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Submicromolar, Selective G-Quadruplex Ligands from One Pot: Thermodynamic and Structural Studies of Human Telomeric DNA Binding by Azacyanines

Özgül Persil Çetinkol,[a] Aaron E. Engelhart,[a] Rupesh K. Nanjunda,[b] W. David Wilson,[b] and Nicholas V. Hud*[a]

The discovery of G-quadruplex structures in nucleic acid sequences associated with cancers has created intense interest in G quadruplexes as potential drug targets.[1] These four-stranded structures, with planar G tetrads represent appealing DNA targets (Figure 1 A), as they are structurally distinct from the Watson–Crick duplex of most genomic DNA. Small molecules with high affinity and high selectivity for G quadruplexes have even begun to show medicinal promise, although the connection between G-quadruplex binding and in vivo activity might not always be obvious.[1g] As one promising example, quarfloxin, an antineoplastic that targets the rRNA–nucleolin complex, is presently in phase II clinical trials.[2]

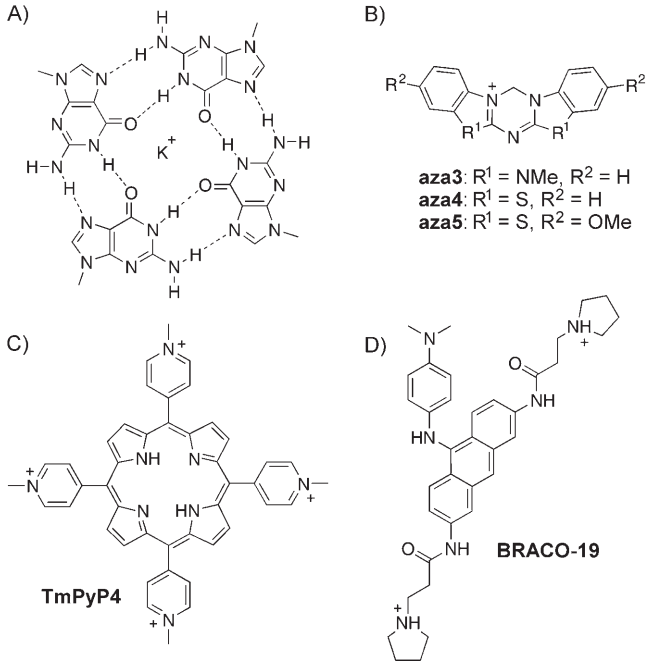

Figure 1. Structures of A) a G tetrad, and G-quadruplex ligands: B) the azacyanines, C) TmPyP4, and D) BRACO-19.

Most investigators who seek new ligands for G-quadruplex DNA have followed two common strategies. First, they have focused on heterocycles with a relatively large and planar surface area, which maximizes stacking with the about 1 nm^2 surface of a G tetrad (e.g., TmPyP4, Figure 1 C).[3] Second, many have used multiple charges to increase electrostatic interactions with the high-charge density G quadruplex (e.g., BRACO-19, Figure 1 D) and to enhance the solubility of potential ligands (often necessary for ligands with large hydrophobic surfaces, e.g., TmPyP4).[4] While these strategies have resulted in several high affinity ligands for G quadruplexes, most ligands do not exhibit high selectivity over duplex DNA.

Recently, several metallated quadruplex ligands have been reported with substantial selectivity for quadruplex DNA.[5] As the authors have suggested, this could be, in part, due to interactions between the metal ion and the lone pairs of the carbonyl oxygen atoms of the exterior quartets, which are not accessible in duplex DNA. Higher-throughput, more combinatorial approaches have begun to show promise as well. For example, Balasubmaranian and co-workers have recently demonstrated dynamic combinatorial selection of quadruplex ligands by disulfide bond formation with a thiol-containing scaffold and side chains.[6]

We have taken a different approach to targeting the G quadruplex. We previously discovered that a planar molecule larger than a typical DNA intercalator can selectively bind purine–purine base pairs.[7] Based upon this discovery, we hypothesized that planar, monocationic molecules that are marginally too large to intercalate a Watson–Crick duplex, such as bispurine analogues, might selectively bind G quadruplexes. We report a new class of selective, submicromolar quadruplex ligands with a facile synthetic route: the azacyanines (Figure 1 B). The synthesis of azacyanines was previously reported by Kurth and co-workers; the route is one-pot and workup is by filtration.[8] The route is general and succeeds for aminobenzimidazoles and aminobenzothiazoles. The synthetic ease makes the class amenable to library preparation for high-throughput screening.

