CHCA) m/z = 687.9 (theory for C₃₈H₅₀N₆O₆ + H⁺ = 687.4). ¹H-NMR (500 MHz, CDCl₃): δ= 1.21 (4H, m), 1.32 (2H, t), 1.55 (4H, m), 1.80 (8H, m), 1.94 (10H, m), 2.19 (4H, t), 2.44 (4H, t), 3.15 (4H, m), 4.34 (4H, m), 7.88 (2H, bs), 8.68 (4H, s) ppm. ¹³C-NMR (100 MHz, CDCl₃): δ = 21.05, 25.08, 25.49, 25.0, 35.56, 36.29, 37.84, 39.10, 41.04, 45.81, 45.85, 55.73, 56.21, 126.00, 130.41, 162.50, and 170.74 ppm. HRMS Calcd. for C₃₈H₅₁N₆O₆: M, 687.3792. Found: m/e 687.3864.

Figure S2. Reversed phase HPLC of 1. The concentration of acetonitrile was changed linearly to 90% from 10% in water containing 0.1% trifluoroacetic acid over 30 min at 40 °C
Figure S3. $^1$H-NMR chart of 1 in CDCl$_3$ using TMS as internal standard.
Figure S4. $^{13}$C-NMR chart of 1 in CDCl$_3$ using TMS as internal standard.
Figure S5. High-resolution mass spectra (HRMS-FAB) of 1.
Synthesis of 2

A suspension of 5 (0.396 g, 0.47 mmol), 1,1-cyclohexanedicarboxylic acid (0.0945 g, 0.47 mmol), 1-Hydroxybenzotriazole Monohydrate (0.217 g, 1.42 mmol) and (Benzotriazol-1-yl oxy)tripyrrolidinophosphonium hexafluorophosphate (0.737 g, 1.42 mmol) was dissolved in mixture of chloroform (500 ml) and triethylamine (15 ml) and the mixture was stirred at room temperature for 42 h. The reaction mixture concentrated to around 50 ml was washed with saturated sodium hydrogen carbonate (50 ml each), and dried over MgSO₄. The solvent was removed and the residue was chromatographed on silica gel with chloroform/diethylamine = 1/0.02 as eluent. The fraction showing Rf of 0.21 on TLC with the same solvent was collected and the solvent was removed under reduced pressure. The residue was taken up in a small amount of methanol, sonicated, and then the solvent evaporated. This process was repeated several times until the orange product (6) solidified. 2 was utilized in all of the experiment after reversed phase HPLC.

Yield: 0.054 g (14%), MALDI-TOFMS (positive mode, α-CHCA) m/z = 798.70 (theory for C₄₄H₆₀N₈O₆ + H⁺ = 798.0). ¹H-NMR (500 MHz, CDCl₃): δ = 1.32 (4H, m), 1.45 (10H, m), 1.66 (4H, m), 1.93 (4H, t), 1.98 (4H, t), 2.16-2.25 (16H, m), 2.48 (4H, m), 3.13 (4H, m), 4.38 (4H, t), 6.90 (2H, bs), 8.76 (4H, s) ppm. ¹³C-NMR (100 MHz, CDCl₃): δ = 21.57, 23.62, 25.89, 26.01, 36.20, 36.77, 37.38, 39.66, 44.05, 52.94, 52.94, 55.63, 56.56, 126.73, 126.78, 132.04, 163.17, and 171.68 ppm. HRMS Calcd. for C₄₄H₆₁N₈O₆: M, 797.4678. Found: m/e 797.4725.

![Figure S6. Reversed phase HPLC of 1. The concentration of acetonitrile was changed linearly to 70% from 21% in water containing 0.1% trifluoroacetic acid over 25 min at 40 °C.](image-url)
Figure S7. $^1$H-NMR chart of 2 in CDCl$_3$ using TMS as internal standard.
Figure S8. $^{13}$C-NMR chart of 2 in CDCl$_3$ using TMS as internal standard.
Figure S9. High-resolution mass spectra (HRMS-FAB) of 2.
Experimental procedures

Topoisomerase I assay

Topoisomerase I assay was carried out according to the method previously reported [3]. Briefly, 0.25 μg of pUC19 were incubated with 5 U of topoisomerase I in 0.1% bovine serum albumin (BSA) and 1 × reaction buffer, composed of 35 mM Tris-HCl (pH 8.0), 72 mM potassium chloride, 5 mM magnesium chloride, 5 mM dithiothreitol (DTT) and 5 mM spermidine at 37 °C for 5 min. A various concentration of 1-3 was then added, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by adding 2 μl of 10% sodium dodecylsulfate (SDS) and 0.5 μl of 20 mg/ml proteinase K, and the solution was incubated at 37 °C for 15 min. It was then extracted with phenol containing chloroform and isoamyl alcohol, and then with chloroform containing isoamyl alcohol. After ethanol precipitation and dissolution, it was analyzed by gel electrophoresis on 1% agarose in 1 × TAE at 18 V for 3.5 h. The gel was stained with Gelstar (Takara Bio, Shiga, Japan) for 30 min in 1 × TAE.


