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Development of a fluorescent intercalator displacement assay (G4-FID) for establishing quadruplex-DNA affinity and selectivity of putative ligands

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Abstract—A fluorescent intercalator displacement assay (G4-FID) has been designed based on the displacement of thiazole orange (TO) positioned onto a quadruplex-forming oligonucleotide by putative ligands. This technique was validated by the use of a set of representative and fully characterized G-quadruplex binders (ranging from pyridodicarboxamide to macrocyclic ligands). To further extend its applicability, a comparative version has been developed which allows a rapid and viable determination of quadruplex- over duplex-selectivity.

A wide variety of techniques are currently available to establish the DNA binding properties of small molecules, but their application to other DNA structures than duplex-DNA is sometimes difficult. Concerning the G-quadruplex DNA (noted hereafter G4-DNA), which is currently the focus point of much attention,[1] only a limited number of methods are employed. Among these, FRET-melting assay,[2] SPR (biacore) technique[3] and fluorimetric titrations[4] are the most widely used. However, the two former are somewhat technically challenging and/or require specialized equipment whereas the latter is limited to fluorescent ligands. Consequently, the development of easy-to-use methods is still needed. The FID (fluorescent intercalator displacement)[5] is a well-known assay based on the loss of fluorescence of a DNA-bound intercalator (ethidium bromide, thiazole orange (TO, Fig. 1)) or minor-groove binder (Hoechst 33258) upon displacement by a DNA-binding molecule. FID has been recently used on DNA hairpins to establish binding affinity and sequence selectivity of small molecules, proteins and triplex forming oligonucleotides.[6]

In this context, we have been interested in the development of an alternative FID assay (G4-FID) based on the displacement of TO from the human telomeric derived quadruplex-DNA, which requires neither modified oligonucleotides nor specific equipments.

Preliminary studies have demonstrated that TO binds the quadruplex-forming oligonucleotide 22AG (Fig. 1) in a single-site manner, with high affinity ($K_a = 3 \times 10^6\ M^{-1}$);[7] this recognition efficiency explains our preference for TO over ethidium bromide (known to weakly bind quadruplex)[8] and ensures probing of a high affinity site in G4-DNA. More importantly, TO is highly fluorescent upon interaction with G4-DNA (~500- to 3000-fold exaltation) whereas totally quenched when free in solution (quantum yield (ϕ) = 2×10^{-4}); consequently, the displacement ability of a given ligand can be easily monitored by the decrease of TO fluorescence (λ_{max} = 539 nm) upon selective excitation at 501 nm.[9] This test has been designed to be performed with inexpensive material (22AG and thiazole orange) in a non-demanding manner (0.75, 1.5 and 7.5 nmol/test

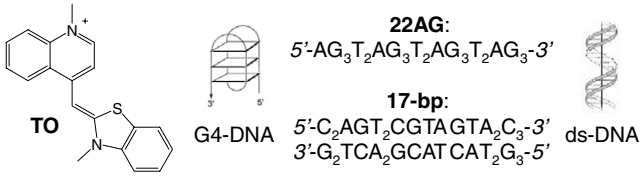

Figure 1. Structure of thiazole orange and oligonucleotides 22AG and 17-bp ds-DNA (counter-ion is p-$CH_3(C_6H_4)SO_3^-$ for TO).

Keywords: G-quadruplex; Thiazole orange; Fluorescent intercalator displacement.
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for 22AG, TO and tested ligand, respectively) with a standard spectrofluorimeter. Additionally, it is neither technically demanding nor time consuming (~40 min/sample).

In the course of our long-time program of developing new G-quadruplex ligands, we have designed and obtained a family of quinacridine ligands (Fig. 2).[4,10] Full investigations (syntheses, biochemical evaluations and NMR studies) will be reported elsewhere.[11] These compounds have been initially evaluated for their ability to bind G-quadruplex DNA by the FRET melting assay recently developed by Mergny and Maurizot.[2] This test is based on the monitoring of melting profile of a quadruplex-forming oligonucleotide labelled with FRET fluorophores, and allows to sort ligands with respect to their quadruplex-stabilization ability (ΔT_m). Up to now it has been successfully applied to a wide panel of ligands, including the high-affinity G4-binders telomestatine,[12] pyridodicarboxamide[13] or bisquinacridine.[10] Eight of the most representative quinacridines (Fig. 2) have been used to validate the FID assay, ranging from polyammonium (**1**–**4**) to N-methylated quinacridines (**5**–**7**) as well as macrocyclic bisquinacridine (**BOQ$_1$**).