We investigated the binding of azacyanines to a G-quadruplex sequence, based on the human telomeric repeat d(TTAGGG)$_n$, for which a solution-state structure has been reported: tel24 [d(TTGGG(TTAGGG)$_3$A)].[9] To ascertain discrimination against duplex DNA, we also conducted binding studies with calf thymus DNA and two isomeric oligonucleotides, dd1 [d(GCGCATATATGCGC)] and dd2 [d(GCGCAAATTTGCGC)]. Oligo-

[a] Ö. Persil Çetinkol,[+] A. E. Engelhart,[+] Prof. N. V. Hud
School of Chemistry and Biochemistry
Parker H. Petit Institute for Bioengineering and Bioscience
Georgia Institute of Technology
Atlanta, Georgia 30332-0400 (USA)
Fax: (+1) 404-894-2295
E-mail: hud@chemistry.gatech.edu

[b] R. K. Nanjunda, Prof. W. D. Wilson
Department of Chemistry and Center for Biotechnology and Drug Design, Georgia State University
Atlanta, Georgia 30303-3083 (USA)

[+] These authors contributed equally to this work.

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nucleotide dd2 was specifically designed with an A-tract sequence element that is associated with a narrow minor groove, which typically favors interaction with groove-binding ligands.[10]

The binding of aza3 to tel24 was first examined by UV/Vis and CD spectroscopy. In the presence of tel24, the longest wavelength absorption band of aza3 was red-shifted and hypochromic (Figure 2A), and a weak induced band was observed in the CD spectrum (Figure 2B). Assessment of the UV/Vis absorption of the G quadruplex as a function of temperature also revealed that the presence of one equivalent of aza3 enhanced the thermal stability of tel24 relative to the unliganded species, and raised the T_m from 65 to 67 °C. The enhanced thermal stability of tel24 in the presence of aza3 and the spectral changes observed for aza3 in the presence of tel24 are all characteristic of ligand binding in the chiral environment of DNA.

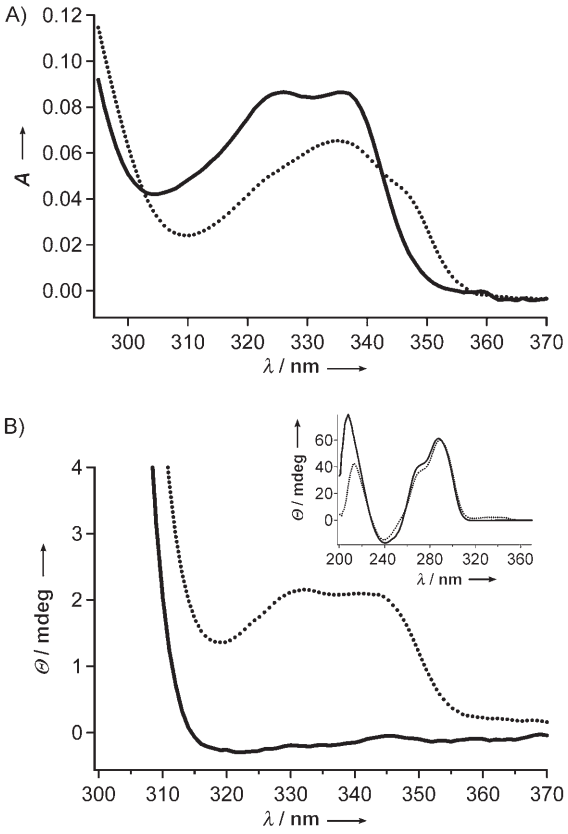

Figure 2. Long wavelength regions of UV/Vis and CD spectra of aza3 with tel24. A) UV/Vis spectra of aza3 and tel24 (1:1) at 5 °C (•••••) and 95 °C (——). B) CD spectra of aza3 and tel24 at 5 °C (1:1; •••••) and of tel24 in the absence of aza3 (——). Insert shows full CD spectra; aza3 gave no CD signal in the absence of DNA.