Evaluation of the binding abilities of 1-3 with a-core or dsOligo

Binding of 1 to A-core or dsOligo was studied spectroscopically on a Hitachi U-3310 spectrophotometer (Tokyo, Japan). Aliquots of 200 μM a-core or 50 μM dsOligo were added to 4-5 μM 1-3 in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl and spectra taken at 25 °C. The absorbance at various DNA concentrations were analyzed by Scatchard formulation: \( \frac{r}{L} = K(n-r) \), where \( r \) is the stoichiometry (the moles of ligand bound per DNA molecule), \( L \) is the free ligand concentration, \( K \) is the observed binding constant, and \( n \) is the number of bound ligand per DNA or Benesi-Hilidebrand formulation: \( \frac{1}{\Delta \text{Abs}} = \frac{1}{l(D\varepsilon \text{ [ligand]})} + \frac{1}{(nKI\Delta\varepsilon \text{ [ligand]})} \times (1/\text{DNA}) \), where \( \Delta\varepsilon \) is molar absorptivity change of ligand and \( l \) is 1 cm.

Circular dichroism (CD) spectral measurements

Various concentrations of 1-3 were added to 1.5 μM a-core or dsOligo in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl at 25 °C, and CD spectra taken at a scan rate 50 nm/min on a Jasco J-820 spectropolarimeter (Tokyo, Japan). Other conditions were: response 2 s, data interval 0.1 nm, sensitivity 100 mdeg, bandwidth 2 nm, and scan number 4 times.

Melting curve measurements

Melting curves of A-core or dsOligo were measured on a Hitachi 3300 spectrophotometer (heating
rate of 0.5°C/min to 90°C) or Jasco J-820 spectrophotometer (response, 100 mdeg; temperature gradient, 30°C/h; response, 8 s; data collecting interval, 0.5 °C; bandwidth, 2 nm) equipped with a temperature controller, respectively. The melting curves based on circular dichroism (CD) at 292.6 nm of A-core or absorption change at 260 nm of dsOligo were measured in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl or KCl. A mixture of 1.5 μM A-core or dsOligo and 1.5 μM of 1-3 was placed in a cell of 1 cm in light path length (total 3 ml).

Isothermal Titration Calorimetry (ITC) measurements
Before measurement, A-core DNA and HP-27 (5′- GCG ATT CTC GGC TTT GCC GAG AAT CGC - 3′) were annealed by heating to 95°C for 10 min, cooled to 25°C at 0.5°C/min. Binding studies were performed using a low volume nano ITC (TA instruments, USA) with a cell volume of 190 μL at 25 °C. Samples were degassed for 5 min prior to use. The sample cell was filled with the 10 μM A-core DNA or HP-27 in 50 mM sodium or potassium phosphate buffer. One μL of 250 μM 1, 2 or 3 in 50 mM sodium or potassium phosphate buffer was added into the thermostated cell by means of a syringe.

Atomic Force Microscopy (AFM) of pUC19
The mica surface was exfoliated with adhesive tape, 40 μl of 10 ppm poly-L-lysine placed on it and allowed to stand for 5 min. They were washed twice with Milli-Q water (100 μl), dried with an N₂ gas for 30 min. Two concentrations (2.0 and 16 μM) of 1, 2 or 3 were added to pUC19 (1.3 ng/μl, 2.0 μM-bp) in MilliQ. They were placed on the substrate, and washed twice with Milli-Q water (100 μl), dried overnight in a desiccator and finally with an N₂ gas for 30 min. AFM images were taken on an Nanonavi IIIs station and nanocute in the dynamic force mode with a cantilever (PRC-DF40P, SII Nanotechnology, Tokyo, Japan).

