Articles

Selective Interaction of Ethidium Derivatives with Quadruplexes: An Equilibrium Dialysis and Electrospray Ionization Mass Spectrometry Analysis†

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ABSTRACT: The telomeric G-rich single-stranded DNA can adopt in vitro an intramolecular quadruplex structure, which has been shown to directly inhibit telomerase activity. The reactivation of this enzyme in immortalized and most cancer cells suggests that telomerase is a relevant target in oncology, and telomerase inhibitors have been proposed as new potential anticancer agents. In this paper, we have analyzed the selectivity of four ethidium derivatives and ethidium itself toward different G-quadruplex species, with electrospray mass spectrometry and competitive equilibrium dialysis and evaluated their inhibitory properties against telomerase. A selectivity profile may be obtained through electrospray ionization mass spectrometry (ESI-MS), which is in fair agreement with competitive equilibrium dialysis data. It also provides unambiguous data on the number of binding sites per nucleic acid (maximal number of two ethidium derivatives per quadruplex, in agreement with external stacking). Our experiments also demonstrate that one compound (**4**) is the most active and selective G-quadruplex ligand within this series and the most selective telomerase inhibitor in a modified TRAP-G4 assay.

Telomeres protect chromosomal ends from fusion events and provide a mean for complete replication of the chromosome. The study of telomeres and the telomerase enzyme that maintains their length has acquired importance through the discovery of telomerase activity in most types of cancer cells. The enzyme is normally inactive in somatic cells, and thus, presents a new and potentially very specific therapeutic target for drug development. Chromosomal DNA of ciliates, yeasts, and vertebrates ends in a 3′ single-stranded overhang that is relatively long in vertebrates. These overhangs may be involved in different DNA conformations such as T-loops (*1*), triplexes (*2*), or G-quadruplexes (G4) (*3*, *4*). The presence of telomeric quadruplexes has been recently demonstrated in the macronucleus of a ciliate, *Stylonychia lemnae* (*5*), and there is a renewed interest for G4 structures because of their putative biological regulatory function during transcription and replication (*6*, *7*).

Folding of the telomeric G-rich single strand $(GGGTTA)_n$ into G4 DNA has been found to inhibit telomerase activity (*8*). It was deduced from this observation that a molecule that favors G4 formation locks the telomeric substrate into an inactive conformation that is no longer recognized nor extended by the enzyme (*9*). Stabilization of G-quadruplexes can then be considered an original strategy to achieve antitumor activity (*10*). G-quadruplexes are a family of secondary DNA structures formed in the presence of monovalent cations that consist of four-stranded structures stabilized by G-quartets (Figure 1) (*11*−*15*). G4 ligands require a structural selectivity (i.e., preferential binding to G4 over duplexes and single strands). The quadruplex itself, which is very different from classical double-stranded B-DNA, provides a good structural basis for selective recognition, and several classes of small molecules that selectively bind to G4 DNA and inhibit telomerase activity have been described such as porphyrins (*16*−*18*), perylenes

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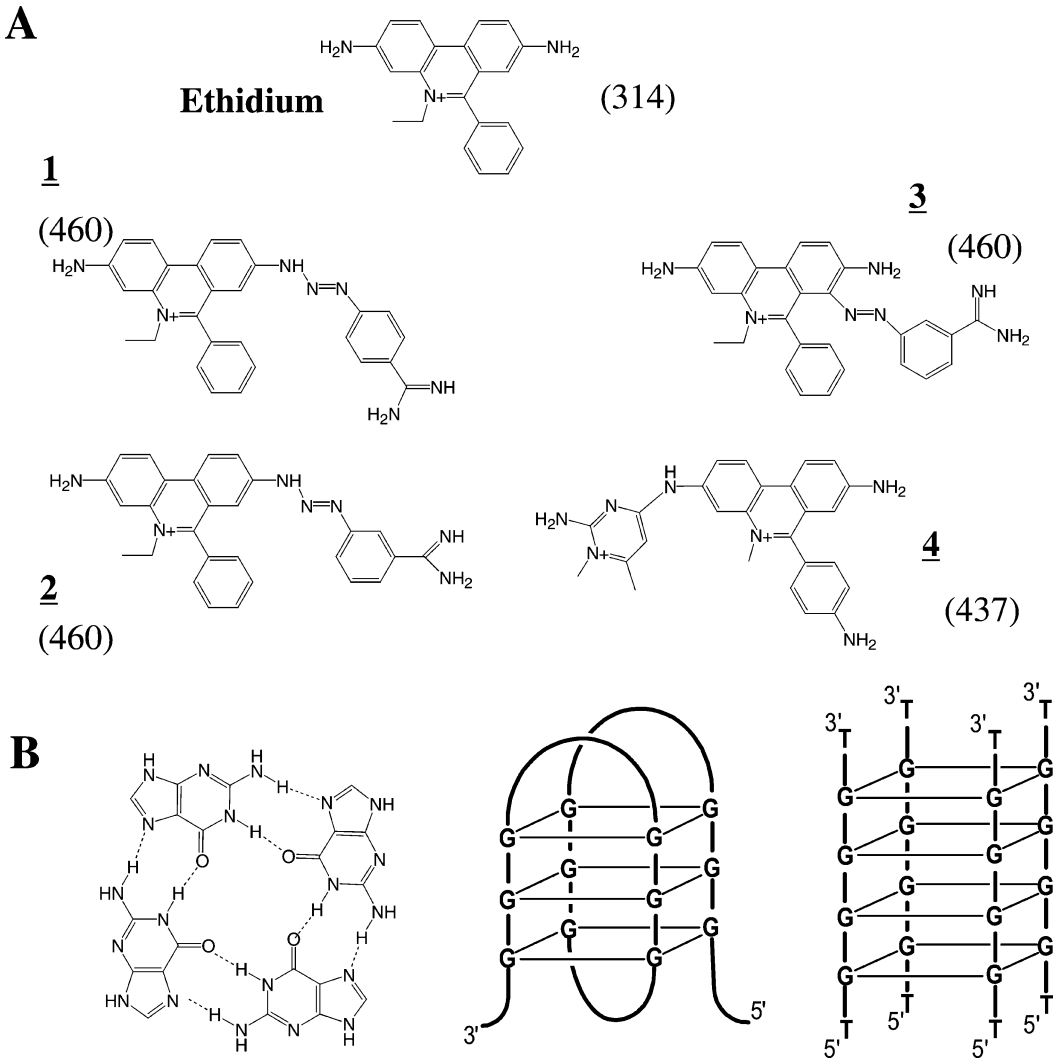

FIGURE 1: (a) Structure of the ethidium derivatives. The masses (Da) are noted in parentheses. (b) Schematic drawing of a G-quartet (left), an antiparallel unimolecular G-quadruplex (center), and a parallel quadruplex (right).

