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G-Quadruplex DNA Binding by a Series of Carbocyanine Dyes

Sean M. Kerwin,* Daekyu Sun, Jonathan T. Kern, Anupama Rangan and Pei Wang Thomas

Division of Medicinal Chemistry, College of Pharmacy, University of Texas at Austin, Austin, TX 78712, USA

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Abstract—We have examined a number of carbocyanine dyes for their ability to bind intramolecular G-quadruplex DNA structures (G4′-DNA) using a Taq polymerase stop assay. Of the five dyes examined, only one, *N,N'*-diethylthiacarbocyanine iodide (DTC), was found to bind to G4′-DNA. DTC was also the only dye found to inhibit human telomerase at 50 μM concentration.

Telomerase is present in the vast majority of cancer cells and largely absent in normal somatic tissue, and is thought to play an important role in the maintenance of telomeres in cancer cells. Inhibitors of telomerase are being examined as potential anticancer agents.[1,2] One such class of telomerase inhibitors are compounds that bind to G-quadruplex DNA structures. These four-stranded DNA structures are formed from a variety of G-rich DNA sequences through the association of guanosine residues into G-tetrads (Fig. 1A).[3] In particular, telomeric DNA sequences [e.g., 5′-d(TTAGGG)$_n$ in humans] have been shown to form four-stranded G-quadruplex structures in vitro.[4,5] There are three major classes of G-quadruplex structures (Fig. 1B): those formed from four separate G-rich DNA strands (G4-DNA), those formed from two DNA strands (G′2-DNA), and those formed from a single strand of DNA (G4′-DNA). Although ligands that bind to G-quadruplex DNA have been shown to inhibit telomerase,[3,6–8] there has not been any attempt to relate the selectivity of these ligands for the different forms of G-quadruplex DNA such as G4-, G′2- and G4′-DNA with their telomerase inhibitory properties. Here, we report our studies of the G-quadruplex DNA binding and human telomerase inhibition properties of a series of carbocyanine dyes. These data demonstrate the importance of G4′-DNA binding in the ability of G-quadruplex DNA ligands to inhibit telomerase.

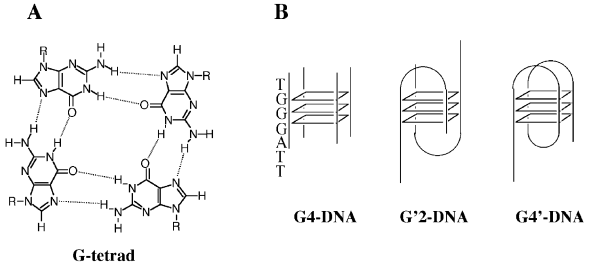
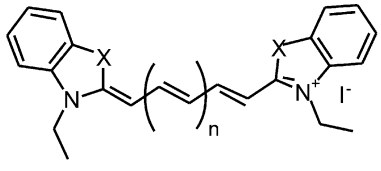


Figure 1. (A) Structure of a G-tetrad; (B) different forms of G-quadruplex DNA.

Shafer and co-workers have previously reported the G-quadruplex DNA binding by the carbocyanine DODC.[9] They characterized the interaction of DODC with a variety of G-quadruplex and duplex DNA structures by fluorescence, absorption, and CD spectroscopy, as well as satellite hole spectroscopy.[10] DODC interacts specifically with G′2-DNA structures. Binding constants determined from absorption spectroscopic titrations reveal that the binding affinity of DODC for the G′2-DNA form of [d(G$_4$T$_4$G$_4$)]$_2$ is 5 times higher than for double-stranded DNA. Equilibrium dialysis experiments, however, demonstrate a preference for DODC binding to triplex DNA over G-quadruplex DNA.[11]



X = O, n = 0 - **DOC** X = S, n = 0 - **DTC**
X = O, n = 1 - **DODC** X = S, n = 1 - **DTDC**
X = S, n = 2 - **DTTC**

We examined a series of carbocyanine dyes for their ability to interact with G4′-DNA structures using the

*Corresponding author. Tel.: +1-512-471-5074; fax: +1-512-232-2606; e-mail: skerwin@mail.utexas.edu

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polymerase stop assay.[12] This assay utilizes a template DNA strand containing four repeats of the human telomeric DNA sequence d(TTAGGG) (Fig. 2). DMS protection experiments demonstrate that this G-rich region of the template strand forms a G4′-DNA structure under the assay conditions.[12] This G4′-DNA structure serves as a block to transcription by Taq polymerase. Ligands that bind to and stabilize the G4′-DNA structure in the template strand cause an increase in abortive transcripts corresponding to polymerase blockage just upstream of the G-rich sequence.

