G-Quadruplex DNA

DOI: 10.1002/anie.200805613

Engineering Bisquinolinium/Thiazole Orange Conjugates for Fluorescent Sensing of G-Quadruplex DNA**

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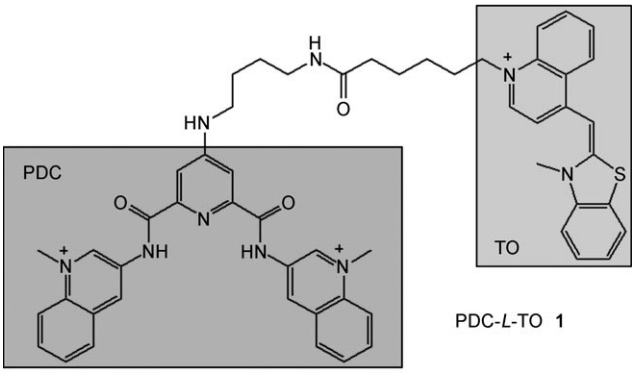
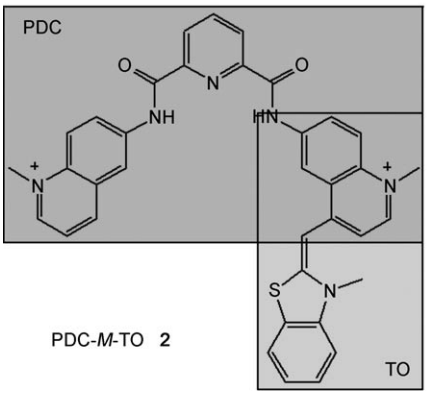
Whereas the in vitro existence of G-quadruplex DNA has been thoroughly studied during the past decades,[1] its in vivo relevance is still a matter of controversy.[2] The indirect nature of the provided evidence has led to scepticism about whether G-quadruplex DNA actually forms in cells.[3] To address this issue, chemical, biophysical, and biochemical tools that will detect G-quadruplex structures in cells must be developed. Interesting impetus has been given recently by a chromosomal radiographic study using the tritiated G-quadruplex ligand [^{3}H]-360A;[4] nevertheless, the handling of radioactive probes requires specific conditions and hence cannot be extended to routine applications. An alternative approach relies on the conception of fluorescent probes which are increasingly used in biology owing to the rapid evolution of detection systems. Ideally fluorescence detection requires a probe whose fluorescence is significantly enhanced upon binding to a given target; this task is particularly complicated in the case of G-quadruplex DNA since most organic fluorophores are quenched by guanines.[5] We thus decided to further investigate this challenging approach, particularly since very few examples of fluorescent G-quadruplex ligands have been reported to date.[6]

The pyridodicarboxamide (PDC) bisquinolinium series appears as one of the most attractive G-quadruplex ligands because of its high affinity and selectivity, along with its rapid and convenient synthetic access.[4,7] Unfortunately, PDC derivatives are only weakly fluorescent and therefore not usable for G-quadruplex detection. On the other hand, thiazole orange (TO) is an exceptional DNA probe, since its fluorescence is greatly increased ($\approx$500-fold) upon binding to DNA, whereas it exhibits a very low quantum yield when free in solution.[8] Interestingly, TO has been shown to bind G-quadruplex DNA with a high affinity ($K_a \approx 10^6\,M^{-1}$) but to display poor, if any, selectivity with regard to other forms of DNA.[6d] We thus decided to assemble the PDC and TO motifs in a single scaffold, hoping to combine their unique characteristics. A first classical design consisted in linking the two components by a flexible linker to afford PDC-*L*-TO **1** (Scheme 1). A second and more innovative design benefited from the presence in both compounds of a *N*-methylquinolinium moiety, enabling us to build the merged system PDC-*M*-TO **2** (Scheme 1). The synthesis of PDC-*L*-TO **1** was achieved according to standard procedures, and PDC-*M*-TO **2** was obtained by a one-step condensation reaction of the parent PDC and the methylbenzothiazole (see the Supporting Information). In the case of PDC-*L*-TO **1** the maintenance of the desired dual activity (binding/labeling) was a priori conceivable since the compound results from the assembly of two entities whose structural integrity is preserved. This





Scheme 1. Structures of PDC-*L*-TO **1** and PDC-*M*-TO **2**, with I^- as the counterion for both probes.

