Telomerase inhibitors based on quadruplex ligands selected by a fluorescence assay

Jean-Louis Mergny*†, Laurent Lacroix*‡, Marie-Paule Teulade-Fichou§, Candide Hounsou§, Lionel Guittat*, Magali Hoarau*, Paola B. Arimondo*¶, Jean-Pierre Vigneron§, Jean-Marie Lehn§, Jean-François Riou‖, Thérèse Garestier*, and Claude Hélène***

*Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, Institut National de la Santé et de la Recherche Médicale Unité 201, Centre National de la Recherche Scientifique Unité Mixte 8646, 43 Rue Cuvier, 75005 Paris, France; §Laboratoire de Chimie des Interactions Moléculaires, Collège de France, Centre National de la Recherche Scientifique Unité Propre de Recherche 285, 11, Place Marcelin Berthelot, 75005 Paris, France; and ‖Centre de Recherche de Vitry-Alforville, Aventis, 94400 Vitry-sur-Seine Cedex, France

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The reactivation of telomerase activity in most cancer cells supports the concept that telomerase is a relevant target in oncology, and telomerase inhibitors have been proposed as new potential anticancer agents. The telomeric G-rich single-stranded DNA can adopt *in vitro* an intramolecular quadruplex structure, which has been shown to inhibit telomerase activity. We used a fluorescence assay to identify molecules that stabilize G-quadruplexes. Intramolecular folding of an oligonucleotide with four repeats of the human telomeric sequence into a G-quadruplex structure led to fluorescence excitation energy transfer between a donor (fluorescein) and an acceptor (tetramethylrhodamine) covalently attached to the 5′ and 3′ ends of the oligonucleotide, respectively. The melting of the G-quadruplex was monitored in the presence of putative G-quadruplex-binding molecules by measuring the fluorescence emission of the donor. A series of compounds (pentacyclic crescent-shaped dibenzophenanthroline derivatives) was shown to increase the melting temperature of the G-quadruplex by 2–20°C at 1 μM dye concentration. This increase in T_m value was well correlated with an increase in the efficiency of telomerase inhibition *in vitro*. The best telomerase inhibitor showed an IC_{50} value of 28 nM in a standard telomerase repeat amplification protocol assay. Fluorescence energy transfer can thus be used to reveal the formation of four-stranded DNA structures, and its stabilization by quadruplex-binding agents, in an effort to discover new potent telomerase inhibitors.

telomere | DNA structure | G-quartet

Telomerase was first identified in ciliates (1). This enzyme is an essential factor in immortalization and tumorigenesis (2–4). Furthermore, telomerase is active in most human tumor cells and inactive in most somatic cells and is therefore an attractive target for the design of anticancer agents. Most human telomeric DNA is double-stranded and contains $(TTAGGG/CCCTAA)_n$ repeats except for the extreme terminal part where the 3′ region of the G-rich strand is single-stranded (5). For human cells, these 3′ overhangs are surprisingly long (averaging 130–210 bases in length), exist during most of the cell cycle, and are present on all chromosomal ends. The G-rich single-stranded DNA can adopt an unusual four-stranded DNA structure involving G-quartets (6–8) (see Fig. 1) or it might fold back and displace one strand of a telomeric duplex to form a so-called T-loop (9). Optimal telomerase activity requires the nonfolded single-stranded form of the primer and G-quartet formation has been shown to directly inhibit telomerase elongation *in vitro* (10). Therefore, a drug that stabilizes quadruplexes could interfere with telomerase and telomere replication (11–13).

The peculiar geometry of the quadruplex structure should allow its specific recognition by small synthetic ligands, and previous experiments have shown that this assumption is correct (14). Many of the G4 ligands were shown to have antitelomerase activity *in vitro*, with IC_{50} in the low micromolar range (15). Fluorescence resonance energy transfer (FRET) has given valuable information on the structure of nucleic acids (17, 18), because of its distance and orientation dependence (19). Concerning multistranded nucleic acids structures, FRET has been successfully applied to triple helices (20, 21), C-rich quadruplexes (22) (the so-called i-motif) and a *c*-myc G-rich repeat (23).

FRET can be used to probe the secondary structure of oligodeoxynucleotides mimicking repeats of the guanine-rich strand of vertebrate telomeres, provided a fluorescein (donor) molecule and a tetramethylrhodamine (acceptor) derivative are attached to the 5′ and 3′ ends of the oligonucleotide, respectively. The melting temperature of such fluorescent oligonucleotides was then measured in the presence of different molecules. A family of crescent-shaped planar aromatic molecules was shown to specifically interact with an intramolecular quadruplex. The ligand-induced stabilization of the quadruplex showed a good correlation with an increased inhibition of telomerase activity.

