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Cooperative hetero-ligands interaction with G-quadruplexes shows evidence of allosteric binding

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Abstract

Although allosteric binding of small molecules is commonplace in protein structures, it is rather rare in DNA species such as G-quadruplexes. By using CD melting, here, we found binding of the small-molecule ligands PDS and L2H2–6OTD to the telomeric DNA G-quadruplex was cooperative. Mass spectrometry indicated a 1:1:1 ratio in the ternary binding complex of telomeric G-quadruplex, PDS, and L2H2–6OTD. Compared to the binding of each individual ligand to the G-quadruplex, single-molecule mechanical unfolding assays revealed a significantly decreased dissociation constant when one ligand is evaluated in presence of another. This demonstrates that cooperative binding of PDS and L2H2–6OTD to the G-quadruplex is allosteric, which is also supported by the mass spectra data that indicated the ejection of coordinated sodium ions upon binding of the hetero-ligands to the G-quadruplex. The unprecedented observation of the allosteric ligand binding to higher ordered structures of DNA may help to design more effective ligands to target non-B DNA species involved in many critical cellular processes.

Graphical Abstract

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Author contributions

H.M initiated the research, did data analyses, and gave comprehensive guidelines to the research. S.P. collected and analyzed the single-molecule data, Y.L. collected the analyzed single-molecule data, M.D.Y. did mass spectroscopy, S.M. helped in synthesizing the DNA construct, L.L. assisted in the data analysis, J.T.S. assisted in the data analysis. All the authors contributed to the writing of the manuscript.

The authors declare no competing financial interests.

Supporting Information

Preparation of DNA construct for single-molecule experiments. Evaluation of thermodynamic stability of the Telo-4G with and without ligands. Full mass spectra of telo-4G and telo-4G with PDS. Blown-up nESI-Mass Spectra of telo-4G with individual ligand (PDS, L2H2–6OTD, Phen-DC3, and Braco-19) and the mixture of PDS and L2H2–6OTD. Unfolding force and change-incontour-length histograms of the Telo-4G with the L2H2–6OTD or the PDS at difference concentrations. Diagrams for the force-pumping/force-probing experiments during ligand binding. Table for overall ligand-bound fractions by including non-unfolded features. The average change-in-contour-length (ΔL) during the mechanical unfolding of the G-quadruplex both with and without ligands. Different species observed in HRMS experiments and their m/z ratios. Quantification of Na⁺ adducts in the ligand-bound Telo-4G species.

Keywords

G-quadruplex; Allostery; Hetero-ligands; Single molecule; Telomestatin; Pyridostatin; Cooperativity

INTRODUCTION

Cooperative binding is a prevalent strategy used by biological systems to alter the binding efficiency of different ligands to the sample substrate.² One mechanism of cooperative binding is allosteric binding in which tertiary conformation of the substrate is changed upon binding of the first ligand, which facilitates the binding of the second ligand.^{3–5}

Due to the plasticity in a macromolecule, binding of one substrate may change the conformation of the macromolecule, facilitating the binding of other ligands by an effect known as allostery.^{6–8} Allostery is often observed in proteins with multi-tiered structures.⁹ However, for nucleic acid structures, allostery is rare.¹⁰ Although some synthetic and natural nucleic acid fragments have shown allosteric effects,^{11–14} higher order DNA structures have not demonstrated this behavior to bind small-molecule ligands.

