

Targeting G-quadruplex Structures with Extrinsic Probes: Prospect of Selective and Efficient G-quadruplex Inducers in Therapeutics

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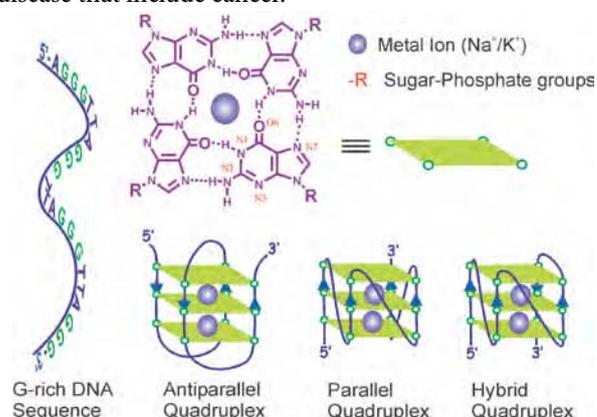
Abstract: The research on the G-quadruplex DNAs has received much attention in recent years and numerous reports appeared probing its detection, structure, stability, reactivity and selectivity, aimed mainly for the chemical intervention of specific biological activity or sensor applications. Our study has demonstrated the use of a fluorogenic dye, Thioflavin T, to stabilize the telomeric DNA strand into a G-quadruplex structure under physiological conditions. We have assessed several other DNA forms and set appropriate conditions to achieve highly selective interaction of the probe with the G-quadruplex structure. In addition, the dye becomes highly fluorescent, more than 2000 times brighter, increasing the sensitivity for the optical detection of the G-quadruplex in the cellular environment against other DNA forms. This work reveals the applicability of Thioflavin T to inhibit telomerase activity and opens up avenues to explore new dyes/drugs based on ThT scaffold, which have the potential to highly specific quadruplex inducing/sensing agents for cancer therapeutics.

Key Words: G-quadruplex, human telomeric DNA, Fluorescence, Thioflavin T.

Introduction

Nucleic acids are capable of forming a wide variety of structures, far indifferent from the Watson-Crick double helix. Among various non-canonical nucleic acid structures, the G-quadruplex motifs have attracted immense research attention as prospective targets for chemical intervention of biological functions. G-quadruplexes (Scheme-1) are formed in guanine rich telomeric and oncogene regions and are implicated in the expression of growth factors. G-quadruplexes are four stranded nucleic acid structures (Scheme-1) formed by stacking of Hoogsteen base paired G-quartets, which are prevalent in G-rich sequences. G-quadruplex structure is considered to be functionally important in the mammalian genome for transcriptional regulation, DNA replication and genome stability and their presence can be explored in assessing cellular functions, or can be utilized for chemical intervention of biological activities. Because of their abundance in functional genomic regions, especially at the end of eukaryotic chromosomes (telomeres), in the promoter regions of important protooncogenes, and in the untranslated regions of mRNAs, they have been recognized as significant drug

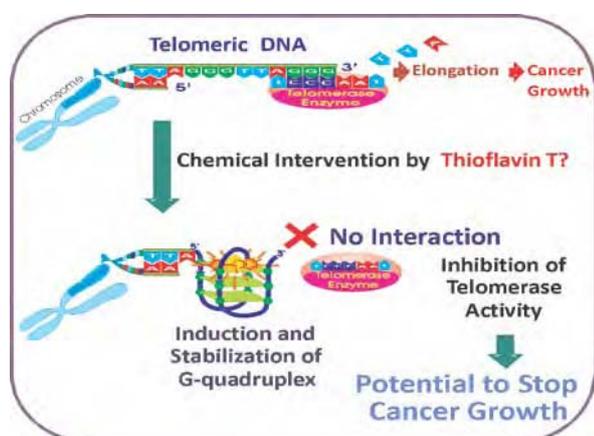
targets to halt the function of telomerase and regulate gene expression. Thus, the functional relevance of G-quadruplexes in human genome is of fundamental importance to life and may well hold the key to new therapeutic approaches in numerous areas of human disease that include cancer.



