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Benzoindoloquinolines Interact with DNA Tetraplexes and Inhibit Telomerase

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Abstract—Telomeric G-rich single-stranded DNA can adopt a G-tetraplex structure which has been shown to inhibit telomerase activity. We have examined benzoindoloquinolines derivatives for their ability to stabilize an intramolecular G-quadruplex. The increase in T_m value of the G-quadruplex was associated with telomerase inhibition in vitro.

Telomerase is active in most human tumor cells and inactive in most somatic cells. Several laboratories have recently synthesized potent inhibitors of this enzyme which is an attractive target for the design of anticancer agents.[1,2] The 3′-terminal region of the G-rich strand of human telomeres is single-stranded and this surprisingly long 3′ overhang may adopt an unusual four-stranded structure involving G-quartets[3] (see Fig. 1A and B). The non-folded single-stranded form of the primer is required for optimal telomerase activity and G-quadruplex formation has been shown to directly inhibit telomerase elongation in vitro.[4] Therefore, a drug that stabilizes quadruplexes could interfere with telomerase and telomere replication. The unique geometry of the G-quadruplex structure allows its specific recognition by small synthetic ligands.[5] The recent discovery that positively-charged aromatic molecules such as amidoanthraquinones,[5,6] ethidiums,[7] amidofluorenones[8] and acridine dyes[9] interact with quadruplex DNA [quadruplex DNA based on guanine-quartets (G4-DNA)] (for a review, see refs 10 and 11) prompted us to test for binding of related compounds to G-quadruplexes. Of particular interest was the observation that tetracyclic or pentacyclic aromatic molecules such as 10*H*-indolo-[3,2-*b*]quinolines[12] or dibenzophenanthrolines[13] were potent inhibitors of telomerase activity

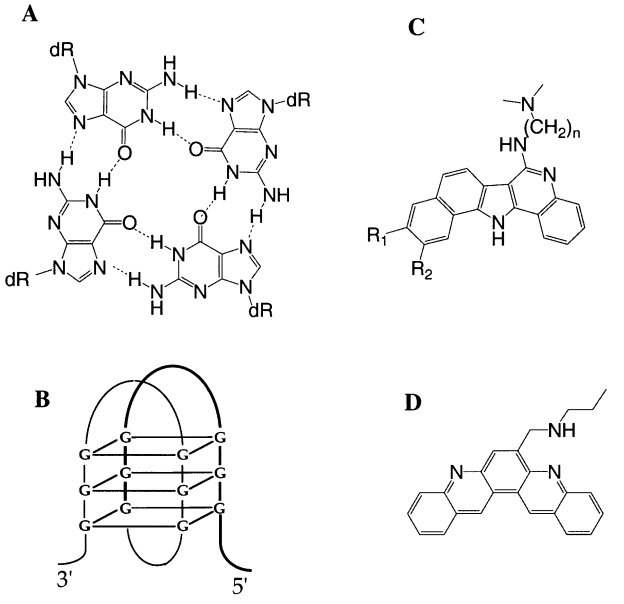

Figure 1. Presentation of the system. (A) A G-quartet involving four guanines. (B) Possible folded structure of the G-rich strand of human telomere. Formation of an intramolecular structure leads to a close proximity of the oligonucleotide 5′ and 3′ ends. The 5′ and 3′ ends of the quadruplex are shown across a diagonal, in agreement with the NMR structure of the human telomeric G-rich repeat.[22] (C) General formula for the BIQ derivatives. **PSI99A**: $n=3$ $R_1=H$, $R_2=NO_2$; **PSI111**: $n=3$ $R_1=H$, $R_2=-OCH_3$; **PSI109**: $n=2$ $R_1=H$, $R_2=-OCH_3$; **SD27**: $n=3$ $R_1=-OCH_3$, $R_2=H$. (D) Formula of a dibenzophenanthroline molecule shown for comparison.

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via a quadruplex-stabilization mechanism. 13*H*-Benzo-[6,7]-indolo[3,2-*c*]quinolines (later referred to as benzoindoloquinolines or BIQ) are pentacyclic aromatic molecules that carry a dimethylamino substituted side chain which is susceptible to protonation[14] (Fig. 1C and D) and were therefore good candidates for quadruplex recognition. The synthesis of **SD27**, **PSI99A** and **PSI111** has been described previously.[14,15] **PSI109** was prepared according to the following protocol: the reaction of 6-chloro-11-methoxy-13*H*-benzo[6,7]indolo[3,2-*c*]quinoline with *N*,*N*-dimethylethylenediamine according to established procedure[14] yielded the free base of the title compound [73% yield; mp 172 °C. ^{1}H NMR (200 MHz, DMSO-d_6, 25 °C); δ: 12.83 (br s, 1H), 8.50 (d, J = 7.9 Hz, 1H), 8.25 (m, 2H), 8.04 (d, J = 9.0 Hz, 1H), 7.74 (m, 2H), 7.54 (m, 1H), 7.40 (m, 1H), 7.25 (dd, J = 8.8 and 2.4 Hz, 1H), 6.70 (br s, 1H), 4.08 (s, 3H), 3.85 (m, 2H), 2.75 (m, 2H), 2.37 (s, 6H). Analysis ($C_{24}H_{24}N_4O$ $(H_2O)_{0.67}$; calcd C 72.70, H 6.44, N 14.13; found: C 72.83, H 6.22, N 13.93].

