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An unprecedented knot-like G-quadruplex peripheral motif

Thi Hong Anh Truong[#], Fernaldo Richtia Winnerdy[#] and Anh Tuấn Phan^{*}

Abstract: We discovered the formation of a knot-like G-quadruplex peripheral structure by a 7-nt DNA sequence DL7: 5'-d(TGTTGGT)-3', whereby six out of its seven nucleobases participate in compact base pairing interactions. Here, solution structure of a 24-nt DNA oligonucleotide containing the DL7 sequence shows the interaction between a two-layer anti-parallel G-quadruplex core and the peripheral structure, including the construction of two sharp turns from the DNA backbone. The formation of this novel structural element highlights the intricate property of single-stranded DNA folding in presence of G-quadruplex-forming motifs. We demonstrated the compatibility of the DL7 knot-like structure with various G-quadruplexes, which could have implications in drug design and DNA engineering.

Deoxyribonucleic acid (DNA) is a biopolymer which carries the genetic information of the living organisms.^[1] Although most biological functions are regulated by the primary sequence of DNA,^[2] it was established that the secondary conformations – the most common being the DNA double helix^[3] – are also vital in controlling gene expression.^[4] The secondary structures that might be adopted by a strand of DNA are complex, as the existence of structures such as unconventional hairpins,^[5] triplexes^[6] and quadruplexes^[7] were discovered. Here, we report a unique secondary structure formation from a 7-nt DNA sequence d(TGTTGGT) (named DL7) in presence of a G-quadruplex (G4). Six out of seven nucleotides in the sequence are engaged in compact base pairing interactions, constructing a knot-like DNA structure stacking onto the G4 planar surface. The structure serves as a G4-stabilizing framework and potential drug target.

G4s are secondary structures of nucleic acids that can form in guanine-rich sequences under physiological condition.^[8] The core structure of G4 is normally composed of two or more stacking G-tetrads with coordination cations at the center. Each G-tetrad comprises of four guanine bases in a square-planar arrangement with eight Hoogsteen hydrogen bonds.^[9] G4-forming motifs^[10] are most abundantly found in biologically-relevant sites such as telomeric^[11], gene promoter^[12] and replication initiation regions.^[13] G4 structures highly influence some regulatory functions such as telomerase inhibition,^[14] gene expression^[12b, 15] and DNA replication.^[16] Polymorphism of G4s^[7, 17] remains a prominent topic of study, following the introductions of bulges,^[18] duplex-loops,^[19] triad-stacking,^[20] non-canonical tetrad,^[21] left-handed fold^[22] and many others. In this work, the solution structure of a

DL7-containing DNA sequence is resolved, revealing the detailed interactions between the knot-like structure and the G4 core. Based on this structure, a cut-and-paste study^[23] was performed on two other G4 structures, Pu24T from the *c-myc* promoter^[24] and an artificially designed G4 (AQ),^[17c] whereby the DL7 sequence was inserted as diagonal loops. Our results suggest that the DL7 knot-like structure formation is compatible with various G4s.

NMR spectrum of a modified anti-cancer oligonucleotide reveals a unique pattern. An anti-cancer DNA aptamer containing 17 guanines AGRO100 (also known as AS1411) was shown to adopt multiple G4 conformations.^[25] Single G-to-T substitutions at various positions of AGRO100 have resulted in the formation of distinct G4 structures.^[22a, 26] The substitution at position 11 (AT11) generated a four-layer parallel G4, while another substitution at position 27 (AT27) resulted in a four-layer parallel left-handed G4. A G-to-T substitution in position 21 was attempted, giving the AT21 sequence (Figure 1A). The one-dimensional (1D) NMR spectrum of AT21 showed 12 sharp imino proton peaks including an unusual three-peak pattern at ~9-10.5 ppm (Figure 1B), suggesting a possible formation of unique base-pairing assemblies outside of G-tetrads. The CD spectrum, displaying a trough at ~265 nm and positive peaks at ~245 and ~290 nm (Figure 1C), indicated an anti-parallel G4 formation.^[27]

DL7 sequence is responsible for unprecedented base pairing interactions. To identify the guanine and thymine bases responsible for the AT21 structure folding, we performed ¹⁵N-¹H

Figure 1. Spectroscopy of AT21. (A) DNA sequence of AT21 with the DL7 motif highlighted in red box. (B) 1D NMR spectrum of AT21 showing 12 well-resolved imino proton peaks with a unique three-peak pattern shown in dashed box. (C) CD spectrum of AT21 showing a typical profile of an anti-parallel G4 fold.