Electrochemical Telomerase assay by using chronocoulometry (CC) [4]
Disposable chips carrying a gold electrode (Hano Manufacturing, Fukuoka, Japan) were prepared as described previously [5]. Three hundred microliters of 1.0 mM 6-mercaptopentanol were added on the gold electrode and kept at 45°C for 1 h, and washed with 300 μL of Milli-Q water. Oligonucleotide TS-primer 5′-HO(CH₂)₆-SS-(CH₂)₆-TTT TTT TTA ATC CGT CGA GCA GAG TTA GGG-3′ was custom-synthesized by Genenet (Fukuoka, Japan). Ten microliters of 25 nM TS primer in 50 mM NaCl were added on the 6-mercaptopentanol immobilized gold electrode and kept at 37°C for 30 min. After the addition of 300 μL of 1 mM 6-mercaptopentanol, the chip was incubated at 37°C for 1 h and washed with 300 μL of Milli-Q water twice to yield TS primer-immobilized electrodes.
The electrochemical measurement was made with a three-electrode configuration in a portable apparatus described previously [5], where a platinum electrode acted as the counter electrode, Ag/AgCl as the reference electrode, and the TS primer-immobilized electrode as the working electrode. CC was measured in 10 mM Tris-HCl (pH 7.4) containing 50 μM RuHex and the electrode was washed with 300 μL of Milli-Q water twice and subsequently washed with 300 μL of Milli-Q water containing 40 units/mL RNase Inhibitor. For the telomerase reaction, 20 μL of a reaction solution prepared above was placed on the electrode and incubated at 37°C for 30 min. After washing the electrode with 300 μL of Milli-Q water twice, multipotential step and CC were taken again under the same conditions.

In the Cottrell equation (1) for chronocoulograms (plot of Q vs. t^{1/2}) in the absence and presence of RuHex, the y-intercept at time zero represents Q_{dl} or Q_{before} and Γ_{DNA} is derived from equations (2) and (3) using the obtained Q_{dl} and Q_{before}. After telomerase reaction of this electrode, the y-intercept at time zero in the chronocoulogram in the presence of RuHex gives Q_{after}. Accordingly, the elongation time x in (TTAGGG)_x is given by equations (4) and (5) using the obtained Q_{before} and Q_{after}.

\[
Q = (2nFAD_{0}^{1/2}C_{0}*/r^{1/2})t^{1/2} + Q_{dl} + nFA\Gamma_{0} \quad (1)
\]

\[
\Gamma_{0} = (Q_{before} - Q_{dl})/nFA \quad (2)
\]

\[
\Gamma_{DNA} = \Gamma_{0}(z/m)N_{A} \quad (3)
\]

\[
\Delta\Gamma_{0} = (Q_{after} - Q_{before})/nFA \quad (4)
\]

\[
(\text{TTAGGG})_{x} = \Delta\Gamma_{0}(z/6)N_{A}/\Gamma_{DNA} \quad (5)
\]

The parameters used are as follows: n, number of electrons per molecule for reduction (n = 3); F, Faraday constant (C/equiv); A, electrode area (cm^2); Q_{dl}, capacitive charge (C); \Gamma_{0}, amount of redox marker, RuHex (mol/cm^2); Γ_{DNA}, probe surface density (molecules/cm^2); m, the number of bases in the probe DNA (m = 30); ΔΓ, elongated products per electrode area (mol/cm^2); z, charge of the redox molecule (z = 3); N_{A}, Avogadro’s number (molecules/mol); Q_{before}, charge before elongation reaction; Q_{after}, charge after elongation reaction, and (TTAGGG)_x, average elongation time per hexanucleotide per primer molecule.

The condition of multi potential steps was as follows: potential 1 = -0.16 V, time 1 = 1.0 s, potential 2 = -0.55 V, time 2 = 1.0 s, potential 3 = -0.16 V, time 3 = 1.0 s, sample interval = 2.0 ms, quiet time = 2.0 s, sensitivity = 5e-4A/V. The condition of CC measurements was as follows: potential step from 0.1 to -0.4 V, step = 1, pulse width = 0.25 s, sample interval = 5.0 ms, quiet time = 2.0 s, sensitivity = 1e-5 A/V. The telomerase reaction solution (20 μL) consisted of 20 mM Tris-HCl (pH 8.5), 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 20 μM dNTP mixture, 25 HeLa cell extracts, and 2.5 μM 1, 2 or 3.

