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A Model of Smart G-Quadruplex Ligand

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Supporting Information

ABSTRACT: An unprecedented strategy to control the quadruplex- vs duplex-DNA selectivity of a ligand is reported. We designed a compound whose structure can rearrange when it interacts with a G-quadruplex, thereby controlling its affinity. Thus, the first "smart G-quadruplex ligand" is reported, since this ligand experiences a structural change in the presence of quadruplexes but not in the presence of duplexes, ensuring a high level of quadruplex selectivity.

Small molecules that interfere with DNA replication trigger genomic instability, making them invaluable in the search for anticancer agents.[1] Genomic DNA assumes locally and transiently tertiary structures that are known to plague the replication process;[2] therefore, small molecules that promote and/or stabilize higher-order DNA folding are promising as cytotoxic agents.[3]

G-quadruplex-DNAs epitomize such tertiary structures, arising from the self-assembly of G-rich sequences upon formation of intramolecular G-quartets (an array of four guanine residues held together by Hoogsteen H-bonds) nucleated by physiologically relevant cations (e.g., K^+).[4] Compounds that interact with the G-quadruplex—so-called G-quadruplex ligands[5]— thus show promise to impede DNA replication, jeopardizing cancer cell integrity and survival.[6]

Recent computational investigations to determine the occurrence of G-quadruplex-forming sequences in the genome found that they are not only abundant (over 350 000)[7] but also distributed in a nonrandom way[8] (telomeres,[9] promoter regions of genes).[10] This makes it a particular challenge to firmly establish their biological functions (i.e., chromosomal stability and transcriptional regulation, respectively). The past years have seen real progress in delineating them,[11] notably via the use of ligands that can regulate and/or visualize these processes.[12] The 15-year quest for such valuable molecular tools[13] has progressively revealed its principles: to be efficient, a ligand must display both high affinity for G-quadruplex and high selectivity over duplex-DNA. While a clear strategy was implemented to improve the ligand's affinity (mostly through an optimized overlap between the aromatic surfaces of the ligand and the accessible G-quartet of the G-quadruplex), ways to hamper duplex interaction are in their infancy, being more a matter of empirical observation than of meticulous molecular design. Here we report an unprecedented strategy to fully control quadruplex- vs duplex-DNA selectivity. Instead of artificially dampening duplex-DNA interactions (via, e.g., steric hindrance), our approach aims at designing a ligand whose structure can rearrange when it interacts with a G-quadruplex (Figure 1). To be effective, the ideal candidate must assume two reversible conformations: an off-state devoid of ability to interact with DNA, and an on-state promoted by and displaying affinity for its quadruplex target.

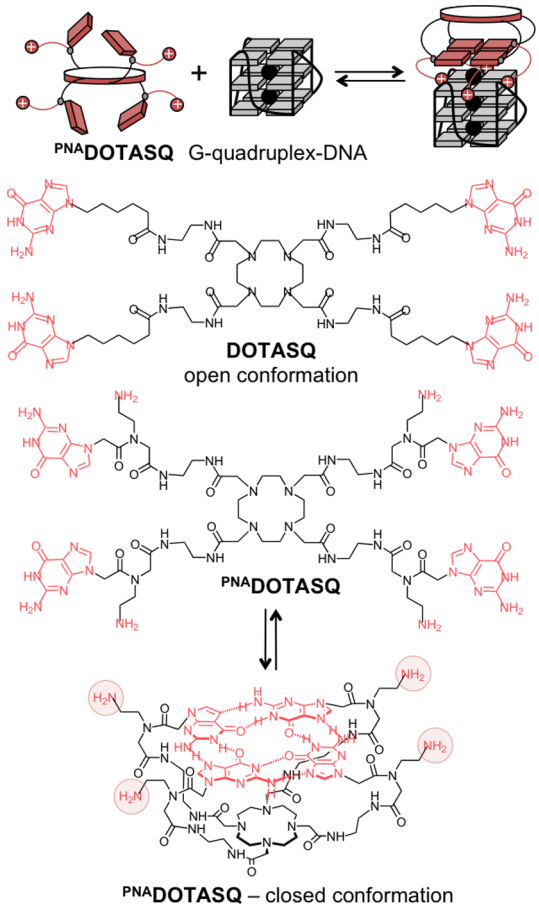

Figure 1. (Top) Smart G-quadruplex ligand principle and (bottom) chemical structures of DOTASQ and ^{PNA}DOTASQ in open and closed conformations.

