Small-Molecule Selectively Recognizes Human Telomeric G-Quadruplex DNA and Regulates Its Conformational Switch

Mingli Chen, Guangtao Song, Chunyan Wang, Dan Hu, Jinsong Ren,* and Xiaogang Qu
State Key Laboratory of Rare Earth Resources Utilization, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, People's Republic of China; and Graduate School of the Chinese Academy of Sciences, Beijing, People's Republic of China

ABSTRACT Structural complexity is an inherent feature of the human telomeric sequence, and it presents a major challenge for developing ligands of pharmaceutical interest. Recent studies have pointed out that the induction of a quadruplex or change of a quadruplex conformation on binding may be the most powerful method to exert the desired biological effect. In this study, we demonstrate a quadruplex ligand that binds selectively to different forms of the human telomeric G-quadruplex structure and regulates its conformational switch. The results show that not only can oxazine750 selectively induce parallel quadruplex formation from a random coil telomeric oligonucleotide in the absence of added cations, it also can easily surpass the energy barrier between two structures and change the G-quadruplex conformation in Na^+ or K^+ solution. The combination of its unique properties, including the size and shape of the G-quadruplex and the small molecule, is proposed as the predominant force for regulating the special structural formation and transitions. These results may stimulate the design of new quadruplex binders that would be capable of discriminating different G-quadruplex structures as well as controlling biological phenomena, functional molecules, and nanomaterials.

INTRODUCTION

Chromosomes in human cells are capped at both ends with noncoding tandem $(TTAGGG)_n$ tracts called telomeres. DNA replication produces a blunt-ended leading strand telomere and a lagging strand telomere carrying a single-stranded G-rich overhang of 100–200 nucleotide (nt) at its end (1,2). The 3′G-rich overhang can fold to form a unique secondary DNA structure that has three stacked guanine tetrads, which is termed an intramolecular G-quadruplex (3–8). Since the substrate of telomerase is the 3′-single-stranded overhang of telomeric DNA, the formation and function of these G-quadruplexes are currently of great interest because of their roles in important biological processes, such as aging and cancer, and their potential as therapeutic targets for cancer (9–12). Furthermore, ligands that selectively bind to G-quadruplex motifs have become the focus of attention in recent years because they may interfere with telomere structures, telomere elongation/replication, and proliferation of cancer cells. To date, a diverse array of G-quadruplex-stabilizing compounds have been identified (13–24), and convincing reports on the efficiency of quadruplex interacting molecules as therapeutically active reagents are beginning to appear in the literature. Some of these compounds have shown encouraging biological effects in vitro that go beyond telomerase inhibition, including telomeric disruption, formation of anaphase bridges, apoptosis, and in vivo activity in mouse xenograft models (25–27). However, there is no general code that explains the ligand-quadruplex binding, and a sequence/conformation-dependent interaction may also be important.

G-rich oligonucleotides can be very polymorphic, and the adopted structures are dependent on several factors, including the base sequence, strand concentration, loop connectivities, and cations present. In the case of a human telomere DNA repeat, there are 208 possible structures when the eight possible quartet orientation combinations are considered with the 26 possible folds (28,29). Experimentally, only four actual structures have been determined. The first of these was observed by NMR spectroscopy in Na^+ solution, which revealed an intramolecular antiparallel basket-type G-quadruplex structure with alternating directions of the G3 runs (3). The basket-type G-quadruplex has one diagonal and two lateral or edgewise loops. In 2002, a very different G-quadruplex structure of the same sequence was observed in a K^+-containing crystal (4). In this propeller-type structure, all four strands are parallel and the connecting TTA loops are double chain reversal loops. More recently, an NMR-derived hybrid, or mixed conformation with three parallel and one antiparallel orientation of the guanine columns, was reported (6,7) (Scheme 1). These different types highlight the remarkable diversity found in the single-chain topology.

Although recent biological investigations confirmed the efficiency of quadruplex ligands as antiproliferative agents, they also showed that the mechanisms underlying the drug action can be drastically more complex (30,31), as the biological and biochemical consequences of binding of ligands to G-quadruplex structures found in telomeres and promoter regions of certain important oncogenes go beyond mere stabilization of these structures (16). It has also been pointed out that it is not necessary for a compound to target an existing cellular quadruplex to have biological activity; the induction of a quadruplex or change of a quadruplex

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*Correspondence: jren@ciac.jl.cn

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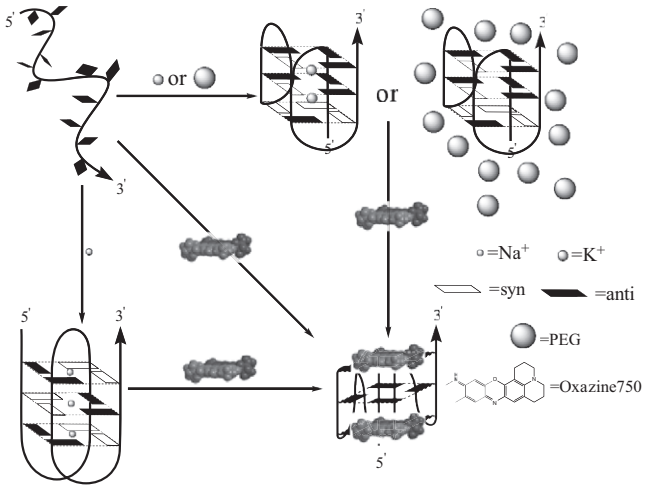

SCHEME 1 Schematic representation of oxazine750 bound selectively to different forms of human telomeric G-quadruplex DNA, structures of oxazine750, and the three types of intramolecular human telomeric G-quadruplex.

conformation on binding may be the most powerful method to exert the desired biological effect (32). Furthermore, switching between antiparallel and parallel G-quadruplexes of guanine-rich telomere DNAs plays a vital role in chromosome association (33). It has been observed that molecular crowding with neutral polymers induced transition from the antiparallel to the parallel G-quadruplex, or from the intramolecular G-quadruplex to a long, multistranded G-wire structure, and the formation of parallel G-quadruplex (34–36). Some divalent metal ions have also been reported to affect the stability of the antiparallel G-quadruplex and induce its structural transition to the parallel form (37,38). Although many elegant studies on G-quadruplex binders have appeared, it would be interesting to determine whether G-quadruplex ligands stabilize the hybrid-type, parallel/antiparallel, or other type of G-quadruplex folding. To date, only slight progress has been made in discriminating specific quadruplexes, and only a few small molecules have been reported to influence the structural transition of G-quadruplexes (16,18–20,22,23,39). Telomestatin, a natural product isolated from *Streptomyces amulatus* 3533-SV4, is the most potent inhibitor of telomerase, and under certain conditions it can convert the preformed mixed-type hybrid human telomeric G-quadruplex to the antiparallel basket form (16). Under the same conditions, the synthetic, expanded porphyrin compound Se2SAP can convert the preformed basket-type to a hybrid G-quadruplex (16). Recently, a diethylene triamine modified anthracene compound was reported to induce the folding of G-quadruplex from single-stranded DNA under salt-deficient conditions through a threading and stacking mode (19). Oxazine dyes are an important group of chromophoric compounds that are used as biological stains (e.g., to monitor Alzheimer's disease), fluorescence standards, and laser dyes (40–42). Recently, we found that a member of this family, oxazine170, a small crescent-shaped cationic molecule with four fused aromatic rings, could selectively induce the formation of a hybrid triplex structure, poly rA:(poly dT)$_2$, under solution conditions in which the triplex would not otherwise form (43). In this study, we demonstrate for the first time (to our knowledge) that its analogue, oxazine750 (Scheme 1), shows preferential binding to the parallel form of human telomeric G-quadruplex. Oxazine750 is unique in that not only can it selectively induce parallel quadruplex formation from a random coil telomeric oligonucleotide in the absence of added cations such as K^+, and Na^+, it can also regulate the transition of the preformed antiparallel or mixed-type hybrid G-quadruplex to the parallel form.