Experimental Procedures

Oligonucleotides. All oligonucleotides and their fluorescent conjugates were synthesized and purified by Eurogentec, Brussels. The primary sequences and names of the fluorescent oligonucleotides are given in Fig. 1 *Middle*. dS26 is a self-complementary oligonucleotide.

Compounds. The synthesis of crescent-shaped dibenzophenanthrolines **1**-**6** has been described (24). Compound **8** was reported in a previous article (25). The synthesis of compounds **7** and **9** is briefly described as follows:

6-[(2-(Piperidin-1-yl)ethyl)aminomethyl]dibenzo[*b*,*j*] (4,7)phenanthroline (compound **7***)* was synthesized following the procedure described for compounds **2** and **3** (25): 6-bromomethyl dibenzo[*b*,*j*] (4,7)phenanthroline (130 mg, 0.35 mmol) was stirred with 1-(2-aminoethyl)piperidine at 60°C for 30 min. The mixture was cooled, water was added, and the aqueous solution was extracted with CH_2Cl_2. After drying over Na_2SO_4, the solvent was removed under vacuum to give a brown oil. HCl (1 M) and H_4folate were added and the precipitate was filtered,

Abbreviations: FRET, fluorescence resonance energy transfer; G4-DNA, quadruplex DNA based on guanine quartets; TRAP, telomerase repeat amplification protocol.

†To whom reprint requests should be addressed. E-mail: mergny@vnumail.com.

‡Present address: Groupe de Biophysique, Ecole Polytechnique, 91128 Palaiseau, France.

¶Present address: UMR 176 Centre National de la Recherche Scientifique, Institut Curie, Paris, France.

**Present address: Centre National de la Recherche Scientifique, FRE 2141, Université de Reims, Reims, France.

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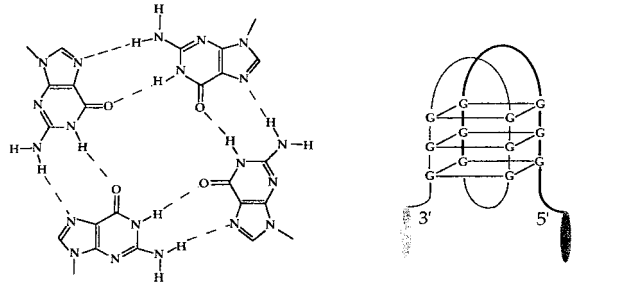

Oligonucleotide name	Sequence
F21T	fluo-GGGTTAGGGTTAGGGTTAGGG-tamra
22A	AGGGTTAGGGTTAGGGTTAGGG
28G	GGGGTTTTGGGGTTTTGGGGTTTTGGGG
TRr2	TCAGATAGTTAGGGTTAGGGTTA

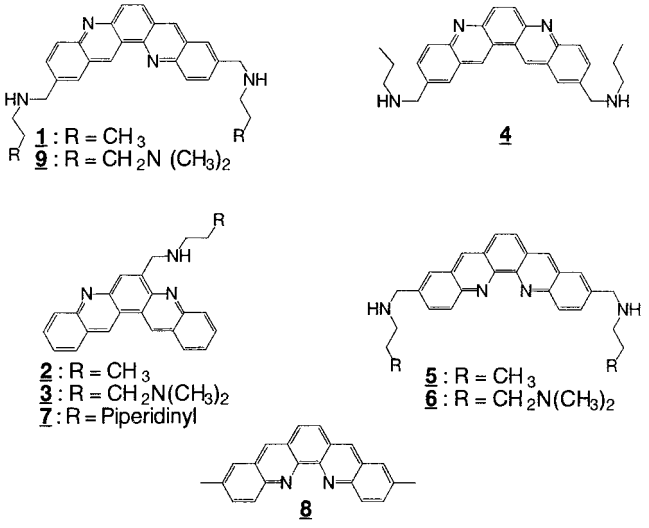

Fig. 1. (*Top*) G-quartet (*Left*) and an intramolecular quadruplex (*Right*). The 5′ fluorescein and 3′ tetramethylrhodamine groups are depicted by dark gray and light gray ovals, respectively. (*Middle*) List of the oligonucleotides used in the present study. Abbreviations used for the fluorescent oligonucleotide are indicated, with the following convention: the dye is written first if it is linked to the 5′ end of the oligonucleotide; when linked to the 3′ end, it is written last. fluo: fluorescein; tamra: tetramethylrhodamine. (*Bottom*) Chemical formula of dibenzophenanthroline derivatives tested as G4 ligands.

