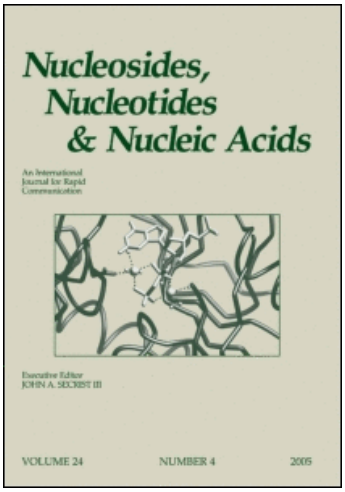

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THIAZOLE ORANGE: A USEFUL PROBE FOR FLUORESCENCE SENSING OF G-QUADRUPLEX–LIGAND INTERACTIONS

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□ *Fluorimetric titrations were performed to gain insight into parameters that govern the association of thiazole orange (TO) and G-quadruplex-DNA (G4-DNA). Use of loop-containing and loop-lacking quadruplexes evidenced the critical influence of the loops on the stoichiometry of the association and on the fluorescence exaltation of TO. We subsequently tried to benefit from this sensitivity to evaluate the influence of G4-DNA cationic environment on ligand binding via a recently reported G4-FID assay.*

Keywords Thiazole orange; G-quadruplex-DNA; fluorescent probe

thiazole orange (TO, Figure 1) is a very interesting DNA-probe since it is highly fluorescent upon complexation with DNA, whereas totally non-fluorescent when free in solution.[1] This unique property makes TO particularly useful for labelling various DNA structures (single-stranded, duplex-, or quadruplex-DNA, noted hereafter G4-DNA). G4-DNA is a high-order DNA-structure that is currently a topic of widespread scientific interest. Indeed, recent investigations suggest its potential implication in critical biological events such as the maintenance of the chromosomal structural integrity or the control of oncogene expression.[2] However, fluorescent studies with G4-DNA are poorly developed; it, thus, was appealing to further study the possibility of labelling G4-DNA with TO.

We previously demonstrated that TO can be a useful fluorescent probe in a new fluorescent intercalator displacement assay (G4-FID assay).[3] G4-FID assay allows the ranking of putative ligands in function of their affinities and selectivities for quadruplex-DNA structure. TO was also used in an original bimolecular fluorescent system for evidencing a specific FRET effect

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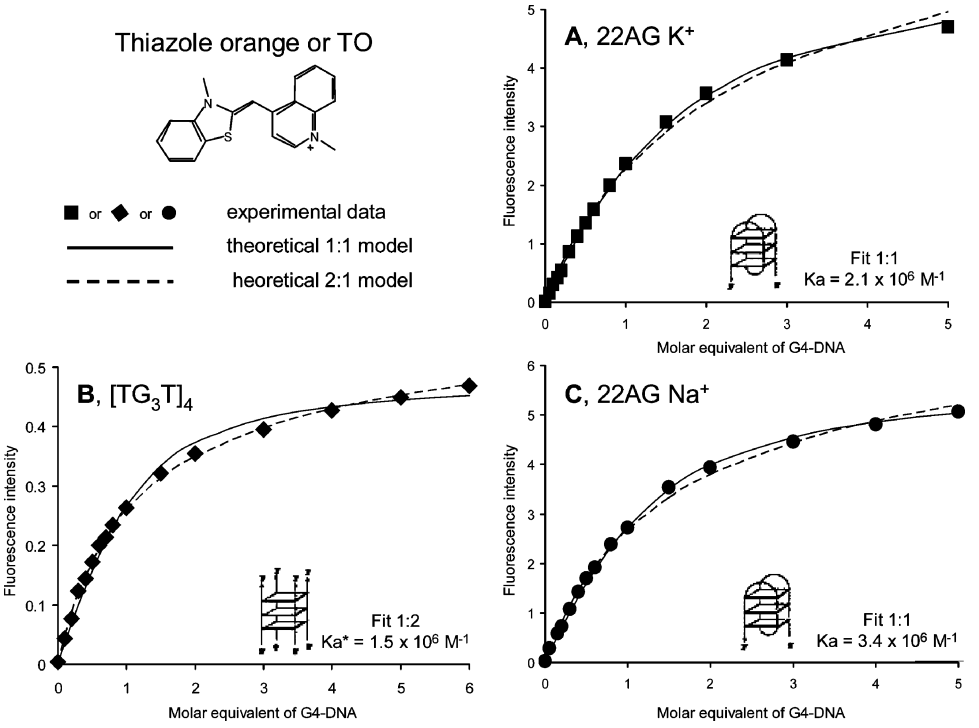

FIGURE 1 Structure of thiazole orange (with p-$CH_3(C_6H_4)SO_3^-$ as conterion) and fluoresence intensity (λ_{ex} = 501 nM) of TO (0.5 μM) under addition of increasing amounts of G4-DNA: 22AG in K^+ buffer (A), $[TG_3T]_4$ (B) and 22AG in Na^+ buffer (C) with experimental data and calculated (pain and dotted lines) titration curves (Specfit 32 version 3.0, *Spectrum Software Associates,* Marlborough, MA, USA) Ka* stands for Ka per binding site.

based on G4-DNA templating.[4] In both cases, studies were carried out with a G4-forming oligonucleotide which mimics the human-telomeric sequence (22AG, [5′-$AG_3(T_2AG_3)_3$-3′]) and it was shown that TO/22AG association occurs in a single-site manner, with a high affinity.[4]

We were then interested in investigating the influence of tetrad environment onto TO/G4-DNA association. Intramolecular 22AG G4-DNA is known to be highly polymorphic,with various structural arrangements which mainly differ by loop connectivities.[2,5–7] Taking into account the knowledge concerning the association of fluorescent dyes with G4-DNA,[8] it, thus, was interesting to evaluate the sensitivity of TO to the presence of loops. For that purpose, tetramolecular quadruplex [5′-TG_3T-3′]$_4$ was studied. Its structure is based on three stacked tetrads as central core but it is totally loop-free. Moreover, due to the symmetrical nature of TG_3T oligonucleotide, it is assumed to present two structurally equivalent external quartets. A series of fluorimetric titrations was thus undertaken to monitor the association of TO with 22AG in K^+ buffer (Figure 1A) and [5′-TG_3T-3′]$_4$ (Figure 1B).