MATERIALS AND METHODS

See the Supporting Material.

RESULTS AND DISCUSSION

Oxazine750 facilitates the formation of parallel human telomeric G-quadruplex

G-quadruplex can exist in parallel, antiparallel, and mixed-type parallel/antiparallel structures depending on the different cations present (3–8). Although the relation between circular dichroism (CD) spectra and topology is not very clear, it has been suggested that, for the most part, the various topologies of quadruplex DNA can be correlated with specific CD signatures (19,44,45). An antiparallel structure is characterized by a negative peak near 260 nm and a positive peak near 295 nm, whereas a parallel structure displays a negative peak near 240 nm followed by a positive peak near 264 nm (16,19,36,37), and a mixed-hybrid structure has two positive maxima at 295 and 268 nm (16). In the absence of ligand, the CD spectrum of human telomeric d[$AG_3(T_2AG_3)_3$] oligonucleotide was found to have a negative band centered at 238 nm, a major positive band at 257 nm, and a minor negative band at 280 nm, consistent with an unstructured single strand. However, upon addition of excess oxazine750 to the solution at room temperature, a dramatic change in the CD spectrum was observed. The bands at 238, 257, and 280 nm disappeared, and negative and positive CD bands at 241 and 263 nm appeared (Fig. 1 *A*). The resultant CD spectrum is characteristic of a parallel quadruplex and indicates that oxazine750 binds to DNA and causes substantial changes in the conformation of the DNA. Furthermore, in the presence of oxazine750, an induced CD signal for the ligand was observed between 520 and 710 nm (Fig. 1 *B*, *square*). This induced signal is additional evidence for the interaction between the DNA and ligand, since its appearance in the CD spectra is indicative of the change in the chirality of the proximal chemical environment of oxazine750. For comparison, another two parallel G-quadruplex structures that were formed in Na^+

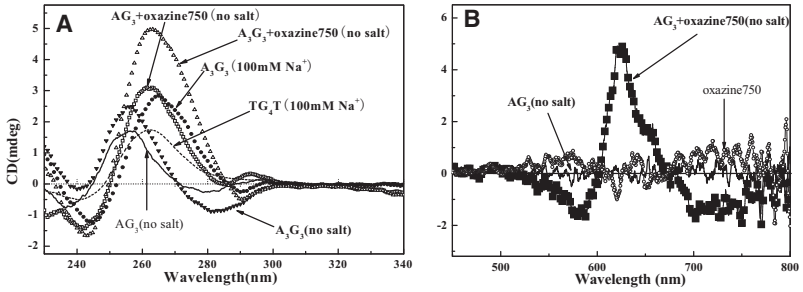

FIGURE 1 (*A*) CD spectra of d[$AG_3(T_2AG_3)_3$] with oxazine750, d[$AG_3(A_3G_3)_3$] with Na^+ and oxazine750, and d[$TG_4T]_4$ with Na^+ in 10 mM Tris-HCl, pH 7.4. (*B*) Induced CD signal for d[$AG_3(T_2AG_3)_3$] with oxazine750 is observed between 580 and 710 nm in 10 mM Tris-HCl, pH 7.4. DNA concentrations were 2 μM/strand for all of the sequences.

solution by the d[$TG_4T]_4$ oligonucleotide (46) and the mutant d[$AG_3(A_3G_3)_3$] oligonucleotide (47,48) are also included in Fig. 1 *A*, further supporting the notion that d[$AG_3(T_2AG_3)_3$] adopts a parallel-stranded topology in solution, as in the K^+ crystalline state (4). The CD spectrum of the ligand-induced human telomeric G-quadruplex d[$AG_3(T_2AG_3)_3$] under salt-deficient conditions best matches the signature patterns of the parallel G-quadruplex. In addition, once the parallel structure is formed, this conformation is not changed upon the addition of even 100 mM Na^+ (see Fig. S3 in the Supporting Material). Since factors such as the base sequence, strand concentration and/or orientation, and loop connectivities may all influence the ligand binding, we also examined the effect of oxazine750 on other distinct G-quadruplex DNAs: the two-repeat Oxytricha nova telomeric sequence d[$G_4T_4G_4]_2$, the 24 nt monomeric Oxytricha nova telomeric sequence d[$(T_4G_4)_4$], and the tetramolecular d[$TG_4T]_4$ oligonucleotide. It has been shown that d[$TG_4T]_4$ forms a parallel G-quadruplex (46), and the d[$G_4T_4G_4]_2$ and d[$(T_4G_4)_4$] sequences form an antiparallel G-quadruplex in Na^+ (23,38). However, oxazine750 does not induce either the parallel or antiparallel G-quadruplex for these sequences under salt-deficient conditions (Fig. S1 *C*). We further investigated the effect of the flanking sequence d[$(T_2AG_3)_4$] and the mutant sequence d[$AG_3(A_3G_3)_3$] on formation of the G-quadruplex. For the flanking sequence that has two extra Ts in the 5′ end compared to the 22 nt human telomeric oligonucleotide, the same CD bands appeared (data not shown), indicating that an identical parallel G-quadruplex structure is formed. Our data show that the 5′-end capping does not influence the induction of the parallel human telomeric G-quadruplex by oxazine750. Of interest, for the mutant sequence in which the thymine residues in the loop region were mutated to adenines, a characteristic major positive band at 263 nm and a minor negative band at 243 nm appeared upon the addition of oxazine750 (Fig. 1). This phenomenon was previously reported for the parallel structure of d[$AG_3(A_3G_3)_3$] (47,48), which suggests that oxazine750 converts single-stranded mutant telomeric DNA into a parallel G-quadruplex in the absence of added salt. In addition, the intensity of the positive band at 263 nm in the presence of oxazine750 is almost twice as great as that in 100 mM Na^+, indicating that oxazine750 alone has a much more strong stabilizing effect than 100 mM Na^+ for the mutant sequence. These results demonstrate that oxazine750 is capable of discriminating between quadruplexes, and the specific selectivity of the human telomeric G-quadruplex is related not only to the G-quadruplex topology, but also to composition of the sequence and loop.