(*19*), amidoanthracene-9,10-diones (*20*), 2,7-disubstituted amidofluorenones (*21*), acridines (*22*), disubstituted-triazines (*23*), telomestatin (*24*), and indoloquinolines (*25*) (for a review, see refs *26* and *27*). General features of molecules that bind to G-quadruplexes include a large flat aromatic surface and cationic charges or a planar and circular structure that could partially mimic a G-quartet. The former category of compounds is generally similar to intercalators or corresponds to derivatives of well-known intercalators. Such a molecular motif seems suitable for stacking with the terminal G-quartets and formation of specific electrostatic contacts. Unfortunately, many compounds studied so far that adopt the terminal stacking mode suffer from insufficient preference for G4 over duplexes. The discovery of new G4-specific compounds is thus of utmost importance for a more comprehensive understanding of the biological implications of these structures and for designing new drugs with enhanced activity and minimized undesired toxicity.

Ethidium itself binds to duplex DNA by intercalating between adjacent base pairs (*28*). Ethidium derivatives were chosen because their parent molecule, ethidium bromide, has been reported to interact with triplexes (*29*, *30*) and G4 (*31*, *32*). This interaction might result from the stacking between the planar rings of the dye and the G-quartet. Original work by Guo et al. (*31*) established that one ethidium bromide molecule could bind to a $[T_4G_4]_4$ quadruplex, with a slight preference over duplex DNA. However, other experiments demonstrated that ethidium affinity for G4 is rather weak as compared to triplexes (*33*). This observation prompted us to search for analogues that would provide better interaction. Four derivatives bearing additional side chains were shown to exhibit high affinity toward G-quadruplexes (*34*). The ligand-induced stabilization of the quadruplex was associated with anti-telomerase activity and a large enhancement of fluorescence quantum yield. However, little data were available concerning the selectivity of these molecules relative to G4 and other nucleic acids. In this study, we have used a variety of biochemical and spectroscopic methods to assay the selectivity of these compounds. Dialysis competition assay and mass spectrometric experiments were used to determine the affinity of these ligands for different DNA structures (duplexes, triplexes, quadruplexes, and single strands). FRET melting studies were performed in the presence of an excess of competitor double-stranded DNA to confirm the selectivity. Telomerase assay was also performed in the presence of an internal control (ITAS) and a telomerase primer (TSG4) able to fold into a G4 to evaluate both anti-telomerase activity and selectivity of the ligands relative to Taq polymerase inhibition. All methods concur to demonstrate that compound **4** (Figure 1A) is, among all

Table 1: List of the Nucleic Acids Used in Equilibrium Dialysis Experiments[a]

number	name	type[b]	structure	T_m (°C)[e]
1	(T,C) triplex	oligos	triplex	38
2	(G,A) triplex	oligos	triplex	53
3	(G,T) triplex	oligos	triplex	53
4	poly dA.2polydT	poly	triplex	71
5	24GA duplex	oligo	"duplex"[c]	37
6	psD duplex	oligos	"duplex"[c]	39
7	24CTG	oligo	"duplex"[c]	64
8	poly d(A−T)	poly	duplex	66
9	poly d(G−C)	poly	duplex	>90
10	calf thymus DNA	poly	duplex	86
11	ds 26	oligo	duplex	75
12	poly dC	poly	i-DNA	51[f]
13	22CT	oligo	ss/i-DNA[d]	13[f]
14	22AG	oligo	G4	62
15	24G20	oligo	G4	>90
16	poly dT	poly	single-str	−[g]
17	poly dA	poly	single-str	−[g]
18	poly rU	poly	single-str	−[g]
19	poly rA	poly	single-str	−[g]

[a] 19 different nucleic acids structures are used (samples labeled 1−19, left column). [b] poly = polynucleotide; oligo = oligonucleotide; oligos = structure formed by the association of two different oligonucleotides. Polynucleotides are >100 bases long. [c] These three duplexes are unusual as they involve the formation of nonclassical base pairs. [d] 22CT may form an i-DNA structure but is mainly single stranded at room temperature. [e] Obtained in an buffer identical to the equilibrium dialysis protocol. [f] Hysteresis. T_m obtained while heating. [g] No transition.

ethidium derivatives tested so far, the most active and selective G4 ligand inhibitor of telomerase.

EXPERIMENTAL PROCEDURES

Materials. All oligonucleotides were synthesized and purified by Eurogentec, Belgium.

Ethidium derivatives **1**−**4** have been previously described (see Figure 1A) (*34*, *35*) (patent WO 0212194). **1** was similarly prepared from 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium iodide, using 4-benzamido-diazonium salt in place of 3-benzamido-diazonium salt for the synthesis of **2**. Compound **2**, also known as isomethamidium, was previously prepared and identified as a trypanocidal drug (*36*, *37*). Compound **3** was identified as the main byproduct in the synthesis of **2**. It occurred through azo-coupling of 3-benzamido-diaazonium salt at position 8 of 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium iodide, ortho to the 7-amino group. Compound **4** was identified as the main byproduct in the synthesis of prothidium iodide according to the May and Baker patent (GB 816236). Solutions of all derivatives were kept at −20 °C in the dark between experiments.

Equilibrium Dialysis. The initial dialysis protocol was defined by Ren and Chaires (*33*). We have adapted this test to accommodate a different set of 19 nucleic acid structures (see Tables 1 and 2) (*38*−*40*). The TC, GA, and GT triplexes result from the association of two strands of different lengths (13 and 30 nucleotides) (*41*). The 24GA duplex results from the self-association of a $(GA)_{12}$ oligonucleotide. The parallel-stranded duplex (psD) results from the association of two AT strands. The 24CTG mimics eight repeats of the trinucleotide unit CTG. ds26 is a duplex formed with a self-complementary oligonucleotide. 22CT is an oligonucleotide that mimics the cytosine-rich strand of human telomeres (*42*), whereas 22AG is an oligonucleotide that mimics the guanine-rich strand of human telomeres and adopts an intramolecular quadruplex structure (*3*). 24G20 may form an intermolecular G-quadruplex, whereas poly(dC) may form an intermolecular i-motif.

All structures used in these experiments were stable at room temperature under the chosen experimental conditions (15 mM sodium cacodylate pH 6.5, 10 mM $MgCl_2$, and 185 mM NaCl, 20 °C) except 22CT (see Table 1) (*39*). The presence of 185 mM sodium chloride promotes quadruplex formation (*39*). A total of 400 mL of the dialysate solution containing 1 μM ligand was used for each competition dialysis assay. A volume of 200 μL at 75 μM monomeric unit (nucleotide, base pair, base triplet, or quartet) of each of the nucleic acids samples was pipeted into a separate Dialyzer unit (Pierce). All 19 dialysis units were then placed in the beaker containing the dialysate solution. The beaker was covered with Parafilm and wrapped in aluminum foil, and its contents were allowed to equilibrate with continuous stirring at room temperature (20−22 °C) overnight. This long incubation time is likely to allow proper thermodynamic equilibrium, as most drug−DNA interactions occur on a faster time scale in the μM concentration range. At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes and treated with 1% SDS. The ligand concentration in each sample was determined by fluorescence or absorbance spectroscopy. The amount of dye in each dialysis unit is directly proportional to the absorbance signal of the sample as the nucleic acid−compound complex is dissociated by SDS. This quantification procedure has been validated in a number of independent studies by several groups including ours (*33*, *38*, *43*−*47*).