The binding and structural selectivity of aza3 was characterized quantitatively by fluorescence titrations of aza3 with various nucleic acids (Figure S1 in the Supporting Information). The ligand exhibited a profound structural preference, with about 100-fold selectivity for G quadruplex versus duplex DNA (Table 1). Compound aza3 was near what is considered the low end of binding constants (10^3–10^4 M^{-1}) for Watson–Crick ligands and near the high end for quadruplex ligands (10^6–10^7 M^{-1}); this result supports our size-selection hypothesis.[11] Additionally, aza3 bound more weakly to dd2 than dd1, which provides some evidence against groove binding.

Table 1. Association constants determined by fluorescence spectroscopy for aza3 with various DNA sequences.

Sequence	Association constants [M^{-1}]
tel24 [d(TTGGG(TTAGGG)$_3$A)]	$1.28\times10^6\pm(2.5\times10^5)$
calf thymus DNA	$9.2\times10^3\pm(1.7\times10^3)$
dd1 [d(GCGCATATATGCGC)]	$6.13\times10^3\pm(1.2\times10^3)$
dd2 [d(GCGCAAATTTGCGC)]	$1.14\times10^4\pm(2.0\times10^3)$

Titration of aza3 into a sample of tel24 (2 mM) revealed that the ligand was in intermediate exchange on the NMR timescale. A number of resonances broadened in a site-specific fashion; this is consistent with the local chemical environment of various residues being perturbed differentially by ligand binding. The 1D ^{1}H NMR spectra of tel24 in the presence of increasing concentrations of aza3 are shown in Figure 3. Resonance line broadening was characterized by the fitting of Lorentzian functions to resolved resonances in 1D spectra, and by measuring the heights of aromatic-H1′ cross-peaks in 2D NOESY spectra (Figure S2). As a qualitative method of determining the ligand-binding site, the rate of decrease of the NOESY aromatic-H1′ cross-peaks intensities as a function of aza3 concentration was examined (Figure S3). The residues were separated into three groups based on their apparent sensitivity to the presence of aza3. The solution structure of tel24 determined by Luu et al.[9b] is shown in Figure 4 with the residues colored according to these three groups. This representation illustrates the localized binding of aza3 to tel24.

Luu et al. reported that an A·T pair is formed by T1 and A20, and a reverse A·T pair is formed by T13 and A24—each being stacked on G tetrads on opposite sides of the quadruplex. Interestingly, broadening occurs for both the aromatic residues of the external G tetrads and these A·T base pairs (Figure 4); this provides support for a mixed intercalation–exterior-stacking mode of binding (i.e., between the exterior tetrads and the capping A·T base pairs of the loops). The NMR spectroscopy data indicate that the primary binding site lies between the G tetrad and T13·A24 base pair (Figure 4B). A secondary weak binding site is also observed on the opposite side of the quadruplex between the G tetrad and the T1·A20 pair. The NMR spectroscopy data do not support a groove-binding mode of interaction; this is consistent with our results from fluorescence titration of aza3 with dd1 and dd2.

"Model-free" ITC titrations (i.e., at very low ligand:binding site ratios) of aza3 into tel24 were performed to better characterize the thermodynamics of the interaction. A ΔH of -2.7 ± 0.14 kcal mol^{-1} was obtained (Figure S4). With the 1.3×10^6 M^{-1} association constant determined by fluorescence titrations, this datum implies a ΔG of -8.3 kcal mol^{-1} and a remarkable $-T\Delta S$ of -5.6 kcal mol^{-1}. There is a paucity of thermodynamic data on quadruplex–ligand interactions, but such entropy-driven