Scheme 1 Structures of the G-quartet/tetrad and the various common folding topologies found in G-quadruplexes

Even though G-quadruplexes have been extensively studied for more than 20 years, the exact nature of their

biological significance, apart from the telomerase activity of the single strand telomere overhang, is still poorly understood. The discovery in the mid 1990s that telomeric DNA is maintained in majority of cancer cells by the action of the telomerase enzyme complex, which synthesizes telomeric DNA repeats and maintains the immortalization of cancer cells (Scheme-2). By contrast, in normal somatic cells, telomerase is not significantly expressed and the normal mechanism of DNA replication results in progressive shortening of telomeric DNA and undergoes programmed cell death. This finding developed into the concept of targeting/inhibiting telomerase activity via induction or stabilization of a G-quadruplex as therapeutic strategy for fighting cancer (Scheme-2). This has stimulated intense research in exploring the stabilization of different quadruplex topologies by extrinsic molecular ligands as potential therapeutic agents for anticancer treatment.



Scheme-2: Action of Telomerase enzyme on the Telomeric DNA, causing uncontrolled growth/cancer and the plausible chemical intervention to induce G-quadruplex and stop the action of telomerase enzyme, thus promising to arrest cancer growth.

It has been found that many of the quadruplex binding dyes do equally interact with other DNA forms like the single strand or duplex DNAs, which make them nonspecific. Hence the development of cellular probes for detection of G-quadruplex structures, which can discriminate other DNA/RNA forms in cells, is inherently difficult. Therefore, there is a need to develop quadruplex specific dyes/ligands which can selectively induce and stabilize quadruplex structures and *in-situ* function as selective fluorescent probe for *in vitro* or *in vivo* visualization of key cellular processes. Noncovalent, extrinsic fluorescent probes find extensive usage as local

reporters in many biological applications, especially in various fields of protein/DNA analysis. Here, the specific interaction with the protein/DNA environment may introduce considerable change in the photophysical characteristics of the dye, projecting the details of its local microenvironment.



Thioflavin T (ThT)

Majority of potential G-quadruplex ligands, however, have been developed as telomerase inhibitors by virtue of their ability to stabilize pre-formed G-quadruplex structures in the 3'-telomeric overhang. However, it has been found that many of the quadruplex binding dyes do equally interact with other DNA forms like the single strand or duplex DNAs, which make them nonspecific for quantitative measurements. With this aim, we have investigated the interaction of ThT with human telomeric DNA (22AG: 5' AGGGTTAGGGTTAGGGTTAGGG-3') and compared it with other single stranded (ss-) or double stranded (ds-) DNAs as well as with the calf thymus DNA (ct-DNA).

Gradual titration of the 22AG human telomeric DNA with the ThT solution at specific solution conditions, resulted in significant bathochromic shift in the ThT absorption profile (Fig.1A) with a concomitant enhancement in the fluorescence intensity (I/I_0) to the order of ~1500-fold, as displayed in Fig. 1B. Since the 22AG DNA strand is prone to fold into quadruplex structures in presence of metal ions, it is quite likely that in the presence of ThT and in the absence of any metal ions, the fluorescence enhancement in ThT could originate from its interaction with a quadruplex structure induced by the cationic ThT itself. To explore this, the measurements in presence of K⁺ ions displayed remarkable changes as the titration of pre-folded 22AG quadruplexes (both parallel and antiparallel) with ThT (50 mM KCl, 50 mM Tris, pH 7.2), displayed huge enhancement in the emission intensity to ~2100 fold. In other words, the good agreement among the spectral features of 22AG-ThT system seen both in the presence of K⁺ or in the absence of metal ions, explicitly highlights the role of ThT in inducing quadruplex folding in the

22AG sequence, particularly in the absence of salt. The resulting fluorescence 'light-up' in ThT to ~2100-1700 fold, in the presence and absence of K^+ , respectively, illustrates that ThT acts as an efficient inducer of quadruplex DNA.