The secondary structure of guanine-rich oligodeoxynucleotides may be investigated with fluorescent probes. Fluorescence is a technique that may give valuable information on nucleic acid structures and interactions. Fluorescence can be used to probe the secondary structure of a quadruplex-forming oligodeoxynucleotide provided a fluorescein molecule and a DABCYL (quencher) derivative are attached to its 5′ and 3′ ends, respectively. Such a strategy is reminiscent of 'molecular beacons'.[16] The oligonucleotide chosen for this assay represents 3.5 repeats of the human telomeric motif. The melting of the G-quadruplex is monitored in the presence of putative G-quadruplex-binding molecules by measuring the fluorescence emission of the donor. A quadruplex-specific ligand should increase the melting temperature (T_m) of the quadruplex, displacing the equilibrium single-strand <–> quadruplex to the right. This is a convenient and straightforward method to investigate G4 ligands.[7,13] A fixed 1 μM dye concentration was chosen based on data available for other series of molecules.[13] The sequence of 21G is 5′ GGGTTAGGGTTAGGGTTAGGG. F21GMB is a doubly labelled 21G oligonucleotide, with fluorescein at the 5′ end and DABCYL at the 3′ end, synthesized and purified by Eurogentec (Belgium). The series of BIQ compounds was shown to increase the melting temperature of the G-quadruplex by 3–11 °C (1 μM dye), indicating a moderate to high affinity for quadruplexes as compared to single strands.

To confirm that these compounds interact with G4 DNA, we recorded the spectroscopic properties of these molecules in the presence or in the absence of a DNA quadruplex. All titrations were performed at 20 °C in a 0.1 M KCl pH 7.2 cacodylate buffer. BIQs have a maximum absorbance wavelength in the far ultraviolet (UV) region (261 nm for **PSI99A**; 269 nm for **PSI109** and **PSI111**, 271 nm for **SD27**) as well as a secondary minor peak or a shoulder around 360 nm. The absorbance spectrum of **SD27** (20 μM) was affected in the presence of 22AG (d[A($G_3T_2A)_3G_3$]) (not shown) demonstrating that an interaction with the G-quadruplex-forming oligonucleotide occurred. A significant hyperchromicity (45%) was observed associated with a slight redshift (from 363 to 368 nm, i.e., 5 nm). The variation of the absorbance spectrum levels off at 20 μM oligonucleotide concentration (1:1 molar ratio).

SD27 is strongly fluorescent (max emission at 387 nm), contrary to the three other compounds (quantum yields are less than 5% of that of **SD27**). This observation prevented us from performing quantitative fluorescence titrations with 21G for the other dyes. Nevertheless, sequential addition of a concentrated stock solution of 21G to a 1 μM **SD27** solution led to important modifications of the fluorescence emission spectrum (Fig. 2). The absence of an isomemissive point allowed us to conclude that at least two different bound forms of the dye were present. The lowered emission intensity obtained at low G4-DNA concentration suggests the formation of dye aggregates on the quadruplex. At higher DNA/**SD27** ratios, a strong increase in fluorescence and an important modification of the shape of the spectrum are observed. The emission spectrum is only marginally affected by further additions of quadruplex DNA and could correspond to individual dye molecules—as opposed to dimers or aggregates—bound to G4-DNA.

Furthermore, the BIQ derivatives not only bind to preformed quadruplexes, but also accelerate the formation of quadruplexes starting from single strands, as demonstrated by an acrylamide-based electrophoresis assay.[17] The purified TR2 oligonucleotide (TACAGATAGTT-AGGGTTAGGGTTA) was 5′-end labelled and used at a final strand concentration of 8 μM. The solution was heated to 95 °C for 5 min in a TE buffer containing 0.1 M KCl and slowly cooled to room temperature. BIQ derivatives were added (20 μM), reaction mixtures were then incubated for 1 h at room temperature and loaded on a native 12% acrylamide vertical gel in a 0.5× TBE

