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Aggregation and G-quadruplex DNA-binding study of 6a,12a-diazadibenzo-[*a*,*g*]fluorenylium derivative

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Abstract—The aggregation and DNA binding behavior of a new G-quadruplex selective ligand, 6a,12a-diazadibenzo-[*a*,*g*]fluorenylium derivative, was studied by UV–vis absorption and fluorescence spectroscopy. The formation of ligand aggregates with different spectral characteristics was observed at low and high concentration of NaCl, respectively. The ligand binds to G-quadruplex with much higher affinity than to single- and double-stranded DNA.

The four-stranded G-quadruplex DNA motif has recently emerged as a biologically important structure.[1] The quadruplex has been linked to mechanisms that relate to a number of disease states, most notably cancer, via interfering with telomere maintenance by telomerase.[2–4] Telomerase activity has been detected in some 80–90% of all human cancers but not in adjacent normal cells.[5] Telomerase's endogenous RNA template requires hybridization with the nonfolded 3′-end overhang of telomeric DNA for effective addition of telomeric repeats.[6] However, when G-rich telomeric repeats are assembled into four-stranded quadruplex structures (in the presence of metal cations or specific compounds), the RNA template cannot hybridize with 3′-end overhang that results in inhibition of telomerase activity.[6,7] In vitro, G-quadruplexes exhibit four-stranded structures containing one or more nucleic acid strands, in parallel or antiparallel orientations, with central channel able to accommodate metal cation.[1] Four guanines on a plane, interacting via Hoogsteen bonding, form a G-quartet. Typically, three or four G-quartets are stacked within a quadruplex and held together by π–π nonbonded attractive interactions (Fig. 1A).

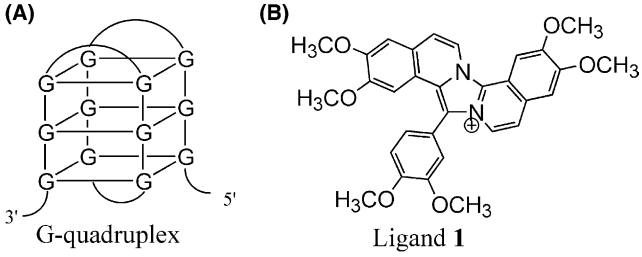

Figure 1. (A) A possible folded structure of G-quadruplex DNA, (B) formula for the ligand **1**.

There is now considerable interest in the design of ligands that target G-quadruplex DNA. A number of promising small molecules have been devised to selectively promote the formation and/or stabilization of such higher-order structures, ranging from derivatives of anthraquinones[8] to porphyrins,[9] acridines,[10] and perylene diimide.[11] These ligands have the common feature of extended planar aromatic electron-deficient chromophore with cationic substituents.[12,13]

Here we report spectroscopic studies of the interactions of oligonucleotides related to human telomeric DNA with a new ligand, 13-(3,4-dimethoxyphenyl)-2,3,8,9-tetramethoxy-6a,12a-diazadibenzo-[*a*,*g*]fluorenylium chloride (Fig. 1B). Ligand **1** exhibits structural similarities to previously reported quadruplex-binding

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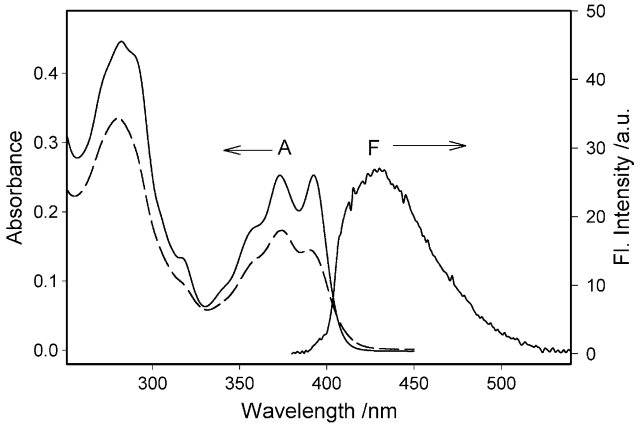

Figure 2. Absorption spectra (A) of 8 μM ligand **1** in methanol (solid line) and in 20 mM Tris buffer, pH 7.2, 100 mM NaCl (dashed line). The fluorescence spectrum (F) was excited at 393 nm.

