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PII: S0040-4039(16)31293-X
DOI: http://dx.doi.org/10.1016/j.tetlet.2016.09.100
Reference: TETL 48170

To appear in: *Tetrahedron Letters*

Received Date: 31 August 2016
Revised Date: 23 September 2016
Accepted Date: 30 September 2016

Please cite this article as: Wang, M-Q., Gao, L-X., Yang, Y-F., Xiong, X-N., Zheng, Z-Y., Li, S., Wu, Y., Ma, L-L., A triphenylamine derivative as a naked-eye and light-up fluorescent probe for G-quadruplex DNA, *Tetrahedron Letters* (2016), doi: http://dx.doi.org/10.1016/j.tetlet.2016.09.100

A triphenylamine derivative as a naked-eye and light-up fluorescent probe for G-quadruplex DNA

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Abstract: G-quadruplex (G4) DNAs have attracted considerable interest because of their important biological functions and medical applications. Searching for highly specific binding molecules is important for the basic research of G4 DNA, as well as the design of novel anticancer drugs. Previous, we have developed a quinolone-substituted triphenylamine probe (**TPA-2b**) with selectivity to G4 DNAs. Herein, we further designed and synthesized a hydroxyethyl functionalized derivative (**TPA-3**) and investigated the interactions with G4 DNAs and living cells. **TPA-3** was found to express significant fluorescence enhancement upon its interaction with G4 DNAs while show almost no response to non-quadruplex DNAs. The distinction can even easily be distinguished by the naked eye under UV light. The spectral analysis showed **TPA-3** bound to G4 DNAs mainly through intercalative binding mode. CD studies results indicated **TPA-3** did not disturb the conformation of G4 structure. Cellular uptake assay suggested that **TPA-3** could pass through membrane and enter living cells. Our results suggested the side chain of core structure could change the binding affinity to G4 DNAs, as well as the interaction with living cells. Thus, this study gives some clues to design new G4 DNA probes with high selectivity, sensitivity and biological imaging applications.

Keywords: G-quadruplex DNA; Fluorescent probe; Naked eye; Bioimaging.

Among various DNA structures, G-quadruplexes (G4s) have received intense research attention in recent years with respect to their biological and medical applications.[1, 2] G4 DNAs are unique structures that formed from the folding of guanine rich DNA sequences by Hoogsteen hydrogen base-pairing.[3] Analysis of the human DNA reveals that a variety of G-rich sequences have been found in some pivotal genomic regions, such as telemetric DNA and promoter regions of some oncogenes.[4, 5] Consequently, numerous biochemical and structural analyses of G4s have been established *in vitro*.[6-8] During the past decades, the existence of G4 structures in mammalian cells remains controversial. To address these fundamental questions, powerful tools for characterizing the G-quadruplexes *in vivo* are desperately needed.

Recently Balasubramanian et al. reported the direct evidence of endogenous G4 DNA formation using a small-molecule ligand.[9] More and more evidences show that G4 DNAs have important roles in various cellular pathways including replication, recombination, transcription and translation, which are highly associated with various human diseases.[10-12] However, the research on the role of G4 DNAs *in vivo* is still at an early stage.[13,14] There is a need to know the formation and location of G4 DNAs in cells at the appropriate time to understand their functions. Therefore, targeting the G4s using

molecule tools has emerged as an alternative strategy for functional exploration of G4s and drug discovery.[15-17]

Fluorescent probes are attractive molecular tools for both analytical sensing and bioimaging because of their high sensitivity, fast response time, and technical simplicity.[18,19] Great efforts have been made to develop fluorescence probes for the detection of G4 DNAs, and many of them have been reported.[20-25] In previous work, we have reported two dyes **TPA-2a** and **TPA-2b** by incorporating a triphenylamine fluorophore into the quinolinium framework (Fig.1).[26] Results showed that **TPA-2b** with an amine-side chain had excellent selectivity to G4 DNAs over other DNA forms *in vitro*. However, **TPA-2b** expressed only about 10-fold increase in fluorescence intensity upon binding to G4 DNA. And it cannot penetrate living cells with an intact membrane, thus the further application in cellular detection was restricted. The direct intracellular detection of G4 DNAs remains a formidable challenge because a limited number of fluorescent G4 probes can penetrate into living cells and their actual targets in cells are not fully known.[13] Inspired by these phenomena, we have great interest in developing a new fluorescence probe based on **TPA-2b** for G4 DNA aiming not only to induce a remarkable fluorescence signal output but also to improve its biocompatibility. Additionally, this will provide deep insights for the structure-based rational design of G4 DNA probes.

