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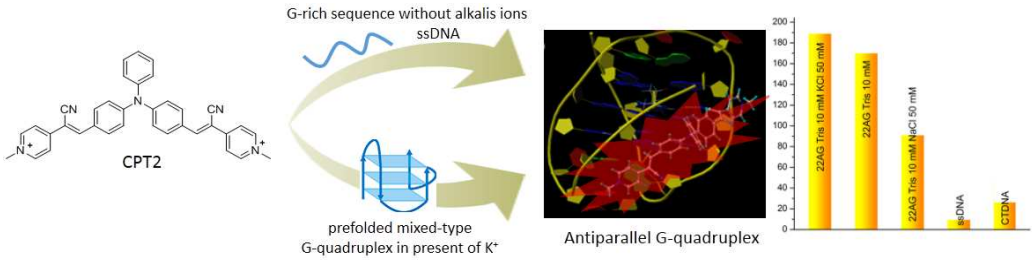

We described a probe based on Cyanovinyl Pyridinium Triphenylamine (CPT) derivative, which showed fluorescent switch-on properties toward antiparallel G-quadruplex.

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Symmetric Cyanovinyl-Pyridinium Triphenylamine: A Novel Fluorescent Switch-On Probe for Antiparallel G-Quadruplex

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Hai Lai [a‡], Yijie Xiao[a‡], Shengyong Yan [a‡], Fangfang Tian[a], Cheng Zhong[a], Yi Liu[a], Xiaocheng Weng[*a,b] and Xiang Zhou[*a, b]

In this study, we present a fluorescent switch-on probe based on cyanovinyl-pyridinium triphenylamine (CPT) derivative that exhibited 190-fold increase in the fluorescence upon binding to G-quadruplex-forming oligonucleotide 22AG. This probe showed specificity and selectivity towards antiparallel G-quadruplex, indicating its promising potential in G-quadruplex imaging.

G-quadruplexes, formed from the folding of guanine (G)-rich nucleic acid sequences by Hoogsteen base-pairing, are of significant interest among non-canonical nucleic acids owing to their applications in cancer and other diseases.[1, 2] These G-rich regions are found to be over-represented in telomeres or transcriptional start sites, indicating their multiple biological functions.[3] In recent years, the studies on the polymorphism and folding topology of G-quadruplexes have shown them suitable candidates for G-quadruplex imaging.[4-8] Recently Balasubramanian et al. reported the direct evidence for endogenous G-quadruplex formation and their stabilization by small-molecule ligands.[9,10] Therefore, the recognition of G-quadruplex has received significant attention for better understanding of their functionality and localization *in vivo*.[11-13]

Numerous fluorescent probes for G-quadruplex have been developed.[14-16] An ideal fluorescent probe should exhibit both high selectivity and appropriate fluorescent intensity when binding to its target.[17] Triphenylamine-based dyes are the common fluorescent molecules used in the photoelectric materials, fluorescence sensing, and cellular imaging.[18,19] Moreover, triphenylamine moiety usually plays the role of donor in various systems and can form a D-π-A system to increase the fluorescence.[20] Some triphenylamines derivatives have demonstrated the ability of interaction with AT regions, indicating their potential for sensing nucleic acid structures.[21,22]

Herein, we designed two cyanovinyl-pyridinium triphenylamine (CPT) derivatives for the purpose of G-quadruplex recognition. CPT1 is designed by linking a cationic pyridinium unit to the triphenylamine core via a cyanovinyl group, whereas CPT2 possesses an additional functional branch than CPT1. Triphenylamine core acts as the electron donor owing to its electron-rich properties, and N-methylated heterocycles are chosen because of the electron-accepting as well as DNA binding properties.[23] The pyridinium triphenylamine (Py-Tp) compounds are non-fluorescent in the free state because of the molecular motions around vinyl bond.[24] Furthermore, the pyridylacetonitrile motif are expected to introduce aggregation-induced emission (AIE) properties to the molecule.[25,26] We envision that the molecule would bind to the G-quadruplex and regain its fluorescence owing to the conformational change.