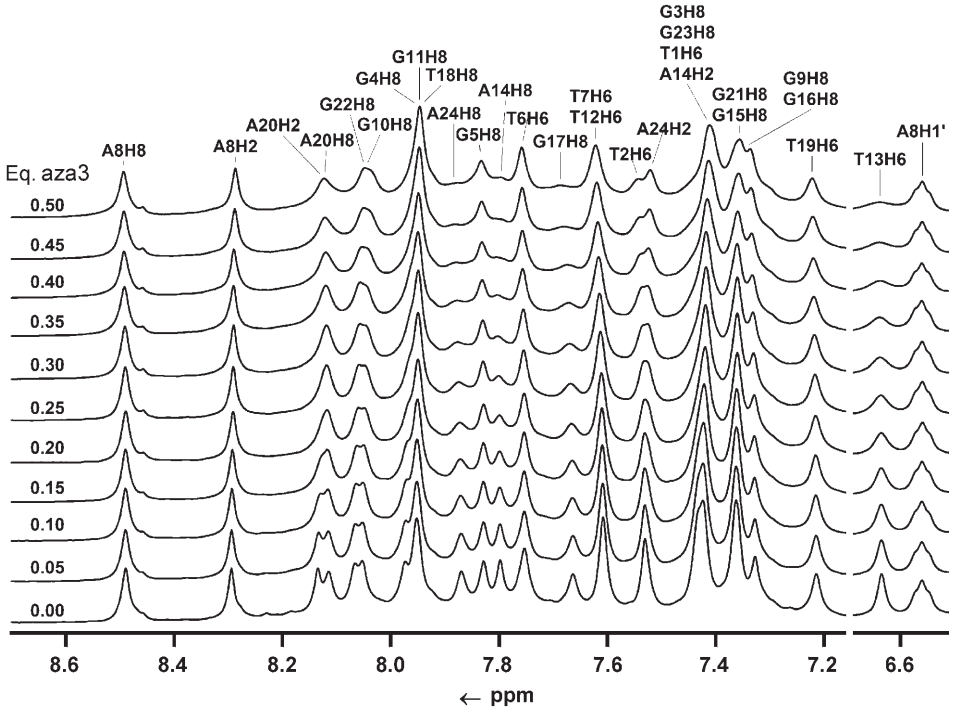

Figure 3. Aromatic region of ^{1}H NMR spectra of tel24 in the presence of 0 to 0.50 molar equivalents of aza3.

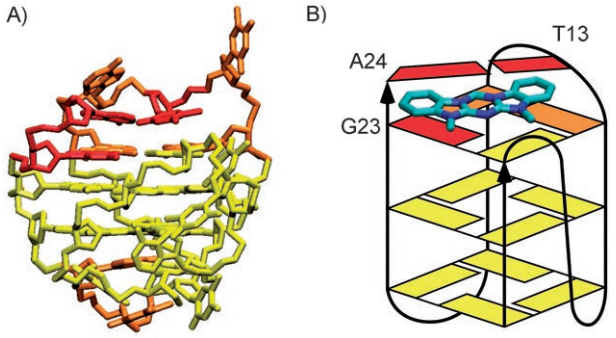

Figure 4. A) The solution state structure of tel24 (PDB ID code: 2GKU); the residues are colored according to the rate of NOE attenuation per equivalent aza3 (red: high; orange: medium; yellow: low). For clarity, hydrogens and phosphate oxygens are not shown. B) A schematic representation of aza3 bound tel24, based on NOE attenuation data, in which aza3 is positioned between the A·T base pair of T13 and A24 and G23 of the terminal G quartet. For clarity, the loop bases are not indicated, except for those of T13 and A24.

binding has been reported previously for a remarkably different, tetracationic ligand, TmPyP4.[12]

Finally, the binding of a broader spectrum of azacyanines (aza3, aza4, aza5) to tel24 was characterized by fitting the steady-state surface plasmon resonance (SPR) response versus ligand concentration for SPR chips with surface-tethered tel24 DNA. All ligands exhibited tight binding; this suggests that G-quadruplex binding is general to this class of compounds. Association and dissociation kinetics were rapid, as expected from small, planar ligands with small substituents. The synthetic accessibility of these compounds suggests substituent variations could be a promising avenue by which to modulate the kinetics for ligand association and dissociation into different ranges for analysis of effects on biological activity.