DNA G-quadruplex (see Figure 1) is a higher order DNA structure found in Guanine (G) rich sequences in vivo.¹⁵ This structure has been shown to participate in many important cellular processes. For example, G-quadruplexes formed in promoter regions can inhibit the expression of oncogenes,¹⁶ which offers a new target for cancer treatment. In one approach exploiting this new target, small molecule ligands have been developed to bind G-quadruplex in the oncogene promoters. Recent studies have indeed demonstrated increased inhibitory functions of a ligand-bound G-quadruplex.¹⁷

The phenomena of cooperativity and allostery are very common in proteins and are investigated in the presence of more than two ligands. However, for a non-canonical nucleic acid structure like DNA G-quadruplex, the investigation of the simultaneous binding of two hetero-ligands on the G-quadruplex structure has not been reported. In this work, we evaluated the binding of two hetero-ligands to a human telomeric DNA G-quadruplex by using CD, mass spectrometry, and force spectroscopy methods. By using CD, we found that

pyridostatin (PDS) and a telomestatin derivative, L2H2–6OTD, bind to the human G-quadruplex cooperatively. Mass spectrometry showed the formation of 1:1:1 ratio of telomeric G-quadruplex, PDS, and L2H2–6OTD in the ternary complex with the ejection of coordinated sodium ions. The binding affinity measurements from single-molecule force ramping assay revealed a significantly increased binding of one ligand to the G-quadruplex in the presence of another ligand, which has been used as the key evidence for allosteric binding.^{10, 18, 19} This strongly suggests that the cooperative binding of L2H2–6OTD and PDS to human telomeric DNA G-quadruplex is allosteric in nature, which is also supported by the ejection of sodium ions revealed by mass spectroscopy. From an in-depth comprehension of allosteric binding of small molecules on G-quadruplex structures, it will provide significant benefit in the development of more selective, potent, and effect allosteric drugs.

MATERIALS AND METHODS

Materials

DNA oligomers were purchased from Integrated DNA Technologies (IDT, IA). Enzymes and plasmids needed for the synthesis of DNA constructs were purchased from New England Biolabs (NEB, England). Streptavidin or anti-digoxigenin coated polystyrene beads were purchased from SpheroTech (Lake Forest, IL). PDS was a gift from Dr. Shankar Balasubramanian at University of Cambridge, telomestatin analogue, L2H2–6OTD, was a gift from Dr. Kazuo Nagasawa at Tokyo University of Agriculture and Technology. Phen-DC3 was a gift from Dr. Laurence Hurley at University of Arizona. Braco-19 was purchased from Sigma-Aldrich [96% (HPLC)].

Preparation of DNA constructs

The Telo-4G sequence (5′-TTAGGGTTAGGGTTAGGGTTAGGGTTA-3′) was sandwiched between two dsDNA handles (2,028 bp and 2,391 bp) (see Figure S1 for details). The 2,028 bp handle was prepared by the PCR of a pBR322 plasmid template using a 5′-end biotinylated reverse primer, 5′-GCA TTA GGA AGC AGC CCA GTA GTA GG and a forward primer, 5′-AAA CCA TAG AGG CTA CAC TAG AAG GAC AGT ATT TG. Then, the PCR product was digested by using XbaI enzyme. The biotinylated 2028-bp handle was ligated with the Telo-4G fragment that was flanked by two oligos (underlined), 5′-CTA GAC GGT GTG AAA TAC CGC ACA GAT GCG TTA GGG TTA GGG TTA GGG TTA GGG TTA GCC AGC AAG ACG TAG CCC AGC GCG TC-3′. The second handle (2391 bp) was prepared by the SacI and BsaI digestions of the PCR amplified λ-DNA (primer sequences: SacI primer, 5′-AAA AAA AAG AGC TCC TGA CGC TGG CAT TCG CAT CAA AG 3′ and BsaI primer, 5′-AAA AAA AAG GTC TCG CCT GGT TGC GAG GCT TTG TGC TTC TC 3′). This handle was labelled with digoxigenin (Dig) at the SacI-digested overhang by using 1nM Dig-dUTP (Roche) and terminal transferase. The 2391 bp handle was finally ligated with the biotinylated DNA through the BsaI site. The DNA construct thus prepared was purified by agarose gel, dissolved in 1 mM Tris (pH 7.4) after ethanol precipitation, and then stored at –20 °C.