In the present study, a new triphenylamine derivative **TPA-3** was designed on the basis of a **TPA-2b** framework by introducing a hydroxyethyl group. The influence on the G4 DNAs binding properties, fluorescence discrimination with other DNA species, and its potential application on live cells staining and imaging were investigated through experimental studies.

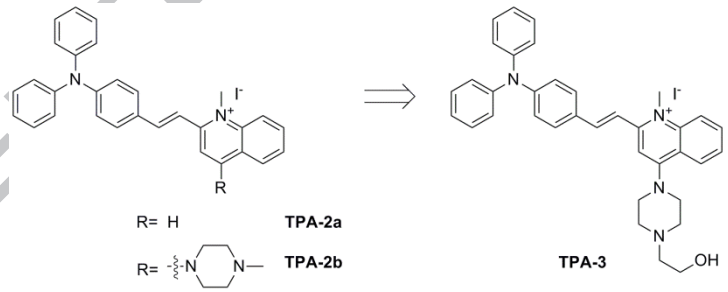

Figure1. Structures of **TPA-2a** and **2b** and the hydroxyethyl group tethered derivative **TPA-3**.

The desired compound **TPA-3** was synthesized according to the method reported by our previous paper.[26] As shown in Scheme S1, **TPA-3** was prepared by condensing 4-formyltriphenylamine, 1, 2-dimethyl-4-chloroquinolin-1-ium iodode and *N*-(2-Hydroxyethyl)piperazine in one-pot. The new compound was characterized by ^{1}H NMR, ^{13}C NMR and MS (see the Supplementary Information).

First, the fluorescence properties of **TPA-3** to different DNA forms, including G4s with different conformations, duplex and single-stranded DNAs, were investigated *via* fluorescence titration assays. As shown in Fig. 2A, free **TPA-3** displayed a weak emission at 550 nm in buffer solution (10 mM Tris-HCl, 60 mM KCl, pH 7.4) when excited at 470 nm. With the gradual addition of G4 DNA 22AG, the fluorescence intensity around 615 nm was remarkably increased. This light-up fluorescence effect was also observed when **TPA-3** was treated with other G4 DNAs, including Ckit1, C-myc, CM22, Scr1,

Ckit3, HTG-21, HRAS, G3T3, which caused 20- to 50-fold fluorescence enhancement. However, the addition of single-strand DNA (ss26, ss22) and duplex DNA (Ct-DNA, ds26, Polyd(A-T)$_9$ and Polyd(G-C)$_9$) under the same conditions induced negligible changes in the fluorescence intensity (Figs. 2B and S1). The distinction between G4 DNA and ssDNA, dsDNA can easily be distinguished by naked eye under UV light (Fig. 2C). The finding indicated that **TPA-3** exhibited excellent selectivity for G4 DNA over single- and double-strand DNA. As we previously reported, the fluorescence emission of **TPA-2b** (Fig. 1) was nearly 10-fold increase could be observed with the addition of G4 DNAs. Thus, **TPA-3** might be better than **TPA-2b**.