Additionally, we have tested the binding affinity of all three azacyanine compounds to tel26 (Table 2), a 26-nucleotide DNA sequence that was also derived from the human telomere sequence and has a similar fold to tel24.[9a] All three azacyanines showed similar strong primary binding affinities to tel26 as well; this indicates that the observed high affinity is general for this particular quadruplex fold (Table 2).

SPR experiments were also performed with intramolecular duplex strands dd3 [d(CGAATTCGTTTTCGAATTCG)], dd4 [d(CGCGCGCGTTTTCGCGCGCG)], and dd5 [d(CCATATATATATATATAGCCCCCGCTATATATATATATATGG)]; the hairpin loop regions are underlined. These strands contain a variety of sites known to favor various modes of ligand binding in duplex DNA, including A-tracts and both pyrimidine–purine steps.

Table 2. Association constants determined by using SPR for aza3–5 with tel24 [d(TTGGG(TTAGGG)$_3$A)], and tel26 [d(AAAGGG(TTAGGG)$_3$AA)].

Compound	Sequence	Association constants [M^{-1}][a]
aza3	tel24	3.1×10^{6}
	tel26	4.1×10^{6}
aza4	tel24	3.0×10^{6}
	tel26	3.0×10^{6}
aza5	tel24	2.9×10^{6}
	tel26	4.7×10^{6}

[a] In addition to the association constants shown for the single strong binding site, all compounds have one or two much weaker binding sites (about $2\times10^{5}\,M^{-1}$). Association constants for duplex sequences were too low to be determined by using the same experimental procedure.

The azacyanine ligands bound poorly to all duplex DNA strands investigated and the SPR data could not be fit to an exponential binding curve. The upper limit for the K^{A} in each aza3– and aza4–dsDNA (double stranded DNA) pair is therefore $10^{4}\,M^{-1}$; this is consistent with our fluorescence data. Remarkably, *no* aza5–duplex interaction was detected under the SPR conditions used (Figure S6). Fluorescence binding data confirm that these ligands exhibit marked selectivity for quadruplex over dsDNA—over 100-fold in the case of aza5.

G-quadruplex ligands hold great promise for use in the treatment of human disease. A common and valid critique of their medicinal utility relates to the gravity and wide range of

off-target effects associated with binding to Watson–Crick DNA. Only by ameliorating such effects is the G quadruplex a viable drug target. We have shown that a bispurine analogue, which is apparently slightly too large to intercalate purine–pyrimidine base pairs, exhibits strong and selective association with a G quadruplex. Kurth's synthetic route to azacyanines provides a general and perhaps the most practical means thus far for the preparation of selective G-quadruplex ligands. A promising avenue for future research will be the decoration of azacyanines with the appropriate functional groups to bind specific promoter-related quadruplexes selectively, as Hurley and co-workers have shown is possible with quarfloxin.[13]

Experimental Section

Oligonucleotides: Unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA) with standard desalting and were filtered before use. For SPR, 5′-biotinylated oligonucleotides were purchased from Midland (Midland, TX, USA) with HPLC purification.

Buffer conditions: NMR spectroscopy, fluorescence, ITC, CD, and UV/Vis experiments were performed in KCl (70 mM), potassium phosphate (25 mM, pH 7). SPR experiments were performed in degassed HEPES buffer (10 mM HEPES, 100 mM KCl, 3 mM EDTA, 5 ppm, *v/v*, of 10% P20 BIACORE surfactant, pH 7.4).

Azacyanines: All azacyanines were prepared as described by Kurth and co-workers[8] and characterized by using ^{1}H NMR and ^{13}C NMR spectroscopy, and HR-ESI-MS. The structure of aza3 was also confirmed by X-ray crystallography. CCDC 671506 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif

Instrumentation: NMR spectroscopy experiments were performed by using a Bruker DRX500. UV/Vis was performed by using an Agilent 8452. CD spectroscopy was performed by using a Jasco J-810. ITC was performed by using a MicroCal VP-ITC. All SPR was performed by using a Biacore 2000.

Detailed information on experimental techniques, where applicable, is provided in the Supporting Information.

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Keywords: cancer · DNA structures · drug design · quadruplexes · telomeres

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