Single-molecule mechanical unfolding of the Telo-4G construct with and without ligands

Single molecular investigations were performed with home-built dual-beam laser tweezers instrument^{20–22}. All the experiments were performed at 23 °C in a 10 mM Tris buffer containing 100 mM KCl at pH 7.4. The antibody-coated 2.10 µm polystyrene bead was incubated with the Telo-4G DNA sample prepared above before the single-molecular experiments. The DNA incubated bead and the streptavidin coated bead were separately captured by two laser tweezers. The DNA construct was then tethered between the two beads through digoxigenin-antibody/digoxigenin and biotin/streptavidin interactions. One of the laser foci was fixed while the other was movable by controlling the laser beam. The tethered DNA was stretched with the two beads moving apart. The tension in the DNA tether was recorded in the force-extension (F-X) curves at 1 kHz with a loading rate of 5.5 pN/s through a LabView program (National Instruments, Austin, TX). Whether a molecule is single or not was determined by the observation of the ~65 pN plateau in F-X curves. The data (F-X curves) were filtered through a function of Savitzky-Golay with a time constant of 10 ms in the Matlab program (The MathWorks, Natick, MA). Mechanical stability of G-quadruplexes formed in the Telo-4G with or without a ligand or a ligand pair was determined by the unfolding force of the G-quadruplex in the F-X curves. The ligand-bound G-quadruplex population was determined by the difference in the mechanical stabilities. To increase the efficiency of data collection for ligand-bound G-quadruplexes, concentration of each ligand (see figure captions for values) was chosen as high as possible (PDS: 100 nM; L2H2–6OTD: 5 nM) without causing noise in F-X curves, which is due to non-specific binding of the ligand to the duplex DNA handles.

Evaluation of thermodynamic stabilities of the Telo-4G with and without ligands

One mM stock solution of oligonucleotides, telomeric 4G (5′-TAGGGTTAGGGTTAGGGTTAGGGTTA-3′), was prepared in DNase-free water. Further dilutions were carried out in phosphate buffer (5 mM K₂HPO₄ and 2 mM KH₂PO₄) containing 100 mM KCl at pH 7.4. Before CD measurement in a JASCO-810 spectropolarimeter (Easton, MD), the DNA samples were heated at 95 °C for 5 min, and gently cooled from 95 °C to room temperature. A 1.0-mm path-length cuvette was prepared by addition of 200 µL (5 µM) of the annealed DNA solution without or with each ligand (5 µM) in a ligand pair, or with both ligands of the same concentration (5 µM each). CD spectra were collected at constant temperatures ranging from 25 to 95 °C. Each measurement was carried out in triplicate. Melting temperature at 290 nm CD signal was calculated according to the procedure described in literature¹.

Force-pumping/force-probing experiments

This approach was designed to investigate the dynamic binding of ligands to the telomeric G-quadruplex²³. First, the DNA construct was stretched mechanically until a G-quadruplex was unfolded. The DNA was then relaxed by a rapid force jump to 0 pN allowing refolding of the G-quadruplex structure in subsequent incubations at 0 pN. The refolding of the G-quadruplex was revealed by a rupture event in the next cycle of the stretching process starting at 10 pN, which was achieved by a second force jump in 10 ms. The two force jumps ensure no refolding of the quadruplex structure during stretching and relaxing at the

low force region (< 10 pN). This procedure can measure events as fast as 10 ms. By differentiating free and ligand-bound G-quadruplexes using rupture forces, we measured the formation kinetics of telomeric G-quadruplexes, as well as the binding kinetics of the PDS (500 nM) and L2H2–6OTD (100 nM) to the G-quadruplex with 0–30s incubation time. During the deconvolution of ligand-free and ligand-bound populations, we first fit the two populations in each rupture force histogram using a two-peak Gaussian function (Figures S8 and S9). The overlapping areas between the two populations were then randomly assigned to each of the species according to the ratio of the two species determined by the two-peak Gaussian fitting in each bin of a particular rupture force histogram.