Alongside the classical design of G-quadruplex ligands (mostly by fine-tuning the helter-skelter duplex intercalators pool, i.e., flat aromatic molecules surrounded by water-solubilizing cationic appendages), recent efforts are aimed at devising nature-inspired ligands. Based on pioneering work by Nikan and Sherman,[14] we reported an original molecule based on the formation of an intramolecular template-assembled synthetic G-quartet

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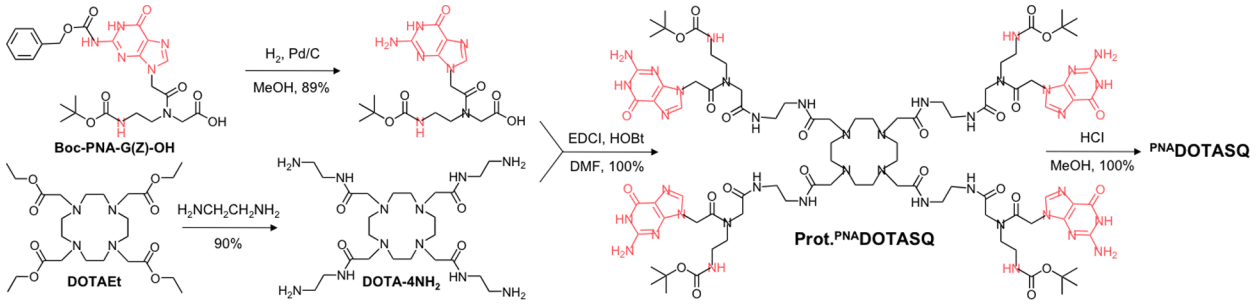

Figure 2. Chemical synthesis of ^{PNA}DOTASQ (see the Supporting Information (SI) for full details of the synthetic pathway).

(TASQ), templated by a water-solubilizing macrocycle, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*′′,*N*′′′-tetraacetic acid (DOTA).[15] This molecule, named DOTASQ (i.e., DOTA-templated synthetic G-quartet, Figure 1), was designed to interact with quadruplex via a nature-mimicking process, based on a "*like likes like*" association between two G-quartets, one native (quadruplex) and the other artificial (ligand). The DOTASQ scaffold can adopt a closed conformation (Figure 1), which is assumed to elicit quadruplex-stabilizing properties (on-state), while the open conformation lacks affinity (off-state). However, DOTASQ was unable to bind to quadruplex-DNA, mainly because its open conformation prevails in solution. We circumvented this by inserting a metal, terbium, within the DOTA cavity, since metal coordination favors the DOTASQ closed conformation. The corresponding metal complex, Tb·DOTASQ, indeed stabilizes quadruplexes, providing a proof-of-concept for nature-inspired quadruplex ligands.[15] To promote the closed conformation in a metal-independent manner, we subsequently modified the nature of the template (porphyrin vs DOTA), but the results were rather disappointing.[16] We thus followed an alternative way, reported herein, in which the nature of the guanine arms is modified (with vs without pendant amine side chains). This apparently trivial modification has crucial implications for the quadruplex binding mode of the resulting TASQ, notably in light of the structural features that make the quadruplex ligand binding site so unique: the quartet is surrounded by phosphodiester bonds that bring a wreath of negative charges around the nucleobases; therefore, a synthetic quartet surrounded by positive appendages (e.g., protonated amines) is most likely to fit snugly into the binding pocket based on both strong electrostatic interactions and shape recognition. Even more interesting, this association may itself promote the TASQ structural switch, thereby controlling its quadruplex affinity (Figure 1). We therefore propose an unprecedented strategy in which the ligand affinity for a quadruplex is triggered by the quadruplex itself.

Our synthetic scheme is displayed in Figure 2. The neutral purine arm used for the initial DOTASQ synthesis is replaced by a guanine-PNA monomer. The resulting TASQ, named ^{PNA}DOTASQ (Figure 1), obtained in a straightforward manner (4 efficient steps, see the SI) from commercially available Boc-PNA-G(Z)-OH and DOTAEt, is surrounded by four protonable primary amine side chains (a guarantee of high G-quadruplex affinity).[17] This new cationic character results in improved water solubility (see the Beer–Lambert analysis in the SI). According to the aforementioned theory, the conformational lability of ^{PNA}DOTASQ makes it able to adopt two conformations (Figure 1); in light of the information gleaned from the DOTASQ study,[15] this equilibrium is shifted toward the open form (off-state) when the ligand is free in solution. The situation may thus be drastically different in the presence of the DNA target (Figure 1), since the precisely located positive charges near the guanines make the TASQ most likely to cling firmly to the quadruplex-accessible G-quartet, thereby shifting the equilibrium toward the closed form (on-state).