yielding 108 mg (43%) of a pale yellow powder. ^{1}H NMR (200 MHz, CD_3OD): δ = 1.75 (br s, 2H), 1.96 (t, 4H), 3.25 (br s, 4H), 3.73 (t, 2H), 3.94 (t, 2H), 5.17 (s, 2H), 7.81 (td, 1H), 7.95 (td, 1H), 7.99 (td, 1H), 8.16 (td, 1H), 8.30 (d, 2H), 8.47 (d, 1H), 8.49 (d, 1H), 8.55 (s, 1H), 9.96 (s, 1H), 10.36 (s, 1H) ppm.

2,10-Di[(3-dimethylaminopropyl)aminomethyl]dibenzo[*b,j*] (1,7)phenanthroline (compound **9**) was synthesized following the procedure described for compound **1** (25): dibenzo*[b,j]* (1,7) phenanthroline-2,10-dicarboxaldehyde (150 mg, 0.446 mmol) was dissolved in CH_2Cl_2/CH_3OH (4/1), 3-dimethylaminopropylamine (3.65 ml, 29 mmol) was then added dropwise, and the mixture was stirred overnight. The solvents were distilled off. The solid residue was thoroughly washed with pentane and then dried. The crude diimine (181 mg) was dissolved in methanol (50 ml) and cooled to 0°C. $NaBH_4$ (66 mg, 1.8 mmol) was added and the solution was stirred at 0°C for 2 h, then at room temperature for 30 min. The solvents were distilled off, water was added, and the product was extracted with dichloromethane. The organic layer was dried over $MgSO_4$ and evaporated. The crude extract then was dissolved in a solution of HCl in CH_3OH and precipitated with H_4folate. The precipitate was collected and recrystallized from CH_3CH_2OH/H_4folate, yielding 52 mg (28%) of a yellow-green powder. ^{1}H NMR (200 MHz, D_2O): δ = 2.15 (m, 4H), 2.8 (s,12H), 3.18 (m,8H), 4.43 (s,2H), 4.53 (s, 2H), 7.76 (d, J = 10Hz, 1H), 7.85 (d, J = 10Hz, 1H), 8.06 (s, 3H), 8.15 (d, J = 9Hz, 1H), 8.31 (d, J = 9.5 Hz, 1H), 8.46 (s, 1H), 8.8 (s, 1H), 10.17 (s, 1H) ppm.

UV Absorption Studies. Spectra were obtained with a Kontron (Zurich) Uvikon 940 spectrophotometer as described (22).

Fluorescence Studies. All measurements were made as described (16, 37).

Assay of Telomerase Activity. Telomerase activity was assayed by using a modified telomerase repeat amplification protocol (TRAP) assay (26) adapted to scintillation proximity assay. An aliquot of 10^5 A549 cells was used as source of telomerase. The TRAP reaction mix was added to the compound and 200 ng of telomerase extract, in a final volume of 50 μl in PCR microplates. After amplification, the telomerase products were transferred into 96-well isoplates (Wallac, Gaithersburg, MD) and 150 μl of streptavidin beads (RPNQ0006, Amersham Pharmacia) at 3.3 mg/ml in 0.53 M NaCl were added to the reaction products and shaked for 30 min at room temperature to allow the binding of streptavidin to biotinylated molecules. Microplates were counted by using a Microbeta Trilux multidetector (Wallac).

***Taq* Polymerase Assay.** The specificity of G4 ligands was assayed against the *Taq* polymerase reaction by using the polylinker from plasmid pCDNA1 as a DNA template. The reaction mixture containing 25 mM *N*-tris[hydroxymethyl]-methyl-3-amino propane sulfonic acid (TAPS) (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 μg/ml pCDNA1, 15 pmol Sp6 primer and 5′ biotinylated T7 primer, 0.2 mM of each dNTP, 32 units/ml *Taq*, and 0.4 μCi [^{3}H]dGTP was added to the compound solution in a final volume of 50 μl in PCR microplates. The reaction was incubated in a thermocycler for 2 min at 92°C followed by 30 cycles of amplification. Samples were treated by streptavidin beads and counted as described for telomerase reaction.