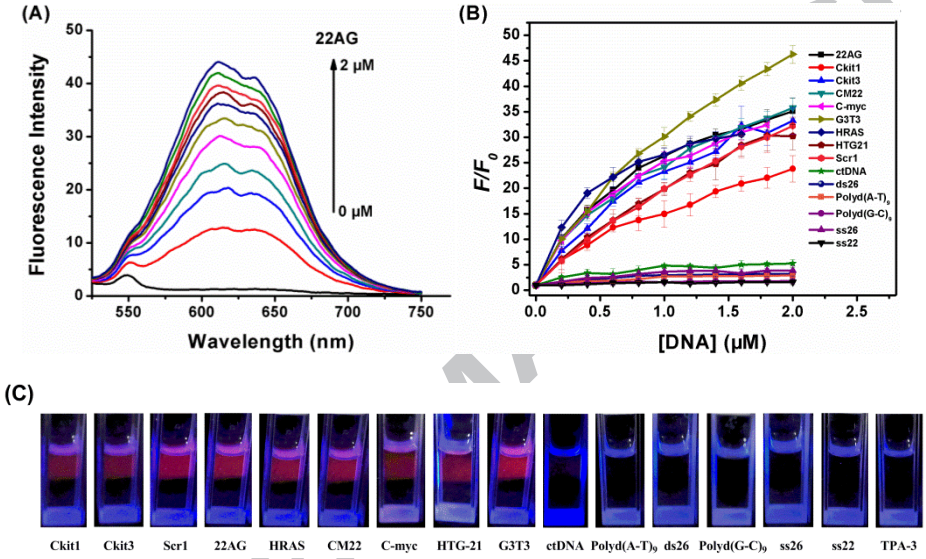

Figure 2. (A) Fluorescence spectrum of **TPA-3** (2 μM) in the absence or presence of 2 μM G4s, duplex and single-stranded DNAs. (B) The fluorescence enhancement of 2 μM **TPA-3** at 615 nm versus the concentration of [DNA]. (c) Visual detection of G4 DNA in the presence of **TPA-3** under the irradiation of 470 nm excitation light.

The sensitivity of **TPA-3** as a "light-up" fluorescence G4 DNA probe was investigated by its fluorimetric titration with 22AG. The concentration-response curve to 22AG (0.2-0.8 μM) showed a good linear relationship with $R^2 = 0.993$ (Fig. S2). The limit of detection (LOD) was estimated to be 34 nM according to the equation $3\sigma/s$ (The σ value represents the standard deviation for multiple measurements of blank solution. The *s* value is the slope derived from the linear range of the fluorescence titrations).[27] It is also noteworthy that the LOD value of **TPA-3** for G4 DNA in solution is significant improved relative to **TPA-2b**.[26] Therefore, the different side chains on the core structure may cause different interaction with G4 DNAs, resulting in the different binding affinity. And the additional hydroxyethyl chain of **TPA-3** was found to be good in the terms of induced fluorescence intensity toward G4 DNAs. Based on the G4 ligands reported previously, we have known that the introduction of side chain would increase the affinity of ligand for G4 DNA because the side chain may dock onto the groove of G4 DNA and interact with the G4 DNA by forming intermolecular hydrogen bond or electrostatic interaction.[28]

In order to examine whether the significant fluorescence enhancement of **TPA-3** was due to its interactions with G4 secondary structure or just simply G-rich sequence, we mixed 22AG and its complementary sequence in a ratio of 1:1 to

form a G-rich duplex structure. Following a similar titration protocol (Fig. S3), the fluorescence intensity of the G-rich duplex structure was found to be very low compared to G4 DNA 22AG, which demonstrated that **TPA-3** indeed interacted with G4 secondary structure instead of simple G-rich sequence.

Besides, although most of G-rich sequences are prone to form monomeric G4s, a small number of them tend to form higher-order G4 structures consisting of consecutive G4 units.[29] In addition, it has been reported that there are some diseases are associated with expansion repeats of G-rich sequences capable of forming multimeric G4s.[30] Thus, the recognition ability of **TPA-3** toward multimeric G4 DNA was studied. As shown in Fig. S4, addition of multimeric G4 DNA Hum45 increased the fluorescence of **TPA-3** steadily, implying **TPA-3** also has the potential to be used for probing multimeric G4 DNA. Collectively, these results demonstrated the promising potential of **TPA-3** to serve as a selective and sensitive probe toward G4 DNAs.