Data analysis

The change in extension (Δx) at a particular force (F) was calculated as the extension difference between the stretching and the relaxing traces at that force. The resulting Δx at this force was then converted to the change in contour length (ΔL) using the following wormlike-chain (WLC) model^{24, 25},

$$\Delta x / \Delta L = 1 - 1/2(k_B T / FP)^{1/2} + (F/S) \quad (1)$$

where Δx is the change in end-to-end distance (or extension) between the two optically trapped beads, ΔL is the change in contour length, k_B is the Boltzmann constant, T is absolute temperature, P is the persistent length of dsDNA (50.8 nm), and S is the stretching modulus (1243 pN)²⁶.

High-resolution mass spectrometry (HRMS) measurements

High-resolution mass spectra of the G-quadruplex forming sequences (5 μ M in 1mM tris-HCl buffer, pH 7.4) and stabilizing ligands (PDS & L2H2–6OTD, each 5 μ M) were obtained with an Orbitrap-based mass spectrometer (Q Exactive, Thermo Scientific, Bremen, Germany) via nanoelectrospray ionization (nESI). Nanospray emitters with tip diameters of *ca.* 0.6 μ m were produced in-house from quartz tubes (1.0 mm o.d., 0.70 mm i.d., Sutter Instruments, Novato, CA) with a laser-based pipette puller (P-2000, Sutter Instruments, Novato, CA). Annealed G-quadruplex and ligands samples were prepared to a final concentration of 5 μ M in 1 mM potassium chloride and 100 mM trimethylammonium acetate (TMAA) buffer solution. Nanoelectrospray was initiated by applying a potential of *ca.* 0.8 kV to a platinum wire inserted into the capillary and in direct contact with the sample solution.

Mass spectra were recorded in the negative-ionization mode with a scan range of m/z 500 – 3000, a mass resolving power setting of 140,000, inlet capillary desolvation temperature of 60 °C, and an automatic gain control (AGC) target value of 1×10^6 ions. To ensure very high mass accuracy, found to be better than 1 mmu, the instrument was calibrated daily. All mass-spectral data were collected and processed with Xcalibur software (version 4.0, Thermo Scientific, San Jose, CA). Presented spectra show the -7 charge-state ions for the G-quadruplex and mixture of G-quadruplex with stabilizing ligands. Other detected analyte-ion charge states (e.g., -6 , -8 , -9 , etc.) showed the same ions with the same relative distribution. See Table S3 for expected and observed m/z for major species indicated in Figure 2.

RESULT AND DISCUSSION

PDS and L2H2–6OTD show cooperative binding to DNA telomere G-quadruplex

The 3' human telomere overhang contains 200 ± 75 nucleotides (nts) of G-rich repeats with a consensus sequence of 5'-TTAGGG.²⁷ Tandem repeats of this G-rich sequence facilitate the formation of telomeric G-quadruplex, which interacts with the telomerase overexpressed in many tumor cells.²⁸ Many potent small molecules have been developed to target the DNA G-quadruplex structures formed in the telomere region.¹⁵ In an effort to develop more effective molecules that can bind to DNA G-quadruplex, we evaluated combination effects of different ligands. It has been found that when G-quadruplex is bound with ligands, its thermodynamic stability often increases^{23, 29}. Therefore, by measuring the melting temperature (T_m) of a DNA G-quadruplex in the presence of ligands, its binding potency to G-quadruplex can be revealed. We performed CD melting for the T_m measurement. First, T_m of only a telomeric DNA G-quadruplex (Telo-4G: 5'-TTA(GGGTTA)₄, 5 μ M) was determined as 59 °C (Figure 1B), which was followed by the T_m measurement of individual ligands (Figures 1&S2). Then, T_m of G-quadruplex in the presence of two different ligands was determined. Among the commonly used ligands, we choose some ligands that were more selective towards G-quadruplex than dsDNA, such as L2H2–6OTD (a telomestatin analogue³⁰), PDS,³¹ Phen-DC3,³² and Braco-19³³ (5 μ M each). We found all individual ligands increased the T_m of the 5- μ M DNA G-quadruplex (Figure 1B&C), indicating they all bind to the G-quadruplex. Importantly, a significantly increased T_m of 67.5 °C was obtained when both L2H2–6OTD and PDS (5 μ M each) were present.