Each carbocyanine was tested at 5, 20, and 50 μM concentration in the stop assay. Two carbocyanines, DTDC and DTTC, have a nonspecific effect of inhibiting Taq polymerase, as evidenced by a complete lack of primer elongation at higher carbocyanine concentrations. The degree of Taq polymerase inhibition at 20 μM carbocyanine concentration was determined by comparing the ratio of band intensities for all of the polymerase products to the intensity of the unextended primer band as compared to the control lane. As shown in Table 1, even at 20 μM concentration, DTDC almost completely inhibits Taq polymerase. DTTC is also a strong inhibitor of Taq at this concentration. The remaining carbocyanines are less inhibitory. Only one carbocyanine, DTC, demonstrated a significant enhancement of the G4′-DNA-induced polymerase pausing (Fig. 2). Quantification of this effect at 20 μM carbocyanine concentration demonstrates over a 4-fold enhancement of the paused products relative to control (Table 1). The G′2-DNA selective G-quadruplex ligand DODC[9] showed only a minor increase in polymerase pausing relative to control.

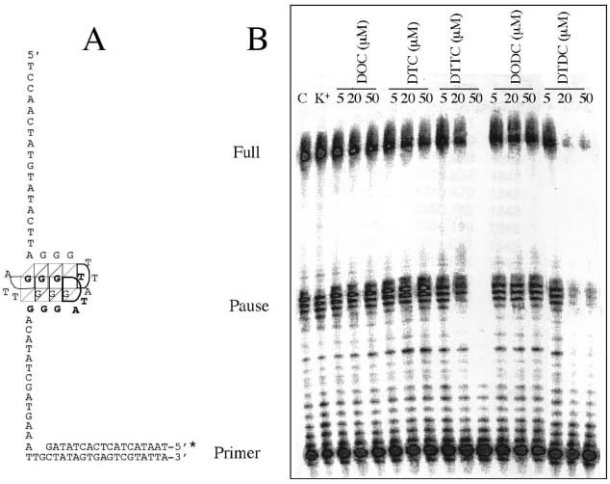

Figure 2. (A) Primer/template used in the polymerase stop assay. The template contains a region consisting of four repeats of the human telomeric DNA sequence d(TTAGGG), which forms a G4′-DNA structure under the assay conditions. (B) Autoradiogram of the polymerase stop assay products. 5′-Labeled primer was annealed with the template strand in 10 mM Tris, pH 8.0, containing 50 mM KCl. The primer/template was incubated with the carbocyanines at the indicated concentrations for 15 min, and the primer extension reactions were initiated by the addition of dNTP (100 μM), $MgCl_2$ (3 mM), and Taq polymerase (2.5 U/reaction). After 15 min incubation at room temperature, stop buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol) was added and the products separated by PAGE.

In order to verify the interaction of DTC with G-quadruplex DNA, we monitored the changes in the visible spectrum of this carbocyanine in the presence of the G4′-DNA form of $d(TTAGGG)_4$ and the G4-DNA form of $[d(TAGGGTTA)]_4$. As shown in Figure 3, the absorption spectrum of DTC undergoes significant changes in the presence of both G-quadruplex DNA structures, indicative of complex formation. Scatchard analysis of spectroscopic titration data provides an estimated binding constant of 4.6×10^5 M^{-1} and a stoichiometry of two DTC molecules per G4′-DNA structure. In contrast to the pronounced spectroscopic changes associated with the interaction of DTC with G-quadruplex DNA, the changes in the DTC spectrum in the presence of excess double-stranded DNA are more subtle (Fig. 3). A clear red shift is observed, demonstrating that an interaction with double-stranded DNA occurs; however, the spectrum is clearly different from the one obtained in the presence of G4′-DNA, suggesting that a different mode of binding is involved.