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Figure 2. AT21 derivative AT21S. (A) The DNA sequence of AT21 and AT21S. Loop A, loop B and DL7 are shown in dashed boxes. The underlined bases indicate the observation of their imino protons. (B) 1D NMR spectra for AT21 and AT21S. Twelve and thirteen imino proton peaks are observed for AT21 and AT21S respectively. Resonance assignments are shown with residue numbers; imino protons from DL7 are indicated in red.

HMQC experiments using multiple site-specific 2% ^{15}N -labeled samples^[28] and obtained full assignments of the twelve observed imino protons (Figure S1 in the Supporting Information). It revealed the participation of G2-G3, G9, G11-G12, G18-T21 and G23-T25 in the base pairing interactions, which included six out of seven nucleobases of DL7 (Figure 2A, top). Interestingly, all three upshifted peaks at ~ 9 -10.5 ppm were identified to be imino proton peaks of the DL7 sequence (Figure 2B, top), suggesting major contribution of DL7 towards the unique non-G-tetrad structure. The two segments in the middle of the sequence, T4-G8 and T13-T16, did not show observable imino protons indicative of base pairing, suggesting their possible roles as loops (Figure 2A).

Mutations of loops preserve the overall structure. The presence of unstructured long loops is known to destabilize G4 structures,^[29] therefore we attempted several loop modifications to get a more stable G4 structure without changing its folding pattern. The loops were designated as loop A (T4-G8) with five nucleotides and loop B (T13-T16) with four nucleotides. We performed various loop modifications on AT21 and recorded 1D NMR and CD spectra for each modified sequence (Figure S2, Table S1). Most mutated sequences showed very similar CD spectra and well-resolved NMR spectra preserving the unusual three-peak pattern. For further structural study, we chose one sequence where loop A was converted to TT and loop B to TTT, designated as AT21S (Figure 2A, bottom), due to its well-resolved NMR spectrum. The melting temperature (T_m) of AT21S was observed to be $\sim 39^\circ\text{C}$, approximately 6°C higher than its predecessor AT21 (Figure S3).

Resonance assignments of AT21S. The 1D NMR spectrum of AT21S showed thirteen imino proton peaks, which corresponded to ten guanines (H1) and three thymines (H3) as identified by the 2D ^{15}N - ^1H HMQC spectrum (Figure S4). The imino proton assignments of AT21S were obtained by comparing NOESY spectra of AT21 and AT21S (Figure S5A, B). Note the different numbering of nucleotides due to the loop shortening (Figure 2A). Guanine aromatic proton (H8) assignments were achieved by a long-range through-bond coupling (via C5) HMBC experiment.^[28] Every aromatic proton of base-pairing guanines was assigned (Figure S5C). The additional imino proton observed for AT21S, but not AT21, was identified to correspond to G13 (Figure S6A). It is likely that the G17 counterpart in AT21 was broadened beyond detection due to motions of the original loops A and B. The complete imino proton assignments of AT21S are presented (Figure 2B).

AT21S forms two-layer G4 accompanied by a compact peripheral structure. The H1-H8 region of the NOESY spectrum obtained on a sample dissolved in H_2O revealed two cyclic patterns: G2•G14•G8•G19 and G3•G6•G9•G13 (Figure 3A), indicating the formation of a two-layer G4 structure. We inferred from the data that the strand orientation of G13-G14 was opposite to those of G2-G3 and G8-G9, while G6 and G19 formed a discontinuous G-tract. In the NOESY spectrum obtained on a sample dissolved in D_2O , strong intra-residue H8-H1' cross-peak were observed for four guanines: G2, G8, G13 and G19, indicating their *syn* glycosidic bond conformations. We also found four rectangular patterns from the intra-residue and inter-residue H1'-H8 cross peaks originated from the following pairs: G2-G3, G8-G9, G13-G14 and G19-G6 (Figure S6B). These rectangular patterns signified *syn-anti* glycosidic bond conformation steps, consistent with the anti-parallel fold.^[28] Overall, the NMR signal of AT21S indicated a chair-type anti-parallel G4 core with one discontinuous G-tract (Figure 3B).