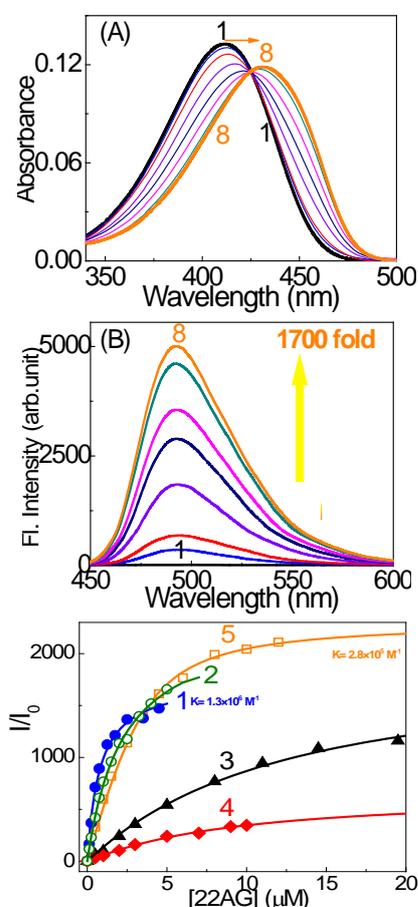


Fig. 1: Absorption (A) and fluorescence (B) spectra of ThT with 22AG DNA at different buffer concentrations. A and B; ThT solution (3.5 μM) containing 5 mM Tris (pH 7.2) with [22AG]/ μM : (1) 0, (2) 0.12, (3) 0.25, (4) 0.75, (5) 1.5, (6) 2.5, (7) 4.0, (8) 5.0. λ_{ex} for B is 425 nm (C): Fluorescence intensity enhancement (I/I_0) of ThT ($\sim 3 \text{ } \mu\text{M}$) at 490 nm plotted against the 22AG DNA at different solution conditions. (1) Solution containing no buffer and salt; (2) buffered at 5 mM Tris, pH 7.2; (3) 50 mM Tris, pH 7.2; (4) solution containing 50 mM NaCl and 50 mM Tris (pH 7.2); (5) 50 mM KCl and 50 mM Tris (pH 7.2).

Saturation in the emission intensity of ThT observed with very low concentrations of the 22AG DNA at different solution conditions (Fig.1C) suggests strong interaction of ThT with the quadruplex DNA. A 1:1 stoichiometric analysis of this binding curve at 490 nm at different solution conditions provided the binding

constants which varied with the change in the surrounding medium. By circular dichroism (CD) measurements, it has been shown that the 22AG DNA in buffered solution (5-50 mM, Tris, pH 7.2) displayed characteristic CD bands at 265 nm and the 240 nm (Fig.2A,B), which matched well with the standard CD bands reported for parallel quadruplexes. Further, on introducing K^+/Na^+ and ThT, the broad multi shouldered band below 350 nm region, gradually transformed to display a distinct positive band at 295 nm and a trough at 265 nm, a clear signature of change over to the antiparallel topology.

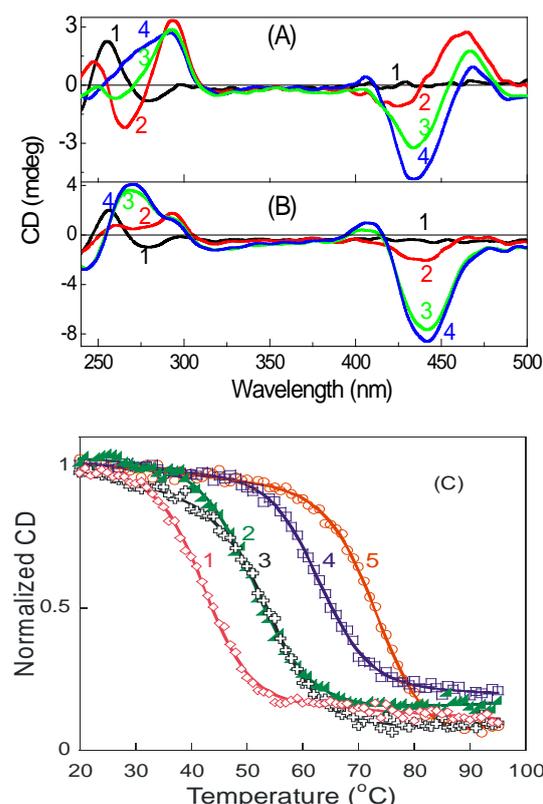


Fig. 2: CD spectra recorded for 22AG DNA (12.5 μM) at different Tris buffer concentrations with ThT. (A) solution buffered with 5 mM Tris (pH 7.2); (B) 50 mM Tris (pH 7.2). ThT: (1) 0 eq; (2) 2eq; (3) 4eq; (4) 8eq. (C) Melting curves for 22AG DNA (12.5 μM) in Tris (50 mM, pH 7.2) in the presence and absence of ThT. (1) 12 eq of ThT; (2) NaCl (50 mM); (3) NaCl (50 mM) with 8 eq of ThT; (4) KCl (50 mM); (5) KCl (50 mM) with 8eq of ThT. Melting of antiparallel quadruplex and parallel quadruplex were monitored at 295 nm and 265 nm respectively.