The carbocyanines were also tested for their ability to inhibit human telomerase activity from S100 HeLa cell extracts. The telomerase TRAP assay consists of a Taq polymerase PCR amplification of telomerase reaction

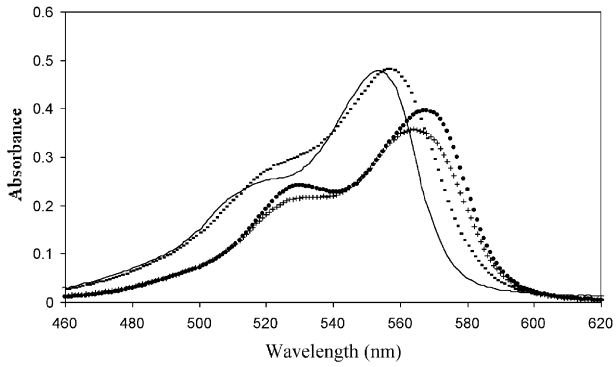

Figure 3. Visible absorption spectra of DTC (3 μM) in 60 mM phosphate buffer/110 mM KCl, 1 mM EDTA, pH 7 (solid line) and DTC in the presence of the G4′-DNA $d(TTAGGG)_4$ (10 μM, +), the G4-DNA form of $[d(TAGGGTTA)]_4$ (10 μM, ●), or double-stranded $d(CGCGCGATATCGCGCG)_2$ (180 μM base pairs, −).

Table 1. Comparison of Taq polymerase inhibition, G4′-DNA binding-dependent Taq polymerase pausing, and human telomerase inhibition for a series of carbocyanines

Compound	Nonspecific Taq inhibition[a] (%)	Normalized ratio of G4′-DNA stop product[b]	Telomerase inhibition[c] (%)
DOC	15	0.9	<10
DODC	27	1.6	<10
DTC	14	4.4	35
DTDC	91	nd[d]	<10
DTTC	70	1.1	<10

[a]Percent decrease in the ratio of total polymerase products to primer at 20 μM concentration of carbocyanine versus control lane.
[b]Ratio of pause/full bands at 20 μM carbocyanine concentration relative to control.
[c]Inhibition of human telomerase activity in S100 HeLa cell extract at 50 μM carbocyanine concentration.
[d]Not determined due to strong Taq polymerase inhibition.

products.[13] Despite the sensitivity of this assay, it cannot be used to establish the telomerase inhibition for compounds that are also potent inhibitors of Taq polymerase,[14] such as the carbocyanines DTDC and DTTC (Table 1). Consequently, we employed modified standard telomerase primer extension assay utilizing 5′-end-biotinylated d(TTAGGG)$_3$ as primer, as described previously.[15] An added advantage of this assay is the ability to ascertain the degree of processivity of telomerase in the presence of the inhibitors; G-quadruplex ligand telomerase inhibitors are known to diminish the apparent processivity of the enzyme.[15] The carbocyanines were tested at a fixed concentration of 50 μM in this telomerase assay. Only one carbocyanine, DTC, inhibited telomerase under these conditions (Table 1), and this inhibitor demonstrated the same pattern of diminished processivity as that observed with the known G-quadruplex DNA interactive inhibitor BSU-1051[6,15] (data not shown).

Certain carbocyanines have been shown to bind to double-stranded DNA.[16–18] In some cases (e.g., DTDC), this DNA binding is associated with the formation of extended aggregates of carbocyanines along the DNA minor groove.[17] These observations may explain the potent Taq polymerase inhibition of DTDC and DTTC; aggregation of these ligands around the primer/template may prevent the association of this substrate with the polymerase. DODC is a selective G′2-DNA binding agent[9] with only weak double-stranded DNA binding properties.[16] Here we verify that DODC weakly interacts with G4′-DNA structures, as determined by the polymerase stop assay. DODC also does not inhibit telomerase at 50 μM concentration. In contrast, DTC both binds to G4′-DNA structures and inhibits human telomerase. This correlation between G4′-DNA binding and telomerase inhibition supports the kinetic modeling of Cathers and co-workers,[19] and is in agreement with the originally proposed mechanism of telomerase inhibition by G-quadruplex ligands.[15]