To gain further insight into the facilitating and stabilizing effects on the parallel G-quadruplex by oxazine750, we carried out thermal denaturation studies (Fig. 2). G-quadruplexes have different stabilities in different concentrations of cations, as reflected by their melting temperature (T_m) (49). Fig. 2 *A* shows that the telomeric sequence exhibited hypochromicity in the presence of oxazine750 without the addition of salt, whereas no transition was seen in the absence of ligand. The T_m value (45.5°C) is close to that obtained in 10 mM Na^+ solution (43.1°C), which indicates that oxazine750 induced the formation of a stable G-quadruplex and mimicked the effect of at least 10 mM Na^+ solution in terms of thermal stability. Furthermore, oxazine750 showed a more dramatic stabilizing effect for the mutant sequence:

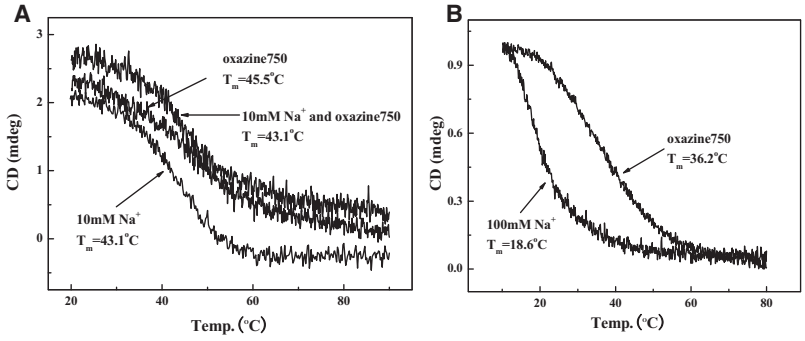

FIGURE 2 (*A*) CD melting curves of 2 μM d[$AG_3(T_2AG_3)_3$] with 10 mM Na^+, with 16 μM oxazine750 in the absence of added salt and with 10 m Na^+ and 16 μM oxazine750. (*B*) CD melting curves of 5 μM d[$AG_3(A_3G_3)_3$] with 100 mM Na^+ and with 40 μM oxazine750 in the absence of added salt . Absorbance changes were collected at 263 nm for the parallel G-quadruplex and 295 nm for the antiparallel G-quadruplex, respectively.

$d[AG_3(A_3G_3)_3]$ formed a parallel G-quadruplex structure with a T_m of <20°C in 100 mM Na^+ buffer (Fig. 2 *B*) (47). In contrast, the oxazine750-induced parallel G-quadruplex had a T_m of 36.2°C. The stabilization induced by oxazine750 far exceeded that by 100 mM Na^+ with a ΔT_m of 18°C, which is fully consistent with the large CD intensity change shown in Fig. 1 *A*.

Oxazine750 affects and regulates the type of G-quadruplex conformation found in solution

To date, several group 1 and 2 metal cations and NH_4^+ ions have been identified as stabilizing the G-quadruplex; of these, K^+ and Na^+ are the most abundant intracellular cations with clear physiological relevance. Therefore, in this section we focus our attention on how oxazine750 might affect the equilibrium between different G-quadruplex structures found in Na^+ and K^+ solutions. We first investigated the structure and stability of $d[AG_3(T_2AG_3)_3]$ with oxazine750 in the presence of Na^+. As shown in Fig. 2 *A*, $d[AG_3(T_2AG_3)_3]$ forms a stable antiparallel structure with a T_m of 43.1°C as described earlier in the presence of 10 mM Na^+ (see Fig. S1 for the CD spectra in 10 mM Na^+). Upon the addition of oxazine750, the CD spectra dramatically changes again, with a disappearance of the maxima at 295 and 243 nm, and the minima at 264 nm that corresponds to antiparallel G-quadruplex structures, and the emergence of parallel conformation characteristics with the distinctive maxima and minima patterns of 263 and 243 nm (shown in Fig. 3). These spectra closely resemble those of the same sequence with oxazine750 in the absence of salt. The results suggest that the structural transition from the antiparallel to the parallel G-quadruplex occurred continuously and cooperatively with the ligand titration (Fig. S2 *A*). In addition, the conformational switch of $d[AG_3(T_2AG_3)_3]$ oligonucleotide by oxazine750 in 10 mM Na^+ was verified by thermal denaturation analysis. As shown in Fig. 2 *A*, the T_m value (44.6°C) is almost the same as that in the presence of ligand alone, which further demonstrates that oxazine750 has the ability to convert either the random structure or the preformed antiparallel G-quadruplex that is present in the 10 mM Na^+ condition exclusively into the parallel G-quadruplex conformation. These results are consistent with the CD spectra obtained at different temperatures under the same conditions (Fig. S2 *B*). The structural transition between the antiparallel and parallel G-quartet of the 24 nt monomeric Oxytricha nova telomeric sequence $d[(T_4G_4)_4]$ has been shown in a phase diagram, in which 225 mM Na^+ or 65 mM K^+ was required for the transition from an antiparallel to a parallel structure (33). Recently, Miyoshi et al. (37,38) showed that a parallel G-quartet of $d[G_4T_4G_4]_2$ was induced from an antiparallel structure by 1 mM Ca^+ in the presence of 7 mM Na^+. However, in the study presented here, only a few micromole ligands could induce the parallel human telomeric G-quadruplex structure in the presence of 10 mM Na^+, indicating that a specific ligand-G-quartet interaction is essential for the structure transition, and that the specific ligand-G-quartet interaction may be stronger than the Na^+ coordination and replace the need for monovalent cations to facilitate the formation and regulation of G-quadruplex structures under certain conditions.

Since it has been shown that G-quadruplexes formed by $d[AG_3(T_2AG_3)_3]$ are more stable at elevated monovalent cations (48), we explored the effect of oxazine750 on $d[AG_3(T_2AG_3)_3]$ in 100 mM Na^+. The samples were heat-denatured and cooled to ensure that the structures were formed under the intended environment before CD recordings were obtained. In the presence of eight equivalents of oxazine750, the CD spectrum showed a dramatic change (Fig. S3). The coexistence of the characteristics of the antiparallel and parallel conformers suggests that the structure formed here is a quadruplex that contains mixed parallel/antiparallel components, or that it is a mixture of G-quadruplex structures that have parallel and antiparallel characteristics. To gain further insight into the ligand-induced structural changes, we carried out ultraviolet (UV) melting under the same conditions. The melting curve of $d[AG_3(T_2AG_3)_3]$ in the presence of 100 mM Na^+ was found to be monophasic (Fig. 3 *B*), indicating the melting of one type of the oligonucleotide, and that the T_m value (58.3°C) was much higher than that observed in 10 mM Na^+ solution (43.1°C). Of interest, upon the addition of four equivalents of oxazine750, an apparently broad and biphasic melting profile was obtained (Fig. 3 *B*), indicating the existence of two species, each of which showed distinct quadruplex melting transitions.

A low melting transition with a T_m value of ~40°C is followed by a higher temperature transition with a T_m value of

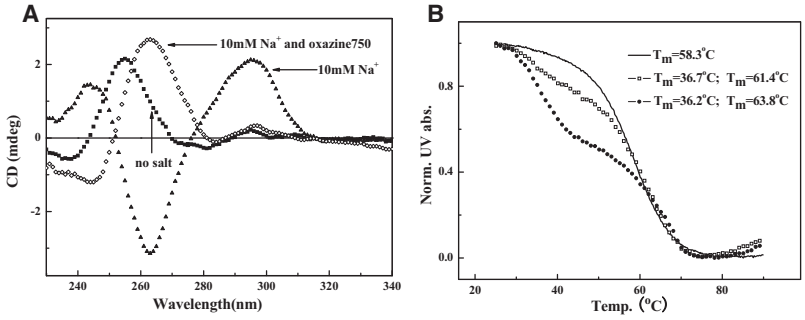

FIGURE 3 (*A*) CD spectra of 2 μM $d[AG_3(T_2AG_3)_3]$ with Na^+ and oxazine750. (*B*) Normalized UV melting curves of 2 μM $d[AG_3(T_2AG_3)_3]$ with 100 mM Na^+ (*black line*), with 100 mM Na^+ and 8 μM oxazine750 (*open square*), and with 100 mM Na^+ and 20 μM oxazine750 (*circle*). Absorbance changes were collected at 295 nm.