Results

Identification of G4-Specific Ligands. The sequence of all oligonucleotides is shown in Fig. 1. Oligonucleotide F21T has a fluorescence energy donor (fluorescein) attached to the 5′ end and an acceptor (tetramethylrhodamine) attached to the 3′ end. In the unfolded form, little transfer is expected, as the average distance of the two chromophores is larger than the Förster critical distance (calculated to be around 5.0 nm). Intramolecular folding into a G-quadruplex brings the two chromophores in close enough proximity to observe energy transfer. Such a strategy is reminiscent of "molecular beacons" (27). Different oligonucleotides (21–26 bases long) have been tested, and a complete description of these oligonucleotides will be presented elsewhere. For comparison purposes, we chose the F21T oligonucleotide (Fig. 1). In a 10 mM sodium cacodylate buffer containing 0.1 M lithium chloride, a melting temperature of 43.3°C was determined by monitoring the quenching of the donor emission at 515 nm, and a similar melting temperature was observed by using UV-absorbance spectroscopy. Known quadruplex ligands, such as 3,3′ diethyloxadicarbocyanine iodide (28) and a 2,6-disubstituted diamidoanthraquinone derivative (14) gave moderate, but significant, stabilization (+4°C, not shown) when added at a final concentration of 1 μM. In this study, different cationic molecules have been tested for their

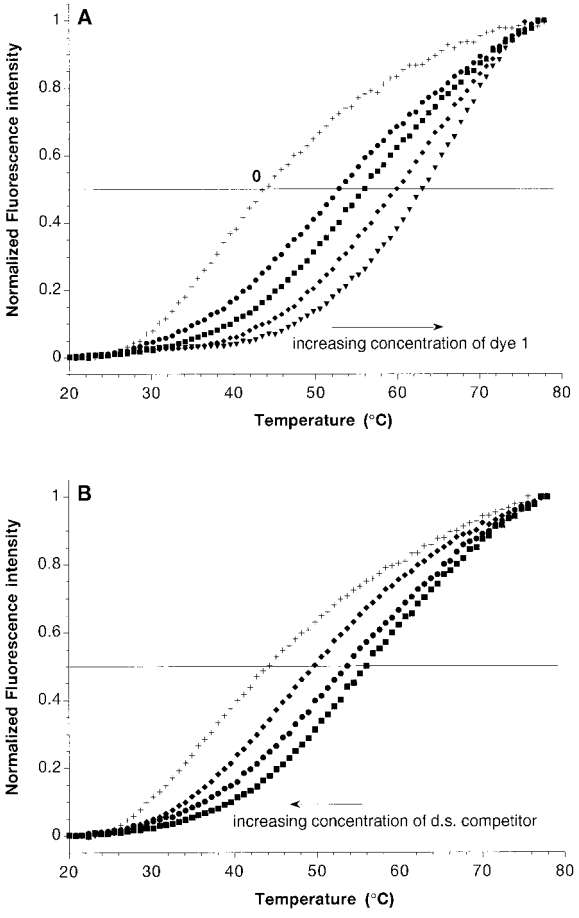

Fig. 2. (*A*) Melting behavior of a fluorescent G-rich oligonucleotide (F21T, 0.2 μM strand concentration) alone (crosses) or in the presence of compound **1** (●, 0.5 μM; ■, 1 μM; ◆, 2 μM,; ▲, 3 μM). Excitation was set at 470 nm, and fluorescence emission (arbitrary units) was recorded at 515 nm. Buffer was 0.1 M LiCl, 10 mM sodium cacodylate, pH 7.3. (*B*) Melting behavior of a G-rich oligonucleotide (F21T, 0.2 μM strand concentration) in the presence of a 100× molar excess of a double-stranded oligonucleotide (crosses) but in the absence of any ligand (the curve is nearly identical to that observed in the absence of double-stranded DNA, see *A*). The three other curves were obtained in the presence of compound **1** (1 μM) with no competitor (■) or a 43× excess of dS26, a double-stranded competitor (●) or a 100× excess (◆). Excitation was set at 470 nm, and fluorescence emission was recorded at 515 nm. Buffer was 0.1 M LiCl, 10 mM sodium cacodylate, pH 7.3.

effect on the melting temperature of a quadruplex-forming oligonucleotide. Planar aromatic chromophores are key structural features in many G4-based telomerase inhibitors. For this reason, nine different dibenzo[*b*,*j*]phenanthroline derivatives, whose formula are shown in Fig. 1 *Bottom* were compared.