This increment ($T_m=8.5$ °C) was even greater than the combined T_m for the two individual ligands (T_m (L2H2–6OTD+PDS) = T_m (L2H2–6OTD) + T_m (PDS) = (3.5 + 1) °C = 4.5 °C). This result suggests the binding of both L2H2–6OTD and PDS ligands to the G-quadruplex. Further, it suggests that the binding is synergistic and cooperative: the binding of both ligands has a more pronounced effect than additive binding of each ligand. In comparison, the T_m of G-quadruplex in the presence of the Phen-DC3 and Braco-19 ligand pair (60.5 °C) was comparable to that of individual ligand (62.5 and 61.5 °C, respectively). This observation suggests that only one ligand, either Phen-DC3 or Braco-19, binds to the telomeric G-quadruplex. The competitive nature of these two ligands may be due to the overlapping binding sites of these two ligands in the G-quadruplex structure.^{34, 35}

The ternary binding complex has 1:1:1 stoichiometric ratio

To provide the evidence for cooperative binding of L2H2–6OTD and PDS to the G-quadruplex, we performed nanoelectrospray mass spectrometry (nESI-MS) experiments (Figure 2). Previously, Gabelica used volatile TMAA buffer to minimize non-specific salt adducts in nESI-MS data and directly observe the stoichiometry of the ligand binding to DNA G-quadruplex.³⁵ Using the same buffer (1 mM KCl/100 mM TMAA, pH 6.9), we found that in the presence of either the L2H2–6OTD or PDS ligand, there was 1:1 binding between each ligand and the G-quadruplex (Figure 2 A&B). When both ligands were present, a ternary complex was observed in which the L2H2–6OTD, PDS, and telomeric G-quadruplex presented a 1:1:1 stoichiometry. This experiment directly proved the dual

binding of the L2H2–6OTD and PDS to the G-quadruplex, which provides a strong support for the cooperative binding observed in the CD melting experiments (Figure 1C).

As a control, when Phen-DC3 and Braco-19 were evaluated (Figure 2C), we found each ligand can bind to the telomere G-quadruplex with a 1:1 ratio. However, no MS signal was observed for the dual binding of both ligands to the G-quadruplex. This data set is in full agreement with previous CD melting results (Figure 1D) that indicate the competitive binding between Phen-DC3 and Braco-19 to the G-quadruplex.

Close inspection on the mass spectra in Figure 2B revealed that in the presence of the L2H2–6OTD, Na⁺ adduct was observed for the telomeric G-quadruplex in addition to K⁺ ions. The trace amount of Na⁺ might come from the containers, the solvent, or from the quartz nanospray emitter itself that can form Na⁺ adducts during the electrospray ionization process. Such an adduct was commonly observed in mass spectrometry due to the negatively charged phosphate backbone of DNA. It is known that L2H2–6OTD binds to the telomeric G-quadruplex by stacking on top of the 5' end G-quartet.³⁶ This stacking may introduce additional interplanar space in which cations such as Na⁺ has shown to fit in by simulation.³⁷ However, when both L2H2–6OTD and PDS bind to the G-quadruplex, the sodium ion was ejected (Figure 2B, bottom panel, see Table S4 for quantification). Such an observation indicates that binding of the second ligand, PDS, changes the interplanar space between the G-quadruplex and L2H2–6OTD, which ejects the Na⁺. Similar ion ejection was observed previously by others.³⁵ This scenario is consistent with the allosteric binding of the two ligands to the G-quadruplex.