~60°C. When the amount of ligand was further increased by 10 equivalents, the T_m values of the two transitions were almost unaffected. However, a significantly increased hypochromicity of the low melting profile was observed (Fig. 3 *B*). Keeping in mind that the ligand-induced parallel G-quadruplex structure has a melting transition at ~43°C in the absence of added cations or in 10 mM Na^+ solution, it can be concluded that the lower temperature transition corresponds to the melting of the parallel quadruplex species, whereas the higher temperature transition is the melting of the antiparallel quadruplex species. The hypochromicity of the melting curve indicates that a more parallel G-quadruplex conformation was formed when the amount of ligand was increased. These results demonstrate that the solution contained mixed parallel/antiparallel components in the presence of oxazine750 at 100 mM Na^+, and further confirm that the small-molecule oxazine750 bound selectively to the parallel G-quadruplex and regulated its structural transition. The structural transition of $d[AG_3(T_2AG_3)_3]$ oligonucleotide induced by oxazine750 in the presence of different concentrations of Na^+ suggests that the effect of the ligand on the transition is competitive with Na^+ in facilitating the formation of the G-quadruplex.

AG_3 forms a mixed conformation in K^+ solution, and K^+ is more potent than Na^+ in stabilizing a G-quadruplex structured as a result of the relative free energies, hydration state, and ionic radius for optimal sphere coordination (19). The unique characteristics of oxazine750 in salt-deficient conditions and in Na^+ solution raise the possibility that it could also affect the equilibrium between different G-quadruplex structures in K^+ solution. Indeed, we found that the addition of the ligand induced the development of a new conformation. In 6 mM K^+ solution, the CD spectrum of $d[AG_3(T_2AG_3)_3]$ showed a distinctive shoulder located near 270 nm on the strong positive band at 289 nm, and a small negative peak near 235 nm (Fig. 4 *A*), similar to previous findings for human telomeric DNA in K^+ solution (16,23). Upon the addition of oxazine750, pronounced and dramatic changes were observed (Fig. 4 *A*). Thus, it seems that the major species induced by oxazine750 in the presence of K^+ is a mixture of species or, alternatively, is a mixed-type hybrid G-quadruplex. Since sodium has a low binding affinity for $d[AG_3(T_2AG_3)_3]$ G-quadruplex, and the ligand had a different effect on the quadruplex in Na^+ and K^+ solution, we analyzed the thermal stability of the quadruplex in the presence and absence of oxazine750 in K^+ solution. The results in Fig. 4 *B* show that the quadruplex had a T_m of 51.0°C in 6 mM K^+, which is more stable than that in 10 mM Na^+ (43.1°C). In contrast, the quadruplex had an unusual stability with a T_m of nearly 77°C in the presence of oxazine750, which far exceeded that by oxazine750 in Na^+ solution with a T_m stability of 44.6°C. In addition, we also investigated the effect of oxazine750 in 20 mM K^+. As shown in Fig. S4, the CD spectrum is similar to that with oxazine750 in 6 mM K^+. The CD melting showed a higher stability, with a T_m of 59°C in the absence of ligand, and again an unusual stability was observed, with a T_m of nearly 79°C in the presence of oxazine750, which is close to that in 6 mM K^+ (Fig. 4 *B*). Compared to the competitive effect of oxazine750 in Na^+ solution, the unusual behavior in K^+ solution indicates that oxazine750 may have a synergetic effect with K^+ on the G-quadruplex transition. Molecular crowding has been reported to induce the transition from antiparallel to parallel quadruplex in Oxytricha nova telomeric DNA, and from intramolecular G-quadruplex to long multistranded G-wire in Tetrahymena $(T_2G_4)_3T_2G_2$ DNA (34,35). Under molecular crowding conditions created by polyethylene glycol (PEG), $d[AG_3(T_2AG_3)_3]$ was shown to form a G-quadruplex free of added cation, and adopt a parallel-stranded conformation in K^+ solution (36). To further understand the interaction between oxazine750 with $d[AG_3(T_2AG_3)_3]$ in solution, we carried out studies using PEG 200 as the crowding agent without adding a cation. The CD spectrum in 40% (w/v) PEG is similar to that previously reported and features a shoulder near 270 nm and a positive band at 289 nm, which are characteristic of a mixture of both parallel and antiparallel G-quadruplexes (Fig. 4 *A*, *triangle*). After the addition of eight equivalents of oxazine750, we observed a strong positive band at 262 nm and negative signal at 242 nm (Fig. 4 *A*, *open triangle*), respectively. A previous report showed that PEG induced a conformational conversion with an increase in PEG concentration in K^+ solution, and a mixed-type hybrid topology and a parallel-stranded topology were formed when PEG reached 30% and 40% (w/v), respectively (36). Our CD results match the key signatures of the parallel $d[AG_3(T_2AG_3)_3]$ quadruplex DNA formed in 150 mM K^+,

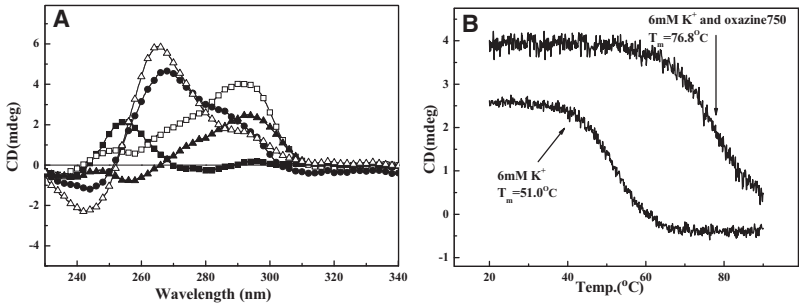

FIGURE 4 (*A*) CD spectra of 2 μM $d[AG_3(T_2AG_3)_3]$ with K^+ and/or PEG and oxazine750: with no salt added (*square*), with 6 mM K^+ (*open square*), with 6 mM K^+ and oxazine750 (*circle*), with 40% PEG 200 (*triangle*), and with 40% PEG 200 and oxazine750 (*open triangle*). (*B*) CD melting curves of 2 μM $d[AG_3(T_2AG_3)_3]$ with 6 mM K^+ and with 6 mM K^+ and 16 μM oxazine750.