A 10 mM sodium cacodylate buffer containing 0.1 M lithium chloride was used to monitor the thermal stability of the G-quartet. These conditions, somewhat less favorable to quadruplex formation, were chosen to detect more easily a stabilization by an interacting ligand. We selected a F21T oligonucleotide strand concentration of 0.2 μM and variable dye concentrations between 0.5 and 3 μM (see Fig. 2*A*). For compound **1**, a concentration-dependent increase in melting temperature of F21T was obtained. In all further experiments a typical dye concentration of 1 μM was chosen. To observe a significant stabilizing effect, dissociation constants in the low or submicromolar range are required. It should be noted that the fluorescence test is sensitive enough for the oligonucleotide concentration to be decreased 1,000 times (data not shown), and

Table 1. Stabilization of G-quadruplexes and inhibition of telomerase activity by dibenzophenanthroline derivatives

Compound no. (see Fig. 1)	ΔT_m G4 (FRET), 1 μM dye	IC_{50} telomerase, μM
1	11.5	0.3
2	5.5	1.45
3	9.5	0.75
4	11.5	1
5	12.5	0.5
6	10	0.9
7	2.5	2
8	4.5	>10
9	19.7	0.028

The compounds used in this study are shown in Fig. 1 *Lower.* The stabilization (in °C) was determined from fluorescence emission measurements of the F21T oligonucleotide (0.2 μM strand concentration + 1 μM compound in a 0.1 M LiCl, 10 mM sodium cacodylate pH 7.3 buffer; λ_{exc} = 470 nm; λ_{emi} = 515 nm). The concentration that gave 50% inhibition of telomerase by TRAP assay is given in μM. Compound **8** gave no inhibition at 10 μM concentration.

if the tested dye concentration is concomitantly decreased by the same factor, one should be able to select ligands with nanomolar dissociation constants. Finally, it should be noted that the same test may be performed in the presence of a large excess of a double-stranded nonfluorescent DNA competitor, allowing to select ligands that show preference for quadruplex vs. duplex structures (Fig. 2*B*). The results shown in Fig. 2*B* suggest that the binding of **1** to a quadruplex is at least 40-fold higher than that to a duplex. The addition of a 43 × molar excess of base pairs induces a decrease in stabilization (Fig. 2*B*, ●), which was slightly less pronounced than the decrease in stabilization obtained upon lowering the concentration of compound **1** from 1 μM (Fig. 2*A*, ■) to 0.5 μM (Fig. 2*A*, ●).

Compounds **1–9** were compared at 1 μM dye concentration, and the results are summarized in Table 1. These ΔT_m values were significantly higher than those obtained with reference compounds (+4°C). The best ligand **9** gave a ΔT_m of +19.7°C, followed by **5,** which gave a ΔT_m of +12.5°C, whereas compound **7** gave a ΔT_m of only +2.5°C. A number of small molecules have been discovered to inhibit the function of telomerase by stabilizing quadruplex DNA (G4-DNA) (29, 30). The ΔT_m effect therefore was compared with telomerase inhibition efficiency. The results are shown in Table 1, and the relation between telomerase inhibition efficiency and ΔT_m is visualized in Fig. 3. The compounds that efficiently inhibited telomerase *in vitro* ($IC_{50} < 1$ μM for **1**, **3**, **4**, **5**, **6,** and **9**) all stabilized G4-DNA by more than 9°C, whereas less effective inhibitors (**2** and **7**) have a lower stabilizing effect. Among this family of compounds, only **8** is completely inactive at inhibiting telomerase; **8** is also the only compound that has no positive charge at neutral pH. The best stabilizer (compound **9**, +19.7°C) is by far the best telomerase inhibitor (IC_{50} of 0.028 μM). Finally, none of the molecules **1**-**8** inhibited *Taq* polymerase at 1 μM concentration (data not shown), showing that the net inhibition obtained in the TRAP assay is the result of telomerase inhibition rather than a trivial inhibition of the amplification step of the test. Compound **9** somewhat inhibited *Taq* (IC_{50} of 0.8 μM) at a concentration 30× higher than the one required for telomerase inhibition, showing that the observed inhibition of telomerase is not an artifact due to interference with the assay itself.

