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Research paper

# Fluorescence intercalator displacement assay for screening G4 ligands towards a variety of G-quadruplex structures

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ABSTRACT

The potential formation of G-quadruplexes in many regions of the genome makes them an attractive target for drug design. A large number of small molecules synthesized in recent years display an ability to selectively target and stabilize G-quadruplexes. To screen for G4 ligands, we modified a G4-FID (G-quadruplex Fluorescent Intercalator Displacement) assay. This test is based on the displacement of an "on/off" fluorescence probe, Thiazole Orange (TO), from quadruplex or duplex DNA matrices by increasing amounts of a putative ligand. Selectivity measurements can easily be achieved by comparing the ability of the ligand to displace TO from various quadruplex and duplex structures. G4-FID requires neither modified oligonucleotides nor specific equipment and is an isothermal experiment. This test was adapted for high throughput screening onto 96-well plates allowing the comparison of more than twenty different structures. Fifteen different known G4 ligands belonging to different families were tested. Most compounds showed a good G4 *vs* duplex selectivity but exhibited little, if any, specificity for one quadruplex sequence over the others. The quest for the "perfect" specific G4 ligand is not over yet!



## 1. Introduction

G-quadruplexes are a polymorphic class of secondary DNA structures in which the structural unit is formed by a planar arrangement of four guanines, known as G-quartets or G-tetrads [1,2]. A vertical stacking of several G-quartets are stabilized by the presence of monovalent cations. These DNA structures can be formed by one, two or four G-rich strands. Tetramolecular quadruplexes generally adopt a well-defined structure, in which all guanines are in the *anti*-glycosidic conformation and all strands are parallel, and might be useful for biotechnology applications. Intramolecular G-quadruplexes formed by single DNA strand have attracted much interest because of their putative biological regulatory function at telomeres, oncogene promoters and other relevant regions of the genome [3–5]. In contrast to tetramolecular quadruplexes, intramolecular structures form faster and are more complex, showing great conformational diversity [3]. Different sequences can adopt distinct topologies, but a given sequence can also fold into various different conformations, which may coexist. One of the best illustrations of this complexity are human telomere sequences, in particular in potassium conditions [6].

The potential formation of G-quadruplexes in many regions of the genome makes them an attractive target for drug design ever since quadruplex ligands were found to inhibit telomerase [7], to induce rapid apoptosis owing to the displacement of telomere-binding proteins and to regulate transcription or translation of genes, especially oncogenes [8–12]. A large number of small molecules synthesized in recent years display an ability to target and stabilize selectively G-quadruplexes [13,14]. Nowadays, the challenge is to develop small molecules capable of selective binding to one quadruplex type over another. To be effective, screening for selectivity has to be performed at the early stage of development and ideally this should be rapid and easy to implement. Several methods are currently used, such as the FRET-melting assays [15], SPR techniques [16], ITC experiments [17], ESI-MS analyses [18], multifluorescent probes assays [19] or equilibrium microdialysis [20]. Although these methods are reliable and powerful, they require specific equipment or buffer conditions, modified or immobilized oligonucleotides, and/or specific ligand properties (*e.g.* fluorescence).

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In this context, we decided to use a simple alternative assay developed by Teulade-Fichou's team since 2006, G4-FID (G-quadruplex Fluorescent Intercalator Displacement) [21–24]. The principle of G4-FID assay is schematically presented in Fig. 1. This assay is based on the displacement of an "on/of" fluorescence probe, Thiazole Orange (TO), from quadruplex or duplex DNA matrices by increasing amounts of a putative ligand. TO being virtually non-fluorescent when free in solution but strongly fluorescent when bound to DNA, the ligand-induced displacement leads to a decrease of the fluorescence which is monitored as function of the ligand concentration. Therefore, the quadruplex-affinity of a candidate compound can be evaluated through its ability to displace TO from quadruplex DNA. Moreover, selectivity measurements can easily be achieved by comparing the ability of the ligand to displace TO from various quadruplex and duplex structures. Moreover, G4-FID requires neither modified oligonucleotides nor specific equipment and is an isothermal experiment. Initially, this assay was performed in a high total volume (3 mL), using quartz cells (referred hereafter as *standard G4-FID assay*). In this work, we developed a low-cost G4-FID assay for high throughput screening onto 96-well plates (which may be easily transposed into 384-well plates) where the total volume does not exceed 25 μL. Notably, we decided to use a qPCR apparatus for all fluorescent measurements. Although it displays a relatively moderate fluorescence sensibility compared to dedicated fluorimeters or microplate readers, it is a widely spread device among research laboratories, already used for FRET-melting measurements.