Encouraged by the strong fluorescence emission enhancement of **TPA-3** with G4 DNAs, it was therefore interesting to understand the details of their interactions. On this basis, we then conducted spectrophotometric titrations of **TPA-3** with different DNAs. As shown in Fig.3, after adding 22AG G4 DNA, the absorption band of **TPA-3** at approximately 447 nm gradually decreased, and a large red-shift in the maximum was observed, which led to a new peak at approximately 493 nm. In addition, the titration spectra exhibited a well-resolved isosbestic point (at 484 nm) revealed the existence of one major binding mode, whereas remarkable hypochromic and bathochromic changes are indicative of an intercalative binding mode.[31] Specifically, the quinoline subunit would interact with G-quartet plane by π-π stacking. Furthermore, we investigated the interaction between **TPA-3** and other DNAs. The changes in the spectral profiles during titration were shown in Fig. S5. Addition of G4 DNAs with different conformations also led to significant hypochromisms and red shifts, which meant **TPA-3** could bind with these G4 DNAs through intercalation. While, in the case of single-stranded DNA (ss26) and double-stranded DNAs (ds26, ct-DNA), the bands showed much smaller redshift (Figs. 3B and S5), suggesting that **TPA-3** had much stronger interaction with G4s than with other DNA forms, which was consistent with the results of fluorescence spectra.

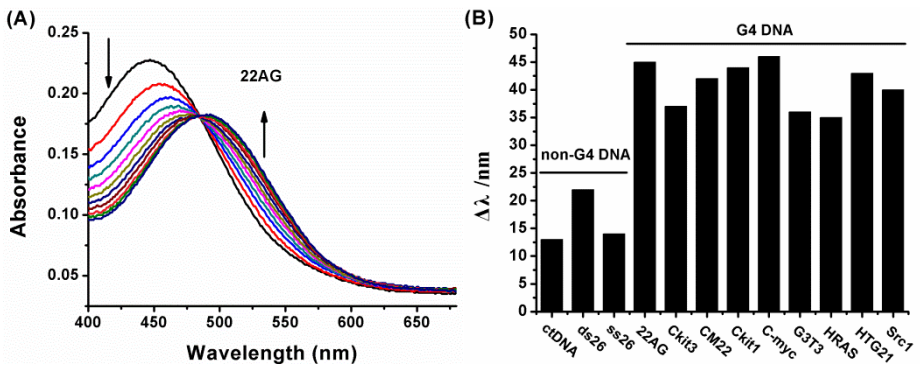

Figure 3. (A) Absorbance spectra of **TPA-3** in buffer (10 mM Tris–HCl, pH 7.4, 60 mM KCl) in the presence of increasing amounts of 22AG. **TPA-3** = 8 μM; [22AG] = 0-2 μM from top to bottom. Arrows indicate the change in absorbance upon increasing the 22AG concentrations. (B) Absorbance response of **TPA-3** to DNAs, the y-axis corresponds to the shift in the absorbance maximum (λ_{max}).

G4 DNA has been recognized as a potential target for anti-cancer drug design. Normal human physiological pH is ~7.4, while tumors generally exhibit acidic microenvironments with pH 6.2–6.9.[32] Considering the practical applications, the G4 recognition fluorescence behavior of **TPA-3** was investigated and compared in the range pH 6.0~7.4 (Fig. 4). Though a change of pH value in this range had a little effect on the G4 recognition, **TPA-3** had a significant fluorescence enhancement to all tested mediums. The titrations resulted in absorption changes in different pH values also displayed similar trends (Fig. S6). The finding shows that detection of G4 DNA is indeed possible under these conditions.

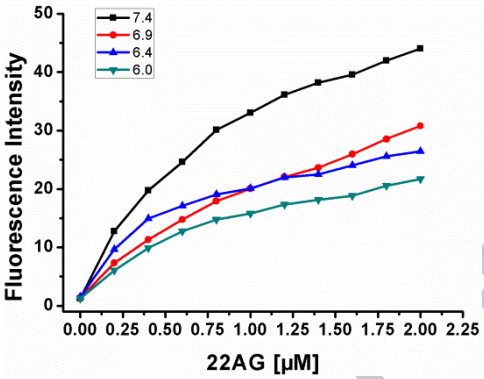

Figure 4. Fluorescence intensity enhancement of **TPA-3** at 612 nm plotted against the concentration of 22AG in different pH solutions.