Further Characterization of G4 Ligands. We tried different approaches to further characterize the interaction of dibenzophenanthrolines with quadruplex DNA. Binding of **1**, **5,** and **9** to quadruplex DNA was confirmed in the presence of physiological concentrations of potassium and sodium. We performed a

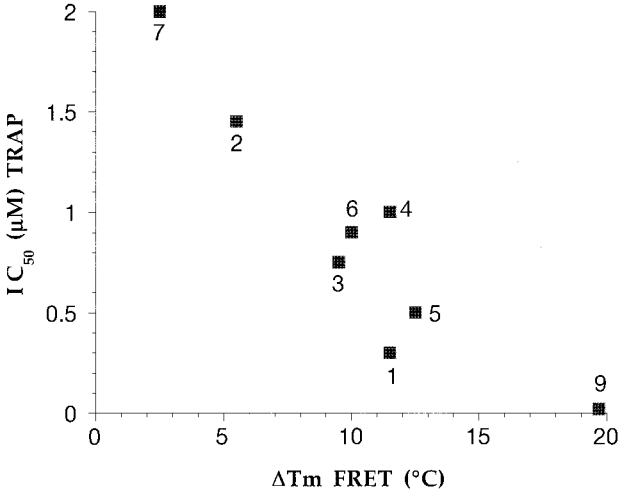

Fig. 3. Correlation between telomerase inhibition (*y* axis; expressed as the concentration necessary to obtain 50% inhibition of telomerase activity in a standard TRAP assay) and G4 stabilization (*x* axis; expressed as ΔT_m of the F21T oligonucleotide). The ΔT_m was measured at 0.2 μM F21T strand concentration, with 1 μM ligand, in a 0.1 M LiCl, 10 mM sodium cacodylate, pH 7.3 buffer. Average of at least two independent experiments. The compound number is indicated for each point. Compound **8** was excluded from this graph as it exhibited no antitelomerase activity at the highest concentration tested (10 μM).

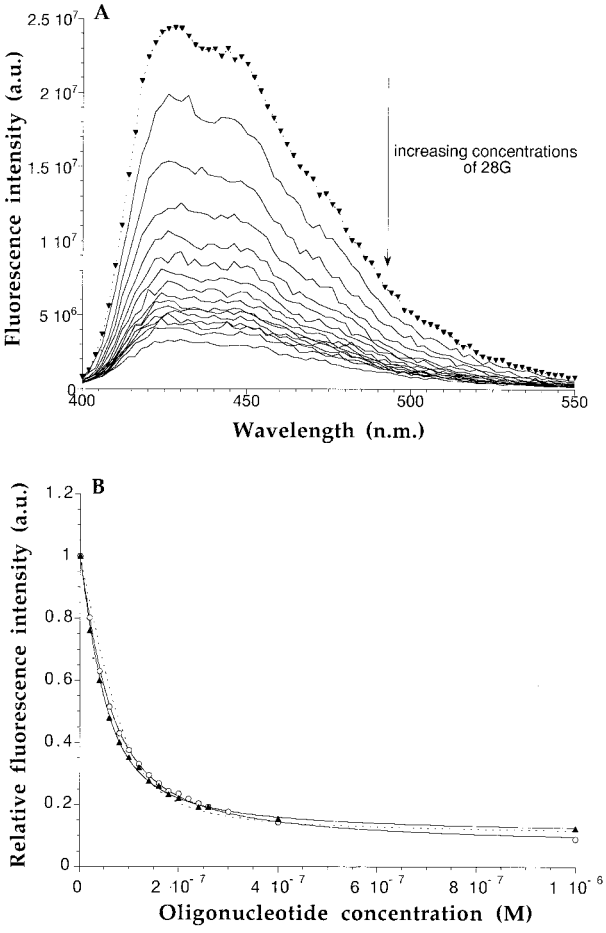

Fig. 4. (*A*) Fluorescence emission spectra of **1** (0.1 μM) in the absence (▼) or in the presence of increasing concentrations of a G4-DNA oligonucleotide (28G: 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 300, and 400 nM). The titration was performed at room temperature, using 1 cm path-length quartz cells, in a 10 mM sodium cacodylate, 0.1 M KCl, pH 7.3 buffer. (*B*) Titration curve of the total fluorescence emission of **1** as a function of G4-oligonucleotide concentration (●: 28G; ▲: 22A). The best fits for both titrations assuming two independent binding sites are shown as solid lines. The best fit for the titration by 28G assuming a single binding site is shown as a dotted line.