The cooperative binding has an allosteric nature

To investigate the mechanism of the cooperative binding of the PDS and L2H2–6OTD ligands, we employed single-molecule techniques such as mechanical unfolding and refolding of the telomere G-quadruplex.²³ Due to its superb signal-to-noise ratio, this technique has demonstrated its capability to deconvolute complex molecular interactions in a solution that contains multiple species, a feat unmatched in bulk based techniques such as CD and NMR.²⁷ To this end, the same Telo-4G DNA fragment was tethered to the two optically-trapped micrometer-sized polystyrene particles through affinity linkages by digoxigenin/anti-digoxigenin and biotin/streptavidin interactions. For mechanical unfolding, one trapped bead was moved away from another by using a steerable mirror (see Materials and Methods)²³. This increased tension in the Telo-4G DNA, causing G-quadruplex structures to unfold.

Two rupture force (R-F) populations centered at 22 ± 2 and 45 ± 2 pN were observed (Figures 3C–E, S8, and S9). The mechanical stability of telomeric G-quadruplex increases significantly due to the ligand binding which has already been established previously.²³ Therefore, we assigned the low force population (22 pN) as a free G-quadruplex and the high force population (45 pN) as the ligand-bound G-quadruplex. The change-in-contour-length (ΔL) (Figures S10) showed average values of 8.0 ± 0.5 nm, which is consistent with folded telomere G-quadruplexes (see SI for calculation).³⁸ In the next step, we used two different ligands to measure mechanical stability of G-quadruplex. Again, two similar populations were observed (Figures 3C–E, S8 and S9) where the 22 ± 2 pN and 45 ± 2 pN

species are assigned as ligand-free and ligand-bound G-quadruplexes, respectively. The fraction of ligand-bound G-quadruplex was then estimated by the 45 ± 2 pN population. By using this method, we calculated the percentage of ligand-bound G-quadruplex in the presence of individual ligand and ligand pairs. During experiments, the concentration of each ligand was kept comparable to or below corresponding K_d value so that binding sites are not fully occupied. This provides the space for another ligand to interact with the G-quadruplex, which is instrumental to elucidate the nature of the G-quadruplex binding between two different ligands.

Comparison of dissociation constants (K_d) of one ligand to the receptor with and without another ligand allows the determination of the allostery in the binding complex.⁹ As such, we measured K_d of one ligand in presence of the other for the PDS/L2H2–6OTD ligand pair.⁹ In the first experiment, we measured K_d of the PDS to the telomeric G-quadruplex in the presence of 5 nM L2H2–6OTD (designated as PDS@L2H2–6OTD; see Materials and Methods, Figure S8, and Table S1 for experimental details; see Supplementary Information for calculation of K_d). We found that the binding affinity of the PDS@L2H2–6OTD ($K_d = 3 \pm 2$ nM; Figure 4A left) was 2 orders of magnitude stronger than the PDS alone ($K_d = 500 \pm 100$ nM; Figure 4A right; see Figure S8 for unfolding histograms). Likewise, binding affinity of the L2H2–6OTD in the presence of 100 nM PDS (L2H2–6OTD@PDS, $K_d = 5 \pm 2$ nM; see Figure 4B left) was significantly higher than that without PDS ($K_d = 14 \pm 1$ nM; Figure 4B right; see Figure S9 for unfolding histograms). Both experiments convincingly demonstrate an allosteric binding between PDS and L2H2–6OTD to the telomeric G-quadruplex; I.E. presence of either of the ligand promotes the binding of the other ligand.

In Figure 4 left panel, we observed a saturation plateau (<50% occupancy) at lower ligand concentrations due to the tighter binding of two potent ligands. The following reasons can explain this behavior. First, before complete unfolding, the G-quadruplex may become loose under mechanical tension generated during the unfolding experiments. This leads to a rupture force lower than expected due to reduced binding affinity of PDS and L2H2–6OTD to the compromised G-quadruplex structure. Second, this ligand pair may bind to a particular G-quadruplex conformation. Third, the PDS and L2H2–6OTD may associate at higher concentrations, thereby reducing the magnitude of the saturation plateau.