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[**] This work was supported by ARC (3365) and EU FP6 "MolCancerMed" (LSHC-CT-2004-502943) grants. Dr. L. Lacroix is acknowledged for helpful discussions and Dr. N. Saettel for the picture of quadruplex DNA.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805613.

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was more difficult to anticipate for PDC-*M*-TO **2** since the presence of a benzothiazole moiety in close vicinity to PDC is likely to alter both the π-stacking interaction with the G-quartets and the coplanar immobilization of the benzothiazole and quinolinium moieties, which is responsible for fluorescence enhancement of TO.[8a–e]

The biophysical properties of PDC-*L*-TO **1** and PDC-*M*-TO **2** were thus investigated. In analogy to what is known for TO (and other cyanine dyes),[8] the absorption of the conjugates was found to be sensitive to the environment commonly attributed to aggregation (see the Supporting Information). Thus, to favor rapid interactions with DNA, low ionic conditions were preferred in studies of the association of both probes with quadruplex and duplex DNA by fluorescence titrations. Like TO, the two newly synthesized probes are virtually nonfluorescent in the free state, and their emission is significantly enhanced upon binding to DNA (Figure 1 A–C and the Supporting Information). As can be seen in Figure 1, the enhancement is more pronounced in duplex DNA (herein ds26, the self-complementary 5′-$CA_2TCG_2ATCGA_2T_2CGATC_2GAT_2G$-3′) for TO and PDC-*L*-TO **1**, while PDC-*M*-TO **2** has a marked preference for quadruplex DNA (22AG, 5′-$AG_3[T_2AG_3]_3$-3′, a quadruplex-forming oligonucleotide that mimics the human telomere). Although the environment of the DNA matrix might strongly influence the quantum yield of the probes, it can be hypothesized that the different behavior of PDC-*L*-TO **1** and PDC-*M*-TO **2** originates in different binding selectivities. This would demonstrate that the quadruplex selectivity of the parent PDC molecule is totally abolished by linkage to the TO moiety in a flexible arrangement, whereas it is retained in the rigid, merged scaffold. Hence, PDC-*M*-TO **2** elicits the essential properties required for G-quadruplex sensing, namely a pronounced fluorescence exaltation upon binding (≈36% of that of TO; see Figure 1 D and the Supporting Information) and a robust ability to discern quadruplex DNA from duplex DNA. Indeed, upon normalization of the duplex-DNA response at 1 (Figure 1 E), PDC-*M*-TO **2** fluoresces approximately eight times more intensively when interacting with quadruplex DNA, while almost no differences are observed with TO and PDC-*L*-TO **1** (1.5 and 0.8-fold intensity).