UV-visible absorbance titration of **1**, **5,** and **9** by increasing concentrations of 22A, an unmodified 22-base-long oligonucleotide whose sequence is shown in Fig. 1. The spectral properties of dibenzophenanthrolines were altered when they interacted with G4-DNA; thus absorbance spectra in the near UV region can provide useful information. The maximum absorption peak of compound **1** (10 μM) was shifted from 327 (328 nm for **9**) to 332 nm upon addition of 22A (5 or 10 μM strand concentration, not shown). A 35–38% decrease in molar absorption coefficient was concomitantly observed. No further changes in absorbance were observed between 5 and 10 μM oligonucleotide concentration. Binding was already complete at 5 μM, and because the concentration of the dye was 10 μM, this result showed that at least two dye molecules could bind to the same oligonucleotide. It is important to note that the experimental conditions of this titration experiment were favorable to quadruplex formation because potassium was present at 0.1 M concentration. In all cases, two binding sites per quadruplex-forming oligonucleotide were detected. Little, if any, modification of the absorbance properties of the dyes was obtained on addition of single-stranded DNA, again arguing against binding to single-stranded DNA (data not shown).

We then investigated whether the fluorescence properties of **1**-**9** were strongly affected by G-quadruplex binding. In these experiments, binding was tested with nonfluorescent oligonucleotides such as 22A and 28G. The fluorescence emission of **1** was almost completely quenched in the presence of a large excess of G4-DNA (28G oligonucleotide, Fig. 4*A*). This variation of fluorescence properties (shown in Fig. 4*B*) allowed us to determine a dissociation constant of $8 \pm 1 \times 10^{-8}$ M and $6 \pm 2 \times 10^{-8}$ M for the complex with 22A and 28G, respectively, with two independent and identical binding sites per oligonucleotide. The data could not be fitted with a single binding site per oligonucleotide (Fig. 4*B*, dashed line). The rather large uncertainty of this K_d is the result of three different technical difficulties encountered while working with this family of molecules: (*i*) aggregation, (*ii*) photodegradation of the dye, and (*iii*) adsorption of the dye to the quartz cuvettes. The titration experiment therefore was repeated several times to obtain reliable results. The stoichiometry (2 dyes/oligo) confirms the results provided by the absorbance titration: two high affinity binding sites are present on each oligonucleotide (22A and 28G). As for the absorbance titration, the fluorescence titration was performed under experimental conditions highly favorable to quadruplex formation (0.1 M KCl), arguing against binding to the single-stranded form of 22A. As expected, the affinity of compound **9** for 22A and 28G was even higher ($K_d \approx 1 \pm 0.1 \times 10^{-8}$ M, not shown).

Finally, compounds **1** and **5**, but not **7** and **8**, were able to promote intermolecular G4-DNA formation in an electrophoresis test recently described by Han *et al.* (31). Under these conditions, bimolecular quadruplex formation is slow, and little, if any, quadruplex structure is obtained in the absence of a G4 ligand. Adding increasing amounts of compound **1** or **5** (from 1 to 20 μM) led to the progressive appearance of a new band of lower mobility, corresponding to the formation of a bimolecular quadruplex structure (not shown). These results demonstrate that dibenzophenanthrolines may act as G-quadruplex chaperones, in a manner similar to *N*,*N*′-bis[2-(1-piperidino)ethyl]-

3,4,9,10-perylenetetracarboxylic diimide (PIPER). These observations are also in good agreement with the G4-stabilizing effects shown by FRET: the ligands that give a significant stabilization of an intramolecular quadruplex (e.g., **1** and **5**) efficiently promote intermolecular quadruplex formation, as revealed by gel electrophoresis.

Discussion

Design of the System. We have chosen an intramolecular G-quadruplex corresponding to approximately four units of the human telomeric repeat sequence. The association/dissociation of the intramolecular quadruplex is fast, as shown by the reversibility of the melting curves (32). All UV and fluorescence melting curves were kinetically reversible and reproducible.

In this article, we have used a method based on FRET to characterize G4 ligands. The FRET approach offers several advantages to identify G-quadruplex stabilizers: (*i*) the technique is extremely sensitive; (*ii*) the dynamic response of most spectrofluorimeters is linear over a wide concentration range; (*iii*) stabilization may be estimated in the presence of a large excess of competitors; and (*iv*) the assay itself can be converted into a high throughput screening test. More than 100 G-quadruplex ligands, belonging to several distinct chemical families, have been identified with this FRET assay and will be described in forthcoming papers.