Mass Spectrometry. Oligodeoxynucleotides d-CGTAAATTTACG (Dk33, M = 3644.45 Da), d-CGCGAATTCGCG (Dk66, M = 3646.44 Da), d-CGCGGGCCCGCG (Dk100, M = 3678.40 Da), d-TGGGGT (M = 1863.26 Da), d-GGGGTTTTGGGG (M = 3788.50 Da), and d-$(GGGTTA)_3GGG$ (M = 6653.35 Da) were purchased from Eurogentec (Angleur, Belgium) and used without further purification. Duplex and quadruplex solutions were prepared, respectively, in 100 and 150 mM NH_4OAc, pH 7.0. Oligonucleotide solutions were heated to 85 °C for 5 min and cooled overnight to 20 °C to form the duplex or quadruplex structures. Ammonium acetate was chosen as the electrolyte for its compatibility with electrospray mass spectrometry. The solution conformation of the oligonucleotide in ammonium acetate was characterized by circular dichroism and UV melting experiments (*48*). Experiments were performed on an LCQ mass spectrometer (Finnigan, San Jose, CA) as described previously (*49*). The experimental conditions were optimized to avoid denaturation of the duplex or quadruplex species: the heated capillary temperature of the electrospray source was set to 185 °C, and a good compromise between sufficient focusing and minimal collisional activation of the ions in the 1 Torr region of the source is achieved with a tube lens offset of 35 V and a voltage on the heated capillary of −11 V (the skimmer is at ground). Full scan MS spectra were recorded in the *m*/*z* range [1000−2000], and 50 scans were summed for each spectrum. Spectra of equimolar mixtures (5 μM) of all possible structure (duplex or quadruplex) + drug combinations were recorded. Methanol (15%) was

Table 2: Sequence of the Oligonucleotides Used in Equilibrium Dialysis[a]

(T,C) TRIPLEX	(#1)	TT-CTTTCTCTCCTCC-3′ *5′-GAAAGAGAGGAGG-3′* CC-CTTTCTCTCCTCC-5′
(G,A) TRIPLEX	(#2)	TT-GGAGGAGAGAAAG-3′ CC-GGAGGAGAGAAAG-5′ *5′-CCTCCTCTCTTTC-3′*
(G,T) TRIPLEX	(#3)	TT-GGTGGTGTGTTTG-3′ CC-GGAGGAGAGAAAG-5′ *5′-CCTCCTCTCTTTC-3′*
24GA (parallel duplex)	(#5)	5′-GAGAGAGAGAGAGAGAGAGAGA-3′
psD duplex	(#6)	5′-AAAAAAAAAATAATTTTAAATATT-3′ 5′-TTTTTTTTTTATTAAAATTTATAA-3′
24CTG	(#7)	5′-CTGCTGCTGCTGCTGCTGCTGCTG-3′
ds 26	(#11)	5′-CAATCGGATCGAATTCGATCCGATTG-3′
22CT	(#13)	5′-CCCTAACCCTAACCCTAACCCT-3′
22AG	(#14)	5′-AGGGTTAGGGTTAGGGTTAGGG-3′
24G20	(#15)	5′-TTGGGGGGGGGGGGGGGGGGGGTT-3′

[a] The name of the oligonucleotides is followed by the structure number (see Table 1) and the sequence. Note that the triplexes correspond to the association of two strands of different lengths. ds 26 is an autocomplementary oligonucleotide.

added to the samples just before injection to obtain a stable electrospray signal. The rate of sample infusion into the mass spectrometers was 4 μL/min. As previously discussed (*49*), the relative intensities of the free and bound DNA in the mass spectra are assumed to be proportional to the relative abundances of these species in solution. As the starting concentrations are known, the concentrations of all individual species at equilibrium (free DNA, 1:1 complex, 2:1 complex, and by difference, the free drug) can be determined from the relative intensities of the free DNA and the complexes. The concentration of bound ligand per DNA molecule or per binding unit give us the affinity of the drug for a given structure. The concentration of bound ligand per DNA molecule is obtained with the following equation (1):

$$[\text{bound ligand}] = C_0(I_{(1:1)} + 2I_{(2:1)})/(I_{\text{DNA}} + I_{(1:1)} + I_{(2:1)})$$

where C_0 is the starting DNA concentration (expressed in structure, duplex, or quadruplex), I_{DNA} is the relative intensity of the free DNA, and $I_{(n:1)}$ are relative intensities of the complexes (*n* drug molecules bound to one DNA target). The relative intensities were obtained from a sum of 50 spectra. The amount of bound ligand expressed in a molecular binding unit (base pair, base triplet, or quartet in the DNA target) is determined by dividing the total amount of bound ligand by the number of monomeric units in the DNA targets.

Fluorescence Melting Experiments. All FRET measurements with the doubly fluorescent (F = fluorescein, T = tetramethylrhodamine) F21T oligodeoxynucleotide (5′ fluorescein-$(GGGTTA)_3$GGG-3′ tetramethylrhodamine, 0.2 μM) were performed on a Spex Fluoromax3 instrument, using a bandwidth of 5 nm and 0.2 × 1 cm quartz cuvettes, containing 600 μL of solution in a pH 7.2, 0.1 M lithium chloride, 10 mM sodium cacodylate buffer. All ligands were tested at 1 μM. In competition experiments, various concentrations of an autocomplementary unmodified 26 base long oligodeoxynucleotide (ds26: d-CAATCGGATCGAATTCGATCCGATTG) or a single-stranded dT_{26} oligodeoxynucleotide were added. All measurements were made as previously described (*50*, *51*).

Circular Dichroism. CD spectra were obtained at 20 °C with a Jobin Yvon CD6 (Lonjumeau, France) circular dichrograph. A quartz cell (Hellma, Inc.) with a 10 mm path length was used to obtain spectra at 0.5 nm intervals from 220 to 640 nm. Spectra result from the averaging of three scans, followed by the subtraction of the CD spectrum of a

solution of 0.15 M aqueous NH_4OAc. The quadruplex concentration was 3 μM, and the drug was added up to a molar ratio r = [drug]/[quadruplex] = 4.2. Results are expressed in molar circular dichroism.

Telomerase Assay (TRAP-G4). Telomerase extract was prepared from A549 cells according to Fu et al. (*52*). TRAP-G4 was performed as described previously (*53*). PCR was performed in a final 50 μL reaction volume containing primers TSG4 (3.5 pmol), TS (18 pmol), CXext (22.5 pmol), NT (7.5 pmol), and TSNT (0.01 amol); 20 mM Tris-HCl (pH 8.0), 50 μM dNTPs, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20, 20 μg/mL bovine serum albumin; 2.5 U Taq DNA polymerase (DyNAzyme II DNA polymerase, Ozyme); 100 ng of telomerase extract; and compounds or distilled water under a volume of 5 μL. Samples were incubated for 15 min at 30 °C and denatured for 1 min at 90 °C and further submitted to 30 PCR cycles: 30 s, 92 °C; 30 s, 52 °C; and 30 s, 72 °C. After amplification, 10 μL of loading buffer containing 20% sucrose, 5X TBE, 0.2% bromophenol blue, and 0.2% xylene cyanol were added to the reaction. After electrophoresis onto a 12% nondenaturing polyacrylamide gel (19:1) in 1X TBE and staining with SYBR Green I (Roche), DNA fragments were digitalized by a CCD camera (Bioprint) and quantified by the BioCapt software. The disappearance of the TSG4 band corresponded to a G-quadruplex stabilization of the TSG4 oligonucleotide and coincided with inhibition of the telomeric repeats extension from either TSG4 or TS oligonucleotides. The disappearance of the ITAS band corresponded to nonspecific inhibition of Taq polymerase activity. For each compound, results were expressed by the IC_{50} of the TSG4 and ITAS band formation and indicated as IC_{50} TRAP G4 and IC_{50} Taq, respectively. The ratio IC_{50} Taq/ IC_{50} TRAP G4 represented the selectivity factor of the compound in this assay.