The test is designed as follows: onto a mixture of prefolded 22AG-quadruplex (0.25 μM) and TO (0.50 μM), in a 10 mM sodium cacodylate, pH 7.3, 100 mM K^+ buffer, addition of increasing amount of ligand (from 0.5 to 10 equiv) is followed by a 3 min equilibration period before the fluorescence spectrum is recorded. The fluorescence area (FA, 510–750 nm), converted in percentage displacement (PD, with PD = 100 – [(FA/FA$_0$) × 100], FA$_0$ being FA before addition of ligand), is then plotted versus the concentration of added ligand.

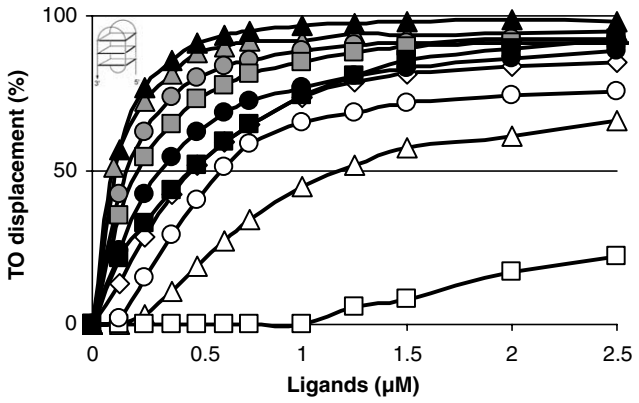

Figure 3. FID results onto 22AG for quinacridines **1** (□), **2** (△), **3** (○), **4** (◇), N-methylated quinacridines **5** (●), **6** (■), **7** (▲), **BOQ1** (■), **360A** (●) and **TMPyP4** (▲).

FID curves are presented in Figure 3. In order to precisely quantify the ligand-induced TO displacement, and in turn to sort ligands with respect to this ability, a $^{G4}DC_{50}$ value was designed as the required concentration to displace 50% TO from 22AG. Values are reported in Table 1.

Remarkably, in most cases FID results are in good agreement with the FRET data. Indeed, in a same series (e.g., polyammonium quinacridines, white motif), the TO displacement ability of quinacridines **1**–**4** parallels their thermal stabilization capacity (Table 1).

Ligand **1** appears poorly efficient which indicates that the FID test displays a high-level of selection for ligands due to the high affinity of the TO probe. In turn, ligands

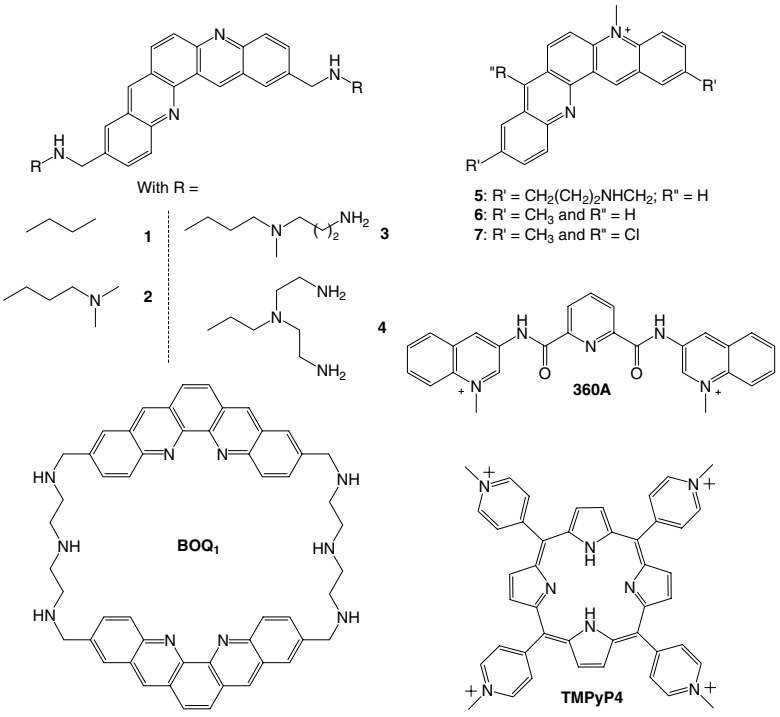

Figure 2. Structure of quinacridine-based ligands (**1**–**7**), macrocyclic bisquinacridine **BOQ$_1$**, pyridodicarboxamide **360A** and **TMPyP4** (counter-ions are $CF_3SO_3^-$ for **5**–**7**, I^- for **360A** and *p*-$CH_3(C_6H_4)SO_3^-$ for **TMPyP4**).