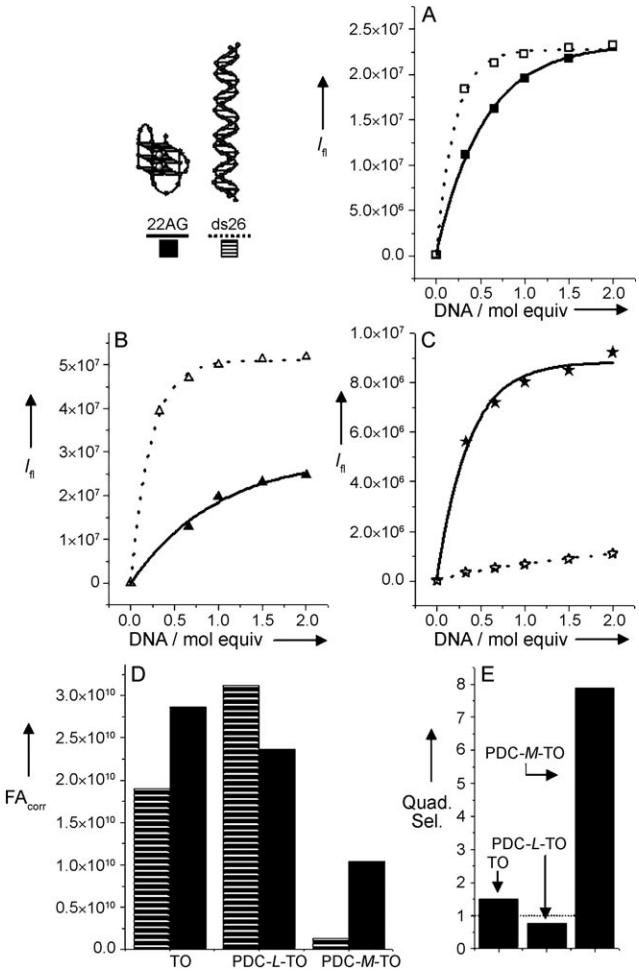

Figure 1. A–C) Fluorescence response of TO (A), PDC-*L*-TO **1** (B), and PDC-*M*-TO **2** (C, 1 μM in 10 mM lithium cacodylate buffer, 1 mM KCl, $\lambda_{ex}=490$ (A, B) and 500 nm (C)) upon addition of increasing amounts (0–2 μM) of quadruplex DNA (22AG, solid line) or duplex DNA (ds26, dotted line). D) Fluorescence areas (corrected with regards to absorbance (FA_{corr}), see the Supporting Information)) of TO, PDC-*L*-TO **1**, and PDC-*M*-TO **2** upon addition of 2 equiv of 22AG (black bar) or ds26 (striped bar). E) Quadruplex/duplex DNA fluorescence selectivity of dyes (normalized at 1 for ds26 fluorescence). I_{fl}: fluorescence intensity, Quad. Sel.: quadruplex selectivity.

To fully confirm that the observed fluorescence increase reflects the binding selectivity, the interaction of PDC-*M*-TO **2** with G-quadruplex DNA was studied by the FRET melting assay.[9] A strong stabilization of F21T (*FAM*-$G_3[T_2AG_3]_3$-*Tamra*) by PDC-*M*-TO **2** was observed since $\Delta T_{1/2}$ values of +18.4 and +25.2 °C were determined in Na^+ and K^+ buffers, respectively (Figure 2 A and the Supporting Information). This reflects a very high affinity for the quadruplex structure albeit somewhat lower than that reported for the parent PDC ($\Delta T_{1/2}=+22.9$ °C in Na^+ buffer conditions, see the Supporting Information). This indicates that the structural inclusion of TO has a cost in terms of target affinity. Similar FRET melting experiments were performed in the presence of various amounts of ds26 as a duplex-DNA competitor (see Figure 2 A and the Supporting Information):[9] PDC-*M*-TO **2** appears highly selective since the F21T stabilization is maintained at a high level (>85% in presence of 10 μM of ds26, regardless of the buffer cation). Remarkably, this level of quadruplex selectivity, rarely reached, is even higher than that of the parent molecule (see the Supporting Information).

The exceptional ability of PDC-*M*-TO **2** to discern quadruplex DNA over duplex DNA was further used and confirmed by preliminary DNA staining gel experiments (see Figure 2 B,C and the Supporting Information). We found that that PDC-*M*-TO **2** fluoresces in quadruplex-DNA-occupied lanes (22AG), whereas it does not within those occupied by duplex-DNA (ds26). Indeed, when the gel is loaded with both DNA matrices (0.3–2 μM) and post-stained with PDC-*M*-TO **2** (5 μM), the probe fluoresces only in quadruplex DNA (Figure 2 B, left lanes). Second post-staining by PDC-*L*-TO **1**, shown to fluoresce almost identically in duplex- and quadruplex-DNA-occupied lanes (see the Supporting Informa-

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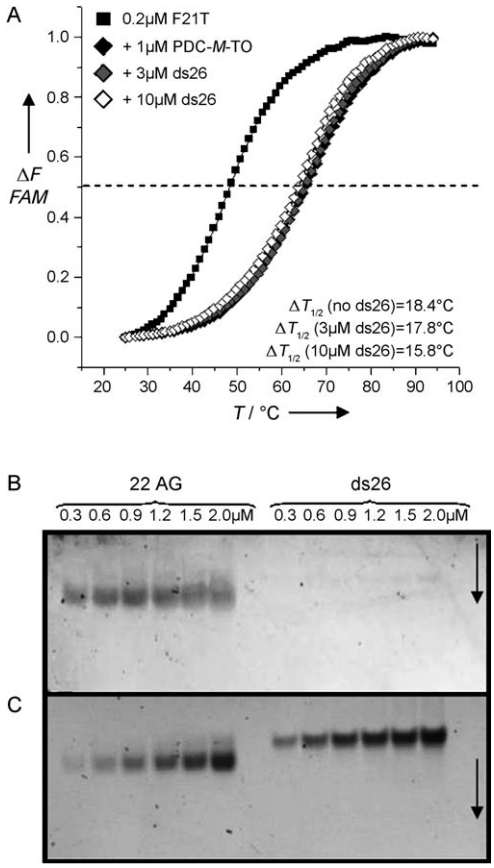