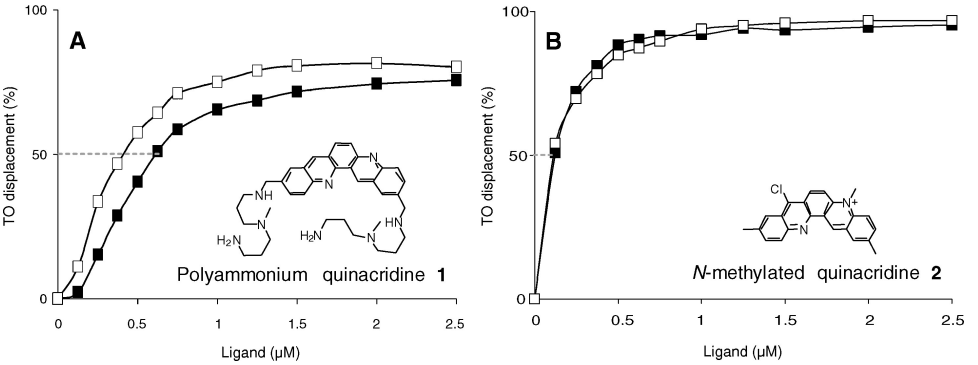

FIGURE 2 G4-FID results of TO from 22AG in K^+ (black squares) or Na^+ (white squares) buffer with polyammonium quinacridine **1** (A) and *N*-methylated quinacridines **2** (B). Dotted lines are intended to serve as visual reference for $^{G4}DC_{50}$ values reported in the text.

TO had similar affinities for both structures (Ka = 2.1 and 1.5×10^6 M^{-1}, respectively). Nevertheless, a clear difference in the stoichiometry of the association emerged: processing the data by least-square fitting evidenced the formation of a 1:1 complex with 22AG and a 2:1 complex with $[5'\text{-}TG_3T\text{-}3']_4$. These observations are consistent with the existence of one tetrad of higher affinity in the 22AG structure and with the equivalence of the two external tetrads of $[5'\text{-}TG_3T\text{-}3']_4$. Additionally, another striking difference stands in the exaltation factor of TO fluorescence, which is much lower in the presence of five equivalents of loop-lacking quadruplex $[5'\text{-}TG_3T\text{-}3']_4$, ~60-fold exaltation) as compared to loop-containing one (22AG, ~400-fold exaltation). This observation underlines the critical role of loops in structural accommodation of TO onto quadruplex-DNA. Altogether these results highlight the TO interest for studying G4-DNA structures, since it allows an easy distinction of quadruplex with one or two sites of high affinity for ligands.

It is worth noting that initial G4-FID experiments with 22AG were carried out in potassium-rich conditions; a rapid glimpse of the literature convinced us to evaluate the impact of the cation exchange (from K^+ to Na^+) on the TO/22AG recognition.[9] As shown in Figures 1A and 1C, the nature of counter-ions (K^+, Na^+) had only a slight influence onto TO/22AG association, since in both cases similar stoichiometries (1:1), affinities (Ka = 2.1 and 3.4×10^6 M^{-1}, respectively) and exaltation factors (>400-fold) were found. These results imply that the fluorescence of bound TO is not responsive to the putative conformational changes induced by the K^+-to-Na^+ exchange. Nevertheless, we were interested in studying the influence of the cation nature on the competitive displacement of TO by a ligand in the G4-FID assay. To evaluate this, two G4-binders have been used: a polyammonium quinacridine (**1**, Figure 2A) and a *N*-methylated quinacridine (**2**, Figure 2B).[10]

The TO displacement ability of **1** and **2** was thus investigated in both buffer conditions (10 mM sodium cacodylate buffer with 100 mM KCl or NaCl, respectively). The TO displacement was quantified by $^{G4}DC_{50}$ values, which represent the concentration of ligand required to displace 50% of TO from the G4-DNA.[3] The TO displacement ability of **1** appeared significantly sensitive to the buffer nature ($^{G4}DC_{50} = 0.61$ and 0.41 μM for **1** in K^+ and Na^+ buffer respectively). In contrast TO displacement with **2** was only slightly affected by the cation exchange since the determined $^{G4}DC_{50}$ values were similar in both buffers ($^{G4}DC_{50} = 0.10$ and 0.11 μM for **2** in K^+ and Na^+ buffer, respectively). These data suggest that differences in the binding mode of ligands might be responsible for the differences in the G4-FID response. Indeed, *N*-methylated quinacridine (**2**) is assumed to stack onto the external quartet of the G4-DNA and it seems reasonable that this unique binding mode is not affected by the loop structural features. In contrast, ligand **1** due to its polyammonium arms is known to establish additional interactions with loops and/or grooves of 22AG and thus binding could be more sensitive to structural modifications of the DNA target.

Altogether these observations suggest that TO alone and TO-based G4-FID assay provide a mean for gaining insight into ligand/G4-DNA interactions both in terms of tetrad accessibility and loop influence. Considerable efforts are currently undertaken to shed light on the sensitivity of this assay, and its potentially further exploitations.

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