Cell Culture Conditions and Survival Assay. A549 human cell lung carcinoma cell line was from the American Type Culture Collection. These cells were grown in DMEM medium with Glutamax (Invitrogen) and supplemented with 10% fetal calf serum and antibiotics.

Cells were seeded in 96-well plates at 3×10^3 cells/well in a final volume of 200 μL. Ligand at final concentrations of 20, 2, and 0.2 μM were added 6 h later under a volume of 20 μL, each concentration in quadruplicate. The MTT survival assay was performed after 4 days of incubation, as recommended by the manufacturer (Sigma). Results were expressed as the percent cell survival relative to untreated control cells. The concentration of the ligand that inhibits 50% of cell growth (IC_{50}) was determined from semi-logarithmic plots.

RESULTS

Equilibrium Dialysis. To evaluate the selectivity of ethidium and its derivatives for different DNA structures, we performed competitive dialysis experiments using 19 nucleic acid structures (described in Experimental Procedures) against a common ligand solution. More product accumulates in the dialysis tube containing the structural form with the highest ligand binding affinity. Results for ethidium, **2**, **3**, and **4** were shown in Figure 2. The spectral properties of compound **1** did not allow a precise quantification of the binding data (not shown). It is possible to correlate the amount of the bound dye to a given structure with the affinity of the dye for that nucleic acid sample. As shown in Figure 2 (top left), ethidium interacts preferentially with duplexes (in black and gray), whereas it shows a weak affinity for i-motif (green), single strands (blue), triplexes (orange), and G4 (red). Interestingly, the preferred structure corresponds to an unusual DNA duplex (24CTG, in gray) resulting from the intramolecular folding of a $(CTG)_8$ repeat oligodeoxynucleotide. Overall, binding to the four regular duplexes (in black) was stronger than to the triplexes or quadruplexes, in agreement with previous reports (*29*, *34*). For **2** and **4**, binding to G4 (in red) is stronger than binding to Watson−Crick duplexes (in black). Binding to single strands (in blue) is virtually nil, demonstrating that these derivatives have some selectivity for quadruplexes over duplexes and single strands. Nevertheless, it is clear from these studies that **2** (upper right) and **3** (lower left) have a lower quadruplex-duplex selectivity than **4** (lower right). For all derivatives, binding to the intramolecular telomeric quadruplex is similar to the binding to the parallel quadruplex, showing that these molecules are not able to discrimimate between these quadruplexes. In principle, competitive dialysis data may be used to calculate apparent binding constants (K_a), using the simple relation K_a = [bound]/([free][NAtotal − bound]), where [bound] is the amount bound to each structure, [free] is the free ligand concentration (kept at 1 μM), and NAtotal is the total nucleic acid concentration (75 μM). It is theoretically possible to calculate the equilibrium constant for each structure (i.e., 5×10^4 and 7×10^3 M^{-1} for compound **4** binding to the parallel quadruplex and the poly d(A-T) duplex, respectively and 8×10^4 and 3×10^4 M^{-1} for compound **3** binding to the telomeric quadruplex and the poly d(A-T) duplex, respectively). These values represent the dissociation constant for a monomeric unit (base pair or quartet). However, these values are poorly reliable as: (i) they are based on a single dye/DNA monomeric unit concentration, (ii) they rely on an accurate determination of the absolute value of bound dye: in our hands, the dialysis profile is reproducible, but the numerical values are not, and (iii) higher affinities should be expected at lower ionic strength. The binding profile of these compounds relative to higher-order DNA structures prompted us to confirm this interaction using different techniques.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) is a highly sensitive method to study drug−DNA interactions, as reviewed recently (*54*, *55*). Stoichiometries and relative binding affinities of DNA complexes with intercalators and minor groove binders have been studied (*56*, *57*). Quantitative determination of binding constants for minor groove binders with different DNA duplexes has been demonstrated (*49*), and the interaction of drugs with DNA triplex and quadruplex structures can also be investigated (*40*, *48*, *58*).

In the present work, electrospray mass spectrometry (ESI-MS) was used to determine the binding affinity and specificity of the ethidium derivatives for DNA duplex and quadruplex forms. Our binding assay uses three 12-base pair duplexes with different GC content and three quadruplexes ($[G_4T_4G_4]_2$, $[TG_4T]_4$, and the human intramolecular telomeric sequence $(G_3T_2A)_3G_3$). The later oligonucleotide is identical to the one used in FRET melting studies and similar to 22AG