We first investigated this via NMR experiments.[4,18−22] We selected the tetramolecular quadruplex-DNA $[T_2AG_3T]_4$, since it gives rise to a rather simple NMR spectrum with a set of three signals that correspond to the three guanine tetrads of the quadruplex (10.90, 11.11, and 11.53 ppm, marked with black * in Figure 3, spectrum B).[18] Adding 0.5−2.0 equiv of ^{PNA}DOTASQ

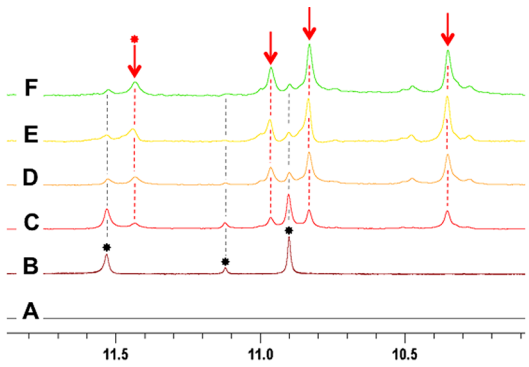

Figure 3. ^{1}H NMR spectra (in Caco·K buffer, 10% D_2O) of ^{PNA}DOTASQ alone (A) and quadruplex $[T_2AG_3T]_4$ alone (B) or with 0.5 (C), 1.0 (D), 1.5 (E), and 2.0 equiv (F) of ligand.

strongly modifies the spectra (spectra C–F): the set of signals corresponding to the quadruplex alone progressively disappears and a new one appears (red arrows), which might correspond to the imino signal of the ^{PNA}DOTASQ/quadruplex assembly. Four new signals are observed: three correspond to the DNA's guanines (10.35, 10.83, and 10.96 ppm, red arrows), which are upfield-shifted relative to the DNA alone, indicating that the ligand interacts strongly with the G-quartets of the quadruplex,[19] and one at 11.43 ppm (red arrow with *) that may be attributed to the synthetic G-quartet based on previous NMR studies of water-soluble TASQ.[15a,20] This may imply that the ligand interacts with its DNA target folded into its closed conformation. As these peaks are not seen without quadruplex-DNA (spectrum A), these NMR experiments support the hypothesis that the synthetic G-quartet formation is templated by the quadruplex itself. Several control experiments have been undertaken to confirm this (see the SI), including NMR titrations: (a) With ^{PNA}DOTASQ and another tetramolecular quadruplex-DNA, i.e., $[T_2AG_3]_4$.[18,21] Upon addition of ^{PNA}DOTASQ, a new set of signal appears corresponding to the ligand/quadruplex assembly, again comprised of four signals, three being the imino signals of

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the DNA's guanines (10.38, 10.85, and 10.97 ppm) upfield shifted upon interaction with the ligand[21] and one at 11.42 ppm that may correspond to the TASQ. (b) With $[T_2AG_3T]_4$ and another quadruplex ligand, the tetracationic porphyrin TMPyP4,[5] selected because it is conformationally inert. Reminiscent of reports by Phan et al.,[22] its interaction with a quadruplex-DNA results in an upfield shift of the DNA's protons (i.e., strong interaction with the G-quartets) but without the appearance of a novel signal, supporting the hypothesis that the additional peak observed with ^{PNA}DOTASQ might originate in the formation of its TASQ. (c) With ^{PNA}DOTASQ and the duplex-DNA ds12 (the self-complementary $CGCGA_2T_2CG$-CG). The addition of the ligand neither modifies the chemical shift of the DNA's signals nor results in the appearance of a new peak, which implies that ^{PNA}DOTASQ does not interact with ds12. Above all, this is a strong indication that the ligand is not folded in the presence of a duplex-DNA and, consequently, that the intramolecular folding is triggered only by quadruplex-DNA.

We subsequently tried to learn more about the nature of the ^{PNA}DOTASQ/quadruplex association via electrospray ionization mass spectrometry (ESI-MS) experiments.[23] As seen in the SI, preliminary ESI-MS experiments with ^{PNA}DOTASQ and the tetramolecular quadruplex $[TG_4T]_4$ revealed a very weak but single 1:1 association in the gas phase (even in the presence of 2 equiv of ligand). The existence of a unique complex implies a well-defined ligand binding mode. Combined with NMR results (i.e., ^{PNA}DOTASQ interacts with the G-quartets), these results advocate for an association based mainly on ligand stacking atop the quadruplex (schematically represented in Figure 1). Interestingly, no associations were detected when ESI-MS experiments were conducted with ds12, again indicating that ^{PNA}DOTASQ does not interact with duplex-DNA. NMR and ESI-MS investigations thus provide valuable insights into not only the affinity but also the selectivity of ^{PNA}DOTASQ for quadruplex-DNA. It was therefore of interest to further investigate these interactions via two well-established and complementary biophysical methods, FRET melting (based on thermal unfolding of doubly labeled oligonucleotides)[24] and G4-FID assays (an isothermal method based on the displacement of a dye from unmodified DNA).[25]