Ap7: 5'-AGGGTT**Ap**GGGTTAGGGTTAGGG

Ap13: 5'-AGGGTTAGGGTT**Ap**GGGTTAGGG

Ap19: 5'-AGGGTTAGGGTTAGGGTT**Ap**GGG

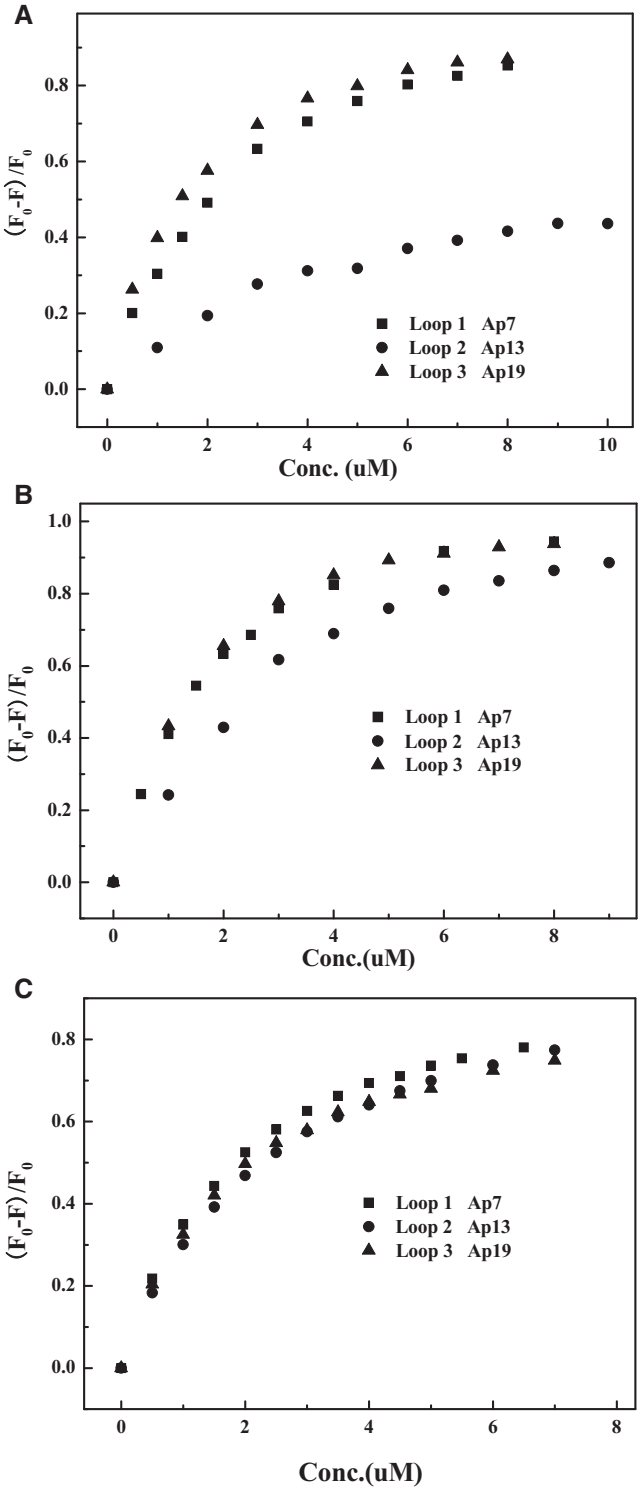

FIGURE 5 Plot of fluorescence intensity at 370 nm of 2-Ap individually labeled $d[AG_3(T_2AG_3)_3]$ versus concentration of oxazine750. DNA concentration was fixed at 1.0 μM (single strand conc.) and titrated by oxazine750 in different buffer at 15°C. Fluorescence emission spectra were recorded from 320 nm to 500 nm with an excitation wavelength at 305 nm. Squares: Ap7 in loop 1; circles: Ap13 in loop 2; triangles: Ap19 in loop 3. (*A*) $d[AG_3(T_2AG_3)_3]$ with 100 mM Na^+. (*B*) $d[AG_3(T_2AG_3)_3]$ with 10 mM Na^+. (*C*) $d[AG_3(T_2AG_3)_3]$ with 100 mM K^+ and 40% PEG 200.

40% PEG solution, suggesting that $d[AG_3(T_2AG_3)_3]$ adopted a parallel-stranded conformation in the presence of oxazine750 under molecular crowding condition, and that oxazine750 has the same ability as a >150 mM K^+ solution in terms of regulating the quadruplex conformation. Although oxazine750 can not totally convert the $d[AG_3(T_2AG_3)_3]$ DNA into a parallel conformation in K^+ solution as it does in 40% PEG, it is worthwhile to note that the CD profile was almost identical to the mixed-type hybrid structure formed in 30% (w/v) PEG (36). This result indicates that the ligand has effects equivalent to those of 30% PEG on the equilibrium between different G-quadruplexes. Collectively, the data shown here support the hypothesis that oxazine750 prefers the parallel conformation and has a synergetic effect with K^+ in stabilizing the G-quadruplex and regulating its structural transition.

Mode of binding

Although the detailed mechanism of how oxazine750 folds and regulates $d[AG_3(T_2AG_3)_3]$ conformation changes remains to be resolved, knowing the binding mode could help us understand the special selectivity of the ligand. Aminopurine (Ap) is a fluorescent adenine isomer that is able to form a Watson-Crick basepair with thymine, and has been widely used to probe local conformational changes in DNA as well as to verify the mode of ligand binding to G-quadruplex and i-motif DNA (23,50,51). In our study, the quadruplex was strategically substituted with 2-Ap at specific adenine residues (Ap7, Ap13, and Ap19; Fig. 5). The fluorescent intensities of the Aps are very similar to each other after added oxazine750 in the absence of added salt, which agrees with the similar local environment for the adenine residues in the random structure (Fig. S5). In contrast, the fluorescent intensity in Na^+ solution followed the order Ap7 > Ap19 > Ap13, as previously reported for the same structure (23,51). The large difference in fluorescence emission between the Aps reflects the heterogeneity of the TTA looping conformations in the Na^+ solution. In the early structure (3), A13 is packed within a diagonal loop at one end of the quadruplex, and A7 and A19 are in separate lateral loops at the opposite end. The titration of oxazine750 into a solution of the Ap-modified $d[AG_3(T_2AG_3)_3]$ oligonucleotide in 100 mM Na^+ shows a decrease in Ap fluorescence following the order A7 ≈ A19 > A13 (Fig. 5 *A*). This indicates that oxazine750 may bind to the end of the G-quartet by external stacking and contacts with the two lateral loops, a binding mode that has been

demonstrated in previous reports (23). In contrast, the fluorescence intensity changes of the Aps became very similar to each other in the absence of added cation or in 10 mM Na^+ (Fig. 5 *B*). Note that for the parallel conformation in K^+ solution, the crystal structure shows that residues A7, A13, and A19 are all in equivalent loop environments, with each intercalated between thymine residues (4). This result reflects the structural conversion induced by oxazine750, in agreement with the structural symmetry for the three external TTA loops. In addition, since the parallel-stranded human telomeric quadruplex has been also observed in K^+ solution under molecular crowding conditions created by PEG (36), we also analyzed the effect of oxazine750 on the Aps of the parallel quadruplex. The results in Fig. 5 *C* show that addition of oxazine750 affected residues to almost identical extents, further supporting the notion that oxazine750 bonds to the end of the parallel G-quartet by external stacking.