Table 1. FID (DC_{50} and selectivity) and FRET (ΔT_m) characterisations of compounds **1**–**7**, **BOQ1**, **360A** and **TMPyP4**

Ligand	$^{G4}DC_{50}$[a] (μM)	ΔT_m[b] (°C)	$^{ds}DC_{50}$[a] (μM)	Sel.[d]	Est. Sel.[e]
1	>2.5	12	n.d.[c]	n.d.[c]	—
2	1.185	19	0.667	0.6	—
3	0.611	21	0.497	0.8	—
4	0.480	32	0.392	0.8	—
BOQ_1	0.475	28	1.862	3.9	—
360A	0.320	26	>2.5	>6.4	43.5
5	0.162	21	0.400	2.5	—
6	0.217	10	0.925	4.3	—
7	0.096	16	>2.5	>25.9	31.7
TMPyP4	0.106	22	0.190	1.8	—

[a] $^{G4}DC_{50}$ and $^{ds}DC_{50}$ stand for the concentration required for 50% displacement of TO onto quadruplex (G4-DNA) and duplex-DNA (ds-DNA) respectively (experimental errors estimated at ±5 %).
[b] $\Delta T_m = T_m$(DNA + ligand) − T_m(DNA); determined by standard FRET measurements.
[c] n.d. for not determined.
[d] Sel. for selectivity G4- versus ds-DNA, established with: Sel. = $^{ds}DC_{50}/^{G4}DC_{50}$.
[e] Est. Sel. for estimated selectivity, see text for explanation.

2–**4** whose affinity was not possible to measure by fluorimetric titration due to their high cationic charge[11] can be evaluated through the present FID assay. Altogether, this means that this test is devoted to the detection of ligands with K_d in the sub-micromolar range. In order to further validate the assay with ligands of various structures, the macrocyclic bisquinacridine **BOQ_1** and the high affinity quadruplex binder **360A** (Fig. 2)[13] have been tested. In both cases, high FID levels are observed ($^{G4}DC_{50} < 0.5$ μM, Table 1). These results confirmed the overall generality of the assay since both ligands are characterized by a high ΔT_m response (>25 °C, Table 1). Interestingly, N-methylated quinacridines **5**–**7** appear to be more potent TO displacers than the polyammonium ligands **2**–**4** (grey motif, $^{G4}DC_{50} < 0.3$ μM) whereas they exhibit lower thermal stabilizations (Table 1). A striking example is ligand **6** which exhibit a $^{G4}DC_{50}$ as low as 0.217 μM and a ΔT_m of only 10 °C. The discrepancy between the two groups of ligands comes obviously from different binding modes and/or sites. Indeed, whereas N-methylated quinacridines are likely to stack on one tetrad (as do pentacyclic acridinium ligands),[14] polyammonium quinacridines, **BOQ1** and **360A** are susceptible to establish additional interactions with the loops and/or the grooves. Hence in the former case a strict competition occurs with the probe whereas in the latter case TO displacement might result from both direct and indirect competition (i.e., fixation to nearby sites). Furthermore, in the N-methylated series, the observation that the electronically poorer the quinacridine (**7** > **5** > **6**) the more efficient the TO displacement also suggest that this displacement highly depends on the quinacridine/quadruplex stacking relationship. Additionally, the well-documented **TMPyP4** (Fig. 2),[15] known to interact via π-stacking, has been tested.[16] Its high TO displacement ability ($^{G4}DC_{50} = 0.106$ μM, Table 1), in the same range as that of N-methylated quinacridines, supports our hypothesis. In that sense, this G4-FID test might complement the use of the FRET assay since the relative weights of the π-stacking and the electrostatic contributions seem to differ for the two methods. Nevertheless, care should be taken when interpreting the data, which should be considered in the context of the experiment.