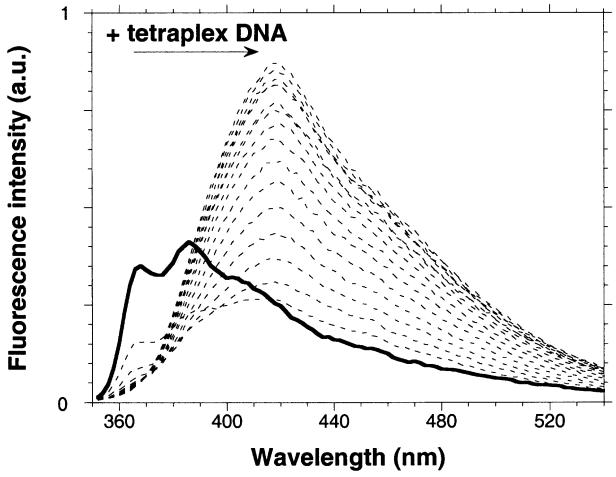

Figure 2. Fluoresence titration of **SD27** with the G-rich strand. Titration was performed at 20 °C. Buffer conditions: 10 mM sodium cacodylate, 100 mM KCl, pH 7.5. Solid line: dye alone (1 μM). Dashed lines: **SD27** + 21G oligonucleotide (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.4, 1.6, 1.8, 2.0 μM strand concentration). Excitation was set at 274 nm. All measurements were performed as previously described.[13,23,24] Titrations were obtained at 20 °C with a Spex fluorolog instrument.[24]

Table 1. Summary of the data

Compd[a]	ΔT_m (G4)[b] (°C)	IC_{50} (TRAP)[c] (μM)	IC_{50} (Taq)[d] (μM)	G4 band[e] (%)	$\lambda_{max}F$[f] (nm)	$\lambda_{max}B$[f] (nm)
PSI99A	+10.5	0.5	5	33	445	443
PSI111	+11	1.1	2.2	27	426	460
PSI109	+3	9.8	>10	30	456	458
SD27	+10	1.0	0.9	14	387	424

[a]General formula shown in Figure 1C.
[b]Stabilization of the F21GMB oligonucleotide (0.2 μM) measured by FRET, for a dye concentration of 1 μM in a 10 mM pH 7.2 sodium cacodylate buffer with 0.1 M LiCl. T_m of F21GMB alone = 43 °C under these conditions.
[c]IC_{50} against telomerase.
[d]IC_{50} against Taq polymerase. This value reflects the unspecific (and undesired) interaction of the dye with DNA duplexes and/or with the polymerase used for the amplification step of the TRAP assay. No inhibition of Taq polymerase was observed with **PSI109** at 10 μM dye concentration.
[e]Accelerated assembly of G-quadruplexes, as shown by non-denaturing polyacrylamide gel electrophoresis.[7,17] % corresponds to the fraction of radioactivity the migration of which corresponds to the quadruplex species formed in the presence of 20 μM BIQ derivative. No quadruplex was obtained in the absence of a BIQ compound.
[f]Position of emission maximum. F = free, B = bound to quadruplex DNA (21G). λ_{exc} = 360 nm.

buffer supplemented with 20 mM KCl. The gel was run at 4 °C for 6 h, dried and analyzed with a Phosphorimager. Table 1 summarizes the data obtained at 20 μM dye concentration. Quadruplex formation is efficiently promoted by all derivatives, although to a different quantitative extent. The most efficient compound is **PSI99A**, whereas **PSI109** is suprisingly effective in view of its poor G4 stabilization. One should remember that this accelerated assembly is measured at a relatively high BIQ concentration and that other factors such as dye aggregation or dimerization could also play a role.

Once G4 stabilization and induction had been established, it was necessary to test whether these molecules inhibit telomerase. A number of small ligands have been discovered to inhibit the function of telomerase by stabilizing G4-DNA structures. These ligands lock a telomeric G-overhang into a folded conformation which cannot be extended by telomerase. The ΔT_m effect was therefore compared to telomerase inhibition efficiency measured by a modified telomerase repeat amplification protocol (TRAP) assay.[7,18] **PSI99A** is a potent inhibitor of telomerase (IC_{50} = 0.5 μM) but a weak inhibitor of Taq polymerization (IC_{50} = 5 μM, a 10-fold higher concentration, Table 1). On the other hand, **PSI109** very poorly inhibits telomerase in agreement with its lower G4-stabilization potency. The situation is a bit more complicated for the two remaining dyes, **PSI111** and **SD27**. These two compounds are active in the TRAP assay (IC_{50} = 1.1 and 1.0 μM, respectively) but in a rather unspecific manner, as Taq polymerase is also affected in a similar concentration range (Taq IC_{50} = 2.2 and 0.9 μM, respectively). Judging from these values, the inhibition of telomerase observed with **SD27** appears to be partially or totally artefactual, due to interference with the amplification step of the TRAP assay. **PSI111** exhibits some specificity towards telomerase (IC_{50} 1.1 vs 2.2 μM).