Two oligonucleotides were used, F21T (FAM-$G_3(T_2AG_3)_3$-TAMRA), corresponding to the doubly labeled quadruplex-forming sequence found in human telomeres, and F-duplex-T, the doubly labeled hairpin duplex-forming sequence FAM-TATAGCTATA-T_6-TATAGCTATA-TAMRA (see the SI for the experimental procedure).[24] As seen in Figure 4, ^{PNA}DOTASQ displays very interesting DNA-interacting properties, efficiently stabilizing F21T in a dose–response manner (with $\Delta T_{1/2}$ up to 21.1 °C at 3 μM ligand, i.e., 15 equiv as compared to DNA; black squares and line), while it interacts only poorly with F-duplex-T (with $\Delta T_{1/2}$ < 2.5 °C even at 3 μM ligand; red squares and line). Interestingly, the $\Delta T_{1/2}$ value obtained with F21T is higher with ^{PNA}DOTASQ (12.5 °C at 1 μM dose, Figure 4) than with DOTASQ and Tb·DOTASQ (2.2 and 9.8 °C, respectively),[15] further supporting our hypothesis. To gain insight into the molecular basis of the ^{PNA}DOTASQ/G-quadruplex recognition, and notably to firmly demonstrate that the concomitant presence of both guanines and charged side chains is mandatory, control experiments were undertaken with two structurally related derivatives, one with guanines but without charge (i.e., Prot·^{PNA}DOTASQ, Figure 2, which can form an intramolecular G-quartet but without protonable amine side chains) and the other one with charges but without guanine

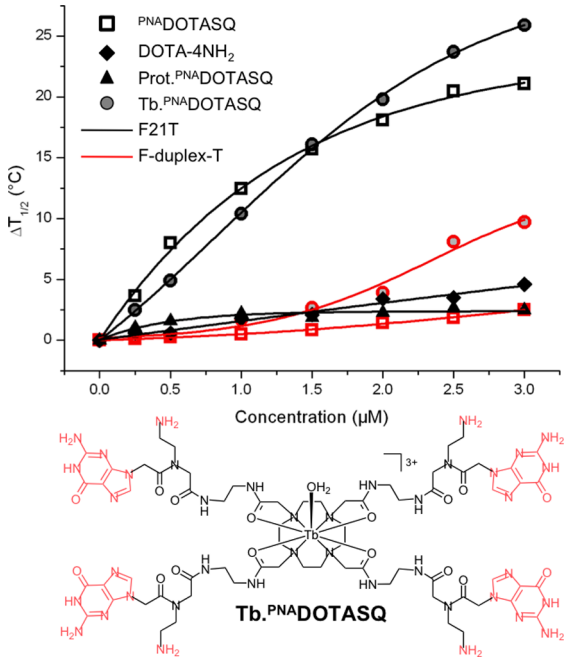

Figure 4. Dose–response curves obtained via FRET-melting assays performed with ^{PNA}DOTASQ (squares), DOTA-4NH$_2$ (diamonds), Prot·^{PNA}DOTASQ (triangles), and Tb·^{PNA}DOTASQ (circles), with F21T (black lines) and F-duplex-T (red lines).

(i.e., DOTA-4NH$_2$, Figure 2, which corresponds to a DOTA ring devoid of guanine moieties but surrounded by four protonable amine side-chains). In Figure 4, the low F21T stabilization monitored in both instances ($\Delta T_{1/2}$ < 2.5 and 4 °C at 3 μM dose for Prot·^{PNA}DOTASQ (triangles) and DOTA-4NH$_2$ (diamonds), respectively) unambiguously demonstrates that the properties of ^{PNA}DOTASQ arise from the wreath of positive charges around its G-quartet.