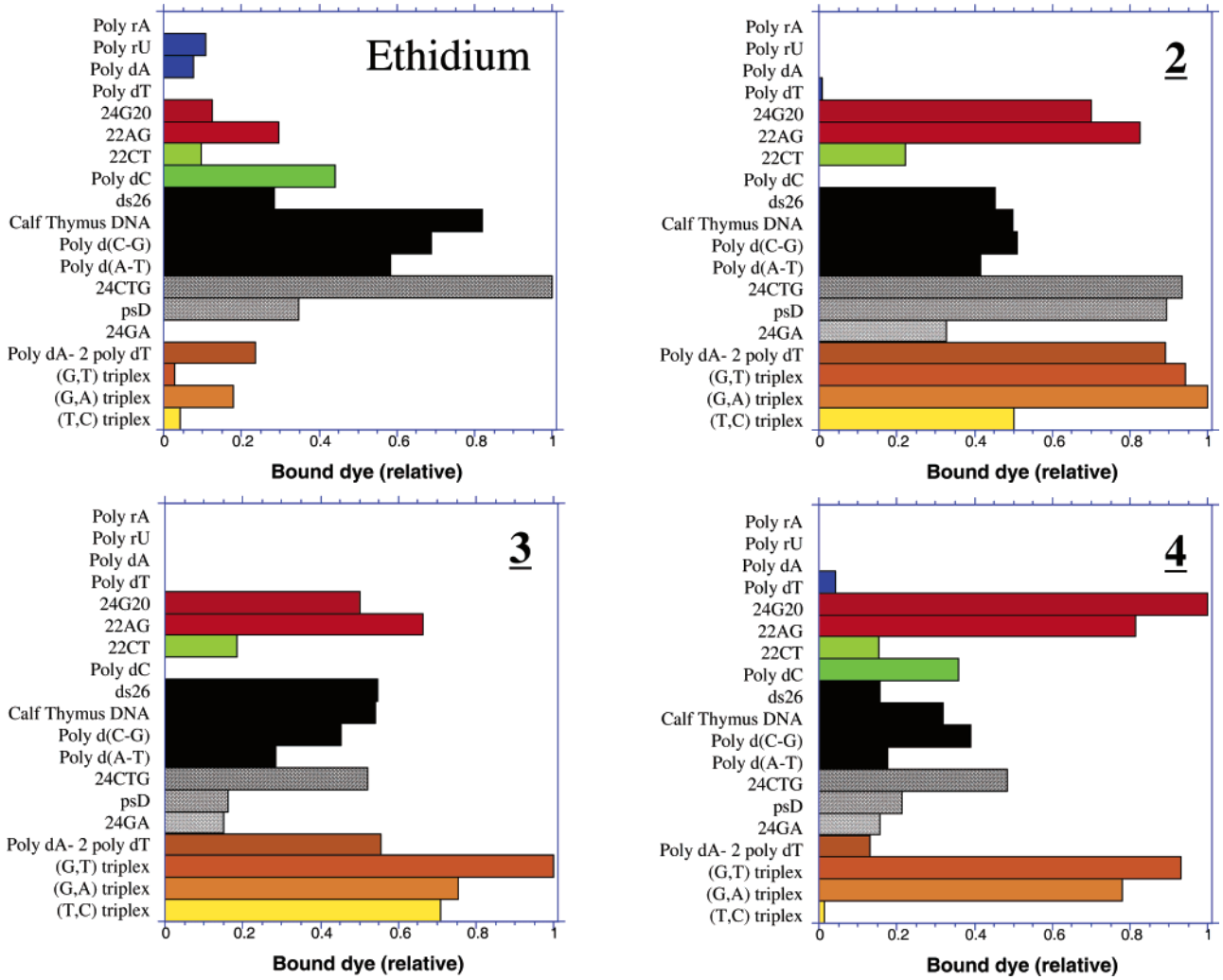

FIGURE 2: Equilibrium dialysis. All measurements were performed in a 185 mM NaCl, 10 mM $MgCl_2$, 15 mM pH 6.5 sodium-cacodylate buffer. The nucleic acid names are given on the left, and structures are described in Tables 1 and 2. The values are normalized for each compound. Maximum amount bound corresponds to 2.1 μM (ethidium); 4.0 μM (compound **2**); 8.6 μM (compound **3**); and 3.5 μM (compound **4**).

$A(GGGTTA)_3GGG$ used for dialysis. Figure 3 illustrates typical ESI-MS mass spectra obtained with an equimolar mixture of the drug **4** with the duplex $[CGCGAATTCGCG]_2$ (Dk66; Figure 3A), the parallel quadruplex $[TG_4T]_4$ (Figure 3C), and the human telomeric sequence (Figure 3B). Drug/DNA (1:1) and (2:1) complexes were observed with all these structures. It has been previously reported that, for the quadruplex $[TG_4T]_4$, specifically three ammonium ions are detected within this particular G4 structure (*48*) and are located between the tetrads. For all compounds bound to this particular quadruplex, the mass of the complexes indicates that the three ammonium ions are conserved in the complex (i.e., a mass shift of 51 Da corresponding to the mass of three ammonium ions). As the ammonium ions between the tetrads are not expelled, this suggests that the drugs are not located between the tetrads.

The largest amount of the complexes (1:1) and (2:1) is observed with the intramolecular telomeric sequence (Figure 3B) followed by the parallel quadruplex (Figure 3C), and the Dickerson duplex (Dk66) (Figure 3A). Nevertheless, to make semiquantitative comparisons with equilibrium dialysis experiments, the amount of drug bound was expressed in terms of the monomeric unit: the amount of bound drug calculated with eq 1 is divided by the number of base pairs or tetrads in the oligonucleotide studied. These results are summarized in Figure 4. Among the duplexes, all drugs interact preferentially with those of high GC content. This is consistent with the GC preference of common intercalators such as ethidium. Concerning the structural selectivity (duplex vs G4), ESI-MS results are in agreement with equilibrium dialysis data: all ethidium derivatives bind preferentially to the G4 structures. The derivative **4** shows the best selectivity for the quadruplex structure. Binding constants obtained with the mass spectrometry experiment were $K_1 = 3.04 \times 10^5$ and $K_2 = 2.0 \times 10^5$ M^{-1} for the vertebrate intramolecular telomeric sequence (two binding sites per quadruplex). The same order of magnitude obtained for the binding constants indicates that the binding sites are equivalent.

FRET Melting Studies. FRET can be used to probe the secondary structure of oligodeoxynucleotides mimicking repeats of the guanine-rich strand of vertebrate telomeres, provided a fluorescein molecule (donor) and a tetramethylrhodamine derivative (acceptor) are attached to the 5′ and 3′ ends of the oligonucleotide, respectively. The melting of the G-quadruplex is usually monitored in the presence of 1 μM dye by measuring the fluorescence of the donor. Ligands known to specifically interact with a quadruplex increased