To further extend the usability of this FID assay, a comparative version has been developed, which allows the estimation of preference for quadruplex- over duplex-DNA, if any. The 'comparative G4-FID' is based on the comparison of results obtained with 22AG and a 17-bp duplex-DNA (or ds-DNA, Fig. 1)[17] under strictly the same conditions (22AG or 17 bp (0.25 μM), TO (0.50 μM), in a 100 mM K^+ buffer, upon addition of increasing amount of ligand (from 0.5 to 10 equiv)). This comparison is feasible since TO has the same level of affinity for duplex- and quadruplex-DNA, and is known to exhibit a poor sequence-specificity.[6] However, considering that the duplex-binding stoichiometry of TO is higher than that of quadruplex (i.e., 3/1 for a 17 bp duplex),[6] it is worth noting that the comparative G4-FID gives the same results when conducted with a higher TO/oligonucleotide ratio (4/1 instead of 2/1). Upon interaction with ds-DNA, a $^{ds}DC_{50}$ is determined, and the ratio between the two DC_{50} (ds vs G4) allows to estimate the ligand selectivity for quadruplex-DNA (Sel., Table 1). Nevertheless, in some cases (**7**, **360A**) we were unable to determine a $^{ds}DC_{50}$ due to the low affinity of these ligands for ds-DNA. In these cases, the selectivity is calculated on the basis of the level of displacement (in %) obtained with 2.5 μM of ligand onto the 17-bp duplex; the concentration required with 22AG to reach the same level (^{G4}C) allows then to determine the estimated selectivity (Est. Sel., Table 1) via the equation: Est. Sel. = $2.5/^{G4}C$. Results are shown in Figure 4 and summarized in Table 1.

BOQ_1 and **360A** are known to be quadruplex-selective, and this preference has been quantified by various techniques: for **BOQ_1**, a 10- and 3.3-fold selectivity have been determined by SPR and dialysis equilibrium,

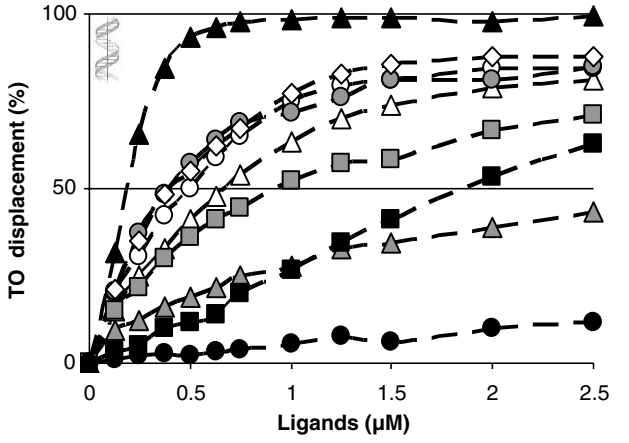

Figure 4. FID results onto 17-bp duplex-DNA for quinacridines **2** (△), **3** (○), **4** (◇), N-methylated quinacridines **5** (●), **6** (■), **7** (▲), **BOQ1** (■), **360A** (●) and **TMPyP4** (▲).

respectively, while a selectivity higher than 50-fold has been measured for **360A** via AlphaScreen test.[13] Herein, a 3.9- and 43.5-fold selectivity is obtained for $\mathbf{BOQ_1}$ and **360A**, respectively. On the opposite, **TMPyP4** is known to be poorly selective.[18] The low selectivity factor evaluated here (Sel. = 1.8, Table 1) confirms the validity of the test. Concerning the quinacridines, while polyammonium ligands appear not selective (Sel. 0.6–0.8 for **2**–**4**), N-methylated quinacridines show a clear preference for the quadruplex-DNA (especially **7** with a 31-fold selectivity). This class of compounds is currently studied, and results will be reported soon.

In conclusion, we have developed a simple G4-FID assay, based on the displacement of TO positioned on a quadruplex-forming oligonucleotide mimicking the human telomeric DNA. The validation of the assay using a set of known and fully characterized quadruplex-ligands was fully satisfactory and indicates that the test ensures a high level of quality for the detected G4-ligands. Additionally, a 'comparative G4-FID' has also been developed, and results obtained are in good agreement with the reported data based on various methods. The structural diversity of tested ligands (from bisquinolinium to 42-membered macrocyclic ligand and condensed heteroaromatic ones) testifies to the wide applicability of the present technique. Indeed, in spite of shortcomings due to direct versus indirect displacement of the probe, the G4-FID method is suitable for rapid examination of putative G4-binders. Consequently, this very 'easy-to-use' test can be of great help in the drug-discovery process. Finally, this FID assay represents a unique opportunity to evaluate ligand affinity for other quadruplex-structures (e.g., bi- or tetra-molecular ones), the protocol being easily adaptable to these nucleic acid structures. These results will be also reported in due time.

Acknowledgments

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16. In this case, a TO fluorescence attenuation effect is due to a minor absorbance of the porphyrin at the excitation wavelength (501 nm). An attenuation factor has been calculated < 10% for a **TMPyP4** concentration of 2.5 μM (10 equiv). Consequently, DC_{50} evaluation is not affected since it is obtained at ligand concentration <0.25 μM (Figs. 3 and 4).
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