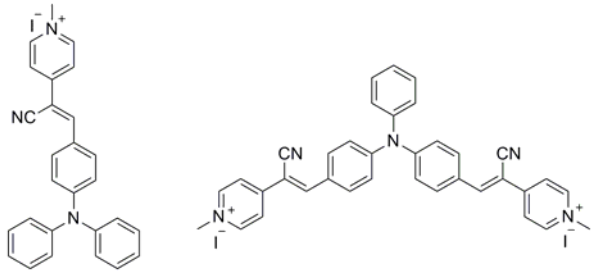

Figure 1. Structures of CPT1 and CPT2

The compounds CPT1 and CPT2 were prepared from commercially available materials using simple coupling and successive methylation procedures. Figure 2 shows that with

gradual addition of 22AG DNA to the CPT2 solution, the fluorescence intensity increased gradually and eventually reached to ~190-fold at the DNA concentration of 10 μM. CPT2 showed negligible fluorescent emission in the buffered solution without any oligonucleotide, whereas CPT2 (10 μM) exhibited high fluorescent emission at 620 nm after adding prefolded 22AG DNA quadruplex in the presence of K^+. Consistent with the change in the fluorescent intensity, the absorption band of CPT2 showed a bathochromic shift of approximately 20 nm after adding 22AG DNA, and exhibited significant hypochromism (nearly 28%, Figure S1), both indicating the strong interaction between CPT2 and G-quadruplex structure of 22AG DNA. The DFT optimization on the excited states of CPT1 and CPT2 at TD-B3LYP/6-31G(d) level using Gaussian 09 program[27] showed that both compounds have a twisted excited state geometry where the donor, diphenyl amino group, and the acceptor groups are perpendicular to each other (see in the supporting information). This phenomenon is also known as twisted intramolecular charge transfer (TICT) state, which predicts the fluorescence quenching and fluorescence recovery in highly polar and less polar solvents or constrained environment, respectively.[28]

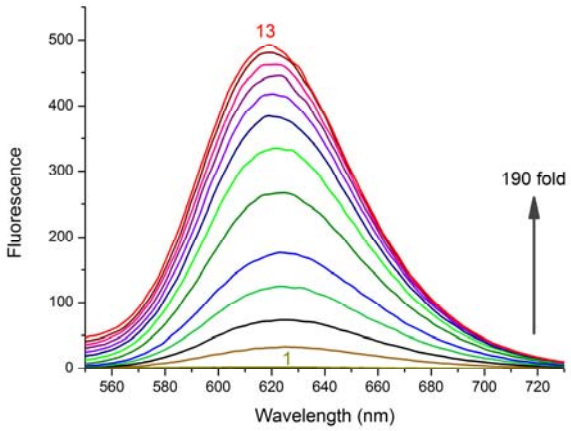

Figure 2. Fluorescence spectra of CPT2 (10 μM) on titration with prefolded 22AG quadruplex DNA in 10 mM Tris (pH 7.4) and 50 mM KCl. [22AG]/μM: (1) 0, (2) 0.5, (3) 1.0, (4) 1.5, (5) 2.0, (6) 3.0, (7) 4.0, (8) 5.0, (9) 6.0, (10) 7.0, (11) 8.0, (12) 9.0, (13) 10.0. λex is 521 nm.

However, CPT1 did not show any significant fluorescence enhancement after adding 22AG DNA. Its absorption band only showed a 10 nm red-shift (Figure S2). The CD spectrum also reflects that the CPT1 molecule interacts with 22AG DNA; however, the effect is less pronounced than CPT2 (Figure S3) because the monocharged compound has a lower affinity towards DNA, and it is relatively harder to bind to the DNA groove. These results indicated that only CPT2 has great potential as the G-quadruplex fluorescent probe.