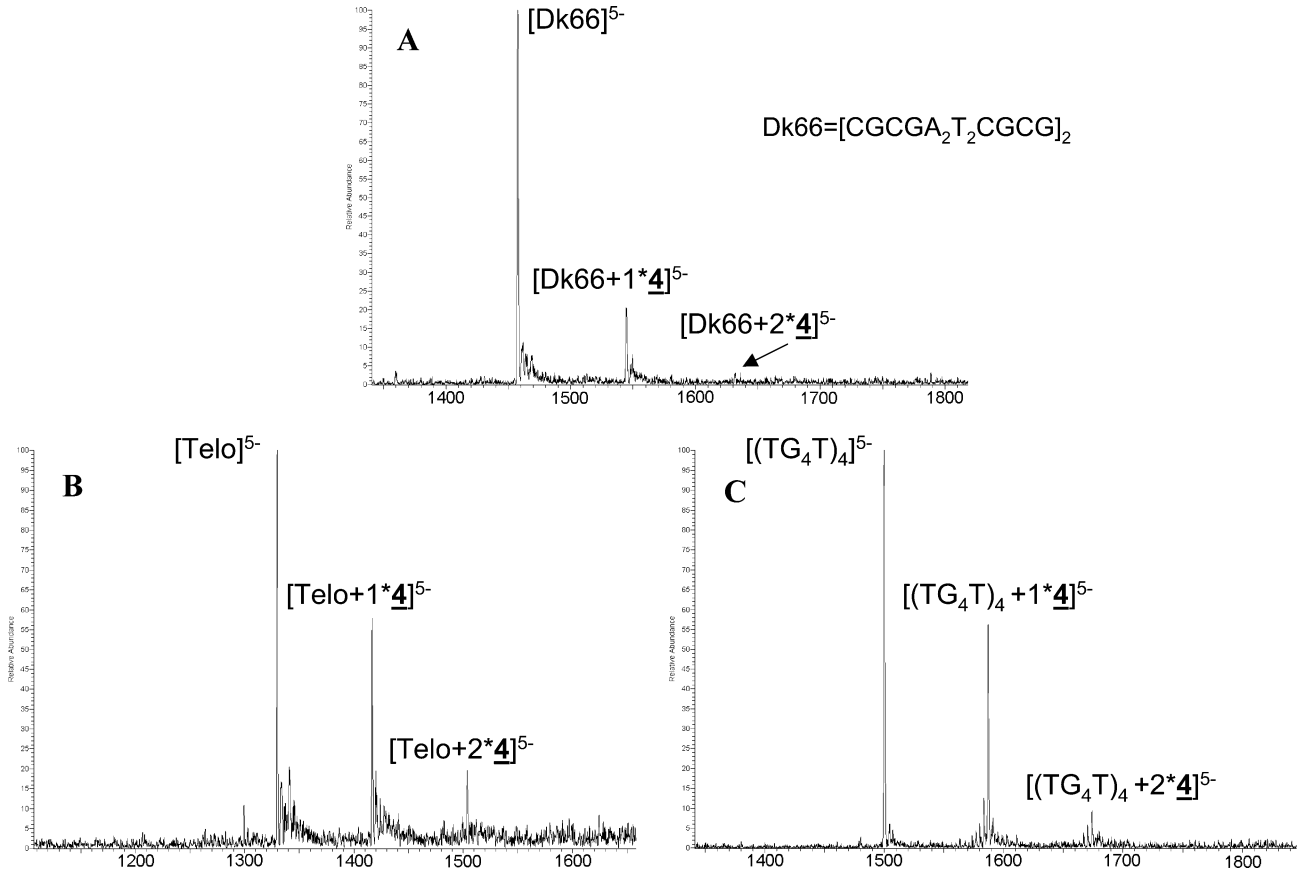

FIGURE 3: ESI-MS full scan spectra of equimolar mixtures ($C_0 = 5\ \mu$M) of **4** with (a) the $[CGCGAATTCGCG]_2$ duplex, (b) the $(G_3T_2A)_3G_3$ antiparallel quadruplex (human telomeric motif), and (c) the $[TG_4T]_4$ parallel quadruplex. The DNA alone, the 1:1, and the 2:1 complexes are indicated.

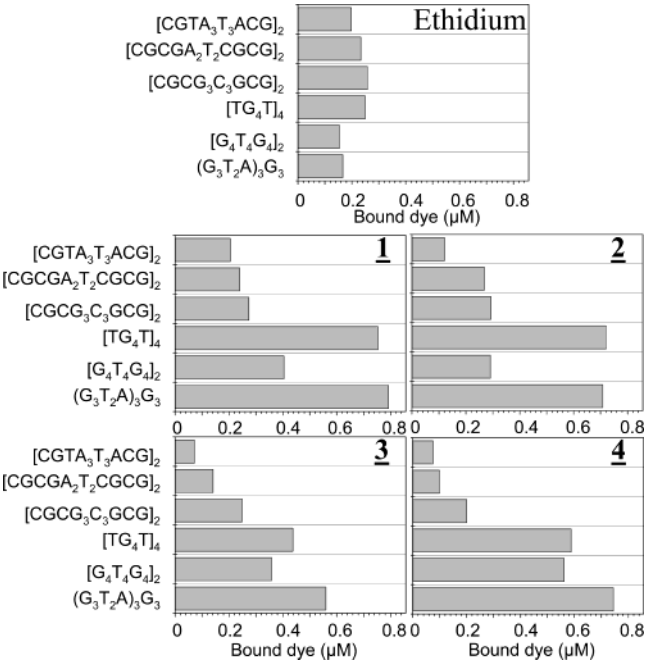

FIGURE 4: Quantification of the ESI-MS scans for ethidium bromide and its four derivatives. Amount of ligand bound (μM) to six different DNA structures (three duplexes, top and three quadruplexes, bottom) determined by ESI-MS expressed in terms of monomeric units (base pair or G-quartet).

the melting temperature of F21T, as shown by a different emission versus temperature profile. Thousands of molecules have been screened, and in addition to ethidium derivatives, at least four independent families of ligands have been evidenced (dibenzophenanthrolines (*59*), triazines (*23*), bisacridines (*47*), and benzoindoloquinolines (*38*)). A variation of this method using a real time PCR instrument allows the simultaneous screening of up to 32 samples (*60*). In this test, ethidium gave little, if any, stabilization whereas **1**−**4** gave $\Delta T_{1/2}$ of 9 °C or more. Judging from these $\Delta T_{1/2}$ values, these derivatives were therefore good G4 stabilizers. The next step was to determine the stabilization in the presence of a large excess of competitor DNA. First, we checked that increasing amounts of competitor DNA have no effect on the melting of the F21T oligonucleotide alone (not shown). In principle, a variety of unlabeled DNA or RNA structures may be added to the solution—and even mixed together—provided they do not interact with F21T. In this study, we restricted the competition assay to single- and double-stranded DNA. Binding of ethidium derivatives to single-stranded DNA was low, as shown by equilibrium dialysis or fluorescence titration. In agreement with this observation, the addition of an excess of single-stranded DNA (dT_{26}) had a limited effect on the stabilization by all compounds, even at 10 μM oligonucleotide concentration (corresponding to 260 μM nucleotide concentration; see Figure 5, light blue bars). In contrast, increasing amounts of a self-complementary double-stranded oligodeoxynucleotide (ds26) led a significant decrease in the melting stabilization of F21T. For **1**−**4**, $\Delta T_{1/2}$ was 0, +4, +6, and +6 °C, respectively, in the presence of 3 μM ds26 (corresponding to 39 μM in base pairs) and +0, 0, 0, and +1 °C, respectively, in the presence of 10 μM ds26 (corresponding to 130 μM in base pairs, see

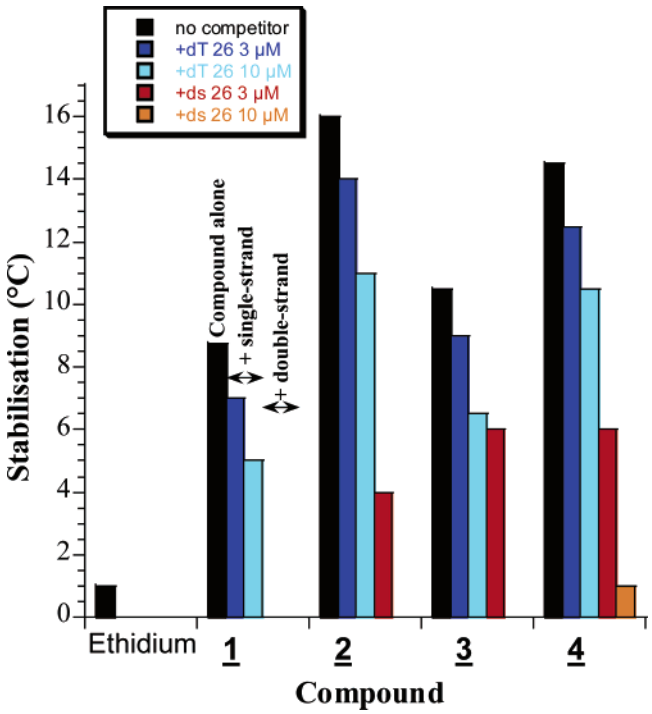

FIGURE 5: Stabilization of the $T_{1/2}$ of the F21T fluorescent oligonucleotide by the ethidium compounds alone (black bars) or in the presence of 3 and 10 μM single-stranded dT_{26} competitor (dark blue and light blue bars, respectively) or in the presence of 3 and 10 μM double-stranded ds26 competitor (red and orange bars, respectively).