The next step was to demonstrate that, unlike DOTASQ, the efficiency of ^{PNA}DOTASQ is metal-independent. To this end, we synthesized the terbium complex of ^{PNA}DOTASQ (named Tb·^{PNA}DOTASQ, see the SI). FRET-melting results displayed in Figure 4 (circles) clearly show that the insertion of a metal within the DOTA cavity of ^{PNA}DOTASQ is no longer required for the ligand to be effective (i.e., high F21T stabilizations, with $\Delta T_{1/2}$ up to 21.1 and 25.9 °C for both ^{PNA}DOTASQ and Tb·^{PNA}DOTASQ). However, the high cationic nature of the terbium complex (up to 7 charges vs 4 for the free base) is detrimental to its quadruplex-selectivity (i.e., high F-duplex-T stabilization, with $\Delta T_{1/2}$ up to 9.7 °C). This certainly originates in random (nonspecific) electrostatic interactions between the highly cationic complex and the negatively charged DNA, whatever its nature (duplex or quadruplex), as usually observed with polycationic ligands.[5,19b,25]

NMR and ESI-MS results indicated an excellent quadruplex-over duplex-DNA selectivity for ^{PNA}DOTASQ. To gain deeper insight into this parameter, which is among the most crucial in the quest for promising ligands, a competitive FRET-melting experiment was carried out with F21T in the presence of 0, 15, and 50 equiv (expressed in duplex motif) of unlabeled 26-bp duplex-DNA (ds26, the self-complementary sequence $CA_2TCG_2ATCGA_2T_2CGATC_2GAT_2G$).[24] This selectivity is quantified via a value defined as $^{FRET}S = \Delta T_{1/2}(+ds26)/\Delta T_{1/2}(-ds26)$. Results obtained with both ^{PNA}DOTASQ and Tb·^{PNA}DOTASQ (see SI) definitively confirm the better

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quadruplex-selectivity of the former (^{FRET}S = 0.84 and 0.74 in the presence of 15 and 50 equiv of ds26) as compared to the latter (^{FRET}S down to 0.59 in the presence of 50 equiv of ds26). The ability of ^{PNA}DOTASQ to interact with quadruplex-DNA, whatever the sequence from which it folds, was also verified. Two other quadruplex-forming sequences were studied, F-myc-T (FAM-$GAG_3TG_4AG_3TG_4A_2G$-TAMRA) and F-kit-T (FAM-$CG_3CG_3CGCGAG_3AG_4$-TAMRA), both of which correspond to the doubly labeled sequences found in the promoter regions of Myc and Kit genes, respectively.[10] As can be seen in the SI, fairly comparable stabilizations were found both at 1 μM dose ($\Delta T_{1/2}$ = 12.5, 12.3, and 15.2 °C with F21T, F-myc-T, and F-kit-T, respectively) and in dose−response experiments (from 0 to 15 equiv of ^{PNA}DOTASQ, with $\Delta T_{1/2}$ up to 21.1, 16.0, and 19.1 °C with F21T, F-myc-T, and F-kit-T, respectively). These results thus confirm the potential of TASQ for the design of omnipotent ligands.

We finally performed G4-FID investigations with ^{PNA}DOTASQ to confirm these quadruplex-interacting properties via an alternative technique (see the SI). G4-FID is an isothermal assay, based on the displacement of a fluorescent probe (either thiazole orange (TO) or TO-PRO-3)[25] from unmodified oligonucleotides. We studied the displacement of TO-PRO-3 from unlabeled DNA corresponding to sequences used for the FRET-melting investigations, i.e., the quadruplexes 22AG ($AG_3(T_2AG_3)_3$), c-kit ($CG_3CG_3CGCGAG_3AG_4$), and c-myc ($GAG_3TG_4AG_3TG_4A_2G$), and the duplex ds26. The results confirm that ^{PNA}DOTASQ elicits a fair affinity for quadruplex-DNA ($^{G4}DC_{50}$ = 3.2, 2.9, and 2.9 μM for 22AG, c-myc, and c-kit, respectively) along with a real ability to discriminate quadruplex- over duplex-DNA ($^{ds}DC_{50}$ >10 μM with ds26).

Collectively, these results unambiguously demonstrate that the introduction of four cationic appendages around a synthetic G-quartet makes TASQ a highly valuable G-quadruplex ligand. The wreath of precisely located cationic charges enables a quadruplex-promoted intramolecular G-quartet folding that triggers its quadruplex-affinity, ensuring an elevated level of quadruplex-selectivity. We thus reported herein on the first example of a G-quadruplex binder that is active to be both a selective and high-affinity ligand (i.e., a "smart" ligand). Our data clearly indicate that an optimized structural design makes possible the advent of a new generation of G-quadruplex ligands.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and characterization of ^{PNA}DOTASQ; preparation of DNA; and protocols and results for UV−vis, FRET-melting, G4-FID, NMR, and ESI-MS experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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