The gel electrophoresis technique is sensitive to structural conformations and has been extensively used to study G-quadruplex folding and the mode of ligand binding (23,51,52). S1 nuclease is widely used as an analytical tool for determining nucleic-acid structure. It binds to an exposed single strand, such as in a hairpin loop, and attacks the O-3′-P bond. The binding mode of oxazine750 is further supported by S1 endonuclease cleavage. Fig. S6 shows the cleavage pattern of 5′-fluorescein-labeled oligomer exposed S1 nuclease for 10 min at 25°C after incubation with different concentrations of oxazine750. All three bands appear to be due to cleavage within TAA loops, consistent with previous studies (23,51,52). The first S1 cleavage occurred in loop 1 near the 5′ end with the shortest length, the second cleavage occurred in loop 2 with intermediate length, and the third cleavage occurred in loop 3 near the 3′ end with the longest length. For the antiparallel G-quadruplex formed in 100 mM Na^+ solution, the lateral loop 1 was the most labile to S1 nuclease in the absence of added ligand (Fig. S6 *A*). Upon addition of increasing concentrations of oxazine750, a decreased cleavage at loop 1 and an increased cleavage at loop 3 were observed, whereas the cleavage at loop 2 was hardly influenced. This cleavage pattern is in accordance with our 2-Ap fluorescence studies and further supports the notion that oxazine750 binds to the end of the G-quartet by external stacking and contacts with the two lateral loops. Note that at higher concentrations of oxazine750 (Fig. S6 *A*, *line 5*), a modestly increased cleavage for loop 2 was observed, which is different from that found at lower ligand concentrations, indicating an increased accessibility of this loop to S1 nuclease. Keep in mind that in our early melting and CD studies, oxazine750 binding at a relative higher concentration resulted in partial parallel G-quadruplex formation at 100 mM Na^+. We speculated that this increased cleavage could be due to the partially formed parallel G-quadruplex structure in which loop 2 was in a propeller-like configuration. Of interest, a different cleavage pattern was found in the absence of added salt: cleavages in loops 2 and 3 were apparently increased upon the addition of increasing concentrations of oxazine750, and at the same time a dramatically decreased S1 cleavage was observed at loop 1 (Fig. S6 *B*). This different cleavage pattern demonstrates that the environments of the three propeller-like loops may be significantly affected by the ligand binding, leading to an increased accessibility of loops 2 and 3 to S1 nuclease. Stacking of the A-T basepairs in loop 1 was especially increased by oxazine750, because this loop was the most labile to S1 nuclease in the absence of oxazine750 but was substantially protected from cleavage after oxazine750 binding. Furthermore, as shown in the melting and CD studies (Figs. 1 and 2), oxazine750 binding resulted in a stable parallel G-quadruplex formation. This result shows a dramatic increasing intensity of the full-length $d[AG_3(T_2AG_3)_3]$ oligonucleotide, demonstrating that the parallel structure was stabilized and greatly protected from cleavage upon the addition of oxazine750. Overall, these results are fully consistent with oxazine750 converting the randomly structured oligomer exclusively into the parallel G-quadruplex and having an end-stacking type of binding propensity.

Although the detailed mechanism of the structural transition of $d[AG_3(T_2AG_3)_3]$ regulated by oxazine750 remains to be determined in further studies, we speculated that the combination of the unique structural character of the parallel G-quadruplex and oxazine750 molecule may be the key factor in quadruplex specificity and discrimination. As mentioned in the Introduction, previous studies with small molecules have shown different effects on the G-quadruplex structural transition and/or the formation of the intramolecular human telomeric sequence (16,18,20,23). Recently, a naphthyridine dimer was shown to act as a molecular glue in the assembly of two telomeric sequences by binding strongly to G-G mismatches in the hypothetical duplex of a telomeric dimmer (39). On the other hand, different G-quadruplex conformations may also contribute to the specificity or selectivity of a ligand binding. For example, a dominant feature of parallel DNA quadruplexes is that they present the large surface area of their terminal G-quartet to the environment (4). In cocrystals of a telomeric oligonucleotide and the anticancer drug daunomycin, three daunomycins were found to stack onto the 5′ end of the quadruplex (46). Overall, these results are supportive of our speculation: the specific oxazine-quadruplex interaction is probably the predominant force for the structural induction and conversion. In support of our reasoning, we found that relatively small changes in the structure of oxazine750 significantly altered binding and selectivity. Nile blue is an analogue of oxazine750. Both of these two benzo[a]phenoxazine dyes have amino or substituted amino groups in the 5- and 9-positions, whereas oxazine750 has two extra rings at the 8-, 9-, and 10-positions (Fig. S7). Under the exact same

conditions used for oxazine750, we found that Nile blue neither induced the G-quadruplex formation nor influenced the structural conformation. UV or CD melting showed that it interacted with the antiparallel-type G-quadruplex. In addition, the titration of Nile blue into a solution of the Ap-modified d[$AG_3(T_2AG_3)_3$] oligonucleotide in 100 mM Na^+ showed a decrease in Ap fluorescence following the order A7 $\approx$ A19 $>$ A13 (Fig. S8), indicating that, like many other G-quadruplex binders, it may also bind to the end of the G-quartet by external stacking and contacts with the two lateral loops (23). Results from the gel electrophoresis studies (Fig. S9) are also fully consistent with the preference of antiparallel conformation for Nile blue. It should be noted that a dominant feature of parallel DNA quadruplexes is that they present the large surface area of their terminal G-quartet to the environment (4,47). Thus, the more-extended surface of the oxazine molecule could fit well and pack tightly onto the end of the quadruplex stack, with the positive side chain forming H-bonding interactions and/or van der Waals contacts with the quadruplex groove. Furthermore, UV-visible (UV-vis) titrations were carried out in the absence of salt to determine the stoichiometry for oxazine750 binding to the human telomeric G-quadruplex structure. A sample of the d[$AG_3(T_2AG_3)_3$] was titrated by oxazine750 with the sum total concentration of ligand and oligonucleotide held constant at 5 μM. After each addition of oxazine750, the reaction was allowed to equilibrate for 20 min and the spectrum was collected. Fig. S10 *A* shows that the d[$AG_3(T_2AG_3)_3$] G-quadruplex bound four molecules of oxazine750. Our observation is not without precedent, although to our knowledge no structural rationalization has yet been presented for these kinds of high-binding stoichiometries. Studies carried out by De Cian et al. (21) using the same human telomeric sequence described the quadruplex-binding properties of new members of the bisquinolinium family: In the presence of Na^+, the ligand bound to the antiparallel G-quadruplex with a binding mode of stacking of the ligand onto the two external G-quartet, whereas in K^+ solution under certain condition, a ligand-induced structural change was observed along with a 4:1 binding stoichiometry. A binding isotherm for the interaction of oxazine750 with G-quadruplex was also obtained by UV-vis measurements (Fig. S10 *B*). An association constant of $3.7(\pm 1.0) \times 10^6\ M^{-1}$ was determined from the titration data. In our case, the ligands could interact with the larger G-quartet involving end stacking and external binding, or through end stacking and ligand aggregation. However, given the larger surface area of the parallel terminal G-quartet, which can simultaneously accommodate three-daunomycin molecules (46), this molecule could also have a binding propensity for stacking two oxazine750 molecules onto the G-quartet. In similarity to the bisquinolinium family (21), the dimension of the dimeric oxazine750 molecules (Fig. S11) is closely related to that of the G-quartet, and thus the selectivity and discrimination could originate from this accurate geometric complementarity. Furthermore, it well explains the structural conversion from the antiparallel to the parallel form, since the presence of diagonal and lateral loops in the antiparallel telomeric quadruplex could weaken the end stacking of oxazine750 molecules. More evidence is necessary to verify the proposed binding mode, and further investigation is under way in our laboratory.