The low specificity of some BIQ towards telomerase inhibition (as compared to Taq polymerase inhibition) prompted us to evaluate the specificity of these compounds for quadruplexes with a competitive dialysis experiment. Different nucleic acids structures are dialyzed against a common ligand solution.[19] More ligand accumulates in the dialysis tube containing the structural form with the highest ligand binding affinity. DNA structural forms included in the assay range from single strands (poly dA), through a variety of duplexes, to a multistrand triplex (poly dA.2poly dT), as well as tetra-

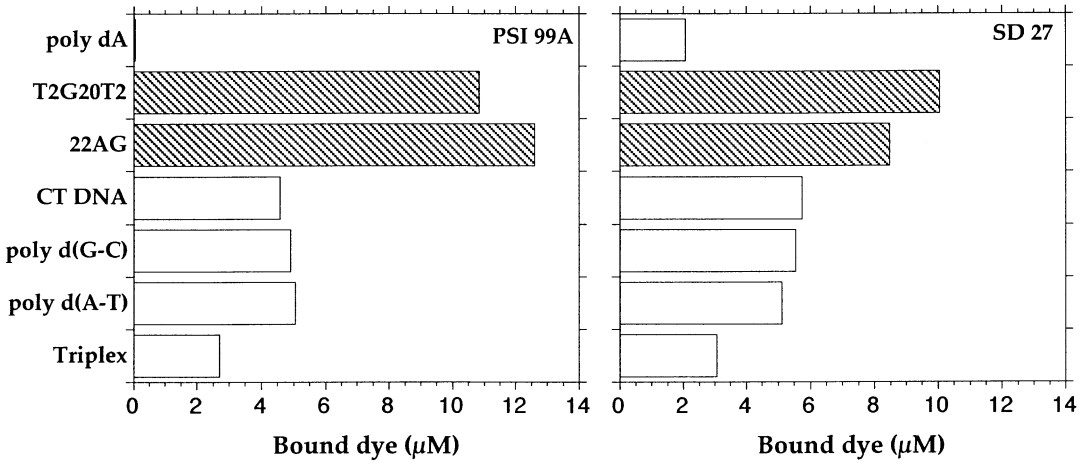

Figure 3. Equilibrium dialysis. Left: **PSI99A**; right: **SD27**. All measurements were performed using a methodology adapted from Chaires and colleagues.[19,20,25] The nucleic acid names are given on the left. $T_2G_{20}T_2$ and 22AG form G-quadruplexes. Chosen buffer: 0.185 M NaCl, 15 mM Na cacodylate pH 6.5 $MgCl_2$ 10 mM. Different nucleic acid (75 μM) were tested against a **PSI99A** or **SD27** solution (1 μM dye) in our dialysis buffer. Hatched bars: quadruplexes; open bars: other structures. Triplex corresponds to poly dA-2.poly dT.

plex forms. Among these samples, two correspond to G-quartet-containing motifs: $(T_2G_{20}T_2)_4$, which forms a parallel-stranded quadruplex and 22AG which forms a folded antiparallel quartet structure. As shown in Figure 3, left, **PSI99A** preferentially interacts with quadruplex-forming DNA samples compared to all other nucleic acids. On the other hand, **SD27** exhibits less structure specificity (Fig. 3, right). This reduced specificity might be related to the low specific TRAP/Taq inhibition index obtained with that compound.

In conclusion, using a fluorescence resonance energy transfer (FRET) method, we have identified a new family of G4-ligands, the benzoindoloquinolines. Evidence for recognition of G4-DNA is also provided by absorbance and fluorescence spectroscopy. Equilibrium dialysis confirms that one of the BIQ derivatives interact preferentially, but not exclusively, with four-stranded structures. This technique is helpful to determine the relative affinities of ligands for a variety of DNA and RNA structures.[20] Figure 3 shows that BIQ derivatives interact with quadruplexes; they also bind to duplexes but very weakly to single strands. External stacking on a terminal G-quartet is a possible mode of interaction, because of the resulting strong π–π stacking between the guanine tetrad and the ligand. However, we cannot exclude that these dyes interact with the G4 structure via the grooves or the connecting loops. Structural methods, such as NMR spectroscopy, will be required to answer this question. The best compound has an IC_{50} of 0.5 μM, which is relatively modest compared to the most potent dibenzophenanthroline ($IC_{50} = 28$ nM). However, this dibenzophenanthroline derivative has two long lateral side chains which can become charged by protonation, whereas **PSI99A** has only one central alkylamine chain. Concerning the dibenzophenthrolines bearing the same number of chains (Fig. 1D), an IC_{50} of 0.75–2 μM was reported for inhibition of telomerase. It will be interesting to synthesize new BIQ derivatives in order to test whether an increase in the number of lateral groups in the BIQ series also provides an increase in telomerase inhibition as recently shown by Read et al.[21]

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