The unusual peripheral structure was analyzed by examining the upshifted imino proton region of the NOESY spectrum. The observed imino-imino and imino-methyl proton cross-peaks indicated the formation of novel structural elements as illustrated, where the T15•T17•G20 triad and G16•T21 base pair assembled in a compact fashion (Figure 3C, D). These structural elements were constructed entirely by the DL7 sequence d(TGTTGGT), with the three upshifted peaks at ~ 9 -10.5 ppm determined to be imino protons of guanines and thymines which were hydrogen-bonded with thymine O2 or O4 atoms. Based on all the NMR data, we found that the peripheral structure is stacked onto the G4 core.

To probe the stabilizing role of the DL7 motif, we substituted DL7 by a three-thymine sequence capable of forming a diagonal loop,^[30] giving rise to the AT21S-3T sequence (Table S2). NMR and UV-melting experiments showed that the structure of the mutated sequence AT21S-3T ($T_m < 25^\circ\text{C}$) was less stable than that of AT21S (Figure S7). These results indicated the stabilizing nature of the peripheral structure of DL7.

Structural analysis reveals a knot-like backbone progression. The structure of AT21S was calculated using constraints generated from the NMR data (Table 1A). One hundred structures were built by simulated annealing and refinement processes, whereby the ten lowest-energy structures were selected (Figure 4A, Table 1B). A representative structure is displayed in cartoon form (Figure 4B), showing the stacking

Figure 3. 2D NOESY (mixing time, 200 ms; temperature, 25°C) analysis of AT21S. (A) H1-H8 region of the NOESY spectrum of AT21S. Indicated in boxes are the H1-H8 NOE cyclic-connectivity patterns indicating the formation of G-tetrads. (B) The schematic of NOE patterns observed in G-tetrads and the proposed G4 core of AT21S. The arrows indicate DNA backbone orientation; cyan and magenta represent *anti*- and *syn*-guanine respectively; greyed area shows the peripheral structure. (C) Different regions of the NOESY spectrum of AT21S. Indicated in boxes are some key NOE cross-peaks used to determine the base interactions. (D) The schematics of NOE peaks seen in the spectra as well as proposed fold of the peripheral structure of AT21S. The arrows indicate DNA backbone orientation; cyan represents *anti*-guanine; orange represents thymine; greyed area shows the G4 core structure.

interaction between the G4 core and the peripheral structure. *Syn-anti* steps are observed for three G-tracts (G2-G3, G8-G9 and G13-G14), as well as one discontinuous G-tract (G19-G6). There are two edgewise loops (T4-T5 and T10-T12), one V-shaped loop^[31] (T7) and one diagonal loop (T15-T18) in the structure. The guanine residue G19, which is a part of both the DL7 motif and one G-tetrad, serves as a bridge between the peripheral structure and the G4 core.

The stacking interfaces between different base pair, triad, and tetrad layers are presented in detail (Figure S8). Both the T•T•G layer formed in the DL7 motif and the T•T•T layer formed between the edgewise loops cap the two G-tetrads and protect their imino protons from exchange with solvent, consistent with the observation of these protons 1 hour after dissolving the sample in D₂O (Figure S9). The overall backbone progression of AT21S, specifically in DL7 region, resembles the shape of a knot with two back-to-back U-turns in a seven-nucleotide span (Figure 4C).

Compatibility of DL7 with various G-quadruplexes. The resolved AT21S structure revealed the importance of the DL7 sequence in forming a G4-stabilizing knot-like structure. To confirm that the DL7 sequence is sufficient for that function, we performed 1D NMR experiments on truncated sequences (Figure S10). The results indicated that the 5'-end thymine (T1) and the 3'-end segment G22-T24 are unnecessary in maintaining the fold. In a broader context, the DL7 motif represents a particular case, where a diagonal loop adopts a well-defined structure. The significance of this knot-like structure was gauged by testing its compatibility with other G4 structures. For this purpose, we chose a G-rich sequence from the *c-myc* promoter Pu24T (Table S3),^[24] which formed a three-layer parallel G4 structure containing a snapback triad-forming diagonal loop. We executed a cut-and-paste study by incorporating the DL7 sequence to the diagonal

loop of Pu24T, designated as Pu24T-mod (Table S3, Figure S11A). The 1D NMR spectrum of Pu24T-mod displayed sharp imino proton peaks corresponding to the G-tetrad core, as well as three ancillary imino proton peaks (Figure 5B) at 9–10.5 ppm. Highly similar spectral patterns of Pu24T-mod with AT21S in this area strongly suggest the formation of a similar knot-like structure by DL7 in Pu24T-mod (Figures 5A and S11B), albeit less robust as shown by the broadened ancillary peaks. A similar cut-and-paste study was also performed on an engineered G4,^[17c] AQ-mod (Table S3, Figure S12A). Three up-field shifted imino proton peaks at 9–10.5 ppm were observed (Figures 5C and S12B). The

Table 1. NMR restraints and statistics of the computed structures of AT21S.