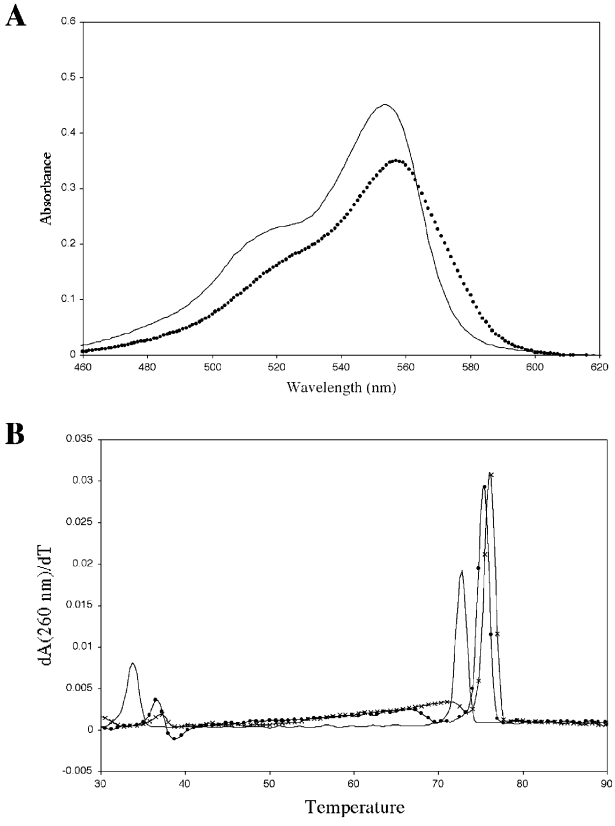

Figure 4. (A) Visible absorption spectra of DTC (3 μM) in 60 mM phosphate buffer/110 mM KCl, 1 mM EDTA, pH 7 (solid line) and DTC in the presence of triple helical poly(dA):[poly(dT)]$_2$ (10 μM base triplet) (dotted line). (B) Derivative plot of the thermal denaturation of triple helical poly(dA):[poly(dT)]$_2$ (10 μM base triplets) in the same buffer as (A) monitored by changes in the absorption at 260 nm as a function of temperature: Triplex DNA alone (—), triplex DNA plus 7.5 μM DTC (●), triplex DNA plus 10 μM DTC (×).

Because the carbocyanine DODC has been shown to bind triplex DNA structures,[11] we examined the ability of DTC to interact with the triple helical form of poly(dA):[poly(dT)]$_2$. The visible spectrum of DTC is both red-shifted and diminished in intensity in the presence of excess triplex DNA (Fig. 4A). These spectral changes are unique to triplex DNA, indicating a mode of interaction that may be different from both G4′-DNA and double-stranded DNA binding. Thermal denaturation experiments of triplex DNA in the presence of DTC show a slight stabilization of the triplex at low DTC concentrations (<1:2 DTC/base triplet, $\Delta T_{m\ Triplex} \sim 3\,^{\circ}C$). At higher DTC concentrations (1:1 DTC/base triplet) the triplex denaturation transition occurs over a broad temperature range, and moves to much higher temperatures ($\Delta T_m \sim 35\,^{\circ}C$) (Fig. 4B). The effect of DTC on the stability of duplex DNA is modest, even at high DTC concentrations ($\Delta T_m \sim -3\,^{\circ}C$).

The identification of DTC as a human telomerase inhibitor may have implications for the design of therapeutic agents. DTC, like other lipophilic cationic carbocyanines, may localize to the mitochondria in cells.[20] This sub-cellular localization of DTC is a liability in terms of the targeting of telomerase, which is present in the nucleus.[21] Suitable modifications of the DTC structure would be required in order to promote nuclear localization. The affinity of DTC for G4′-DNA, its selectivity for G4′-DNA versus triplex DNA, and its ability to inhibit human telomerase are less than optimal for potential therapeutic use. However, the mode of interaction of DTC with G4′-DNA may provide insight into alternative modes of targeting these unusual DNA structures. The G-quadruplex binding telomerase inhibitors that have been previously reported appear to share a common mode of G-quadruplex interaction involving end stack on terminal G-tetrads.[22–24] Shafer and co-workers have proposed that DODC binds to G′2-DNA through interactions with the G-quadruplex DNA grooves.[9] Recently, Mayol and co-workers have reported NMR-based evidence for the binding of two side-by-side distamycin dimers in two opposite grooves of a G4-DNA structure.[25] If DTC binds similarly to G4′-DNA through groove interactions, this ligand could serve as the starting point for the design of a new class of G-quadruplex groove binding telomerase inhibitors.

Acknowledgements

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