The extent of quadruplex stabilization in presence of salt and/or ThT was assessed from their melting temperature (T_m) evaluated from the CD thermal denaturation profiles presented in Fig.2C. In the unbuffered 22AG solution containing ThT, the quadruplex melting curve

monitored at 295 nm (antiparallel folding) provided a T_m \sim 55°C, whereas, the parallel quadruplex formed in buffered solution of 22AG and ThT was found to be much less stable, as the T_m monitored at 265 nm (parallel folding) decreased to 42.6 \pm 0.2°C (Fig.2C).

On the other hand, the antiparallel 22AG quadruplex predominant in presence of Na⁺ provided the T_m as 51.6 \pm 0.1°C, which improved only marginally to 55.0 \pm 0.3°C (ΔT_m \sim 3.4°C) on introducing ThT dye. In presence of K⁺ ions, the melting curve for the 22AG at 295 nm provided the T_m as 63.3 \pm 0.2°C, which further increased to 74 \pm 1°C (ΔT_m = 11°C) in presence of ThT, registering significant improvement in the quadruplex stability imparted by the ThT. The melting temperature and hence the stability of the 22AG quadruplexes in presence of ThT/buffer/salt/ conditions are seen in good agreement with the structural transformations and the corresponding spectral features observed from the absorption, fluorescence and the CD measurements. The finding that even in the absence of salt and buffer, the ThT induced antiparallel 22AG quadruplex DNA exhibited better stability and emission enhancement (T_m \sim 55°C, I/I_0 \sim 1500 fold) is prominent, as it allows to explore the quadruplex dynamics in salt-free conditions too, at par with that possible in presence of K⁺ (T_m \sim 74°C, >2000 fold).

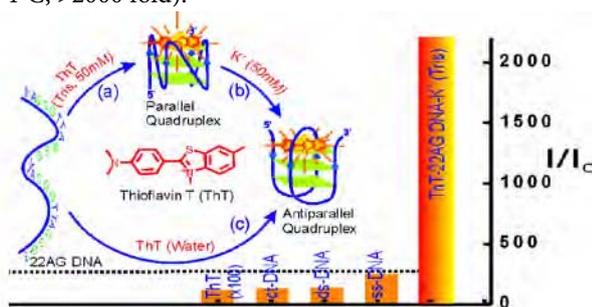


Fig. 3: Emission intensity enhancement (I/I_0) of ThT with various DNAs. Inset: Schematic representation of the topological transformation in the 22AG human telomeric DNA to parallel and antiparallel quadruplex by Thioflavin T (ThT) in the presence of Tris (pH 7.2)/K⁺.

To recognize the selectivity of ThT towards the quadruplex DNA, similar experiments have been carried out with DNAs of nonspecific sequences, as single strands (ss-) and duplex (ds-) DNAs (Fig.3). In both the cases, the fluorescence enhancements were found to be only in the range of 180-220 fold. Particularly, the double stranded calf thymus DNA (ct-DNA) too afforded emission enhancement in ThT \sim 250 fold, which further

decreased in presence of salt. Clearly, the striking dominance of emission enhancement in the 22AG DNA in the absence or presence of salt (especially K⁺) vs other DNA strands, unambiguously establishes the highly selective fluorescence *light-up* of ThT on the quadruplex DNA.

In summary, in this study, we demonstrated the function of a water soluble fluorogenic dye, Thioflavin T (ThT), in a dual role of exclusively inducing quadruplex folding in the 22AG human telomeric DNA and for sensing the same by its remarkable fluorescence *light-up* having emission enhancement \sim 2100 fold in the visible region. This work opens up avenues to explore new dyes based on ThT scaffold, which have the potential to emerge as highly specific quadruplex inducing agent for diagnostic, therapeutic and cation sensing applications. We are optimistic to take up these results to the visualization of G-quadruplex formation in the human transcriptome, and corroborate the selectivity and application of stabilizing ligands that target G-quadruplexes within a cellular context, a positive note on the prospect of intervention in biological processes as an anticancer strategy.

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