A. NMR Restraints		
Distance restraints	H ₂ O	D ₂ O
Intra-residue	4	275
Inter-residue	82	115
Other restraints		
Hydrogen bond		42
Dihedral angle		10
Planarity		4
B. Structure Statistics		
NOE violations		
Number (>0.2 Å)	0.3 ± 0.5	
Deviations from the ideal geometry		
Bond lengths (Å)	0.003 ± 0.000	
Bond angles (°)	0.695 ± 0.006	
Impropers (°)	0.366 ± 0.006	
Pairwise heavy atom RMSD value (Å)		
G-tetrad core	0.726 ± 0.183	
All heavy atoms	1.170 ± 0.248

Figure 4. Solution structure of AT21S. (A) Superimposed ten lowest-energy structures of AT21S. (B) Cartoon representation of the lowest-energy structure of AT21S. Cyan, magenta and orange indicates *anti*-guanine, *syn*-guanine and thymine respectively. (C) Cartoon views of AT21S highlighting DL7 backbone in red, showing two sharp turns constructing a knot-like progression.

comparison between these two sequences (*Pu24T-mod* and *AQ-mod*) and our reference sequence (AT21S) is summarized in Figure 5. We performed unambiguous spectral assignments using site-specific ^{15}N enrichment (Figure S13) and confirmed the identities of the up-field shifted imino proton peaks in each sequence to the respective expected position in the DL7 segment (Figure 5). 2D NOESY of *Pu24T-mod* and *AQ-mod* also revealed the correlations between imino protons of the DL7 residues, supporting the formation of a similar DL7 knot-like structure (Figure S14). The CD profiles of *Pu24T-mod* and *AQ-mod* were consistent with the proposed parallel and (3+1) hybrid G4 folding topologies (Figure S15), which were designed to incorporate the knot-like DL7 structure. In the perspective of G4 engineering, the DL7 sequence can be incorporated into a G4-forming sequence to produce a compact and self-stabilizing structure.

Possible biological relevance of the DL7 motif. Given the short nature of the DL7 sequence (7-nt), we expect a high number of occurrences of DL7 and similar motifs in the human genome. A bioinformatics search on the reference strands of the human genome resulted in ~270,000 exact matches, exceeding the expected number of random occurrences by ~1.5 times (see Supporting Information). The formation of the DL7 knot-like structure could modulate the recognition and stability of adjacent G4s, affecting their role in biological processes involving DNA, such as transcription, replication and repair. A similar knot-like structure could also form and modulate the RNA structure and function. RNA pseudoknot is known to play a role in RNA splicing and ribosomal frame-shifting.^[32]

In summary, the DL7 sequence was established to form a compact structure as a G-quadruplex peripheral. This knot-like structure is comprised of unique base pairing interactions from six out of seven nucleobases and accommodated two sharp turns on the DNA backbone. Its G4-stabilizing property was examined from the resolved AT21S structure, where the T•G base pair and T•T•G triad of DL7 were observed to stack onto the G-tetrad core. We successfully incorporated the DL7 motif into other G4 structures. The DL7 motif is an example of an intricate folding of a short single-stranded DNA in presence of a G4 motif. The compatibility and stabilizing effects of DL7 could prove useful in G4 sequence engineering.

Figure 5. Compatibility of DL7 with various G-quadruplexes. The sequences, imino proton NMR spectra and proposed folds of (A) AT21S, (B) *Pu24T-mod* and (C) *AQ-mod*. Indicated in the sequences with different colours are the DL7 segments. The assigned three-peak pattern is shown in all spectra. The schematics show three folding topologies of anti-parallel, parallel, and (3+1) hybrid respectively.

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COMMUNICATION

A knot-like DNA secondary structure was formed by 7-nt sequence *DL7*, d(TGTTGGT) next to a G-quadruplex, whereby six out of its seven nucleobases participate in compact base pairing interactions.

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**An unprecedented knot-like G-
quadruplex peripheral motif**