The fluorescent measurements were also performed in 10 mM Tris–HCl buffer with K^+ or Na^+, or without any high concentration of alkali metal (Figure 3). It has been reported that G-quadruplex formed in the presence of K^+ is more stable than in Na^+.[29] The binding of CPT2 to K^+ induced G-quadruplex was indeed found to be strong, indicating the higher fluorescence enhancement in the buffered solution with KCl (190-fold) than that with NaCl (90-fold). Interestingly, in the absence of metal ions (curve 2, Figure 3), CPT2-22AG system still shows a fluorescence turn-on up to 160-fold. After adding 22AG DNA without prefolding operation, the absorption spectra showed 15 nm red-shift and approximately 30% hypochromism (Figure S4), which is similar to the result of absorption spectra in the presence of K^+ (Figure 2b). That might indicate the potential of CPT2 to induce 22AG into G-quadruplex.

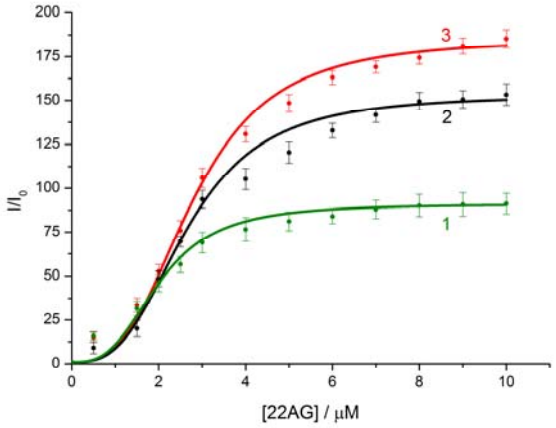

Figure 3. Fluorescence intensity enhancement (I/I0) of CPT2 (10 M) at 620 nm plotted against the 22AG DNA at solution containing (1) 10 mM Tris (pH 7.4) and 50 mM NaCl; (2) 10 mM Tris (pH 7.4); (3) 10 mM Tris (pH 7.4) and 50 mM KCl.

To validate the assumption, the structural changes in 22AG DNA caused by CPT2 were studied using the CD measurements. In the absence of alkali metal ions, the CD signal did not show any characteristic G-quadruplex-forming of 22AG DNA alone (Figure 4, curve 1a). After adding CPT2 to the 22AG DNA solution, two apparent CD bands gradually appeared. Both negative and positive peaks located at 265 and 295 nm, respectively, confirm an antiparallel quadruplex formation. This transformation indicates that CPT2 could bind to 22AG DNA and induces a topological change towards antiparallel quadruplex formation. In the presence of K^+, 22AG DNA exhibited two positive peaks at 265 and 290 nm without adding CPT2, confirming the mixed type of G-quadruplex structures. It was reported that K^+ induces a mixed population of both parallel and antiparallel structures.[30] After adding CPT2, the CD spectra changed from mixed to antiparallel type (Figure 4b), indicating specific selectivity towards antiparallel structure, and the other topological structures of G-quadruplex could adapt antiparallel structure. Meanwhile, in the presence of Na^+, the 22AG DNA maintained its original antiparallel structure after adding CPT2 (Figure S5), which further proves the antiparallel selectivity of CPT2. Moreover, to determine the binding stoichiometry of the Ru-complex with 22AG DNA, continuous variation analysis was carried out (Job Plot, Figure S12). The intersection points obtained in the Job Plot demonstrated 1:1 binding stoichiometries, indicating one CPT2 per G-quadruplex. The CD melting studies also suggested that CPT2 molecules could stabilize the 22AG DNA G-quadruplex

by a Tm of ~8 °C (Figure S6). Note that though the continuous variation analysis implies 1:1 binding stoichiometries, the higher thermal stabilization may attribute to the higher equivalents of CPT2 used in the study.

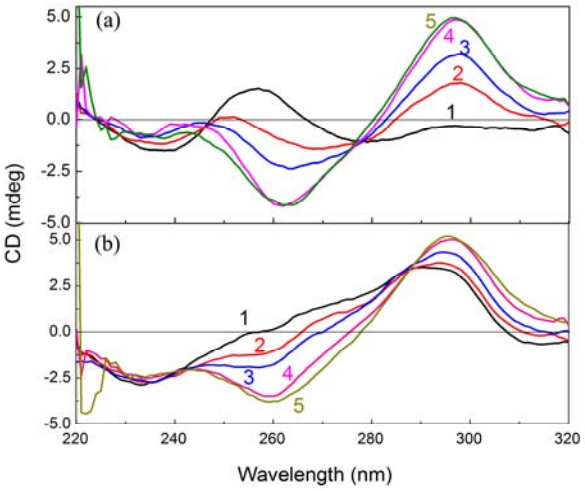

Figure 4. CD spectra for 22AG DNA (10 μM) with or without CPT2. (a): Solution buffered with 10 mM Tris (pH 7.4); (b): Solution buffered with 10 mM Tris (pH 7.4) and 50 mM KCl. [CPT2]/μM: (1) 0 ; (2) 10; (3) 20; (4) 50; (5) 100.