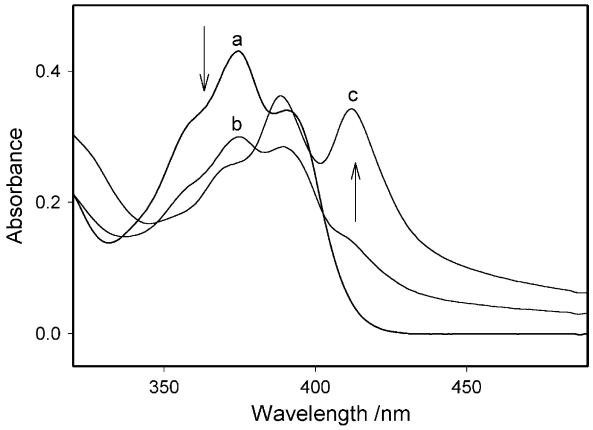

Figure 3. Effect of NaCl concentration on the absorption spectra of **1**. Conditions: [**1**] = 25 μM, Tris buffer (20 mM, pH 7.2); [NaCl] = 0 M (a), 0.4 M (b), 1.0 M (c).

telomerase inhibitors.[8–13] In addition, it possesses strong fluorescence properties that can be potentially useful for the detection of G-quadruplexes. The fluorenylium derivative **1** was prepared by the Gadamer and Schulemann oxidation of papaverine with mercury(II) acetate according to the procedure described elsewhere.[14]

Figure 2 shows absorption spectra of **1** in methanol and in buffered aqueous solution. In the investigated spectral range (250–500 nm), the ligand spectrum exhibits a long-wavelength band with resolved vibronic structure in the visible region and a second absorption band at ca. 280 nm. For the first singlet transition of **1** the 0–0 and 0–1 vibronic components are seen at 393 and 374 nm, respectively, while the 0–2 occurs as shoulder at ca. 360 nm. The peak positions are independent of the solvent, but the peak-to-valley ratio for 0–0 transition (393 nm) increases from 1.03 in buffer to 1.33 in MeOH. The changes in vibrational structure of the long-wavelength band in water suggest that some stacking interactions between ligand molecules occur in an aqueous solution. Hydrophobicity of the rigid aromatic system in **1** can facilitate an intermolecular stacking and the formation of aggregates in aqueous solutions as it was observed for many large molecules.[15–17] It should be noted that spectra recorded in other organic solvents close resembled that in MeOH showing negligible variations in spectral parameters with ligand concentration. On the contrary, an increase in ligand concentration in aqueous solutions caused noticeable spectral changes, supporting plausibility of aggregation phenomena in these conditions.[18] The buffer type (HEPES, Tris, phosphate) and the buffer concentration (10–200 mM) do not affect the spectral properties of the ligand. On the other hand, an increase in pH from 7.0 to 11.0 caused a gradual decrease in intensity of the whole spectrum (ca. 5% per pH unit). This alkaline bleaching was probably caused by hydroxide attack on the heterocyclic iminium group as it was suggested earlier for some benzo[*c*]phenanthridine alkaloids.[19]

Ligand **1** exhibits broad emission band at ca. 430 nm as shown in Figure 2. The same spectrum is obtained independently of the excitation wavelength and solvent used. The fluorescence quantum yield varies with solvent and increases in the following order: $H_2O <$ MeOH < ACN < DMSO < $CHCl_3$. Aggregation significantly decreases fluorescence intensity but there is no sign of excimer fluorescence, which indicates rather tight aggregate formation and means that quantum yield of dimer (aggregates) is close to zero.

Interestingly, high concentrations of neutral electrolytes strongly influence spectral properties of ligand as shown in Figure 3. In the presence of NaCl at moderate concentration (0.4 M), the absorption band exhibits significant hypochromicity without any important wavelength shift (spectrum b in Fig. 3). At the higher chloride concentration (1 M) whole spectrum is red shifted by ca. 1200 cm^{-1}. The position of 0–0 transition underwent remarkable shift from 390 to 412 nm and the peak-to-valley ratio for this band increased from 1.01 (in the absence of chloride) to 1.28 in 1 M NaCl. A slow decrease in absorbance and an appearance of light scattering with time, indicating gradual formation of larger aggregates were observed. An explanation of the observed salt effect may involve a specific arrangement of ligand molecules in aggregates (H-type and J-type aggregates). According to the point dipole model of Kasha et al.[20] H-type and J-type aggregates possess different spectral properties. Hypochromicity and blue shift of absorption band characterize H-type aggregates with a parallel orientation of transition moments, whereas hyperchromicity and bathochromic shift indicate a head-to-tail arrangement of transition dipoles in J-type aggregates. Thus, the observed salt effect on the spectrum of **1** may be explained as follows. The initially formed H-type aggregates with parallel dipole orientation exhibit hypochromicity and negligible spectral shift. The shielding of the positive charges of ligand molecules at high ionic strength favors tight aggregate formation that reduces torsional freedom of the dimethoxybenzene substituent[21] and promotes head-to-tail assembly formation.