The dibenzophenanthroline molecules investigated in the present report are of special interest. They strongly stabilize G-quadruplexes, as shown by FRET melting data. The ΔT_m reported with compounds **1** and **5** and especially compound **9** are much larger than those found with previously described G4 ligands. They have a strong affinity for the quadruplex structure, with submicromolar dissociation constants. The measured K_d values are 1 or 2 orders of magnitude lower than previously described. Finally, they efficiently inhibit telomerase activity *in vitro*, with an IC_{50} of 28 nM for the most active compound **9**.

G-Quartet Ligands and Inhibition of Telomerase. G-quadruplex binding affinity is not expected to be strictly correlated to telomerase inhibition efficiency. Nevertheless, the results shown in Fig. 3 demonstrate that, at least within one family of compounds (dibenzophenanthrolines), an excellent correlation between telomerase inhibition and G4 stabilization was obtained. It should be noted that the ΔT_m values do not depend only on the affinity constant of the ligand, but also on the number of binding sites per oligonucleotide. The development of telomerase inhibitors based on their binding to quadruplex structures of the G-rich telomeric domain raises several questions:

(*i*) The selectivity of G4 ligands remains to be established. The situation could be complicated by the fact that some of the investigated molecules might interact with other nucleic acids structures (33). For example, some of the dibenzophenanthrolines derivatives investigated here have been recently tested for their ability to stabilize triplexes (24). Compound **5** is a weak triplex stabilizer (but a potent telomerase inhibitor) whereas **3** is one of the strongest triplex ligands described to date. In any case, our FRET assay allows us to select for quadruplex ligands that do not bind strongly to other structures and/or sequences, by adding unlabeled nucleic acids as competitors, in a manner similar to the experiment shown in Fig. 2*B* for duplex DNA. The dialysis method proposed by Ren and Chaires (34) also should be helpful to determine the relative affinities of G4 ligands for a variety of DNA and RNA structures.

(*ii*) The binding strength of the complex should be high enough, if *in vivo* applications are considered. Dissociation constants reported so far for quadruplex ligands are in the micromolar range ($K_d = 10^{-6} - 10^{-5}$ M). Compound **9** has a dissociation constant of 10^{-8} M at quasi-physiological ionic strength, showing that a major step toward tight binding has been achieved. Such an improvement also was found for antitelomerase activity: IC_{50} of 0.3 and 0.5 μM were found for compounds **1** and **5**, respectively, and compound **9** is even more efficient (IC_{50} of 0.028 μM). So far, the reported IC_{50} values of G4-based telomerase inhibitors are 1–20 μM for anthraquinones, 6–25 μM for porphyrins, 8–20 μM for fluorenones, and 1–14 μM for acridines (ref. 35; data collected in ref. 15). Therefore, the increase in binding affinity for quadruplexes (10- to 100-fold) observed for the dibenzophenanthroline compounds that we have investigated is associated with a similar increase in telomerase inhibition efficiency.

(*iii*) G4-binding agents may exhibit a difference in affinity for the different types of quadruplexes. We have chosen to test an intramolecular quadruplex, but it is not clear whether telomerase inhibitors should target intramolecular or intermolecular quadruplexes. At least for compounds **1** and **5**, we have data showing that this molecule recognizes both intramolecular and bimolecular quadruplexes.

(*iv*) Although intercalation or terminal stacking seems likely (36), because of the resulting strong $\pi-\pi$ stacking between the guanine tetrads and the ligand, we cannot exclude that dibenzophenanthroline derivatives interact with the G4 structure via the grooves or the connecting loops. It is tempting to speculate that one high affinity binding site is provided at each end of the oligonucleotide, via a terminal stacking mode. Structural methods, such as NMR spectroscopy, will be required to answer this question.

(*v*) Other sequences than telomeres in the human genome do contain repetitive stretches of guanines, and G-quadruplex ligands also might interfere with replication, transcription, or recombination around these DNA regions.

In summary, the development of quadruplex ligands, which was initiated only a few years ago, already has given promising results. The introduction of a fluorescence test to identify G4 ligands might accelerate the discovery of telomerase inhibitors. Some crescent-shaped dibenzophenanthroline compounds have been shown to increase the melting temperature of the quadruplex by more than 10°C and appear to be among the most potent non-nucleoside telomerase inhibitors reported to date (IC_{50} = 28 nM for compound **9**). In this series, a good correlation was found between telomerase inhibition and quadruplex affinity. Molecular modeling based on structure-activity relationships should help us to design second-generation molecules once lead compounds have been isolated. Therefore, the FRET assay that we have described is a promising method to screen molecules for their G4-binding activities.

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