To further prove the potential of CPT2 as a G-quadruplex specific probe, the interactions between CPT2 and other types of DNA confirmations were investigated. From the fluorescence titration experiments (Figure 5a), the fluorescence intensity of CPT2 to a complementary oligonucleotide of 22AG DNA (ssDNA) and calf thymus DNA (CTDNA) were found to be very low compared to other G-quadruplex DNAs. The fluorescence enhancement of ssDNA and CTDNA were only 10- and 17-folds, respectively, at 10 μM DNA concentration, which is less than one-tenth compared to G-quadruplex-forming 22AG DNA. Similarly, the fluorescence enhancement of dsDNA was 45-folds in the presence of K^+, which is one-fourth compared to 22AG DNA. Moreover, CPT2 also showed different fluorescent responses towards other DNA G-quadruplex sequences and RNA G-quadruplexes (NRAS and TERRA). The fluorescent enhancement of 22AG, G3T3, H-RAS, and c-kit DNAs were higher than other oligonucleotides. In the presence of 50 mM KCl, different oligonucleotides showed three main conformations of G-quadruplexes. Therefore, the topologies of G-quadruplexes with CPT2 were studied using the CD studies that revealed great selectivity of CPT2 towards the topology of G-quadruplex. The oligonucleotides G3T3, H-RAS, and c-kit were added to the CPT2 solution, similarly to the 22AG DNA, and the structure of these mixed-type G-quadruplex eventually changed to antiparallel form and the fluorescence enhancement was found to be significant (Figures 5 and S7). However, the oligonucleotides src1, ckit, c-myc, NRAS and TERRA, which formed parallel structures in the presence of K^+, cannot be changed to other topological formation even at a CPT2 concentration of 100 μM. The fluorescence enhancements were found to be less than 50-fold. Notably, although TBA could form an antiparallel structure, its fluorescence enhancement was only found to be 22-fold. This may account for the relatively shorter sequence of TBA, the loop of which may not be large enough to bind CPT2 molecule. In addition, 8TG sequence could form a four strand intermolecular quadruplex and inter1+3 sequence could form a (1+3) intermolecular quadruplex.[31,32] The fluorescence enhancement of 8TG and inter1+3 were only 19- and 18-folds, respectively, indicating that CPT2 had low affinity toward intermolecular quadruplexes. These findings indicate that CPT2 has the potential to be a fluorescent switch-on probe for antiparallel G-quadruplexs. Moreover, in the absence of K^+, none of the sequences showed typical G-quadruplex structure at first (Figure S8); however, after the interaction with CPT2, 22AG, G3T3, H-RAS, and c-kit, all the sequences showed antiparallel structures. There is a clear distinction between antiparallel G-quadruplex DNA and other DNAs, which could be visualized by naked eye (Figure 5b) under UV-light irradiation. These observations were found to be consistent with the results of the titration experiments.