Cationic species especially K^+ and Na^+ ions also play a very important role in the G4s formation. It has been reported that the structure of G4 formed in the presence of K^+ is more stable than that in the presence of Na^+.[33] To explore this, the fluorescence titrations assays were performed with K^+ or Na^+ (Fig. S7). The titrations of **TPA-3** with 22AG in solution containing 60 mM NaCl displayed a relatively small emission enhancement and with a nominal bathochromic shift in the absorption band (Fig. S7). Evidently, **TPA-3** interacted stronger with 22AG/K^+ than that of Na^+, showing a binding specificity to the K^+-induced G4 of 22AG. The structural changes in 22AG caused by **TPA-3** were further studied using circular dichroism (CD) measurements. CD has been widely used as a powerful tool to monitor the conformation of DNA sequence due to its outstanding sensitivity. In the presence of K^+, 22AG sequence has formed a hybrid-type of quadruplex DNA containing parallel and anti-parallel structure, with two positive peaks at 265 and 290 nm, and a negative band near 235 nm. While in the presence of Na^+, 22AG has formed an anti-parallel structure with a positive band around 295 nm and a negative band around 260 nm. Upon additions of **TPA-3**, very little change of the CD spectrum was observed in the solution of K^+ and no apparent spectral change in that of Na^+(Fig. 5A and B), which implied that the conformation of G4 was stabilized by alkali metal ions, and **TPA-3** did not disturb the conformation of G4 at high ionic strength.

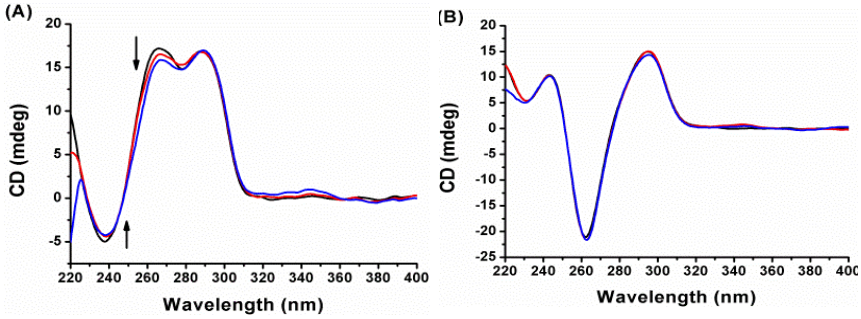

Figure 5. CD titration spectra of **TPA-3** for 22AG DNA (5 μM). (a) Solution buffered with 10 mM Tris (pH 7.4) and 60 mM KCl; (b) Solution buffered with 10 mM Tris (pH 7.4) and 60 mM NaCl. [**TPA-3**]/ μM: 0-5 μM.

The experiments described above showed the **TPA-3** bound to 22AG G4 DNA mainly through intercalative binding mode. To gain further insight into this binding mode, a G4 fluorescent intercalator displacement (G4-FID) experiment was performed. The G4-FID is a well-known method assay based on the loss of fluorescence of a G4-bound intercalator (thiazole orange, TO) upon displacement of a G4-bind molecule.[34] TO binds to G4s in a single-site manner of intercalation with high fluorescence intensity. Molecules that compete with TO for G4 binding are also considered to bind through intercalation and will result in fluorescence intensity decreased. The emission spectra of TO bound to 22AG in the absence and presence of **TPA-3** were shown in Fig. S8. Addition of **TPA-3** to the G4-bound TO solution caused a great reduction in the emission intensity, implying that the molecule could bind to G4 DNA mainly in an intercalative binding mode. Moreover, the results obtained from photo-fluorimetric titrations indicated that **TPA-3** interacted with 22AG in a 1:1 stoichiometry (Fig. S9). Thus, we deduced that one molecular of **TPA-3** might simultaneously interact with one G-quartets of 22AG. According to the 1:1 ratio obtained from the Job plot, the binding constant (K_b) for the **TPA-3**/22AG complexation derived from the fluorescence titration data can be estimated to be 1.21×10^6 (R^2 = 0.997, Fig. S10) by using Benesi−Hildebrand plot.[35]

In addition, to examine the bioactivity of **TPA-3**, the cellular uptake and localization were investigated. The living cells were incubated with **TPA-3** (10 μM) for 2h, and then imaged by under a standard cell staining protocol. The confocal imaging showed that **TPA-3** could pass through membrane and enter living cells (Fig. 6). However, the co-staining with DAPI (duplex DNA probe) showed that **TPA-3** mainly located in cytoplasm. Thus, the fluorescence of **TPA-3** upon binding to G4s might be difficult to observe in cells. Fluorescence from the cytoplasm may be due to **TPA-3** located in an environment of low polarity. Our previous results have shown that **TPA-2b** with amino group could not enter living cells, therefore, the results suggested the hydroxyethyl group of **TPA-3** increased the cellular uptake.