To further investigate this allosteric binding, we performed force-pumping/force-probing (or (FP)2) experiments²³ to separately measure the binding kinetics of the PDS and the L2H2–6OTD to the telomeric G-quadruplex (Figures S11 and S12). After incubating the single Telo-4G with either PDS or L2H2–6OTD, force ramping experiments were carried out to unfold the G-quadruplex (see Materials and Methods for details). The unfolding was followed by rapid force reduction to 0 pN in 10 ms to refold the quadruplex. The refolded quadruplex was determined during a subsequent round of force ramping experiment (probing). The rupture force of the unfolding transition during the probe step reveals whether the G-quadruplex is bound with a ligand (see Figures 3, S8, and S9). The G-quadruplex formation probability during an incubation was calculated by the ratio of the unfolding events observed in subsequent pulling of the same Telo-4G molecule versus the total subsequent pulling curves performed for that incubation time.

As shown in the left panel of Figure 5A, both 100 nM L2H2–6OTD ($k_F = 0.27 \pm 0.02 \text{ s}^{-1}$) and 500 nM PDS ($k_F = 0.16 \pm 0.01 \text{ s}^{-1}$) promoted the formation of G- quadruplex ($k_F = 0.10 \pm 0.01 \text{ s}^{-1}$ for free G-quadruplex). After deconvoluting free G-quadruplex and ligand-bound species (Figure 5A, right), we found that the 100 nM L2H2–6OTD had a binding rate constant (k_b) of $0.12 \pm 0.02 \text{ s}^{-1}$ while the 500 nM PDS had a k_b of $0.05 \pm 0.01 \text{ s}^{-1}$. Given these two different concentrations, the 2nd order binding rate constant for the L2H2–6OTD was estimated to be 12 times faster than the PDS ($1.2 \times 10^6 \text{ M.s}$ vs $1.0 \times 10^5 \text{ M.s}$). Therefore, during the allosteric binding, it is likely that the L2H2–6OTD first associates with human telomeric G-quadruplex via π - π stacking on the top quartet, which leads to a conformation change in the G-quadruplex, facilitating subsequent PDS binding (Figure 5B). It has been found that binding of the L2H2–6OTD can indeed change the structure of telomeric G-quadruplex by dislocating the third loop (counted from the 5' end).^{36, 39} Recent investigations also suggested that the PDS may bind to the G-quadruplex through interaction with the two external G-quartets.⁴⁰ Thus, binding of the L2H2–6OTD to the top G-quartet³⁶ may render more compact stacking among three G-quartets, which leaves the bottom G-quartet more open to access the PDS in solution. On the other hand, binding of the PDS also changes the conformation of the L2H2–6OTD/G-quadruplex complex, which has been confirmed by our mass spectra data that demonstrated the ejection of the sodium ion when both L2H2–6OTD and PDS bound to the G-quadruplex (Figure 2B). Previous studies have shown that the Telo-4G sequence (5'-TTA(GGGTTA)₄ assumed the hybrid-1 conformation.⁴¹ When L2H2–6OTD was bound to the same sequence, same hybrid-1 conformation was also observed.^{30, 39} When we evaluated ΔL values of unfolding features, which also reflects the conformation of a G-quadruplex⁴¹, we found they did not change during the mechanical unfolding of G-quadruplex ($8.0 \pm 0.5 \text{ nm}$) both with and without ligands (Figure S10 and Table S2). This again suggested the same G-quadruplex conformation was maintained with and without ligands. These results provide an evidence for allosteric binding of hetero-ligands to the telomeric DNA G-quadruplex instead of conformational selection.