Computer modeling of the complex of hybrid type tetraplex structure of a-core with 1 or 2
Molecular modeling of these complexes was constructed by MOE [6]. Data of NMR in the aqueous solution of K⁺ (PDBID: 2GKU) [7] was utilized in the structural construction of a-core. Structure of a-core was fixed except for the bases of T1T2T18T19T20 located upper site of three G-tetraplex planes which is expected as the binding site of 1 or 2. Ligand, 1 or 2 was placed on the binding site of a-core and energy minimization of these complexes was carried out. Molecular dynamics calculation of these mineralized complexes was further carried out until 1 or 2 was located in the binding site as stable condition. Finally, energy minimization of the complexes was obtained as shown in Figure 3. These calculations were used the force field of MMFF94x.

Figure S10. CD spectra of 1.5 μM a-core in 50 mM Tris-HCl (pH 7.4), 100 mM KCl (A) or NaCl (B) in addition of 1 (0, 0.38, 0.75, 0.80, 2.25, and 3.00 μM from bottom to top) at 25 °C.
Figure S11. Temperature dependence of 1.5 μM a-core in the absence (A, D) or presence of 1.5 μM 1 (B, E) and 2 (C, F) under 50 mM Tris-HCl (pH7.4) and 100 mM KCl (A-C) or NaCl (D-F).

Figure S12. Temperature dependence of 1.5 μM dsOligo in the absence (A, B) or presence of 1.5 μM 1 (C, D) and 2 (E, F) under 50 mM Tris-HCl (pH7.4) and 100 mM KCl (A, C, E) or NaCl (B, D, F).
Figure S13. Topoisomerase I assay for pUC19 treated with 5.0 U of enzyme. Following incubation a various concentration of 1 (A), 2 (B), and 3 (C) (2.0, 5.0, 10, 20, 30, 40, 50 μM from left to right) was added and the mixture incubated further. After work-up, DNA was electrophoresed Lanes M and Topo I - represent 1 kb size markers and pUC19, respectively. OC and CCC refer to open circler and covalent closed circular, respectively.
Figure S14. Atomic force microscopic images for 2 μM-bp pUC19 in the absence (A) and presence of 2 μM (B) of 1, 16 μM (C) of 1, 16 (D) of 2, 2 μM (E) of 3, and 16 μM (F) of 3. 3 as threading intercalator led to solely plectonemic supercoiling (E and F) of pUC19 as described previously [8], whereas almost no structural changes were observed in the case of 1 and 2.

Figure S15. Calorimetric data for 1-3 binding to a-core. In each cases, 250 μM of 1-3 was titrated into 10 μM A-core at 25°C. Data are shown for Raw calorimetric data (a) and binding isotherm (a, heat change vs ligand/a-core molar ratio) in 50 mM potassium (A, C, E) or sodium (B, D, F) phosphate buffer (pH7.4) with serial injections of 1 (A, B), 2 (C, D) or 3 (E, F).
Figure S16. Calorimetric data for 1-3 binding to HP27. In each cases, 250 μM of 1-3 was titrated into 10 μM HP-27 at 25°C. Data are shown for Raw calorimetric data (A-F) and binding isotherm (G, H, heat change vs ligand/A-core molar ratio) in 50 mM potassium (A, C, E, G) or sodium (B, D, F, H) phosphate buffer (pH7.4) with serial injections of 1 (A, B), 2 (C, D) or 3 (E, F, G, H).
Table S1 Thermodynamic data for the binding of 3 to HP27 in the presence of K⁺ or Na⁺

<table>
<thead>
<tr>
<th></th>
<th>K⁺</th>
<th>Na⁺</th>
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<tbody>
<tr>
<td>10⁻⁵K/M⁻¹</td>
<td>0.71</td>
<td>0.87</td>
</tr>
<tr>
<td>n</td>
<td>2.7</td>
<td>2.3</td>
</tr>
<tr>
<td>ΔH/kcal mol⁻¹</td>
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<td>-8.18</td>
</tr>
<tr>
<td>ΔS/cal mol⁻¹</td>
<td>8.94</td>
<td>-4.84</td>
</tr>
</tbody>
</table>

50 mM potassium or sodium phosphate buffer (pH7.4).

HP27: 5’- GCG ATT CTC GGC TTT GCC GAG AAT CGC-3’

Figure S17. Telomerase inhibition assay by electrochemical method with 2.5 μM 1 – 3 in the presence of 25-cell extracts at 37 °C for 1 hr. Immobilization density of the TS-primer was 1.0×10¹¹ molecules/cm² reported previously [4].