Figure 5, orange bars). Compound **4** is therefore the only ligand that still (slightly) stabilizes the quadruplex in the presence of a very large excess of a double-stranded oligonucleotide, in agreement with a stronger selectivity of this dye toward quadruplexes.

Circular Dichroism. CD experiments were carried out to determine the number of binding sites of one of the drugs (**4**) on the parallel-stranded quadruplex $[TG_4T]_4$. The ligands under investigation do not show intrinsic optical activity but become slightly optically active when they bind to the DNA. They exhibit small induced CD signals between 280 and 310 nm when the parallel quadruplex structure $[TG_4T]_4$ is present in solution. In the case of the $[TG_4T]_4$ + **4** system, single-wavelength (295 nm) titration data extracted from the CD spectra (Figure 6) exhibited one inflection point at a molar ratio [drug]/[quadruplex] of 2.4. This parallel quadruplex structure has approximately two equivalent binding sites, which are compatible with external stacking.

Telomerase Inhibition. The ethidium derivatives were evaluated in the TRAP-G4 assay. As compared to the classical TRAP assay, TRAP-G4 uses a primer able to form a G4 structure as a template for telomerase and allows us to determine the inhibitory properties of G4 ligands against telomerase more accurately. In the presence of a specific ligand, G-quadruplex stabilization inhibits the formation of the TSG4 band in correlation with the telomere ladder inhibition. When used in combination with an internal primer (ITAS), the assay discriminates between G4-based telomerase inhibition and nonspecific Taq polymerase inhibition. A representative experiment of a comparison between ethidium and compound **4** by the TRAP-G4 assay is presented in Figure 7, and the IC_{50} obtained are summarized in Table 3.

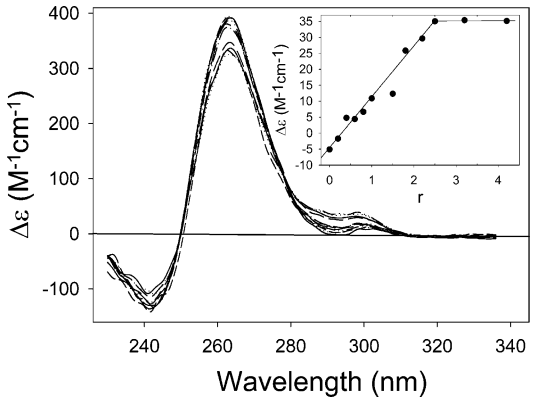

FIGURE 6: CD titration of the $[TG_4T]_4$ quadruplex with compound **4** in 150 mM ammonium acetate at 20 °C. The quadruplex concentration was 4 μM. The ellipticity change at 295 nm is plotted as a function of the drug/duplex fraction r (inset). An inflection point is observed at $r = 2.4$, which corresponds to a 2:1 complex.

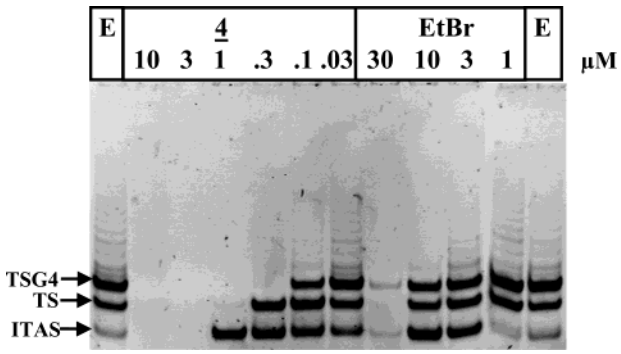

FIGURE 7: Inhibition of telomerase activity by ethidium derivatives in the TRAP-G4 assay. Ethidium bromide (EtBr) and derivative **4** were added to telomerase extracts as described in Experimental Procedures at the indicated concentrations. E, telomerase extract without compound. Arrows, positions of TSG4, TS, and ITAS PCR products. TSG4 band inhibition corresponded to a G4 stabilization of primer TSG4 and correlated with telomere ladder inhibition. ITAS inhibition corresponded to Taq polymerase inhibition.

Table 3: List of of the Compounds Used in This Study[a]

	IC_{50}		selectivity	
compound	G4 (μM)	ITAS (μM)	TRAP	MS
ethidium	10	15	1.5	0.8
1	0.3	1.9	6.2	2.8
2	0.1	0.22	2.2	2.5
3	1.1	2.5	2.4	2.9
4	0.12	2.2	18.3	5.03

[a] IC_{50} against telomerase in the G4 TRAP assay (IC_{50} G4) and against the internal PCR control (IC_{50} ITAS) (average of two to three independent experiments). Selectivity: ratio between the two IC_{50} (TRAP) or between the amounts bound to the three G4 structures vs the three duplexes determined by ESI-MS (MS).

Ethidium (EtBr) was found to be poorly active to block the TRAP-G4 assay; an effect was observed at a concentration that also blocked Taq polymerase activity. Other derivatives were found to be potent inhibitors of the assay (IC_{50} between 0.1 and 1 μM), with derivatives **4** and **2** being the most active and **3** the less active of the series. A good correlation was found between the TRAP-G4 assay results and the FRET melting experiment.

The assay also showed that ethidium derivatives inhibited the activity of Taq polymerase but at different concentrations,

as compared to their IC_{50} in TRAP-G4. Their selectivity ratio was ranging from 2- to 18-fold. Such criterion allowed us to discriminate between the two most active derivatives. Compound **4** was found as the most selective and potent G4 ligand of the series, as compared to **2** that was active but poorly selective. A side chain modification existing between **2** and **1** is able to significantly improve selectivity to a 6-fold ratio but is detrimental to the potency of the compound.

DISCUSSION

Different approaches have been proposed to identify G4 ligands: molecular modeling/docking (*61*, *62*), a DNA polymerase stop assay (*63*), T_m experiments (*18*), and FRET methods (*59*, *60*). However, the specificity toward G4 (vs duplexes or single strands) is a major issue. In our hands, it has been relatively simple to find quadruplex ligands and much harder to evidence G4-specific ligands. A standard dialysis assay may help to evaluate this specificity (*43*, *46*, *58*, *64*). Other techniques, such as fluorescence spectroscopy (*34*) or surface plasmon resonance, are possible (*22*, *58*). In all cases, the binding of a ligand to a G4 structure is compared to the binding to duplexes or single strands under identical conditions. Unfortunately, these methods are somewhat time-consuming and laborious. As a result, little data are available concerning the selectivity of these molecules.