Figure 2. Selectivity of PDC-*M*-TO **2**. A) FRET melting curves for experiments carried out with F21T (0.2 μM in 10 mM lithium cacodylate buffer, 100 mM NaCl (black square)) with PDC-*M*-TO **2** (1 μM) in the absence (black diamond) or presence of competitive ds26 (3 μM (gray diamond) and 10 μM (white diamond)). B,C) Gel electrophoresis (20% acrylamide, in 1×TBE buffer + 20 mM KCl, 5 °C) of DNA (22AG (left lanes) and ds26 (right lanes) at concentrations from 0.3 to 2.0 μM stained by PDC-*M*-TO **2** (B, 5 μM, 5 h) and post-stained by PDC-*L*-TO **1** (C, 2 μM, 1 h) (λ_{ex} = 532 nm, λ_{em} = 550 nm). Δ*F FAM*: normalized FAM fluorescence.

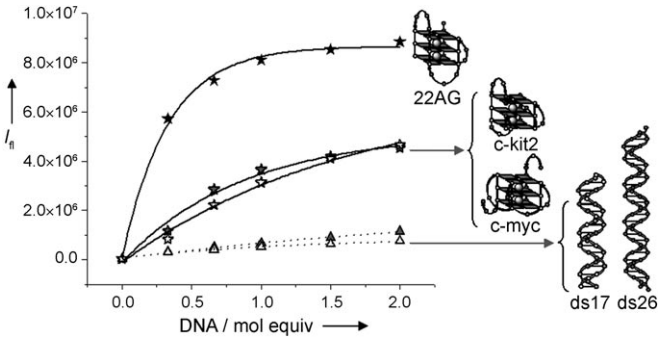

Figure 3. Fluorescence response of PDC-*M*-TO **2** (1 μM in 10 mM lithium cacodylate buffer, 1 mM KCl, λ_{ex} = 500 nm) upon addition of increasing amounts (0–2 μM) of quadruplex DNA (22AG (black star), c-kit2 (white star), c-myc (gray star)) and duplex DNA (ds26 (gray triangle) and ds17 (white triangle)).

tion), confirms the presence of DNA in all lanes, as well as the difference in selectivity between the two dyes (Figure 2C). Altogether, these results confirm that PDC-*M*-TO **2** is a fluorescent and highly specific G-quadruplex ligand, although it is not sensitive enough to allow quantitation of low amounts of DNA.

Finally, in view of the large number of quadruplex-forming sequences in the human genome,[10a,b] it was also of interest to evaluate whether PDC-*M*-TO **2** would be able to discern among various types of quadruplexes. To this end, we selected the two most well-characterized quadruplexes potentially present in the promoter regions of oncogenes, that is c-myc and c-kit (herein c-kit2).[10b,c] Also to ensure that the high selectivity of quadruplex DNA over duplex DNA was not sequence-dependent, another duplex (ds17)[11] was tested. As depicted in Figure 3, PDC-*M*-TO **2** fluoresces only weakly with both duplex DNAs (ds17 and ds26), whereas its fluorescence is greatly enhanced in presence of c-myc and c-kit2. Although the response is weaker than that obtained with 22AG (roughly half), it represents a very promising step toward intra-quadruplex recognition.

In conclusion, we have described a novel quadruplex-selective fluorescent probe, PDC-*M*-TO **2**, whose design originally relies on the structural combination of a highly effective G-quadruplex ligand and of a DNA fluorescent "light up" probe. Its synthetic access is straightforward and it shows exceptional properties in terms of quadruplex recognition and fluorescent discrimination. Given that the quinoline (and quinolinium) moiety is a frequently encountered motif in the design of G-quadruplex ligands,[1b,2d,12] results reported herein represent a solid and innovative basis for the design of novel G-quadruplex-selective fluorescent probes.

Received: November 17, 2008
Published online: February 6, 2009

Keywords: DNA · fluorescence probes · G-Quadruplex · guanine

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