CONCLUSIONS

In summary, we have revealed that the dual binding of the PDS and L2H2–6OTD to the telomeric G-quadruplex is allosteric. We anticipate that the allosteric binding is instrumental to the design of more effective drugs targeting DNA G-quadruplexes, which have demonstrated many biological activities inside cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD melting curves of the DNA Telo-4G fragment with and without ligands. **A**) Structures of the telomestatin analogue (L2H2-6OTD), PDS, Phen-DC3, and Braco-19. **B**) Top, schematic of a hybrid-1 telomeric G-quadruplex structure and a G-quartet plane. Bottom, CD melting curve of the Telo-4G without any ligand. CD melting curves of the Telo-4G with **C**) L2H2-6OTD/PDS ligand(s) (each 5 μ M; note there was significant increase in melting temperature (T_m) when both ligands were present) and **D**) Phen-DC3/Braco-19 ligand(s) (each 5 μ M, note there was no significant increase in T_m in presence of both ligands). The melting data at the 290 nm CD signal were retrieved from Figure S2 in the SI. T_m was determined according to reference.¹

Figure 2. nESI-MS of the -7 charge state of $5\ \mu\text{M}$ Telo-4G with PDS and telomestatin analogue, L2H2-6OTD, in $1\ \text{mM}$ KCl/ $100\ \text{mM}$ TMAA. **A**) Mass spectrum of the Telo-4G. **B**) Mass spectra of the $5\ \mu\text{M}$ Telo-4G in presence of $5\ \mu\text{M}$ each of PDS (top), L2H2-6OTD (middle), and a mixture of PDS and L2H2-6OTD (bottom). Note that -7 charge state of a complex with both ligands bound was observed at m/z 1402.2723. **C**) Mass spectra of the $5\ \mu\text{M}$ Telo-4G in presence of $5\ \mu\text{M}$ each of Phen-DC3 (top), Braco-19 (middle), and a mixture of Phen-DC3 and Braco-19 (bottom). Note that a complex with both ligands at m/z 1380.6908 or 1386.1130 was not detected. See Figure S3 & S4 for full mass spectra and S5–S7 for blown-up mass spectra, and Table S3 for m/z values of all marked species. Shaded regions indicate Telo-4G signals without bound ligands.

Figure 3.

Experimental setup for the mechanical unfolding of the Telo-4G construct with and without ligands. **A)** Single-stranded human telomeric DNA (Telo-4G) is sandwiched between two dsDNA handles. The overall construct is tethered between two optically trapped beads via an antidigoxigenin-digoxigenin and a streptavidin-biotin linkage. **B)** Typical stretching (red) and relaxing (black) force-extension curves of the Telo-4G and the corresponding rupture force (R-F) histogram. Typical stretching (red) and relaxing (black) force-extension curves and the corresponding rupture force (R-F) histogram of the Telo-4G in presence of **C)** 100 nM Pyridostatin (PDS), **D)** 5 nM telomestatin analogue L2H2-6OTD, and **E)** 100 nM PDS and 5 nM L2H2-6OTD. Solid curves depict Gaussian fittings. N and n represent the numbers of unfolding features and molecules, respectively.

Figure 4.

Binding of PDS and telomestatin analogue, L2H2-6OTD, to the telomeric G-quadruplex is allosteric in nature. **A)** Binding curves of the PDS to the G-quadruplex with (left) and without 5 nM L2H2-6OTD (right). **B)** Binding curves of the L2H2-6OTD to the G-quadruplex with (left) and without 100 nM PDS (right). All the experiments were performed in a 10 mM Tris buffer with 100 mM KCl at pH 7.4. A single-site binding model was used for fitting (see Supplementary Information for details). Error bars are one standard deviation from three independent measurements.

Figure 5.

Sequential binding of the L2H2-6OTD and PDS to the G-quadruplex. **A**) Refolding probability of all G-quadruplexes (left) and the ligand-bound G-quadruplex (right). A two-state model (see Supplementary Information for details) is used for fitting to retrieve the folding/binding rate constants (k_F/k_b). Error bars are standard deviations from three measurements. **B**) A schematic diagram for a possible allosteric binding mechanism, which starts with the L2H2-6OTD binding together with a Na^+ ion, followed by the PDS binding with ejection of the Na^+ ion.