In order to get more insight into the structure selectivity of the studied molecules, we have used a large number of oligonucleotides displaying various structures (quadruplexes, duplexes and single-strands). Thus, we have exhaustively characterized the structure and stability of 30 DNA and RNA sequences, by absorbance thermal difference spectra (TDS), circular dichroism (CD), UV melting and PAGE. We have determined which sequences could be reliably used in function of the fluorescence enhancement of bound TO. Finally, we have assayed 15 compounds, displaying different binding properties, using our screening method. All these ligands have already been tested by FRET-melting assay and/or standard G4-FID assay enabling a comparison of the results.

## 2. Experimental conditions

### 2.1. Relevant oligonucleotides and ligands

#### 2.1.1. Oligonucleotides

Oligonucleotides were purchased from Eurogentec (Seraing, Belgium), dissolved in bi-distilled water and stored at −20 °C. Concentrations of all oligonucleotides were determined by UV-absorption using the extinction coefficients provided by the manufacturer. Sequences are given in the 5′ to 3′ direction (Table S1). For spectroscopic measurements, Reverse-Phase Cartridge•Gold™ (RPC) purified oligonucleotides were used without further purification.

#### 2.1.2. Ligands

TrisK3-NH, TrisQ [25], 360A, 832A, 307A, iPDC [26], Phen-DC3, Phen-DC6, Bipy-DC3, Bipy-DC6 [27], and the metallo-organic complex Cu-ttpy [28] have been synthesized and supplied by Teulade-Fichou's team (Fig. 2). BRACO-19 [29] and 12459 [30] were a kind gift from Dr. P. Mailliet (Sanofi). Piper [31] and BSU1051 [7] are commercially available. Stock solutions of these ligands (1–10 mM in DMSO) were used for G4-FID assay and are stored at −20 °C. TO was purchased from Sigma–Aldrich and used without further purification.

### 2.2. Preparation of oligonucleotides

Oligonucleotides (intramolecular quadruplexes, duplexes and single-strands) were prepared by heating the corresponding oligonucleotides at 90 °C for 5 min in a 10 mM lithium cacodylate buffer pH 7.2, 100 mM KCl then slowly cooling to room temperature for 2 h. Oligonucleotide structures were formed at 50 μM strand concentration. Tetramolecular quadruplexes from $TG_4T$, $TG_5T$ and $UG_5U$ were pre-folded at high strand concentration (200 μM), at 4 °C for 48 h, in the same buffer.

### 2.3. Fluorescence enhancement measurements

Experiments were performed with 96-well microplates from Stratagene. Each condition was tested at least in duplicate, in a volume of 25 μL for each sample. Measurements were performed at 25 °C, all G-quadruplex sequences being folded in KCl at this temperature. Two equivalents of TO (2 μM) were added in a solution of 1 μM oligonucleotide. Samples were incubated in 10 mM lithium cacodylate buffer pH 7.2, 100 mM KCl. Fluorescence emission was collected at 516 nm with 8-fold gain after excitation at 492 nm in a real-time quantitative PCR (Stratagene Mx3005P instrument).

### 2.4. G4-FID protocol: isothermal assay

All experiments were performed with 96-well microplates. Each condition was tested in duplicate and at least 3 times, in a volume of 25 μL for each sample. Samples were incubated in 10 mM lithium cacodylate buffer pH 7.2, 100 mM KCl. Pre-folded DNA target (0.5 μM strand concentration for intramolecular G4, 2 μM strand concentration for tetramolecular G4 and 1 μM strand concentration for duplexes) was mixed with 1 μM of Thiazole Orange. Five equivalents of each ligand (2.5 μM) were added for each structure. The fluorescence of samples was measured at 25 °C in a qPCR (Stratagene Mx3005P) instrument. The temperature was kept constant with a thermostat cell holder (Peltier). The Thiazole Orange was excited at 492 nm (±5 nm) and the emission was collected at 516 nm (±5 nm) with 8-fold gain.

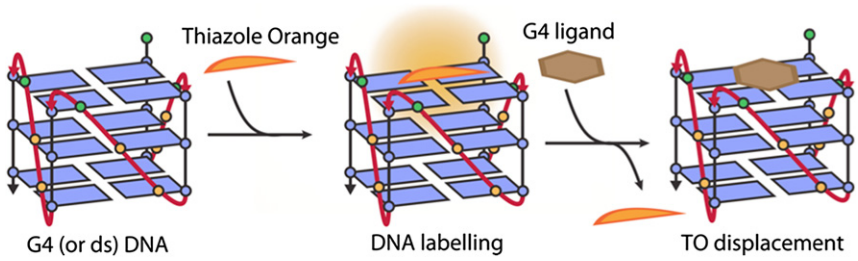


**Fig. 1.** Schematic representation of the two main steps of the G4-FID assay, *i.e.*, (a) labelling of the DNA matrices (quadruplex or duplex DNA) by Thiazole Orange (TO) and (b) displacing the fluorescent probe from the DNA matrices by a small molecule candidate.