The 21-mer quadruplex-forming oligonucleotide $dG_3(T_2AG_3)_3$ (G-4) and its complementary strand

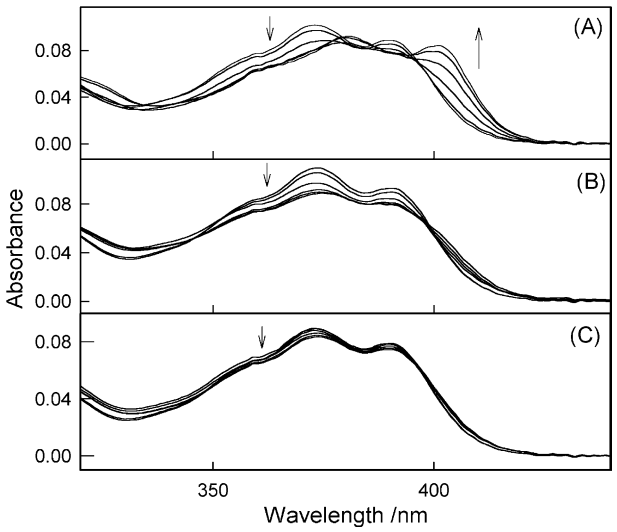

Figure 4. Spectrophotometric titration of **1** with the 21-mer G-4 oligonucleotide $dG_3(T_2AG_3)_3$ (A), the $dG_3(T_2AG_3)_3/d(C_3TA_2)_3C_3$ equimolar mixture that forms double-stranded DNA (B), and $d(C_3TA_2)_3C_3$ representing single-stranded DNA (C). Conditions: [**1**] = 5 μM, Tris buffer (20 mM, pH 7.2); [NaCl] = 100 mM, [oligonucleotide] = 0–5 μM (in strand).

$dC_3(TA_2C_3)_3$ (C4) were purchased from TAG Copenhagen (Denmark). DNA-binding affinity of **1** was studied in three DNA systems: (i) tetraplex DNA—an intramolecular G-quadruplex formed by $dG_3(T_2AG_3)_3$ in the presence of NaCl, (ii) double-stranded DNA—a Watson–Crick duplex $dG_3(T_2AG_3)_3/d(C_3A_2T)_3C_3$, and (iii) single-stranded DNA—$dC_3(TA_2C_3)_3$ that should not produce higher-order structures (e.g., i-motif) in the experimental conditions used (pH 7.2).[22] In the presence of nucleic acids, the absorption bands of **1** exhibit changes but their extent depends on the secondary structure and concentration of oligonucleotide as it is shown in Figure 4A–C. The G-4 DNA (Fig. 4A) produces spectral changes distinctly different from those observed for other systems. With an increase in DNA concentration a new band appears at 402 nm, which is red shifted by ca. 700 cm^{-1} when compared to that of free dye. Subsequent absorbance changes are distinctly different from those at low P/D (DNA phosphate to dye concentration ratio), showing hyperchromicity with the P/D increase. In contrast to the pronounced spectral changes associated with the interaction of **1** with G-4 DNA, the dsDNA (Fig. 4B) and single-stranded DNA (Fig. 4C) cause absorbance decrease without any important wavelength shift. These results indicate that ligand **1** interacts most strongly with G-4 DNA and it has little affinity for ss and dsDNA under experimental conditions used here. All these may suggest different interactions, which can be referred to as the nonspecific in case of single- and double-stranded DNA, contrary to the specific binding mode presented by G-4 DNA. Nonspecific binding may be attributed to condensation and stacking of the dye along the polymer. This cooperative binding originates from electrostatic interactions between positively charged dye and negatively charged phosphate groups and from the aggregation tendency of ligand.[16,17] On the other hand, the specific binding may be attributed to well known end-stacking interactions of monomeric or dimeric dye with G-tetrad.[23,24] Such binding mode requires only one face of molecule to interact with G-quartet; therefore, either monomer and dimeric species are allowed to bind to G-quadruplex. Although there is apparent similarity between spectra for G-4 DNA/ligand **1** complex and those observed for J-type aggregates of **1** in an excess of chloride (Fig. 3), the origin of the bathochromic shift of absorption bands for DNA-bound ligand seems to be different than the extensive aggregation (405 nm vs 412 nm, and negligible light scattering). It can be explained by interactions of **1** with π-electron systems of guanines resulting from end-stacking binding on G-quadruplex. One cannot exclude interactions with loop-forming bases.