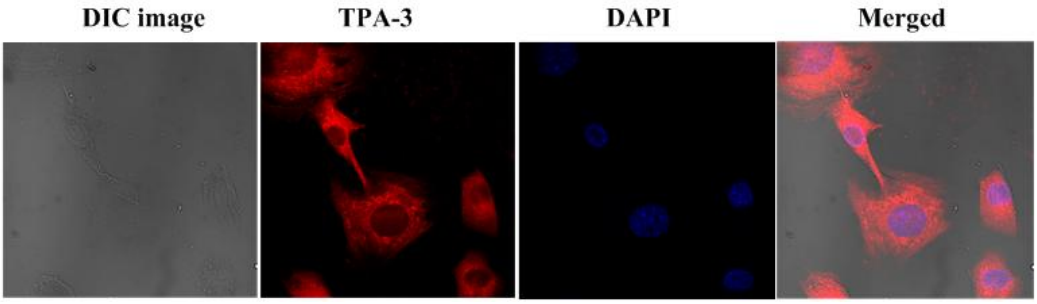

Figure 6. Confocal images of Hep-G2 cells stained by **TPA-3** (10 μM) (λex=470 nm) and DAPI (5 μg/mL) (λex =405 nm).

In summary, a hydroxyethyl-functionalized triphenylamine derivative **TPA-3** has been designed and synthesized. The interactions with G4 DNAs and living cells were investigated. Fluorescence spectra showed that **TPA-3** could serve as a naked-eye and light-up probe for G4 DNAs over ss-/ds-DNA. Absorption and G4-FID assays suggested that **TPA-3** bound to G4 DNA mainly through intercalation binding mode. Furthermore, CD studies showed **TPA-3** did not disturb the conformation of G4 structure. Confocal imaging showed **TPA-3** could enter into cancer cells and mainly located in cytoplasma. These results confirmed that the side chain of the ligand could affect the binding ability to G4 DNAs and change the cellular uptake. These characteristics of **TPA-3** provide some information that may be useful for the design of new specific G4 DNA probes for diagnostic and biosensing applications.

Acknowledgements

This work was financially supported by the Senior Talent Cultivation Program of Jiangsu University (Nos. 15JDG068). This research was also supported by Technical Research Program for Chongqing Education Commission (KJ1600916), Chongqing Research Program of Basic Research and Frontier Technology (No.cstc2013jcyjA50012, cstc2016jcyjA0508), China Postdoctoral Science Foundation funded project (2014M562326, 2016T90851).

Supplementary date

Experimental procedures, fluorescence and absorption spectra of the probe; Copies of ^{1}H and ^{13}C NMR, MS spectra for new compounds.

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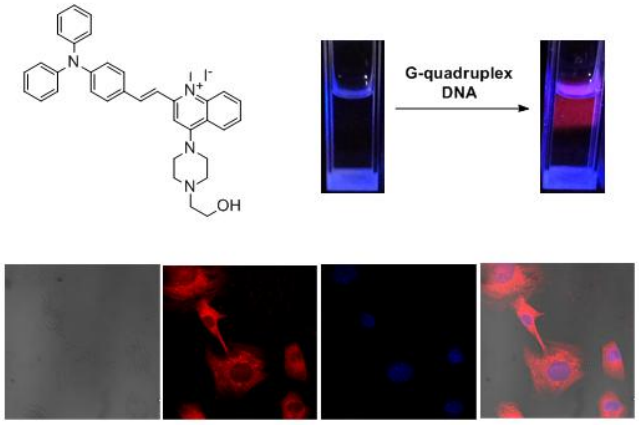

Highlights：

A hydroxyethyl-functionalized triphenylamine derivative was developed.

Significant “light-up” effect was observed when this probe bound with G4 DNAs.

The G4-targeting probe displayed excellent selectivity, and sensitivity.

The cellular application in cancer cells was demonstrated.