CONCLUSIONS

Structural complexity is an inherent feature of the human telomeric sequence, and it presents a major challenge for developing ligands of pharmaceutical interest. To date, only slight progress has been made in achieving specificity between G-quadruplex conformations. In this study, we examined how the addition of the small molecule oxazine750 induced the formation of a parallel human telomeric G-quadruplex and regulated its conformational switch in solution. To our knowledge, our results provide the first insight into the selectivity and discrimination of oxazine750 for the human telomere sequence: not only can the ligand preferentially induce a parallel formation from a random coil telomeric oligonucleotide in the absence of added cations, it also can easily surpass the energy barrier between two structures and change the G-quadruplex conformation in Na^+ or K^+ solution. Of importance, the combination of its unique properties, including the size and shape of the G-quadruplex and the small molecule, is proposed as the predominant force for regulating the special structural formation and transitions. Crowding and crystal packing force can influence the type of G-quadruplex conformation, and it is also likely that small ligands can have equivalent effects on the identity of the conformations found. Given the apparent prevalence of DNA quadruplexes in biology and their applications in nanoscience, ligands such as oxazine750 present the possibility of being able to control biological phenomena, functional molecules, and nanomaterials. For example, if a G-quadruplex-forming sequence or a G-qudruplex is naturally present in genomic DNA, inducing a quadruplex or changing a quadruplex conformation on a specific ligand binding could subsequently change its molecular recognition, such as by disrupting the existing protein interaction and introducing new protein recognition. Furthermore, the specific interaction of oxazine750 with the human telomere sequence as described in this study can be used to gain an understanding of the biological roles played by quadruplexes, and to design next-generation molecules. Small ligands with more selectivity and discrimination for the sequence could be obtained through modification. Finally, our results indicate that a specific ligand-G-quadruplex interaction is essential for the structural transition and may be stronger than the Na^+/or K^+ coordination to the guanine O6, and it should be taken into account when the cellular responses to these agents are being evaluated.

SUPPORTING MATERIAL

Materials and methods and 11 figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01286-7.

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REFERENCES

1. Makarov, V. L., Y. Hirose, and J. P. Langmore. 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell.* 88:657–666.
2. Olaussen, K. A., K. Dubrana, J. Dornont, J. P. Spano, L. Sabatier, et al. 2006. Telomeres and telomerase as targets for anticancer drug development. *Crit. Rev. Oncol. Hematol.* 57:191–214.
3. Wang, Y., and D. J. Patel. 1993. Solution structure of the human telomeric repeat d[AG3(T2AG3)3] G-tetraplex. *Structure.* 1:263–282.
4. Parkinson, G. N., M. P. H. Lee, and S. Neidle. 2002. Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature.* 417:876–880.
5. Ren, J. S., X. G. Qu, J. O. Trent, and J. B. Chaires. 2002. Tiny telomere DNA. *Nucleic Acids Res.* 30:2307–2315.
6. Dai, J. X., M. Carver, C. Punchihewa, R. A. Jones, and D. Z. Yang. 2007. Structure of the hybrid-2 type intramolecular human telomeric G-quadruplex in K^+ solution: insights into structure polymorphism of the human telomeric sequence. *Nucleic Acids Res.* 35:4927–4940.
7. Luu, K. N., A. T. Phan, V. Kuryavyi, L. Lacroix, and D. J. Patel. 2006. Structure of the human telomere in K^+ solution: an intramolecular (3+1) G-quadruplex scaffold. *J. Am. Chem. Soc.* 128:9963–9970.
8. Paeschke, K., T. Simonsson, J. Postberg, D. Rhodes, and H. J. Lipps. 2005. Telomere end-binding proteins control the formation of G-quadruplex DNA structures in vivo. *Nat. Struct. Mol. Biol.* 12:847–854.
9. Zahler, A. M., J. R. Williamson, T. R. Cech, and D. M. Prescott. 1991. Inhibition of telomerase by G-quartet DNA structures. *Nature.* 350:718–720.
10. Gilley, D., H. Tanaka, and B. S. Herbert. 2005. Telomere dysfunction in aging and cancer. *Int. J. Biochem. Cell Biol.* 37:1000–1013.
11. Han, H., and L. H. Hurley. 2000. G-quadruplex DNA: a potential target for anti-cancer drug design. *Trends Pharmacol. Sci.* 21:136–142.
12. Jing, N., W. Sha, Y. Li, W. Xiong, and D. J. Tweardy. 2005. Rational drug design of G-quartet DNA as anti-cancer agents. *Curr. Pharm. Des.* 11:2841–2854.
13. Anantha, N. V., M. Azam, and R. D. Sheardy. 1998. Porphyrin binding to quadruplexed T(4)G(4). *Biochemistry.* 37:2709–2714.
14. Haq, I., J. O. Trent, B. Z. Chowdhry, and T. C. Jenkins. 1999. Intercalative G-tetraplex stabilization of telomeric DNA by a cationic porphyrin. *J. Am. Chem. Soc.* 121:1768–1779.
15. Mergny, J. L., L. Lacroix, M. P. Teulade-Fichou, C. Hounsou, L. Guittat, et al. 2001. Telomerase inhibitors based on quadruplex ligands selected by a fluorescence assay. *Proc. Natl. Acad. Sci. USA.* 98:3062–3067.
16. Rezler, E. M., J. Seenisamy, S. Bashyam, M. Y. Kim, E. White, et al. 2005. Telomestatin and diseleno sapphyrin bind selectively to two different forms of the human telomeric G-quadruplex structure. *J. Am. Chem. Soc.* 127:9439–9447.
17. Moorhouse, A. D., A. M. Santos, M. Gunaratnam, M. Moore, S. Neidle, et al. 2006. Stabilization of G-quadruplex DNA by highly selective ligands via click chemistry. *J. Am. Chem. Soc.* 128:15972–15973.
18. Goncalves, D. P. N., R. Rodriguez, S. Balasubramanian, and J. K. M. Sanders. 2006. Tetramethylpyridiniumporphyrazines—a new class of G-quadruplex inducing and stabilising ligands. *Chem. Commun. (Camb.).* 4685–4687.
19. Rodriguez, R., G. D. Pantos, D. P. N. Goncalves, J. K. M. Sanders, and S. Balasubramanian. 2007. Ligand-driven G-quadruplex conformational switching by using an unusual mode of interaction. *Angew. Chem. Int. Ed. Engl.* 46:5405–5407.
20. Fu, B. Q., J. Huang, L. Ren, X. C. Weng, Y. Y. Zhou, et al. 2007. Cationic corrole derivatives: a new family of G-quadruplex inducing and stabilizing ligands. *Chem. Commun. (Camb.).* 3264–3266.
21. De Cian, A., E. Delemos, J. L. Mergny, M. P. Teulade-Fichou, and D. Monchaud. 2007. Highly efficient G-quadruplex recognition by bisquinolinium compounds. *J. Am. Chem. Soc.* 129:1856–1857.
22. Monchaud, D., P. Yang, L. Lacroix, M. P. Teulade-Fichou, and J. L. Mergny. 2008. A metal-mediated conformational switch controls G-quadruplex binding affinity. *Angew. Chem. Int. Ed. Engl.* 47:4858–4861.
23. Yu, H., X. Wang, M. Fu, J. Ren, and X. Qu. 2008. Chiral metallo-supramolecular complexes selectively recognize human telomeric G-quadruplex DNA. *Nucleic Acids Res.* 36:5695–5703.
24. Izbicka, E., R. T. Wheelhouse, E. Raymond, K. K. Davidson, R. A. Lawrence, et al. 1999. Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. *Cancer Res.* 59:639–644.
25. Heald, R. A., C. Modi, J. C. Cookson, I. Hutchinson, C. A. Laughton, et al. 2002. Synthesis and telomerase-inhibitory activity of methylated pentacyclic acridinium salts. *J. Med. Chem.* 45:590–597.
26. Kim, M. Y., W. Duan, M. Gleason-Guzman, and L. H. Hurley. 2003. Design, synthesis, and biological evaluation of a series of fluoroquinoanthroxazines with contrasting dual mechanisms of action against topoisomerase II and G-quadruplexes. *J. Med. Chem.* 46:571–583.
27. Shammas, M. A., R. J. Shmookler Reis, C. Li, H. Koley, L. H. Hurley, et al. 2004. Telomerase inhibition and cell growth arrest after telomestatin treatment in multiple myeloma. *Clin. Cancer Res.* 10:770–776.
28. Lane, A. N., J. B. Chaires, R. D. Gray, and J. O. Trent. 2008. Stability and kinetics of G-quadruplex structures. *Nucleic Acids Res.* 36:5482–5515.
29. Webba da Silva, M. 2007. Geometric formalism for DNA quadruplex folding. *Chem. Eur. J.* 13:9738–9745.
30. Pennarun, G., C. Granotier, L. R. Gauthier, D. Gomez, F. Hoffschir, et al. 2005. Apoptosis related to telomere instability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands. *Oncogene.* 24:2917–2928.
31. Gomez, D., M. F. O'Donohue, T. Wenner, C. Douarre, J. Macadre, et al. 2006. The G-quadruplex ligand telomestatin inhibits POT1 binding to telomeric sequences in vitro and induces GFP-POT1 dissociation from telomeres in human cells. *Cancer Res.* 66:6908–6912.
32. Wilson, W. D., and H. Sugiyama. 2007. First international meeting on quadruplex DNA. *ACS Chem. Biol.* 2:589–594.
33. Rujan, I. N., J. C. Meleney, and P. H. Bolton. 2005. Vertebrate telomere repeat DNAs favor external loop propeller quadruplex structures in the presence of high concentrations of potassium. *Nucleic Acids Res.* 33:2022–2031.
34. Miyoshi, D., A. Nakao, and N. Sugimoto. 2002. Molecular crowding regulates the structural switch of the DNA G-quadruplex. *Biochemistry.* 41:15017–15024.
35. Miyoshi, D., H. Karimata, and N. Sugimoto. 2005. Drastic effect of a single base difference between human and tetrahymena telomere sequences on their structures under molecular crowding conditions. *Angew. Chem. Int. Ed. Engl.* 44:3740–3744.
36. Xue, Y., Z. Y. Kan, Q. Wang, Y. Yao, J. Liu, et al. 2007. Human telomeric DNA forms parallel-stranded intramolecular G-quadruplex in K^+ solution under molecular crowding condition. *J. Am. Chem. Soc.* 129:11185–11191.
37. Miyoshi, D., A. Nakao, T. Toda, and N. Sugimoto. 2001. Effect of divalent cations on antiparallel G-quartet structure of d(G4T4G4). *FEBS Lett.* 496:128–133.
38. Miyoshi, D., H. Karimata, Z. M. Wang, K. Koumoto, and N. Sugimoto. 2007. Artificial G-wire switch with 2,2′-bipyridine units responsive to divalent metal ions. *J. Am. Chem. Soc.* 129:5919–5925.
39. Nakatani, K., S. Hagihara, S. Sando, S. Sakamoto, K. Yamaguchi, et al. 2003. Induction of a remarkable conformational change in a human