The spectrophotometric titration data for dsDNA and G-4 DNA were analyzed in order to extract DNA-binding parameters according to the simple Scatchard method.[25] Unfortunately, a lack of clear isosbestic points hampered the precise calculations of binding constants. The binding isotherm for G-4/C4 duplex showed pronounced curvature indicative of positive cooperativity. The apparent binding affinity was on the order of $10^4\ M^{-1}$ with saturation binding of ca. 5–10 ligands per duplex. For G-4 DNA titration, the binding isotherm exhibited better linearity giving the binding constant on the order of $10^5\ M^{-1}$ and a binding ratio at saturation of 1.5 ligands per G-4 DNA molecule. It should be noted that the character of metal cation has marginal effect on the ligand/G-4 DNA complex formation. Similar spectral changes were observed when G-4 DNA titration was performed in the presence of 0.1 M KCl.

Another criterion for interaction of a ligand with G-4 DNA is the observation of an increase in the melting temperature (T_m) of the quadruplex. A quadruplex-selective ligand should increase the T_m of tetraplex, shifting the equilibrium ssDNA ↔ G-4 DNA to the right. According to Mergny et al., it is possible to provide evidence for G-4 structure stabilization by recording the absorbance at 295 nm (a quadruplex-induced hyperchromicity).[26] Under experimental conditions used (5 μM G-4, 20 mM Tris buffer (pH 7.2, 10 mM NaCl, 100 mM LiCl)), the melting temperature of quadruplex is ~40 °C in the absence of **1** and ~44 °C at the equimolar concentration of **1**.[27] The G-4 structure stabilization effect of **1** ($\Delta T_m \sim 4$ °C) is comparable to those reported for other ligands.[8,26,28]

To confirm that ligand **1** interacts with G-4 DNA, we recorded the fluorescence spectra of ligand in the presence and in the absence of a DNA quadruplex. Emission spectrum of **1** bound to G-4 DNA resembled that for free ligand with the fluorescence maximum centered at 430 nm. Excitation of the ligand at 393 nm resulted in negligible variation in emission intensity with the concentration of G-4 DNA; however, upon selective excitation at 405 nm, the fluorescence intensity exhibited pronounced enhancement with the DNA concentration. Figure 5 shows changes in fluorescence intensity observed upon the addition of G-4 DNA (curve 1) and those resulted from the presence of G-4/C4 duplex (curve

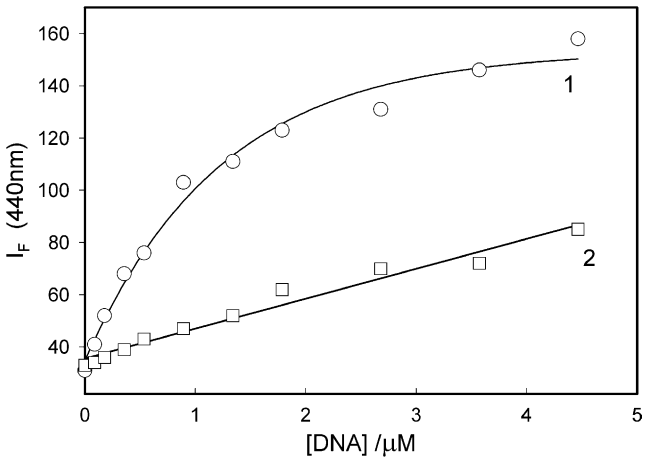

Figure 5. Effect of the concentration of G-quadruplex DNA (circles) and double-stranded DNA (squares) on the fluorescence enhancement of ligand **1**. Excitation was set at 405 nm, other conditions as in Figure 4.

2). A significant enhancement of the ligand emission is evident in the case of G-quadruplex DNA, while dsDNA causes a modest increase in the fluorescence intensity. The G-4-induced fluorescence enhancement may be exploited for the detection of G-4 structures.

In conclusion, the evidence for recognition of G-quadruplex DNA by ligand **1** is provided by UV–vis absorption and fluorescence spectroscopy. Ligand binds preferentially with four-stranded DNA, it can bind weakly to the duplex and very weakly to ssDNA. The plausible binding mode is the end stacking on terminal G-tetrads as evidenced for many G-4 interacting ligands.[11,23,24] Recently, new reports appeared that proposed grooves of G-quadruplex as plausible binding site for some carbocyanines[29] and distamycin[30] but this binding mode seems unlikely to operate in our system (specific structural requirements). As discussed by Kerwin et al.[15,31] the aggregation feasibility and stacking interactions may influence selectivity of end binding on terminal G-tetrads, thus ligand **1** may serve as a new model compound to study effect of certain factors such as salt concentration or presence of organic modifiers. It is necessary to test whether ligand **1** inhibits telomerase. The identification of **1** as a G-4 DNA selective ligand and possibly as a human telomerase inhibitor may have implications for the design of new therapeutic agents.

Acknowledgements

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