Screening of Sequence Selectivity. A number of independent techniques have been implemented to unambiguously demonstrate the interaction of ethidium derivatives with quadruple-stranded nucleic acids. Each assay has different optimal experimental conditions, and these conditions were established previously. It is not possible, for example, to perform the MS analysis in a potassium buffer. As a consequence, buffer conditions may vary between experiments. It would have been better to perform all experiments in a standardized buffer, but this is unfortunately impossible. Despite this limitation, it is interesting to note that we could establish a consistent trend. The mass spectrometric data gave results similar to the dialysis experiments: all the ethidium derivatives have a preference for quadruplex, and the higher binding selectivity is obtained with **4**. Within the quadruplex family, equilibrium dialysis showed that the affinities for the antiparallel structure (22AG) are slightly better than the parallel (24G20) G4 structure except for the drug **4**. MS results also showed a slight preference, if any, for the antiparallel quadruplex $(GGGTTA)_3GGG$ as compared to the parallel $[TG_4T]_4$ structure. These oligonucleotides are reminiscent of quadruplex 22AG and 24G20, respectively, used in the dialysis assay. Even if the experimental conditions (i.e., ammonium acetate vs NaCl for dialysis) and oligonucleotide sequences are different, the overall conclusion is the same.

Binding Mode and Stoichiometry. Almost all G4 ligands described so far have extended planar chromophores, thus favoring $\pi-\pi$ interactions with the G-quartet surface. Some recent papers describe the interaction between small organic molecules with the G-quadruplex by an external stacking (*19*, *65*, *66*). However, at least for some porphyrin molecules, there is still a controversy concerning the mode of recognition. Concerning the ethidium derivatives, some of the experiments presented here (as well as previous observations (*34*)) offer some indications on the binding mode.

In contrast to equilibrium dialysis, mass spectrometry experiments allow the unambiguous determination of the maximum number of dyes bound per structure. For all three quadruplexes tested here, a maximum number of two dyes per quadruplex was found, in agreement with the presence of two external binding sites at both ends of the quadruplex, independently of the number of quartets.

Circular dichroism experiments showing the presence of one inflection point ($r = 2.4$) are consistent with a single binding mode. About two drug molecules bind to the quadruplex $[TG_4T]_4$. This value is in good agreement with the mass spectrometric experiment that showed only 1:1 and 2:1 complexes, even at a high drug concentration (data not shown). The binding of one drug at each end of the quadruplex through external stacking rather than through intercalation between the G-quartets must be considered. Fluorescence energy transfer between the DNA bases and the G4 ligands allowed us to demonstrate a close proximity between the G-quartets and the dye (*34*). However, both intercalation and terminal end stacking would explain such a FRET phenomenon, whereas interaction with G4-DNA via the grooves or the connecting loops would probably not lead to any measurable energy transfer from the bases to the ligand. On the basis of available evidence for planar aromatic molecules, stacking of the drug on the outer planes of the G-quartets might be the most likely model.

To address this question, preliminary ^{1}H-NMR experiments were performed with the 22AG oligonucleotide compound. Without ligand, the 1-D ^{1}H-NMR spectrum of this oligonucleotide in 0.1 M NaCl (pH 7.0, 7 °C) was very similar to previously published data (ref *3*; L. Lacroix and J.-L. Leroy, unpublished results). The addition of an equimolar amount of compound **1**, **2**, or **3** resulted in the alteration of a few peaks in the imino region attributed to guanine 16 and 4 (*3*) and T6.A19 or T18.A7 base pairs. These two guanines are located in a terminal G-quartet, suggesting a preferential external binding of the dye. This mode of binding is in agreement with the mass spectrometry experiment, in which none of the ammonium cations sandwiched between the guanine quartets are evicted upon ligand binding to the parallel quadruplex. In other words, our experiments suggest that these ethidium derivatives interact through external stacking, although we cannot completely rule out intercalation or groove binding.

It is important to note that the human telomeric repeat may adopt an intramolecular parallel quadruplex in potassium (*4*). Most of the data presented here were obtained in buffers that contain little, if any, potassium. This choice was dictated by technical reasons: it is not possible to perform the equilibrium dialysis experiment in a potassium buffer, as SDS (sodium dodecyl sulfate) is required for drug−DNA complex disruption and KDS precipitates (L. Guittat and P. Alberti, unpublished observations). Ammonium is the only choice for ESI-MS experiments. Finally, it is essential to keep the Na^+ (or K^+) concentration low for FRET experiments, to reduce the $T_{1/2}$ of the quadruplex (*51*). Nevertheless, we previously demonstrated that compound **4** could bind to the human telomeric motif in a potassium buffer (*34*). This indicates that recognition of a DNA quadruplex is still observed with the physiological monocation (K^+). Work is currently under progress to determine whether these ligands prefer antiparallel or parallel quadruplexes.

Correlation between Quadruplex Binding and Biological Activity. Ethidium derivatives were previously reported to inhibit telomerase activity (*34*); however, these initial experiments were performed using a classical TRAP assay adapted for scintillation proximity (SPA). Furthermore, Taq polymerase inhibition was performed in a separate assay. Unfortunately, this SPA TRAP assay did not allow a direct visualization of the reaction products. Since the TRAP assay is based on the calculation of the decrease of the TS band and telomere ladder products, it underestimates the inhibitory potency of a selective compound such as **4** (compare inhibition of the TS band and the TSG4 band in Figure 7). As a consequence, the calculation of the compound selectivity is also modified. For these reasons, **4** was not initially evaluated as the most active and selective compound of the series, a situation that is corrected now by the modification in the TRAP-G4 assay and the inclusion of an ITAS (*53*). Interestingly, there is a rather good correlation between data obtained in the TRAP-G4 and measurements obtained by mass spectrometry. A selectivity index could also be calculated from the ratio of the mean amount of ligand bound to G4 over the mean amount of ligand bound to duplexes (Table 3). The selectivity index obtained by mass spectroscopy experiments correlates relatively well with the selectivity obtained from TRAP-G4 experiments, suggesting that mass spectroscopy is an interesting method to predict biochemical activity of G4 ligand inhibitors of telomerase. It is also interesting to mention that, perhaps as a result of a higher in vitro selectivity toward quadruplexes, **4** is less cytotoxic than **1** on A549 human adenocarcinoma cells (IC_{50} of 18 and 4 μM, respectively, as compared to 9 μM for telomestatin; see Experimental Procedures for details).

Significance. The number of G4 ligands has grown rapidly over a few years (*27*). G4 ligands require a structural selectivity (i.e., preferential binding to quadruplexes over duplexes and single strands). Several experiments dealing with the specificity of a family of ethidium derivatives have been performed in this study. A standard dialysis assay helped to evaluate the specificity of these compounds. Electrospray ionization mass spectrometry confirmed that, among these ligands, **4** is the most promising compound. The FRET assay was performed in the presence of an excess of double-stranded DNA thus trapping unspecific G4 ligands, and **4** induces a significant stabilization under these conditions. Finally, the TRAP-G4 assay (*53*), which allows the unambiguous detection of the inhibitory properties of a G4 ligand on telomerase activity, confirmed that **4** is the most selective inhibitor.

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