telomeric sequence by the binding of naphthyridine dimer: inhibition of the elongation of a telomeric repeat by telomerase. *J. Am. Chem. Soc.* 125:662–666.

40. Cincotta, L., J. W. Foley, T. MacEachern, E. Lampros, and A. H. Cincotta. 1994. Novel photodynamic effects of a benzophenothiazine on two different murine sarcomas. *Cancer Res.* 54:1249–1258.
41. Foley, J. W., X. Song, T. N. Demidova, F. Jalil, and M. R. Hamblin. 2006. Synthesis and properties of benzo[a]phenoxazinium chalcogen analogues as novel broad-spectrum antimicrobial photosensitizers. *J. Med. Chem.* 49:5291–5299.
42. Hintersteiner, M., A. Enz, P. Frey, A. L. Jaton, W. Kinzy, et al. 2005. In vivo detection of amyloid-β deposits by near-infrared imaging using an oxazine-derivative probe. *Nat. Biotechnol.* 23:577–583.
43. Song, G., F. Xing, X. Qu, J. B. Chaires, and J. Ren. 2005. Oxazine 170 induces DNA:RNA:DNA triplex formation. *J. Med. Chem.* 48:3471–3473.
44. Rachwal, P. A., T. Brown, and K. R. Fox. 2007. Effect of G-tract length on the topology and stability of intramolecular DNA quadruplexes. *Biochemistry.* 46:3036–3044.
45. Antonacci, C., J. B. Chaires, and R. D. Sheardy. 2007. Biophysical characterization of the human telomeric (TTAGGG)4 repeat in a potassium solution. *Biochemistry.* 46:4654–4660.
46. Clark, G. R., P. D. Pytel, C. J. Squire, and S. Neidle. 2003. Structure of the first parallel DNA quadruplex-drug complex. *J. Am. Chem. Soc.* 125:4066–4067.
47. Li, W., D. Miyoshi, S. Nakano, and N. Sugimoto. 2003. Structural competition involving G-quadruplex DNA and its complement. *Biochemistry.* 42:11736–11744.
48. Risitano, A., and K. R. Fox. 2003. Stability of intramolecular DNA quadruplexes: comparison with DNA duplexes. *Biochemistry.* 42:6507–6513.
49. Risitano, A., and K. R. Fox. 2004. Influence of loop size on the stability of intramolecular DNA quadruplexes. *Nucleic Acids Res.* 32:2598–2606.
50. Li, J., J. J. Correia, L. Wang, J. O. Trent, and J. B. Chaires. 2005. Not so crystal clear: the structure of the human telomere G-quadruplex in solution differs from that present in a crystal. *Nucleic Acids Res.* 33:4649–4659.
51. Li, X., Y. Peng, J. Ren, and X. Qu. 2006. Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation. *Proc. Natl. Acad. Sci. USA.* 103:19658–19663.
52. Xu, Y., K. Kaminaga, and M. Komiyama. 2008. G-quadruplex formation by human telomeric repeats-containing RNA in Na^+ solution. *J. Am. Chem. Soc.* 130:11179–11184.