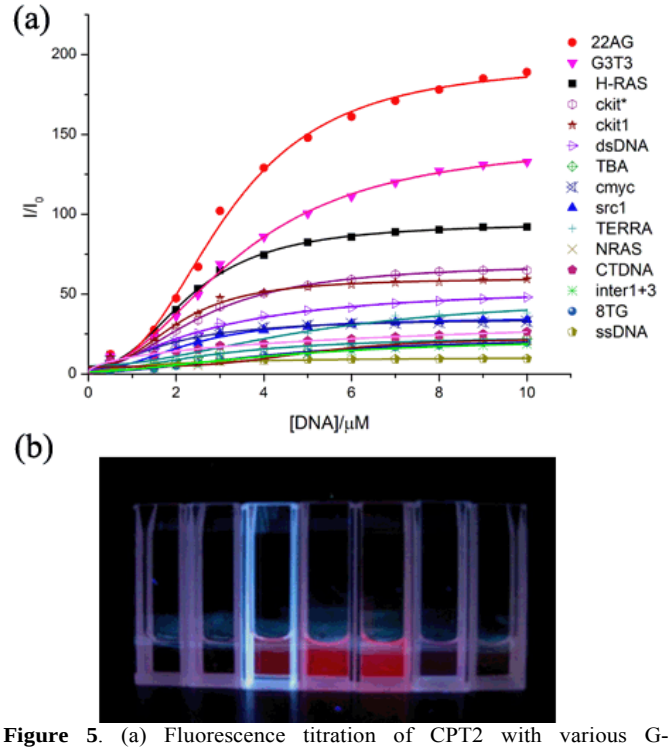

Figure 5. (a) Fluorescence titration of CPT2 with various G-quadruplexes and CTDNA/ssDNA. Solution contains 10 μM CPT2, 10 mM Tris (pH 7.4) and 50 mM KCl. λex is 521 nm and the fluorescence intensity recorded at 620 nm. (b) Fluorescent distinction of CPT2 with different DNAs under irradiation o f UV light, the solution is buffered with 10 μM CPT2, 10 mM Tris (pH 7.4) and 50 mM KCl. From left to right, DNA are as follows: ssDNA, CTDNA, H-RAS, 22AG, G3T3, c-kit*, c-myc. Concentrations of different DNAs are all 10 μM.

The MD simulation shows that CPT2 binds strongly to G-quadruplex DNA than CPT1 and is more stable in antiparallel confirmation (Figure S9, S10). According to the physical meaning of the score in docking, the number presents the negative logarithmic of the dissociation equilibrium constant for ligand-DNA system. Therefore, the binding strength of

CPT2 to 143D is stronger than that of CPT2 to 1KF1, indicating that CPT2 binds selectively to the antiparallel G-quadruplex structure. As showed in Figure S9, CPT2 binds in the groove of anti-parallel G-quadruplex structure(143D) by the way of bending its molecule and fitting into the shape of the groove. Hydrogen binding and van der Waals forces are the dominant driving forces of this binding process, and hydrogen binding also helps to fix the position and configuration of the ligand. The π-π stacking force plays a major role when CPT2 binds to parallel G-quadruplex structure (1KF1). However, this π-π stacking force is crippled by the imperfect overlap of CPT2 to G-quadruplex base-pairs.

Considering the differences between CPT1 and CPT2, CPT2 molecule is bigger than CPT1 and possesses more hydrogen-binding acceptor sites. The hydrogen-binding was observed in CPT2's binding model, whereas no hydrogen-binding was observed in CPT1's binding model. The molecular modelling calculation was found to be consistent with the abovementioned results, and supported that CPT2 could be the probe for antiparallel G-quadruplex.

Conclusions

In conclusion, we synthesized a novel symmetric cyanovinyl-pyridinium triphenylamine derivative that exhibited 190-fold increase in the fluorescence upon binding to G-quadruplex of 22AG DNA. The CD studies showed that CPT2 molecule could induce 22AG DNA sequence to adapt an antiparallel structure. The molecular modelling experiments also indicated that CPT2 could adapt an antiparallel structure, which is more stable than antiparallel G-quadruplex DNA. Furthermore, CPT2 showed a high selectivity towards antiparallel G-quadruplexes, which was observed by naked eye under UV light. These characteristics of CPT2 molecule makes it a promising fluorescent probe for sensing antiparallel G-quadruplexes, and it may provide useful information for future studies.

Notes

[a] College of Chemistry and Molecular Sciences, Wuhan University, Hubei, Wuhan 430072 (P.R. of China). E-mail: xcweng@whu.edu.cn, xzhou@whu.edu.cn

[b] The State Key Laboratory of Natural and Biomimetic Drugs and State Key Laboratory of Applied Organic Chemistry

‡ The three authors contributed equally to this work.

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