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Development of fluorescent probes specific for parallel-stranded G-quadruplexes by a library approach†

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A 241-membered cyanine-based library was constructed by the combinatorial chemistry strategy. Combined with high-throughput screening, we successfully discovered a novel fluorescent probe (CyC-M716) capable of identifying a subset of parallel G-quadruplexes with propeller loops stretching across three tetrad layers with high sensitivity and selectivity.

G-quadruplexes (G4s) are non-canonical, nucleic acid structures formed by G-rich sequences. These structures are highly polymorphic and have been suggested to perform unique functions particularly in human telomeres and gene promoters.[1] Consequently, tremendous efforts are directed towards understanding novel G4 architectures,[2,3] and exploring them for anti-cancer drug development,[4] drug delivery vehicles,[5] and materials for nanodevices.[6] To accelerate this process, there is a strong impetus to develop probes capable of detecting and distinguishing the myriad of G4s with different structural features.[7] Fluorescent probes are attractive and versatile tools for the sensing of G4s because of their technical simplicity, fast response time and high sensitivity. To date, G4 selective fluorescent ligands have been designed based on two binding modes – end stacking and groove embedding.[7,8] These limited structural guidelines impede the ability to discover new fluorescent probes with novel selectivity. Despite being resource intensive, as a complementary approach, screening of fluorescent libraries can produce interesting fluorescent ligands able to discriminate additional structural features.[9] There have been several innovative disclosures of G4 selective fluorescent and luminescent probes.[3,10–13] Although these probes are capable of differentiating G4s from conventional double and single-stranded nucleic acids, these early reports are unable to provide additional structural information. G4s are highly polymorphic,[14] hence, to attain higher selectivity, simultaneous association with multiple structural elements is desired.[15] To achieve this goal, we thus derived a fluorescent library of cyanine dyes, envisioning that the cyanine core can assist binding to G4s while incorporation of additional flexible arm groups can further yield unexpected association and recognition of other G4 structural features. Cyanine dyes, such as the Cy5 core we employed, are known molecular rotors; restriction in rotation reduces non-radiative energy loss thereby enabling more energy to be channeled into fluorescence.[16,17] In a similar manner, we anticipate that the restriction in the overall flexibility of our Cy5-based dyes by selective binding of these arm groups will enhance the fluorescence of the fluorophore thus achieving the sensing of a subset of quadruplexes possessing common structural features.

To enable the facile construction of our library, Cy5 was selected as the core and arm groups were systematically incorporated using simple and robust methods. The synthesis of 241 library compounds was achieved in 4 linear steps and a final diversification step with high purities (Tables S1–S3, ESI†). All of these compounds exhibited photo-physical properties similar to its parent Cy5 fluorophore[18] ($\lambda_{abs} \approx 648$ nm, $\lambda_{em} \approx 670$ nm, $\Phi_F \approx 0.2$–0.3). In addition, these compounds displayed a dramatic increase in fluorescence when dissolved in viscous solvents (which restricts bond rotation) thus validating their potential as fluorescent molecular rotors (Table S4, ESI†). We performed primary screening of the library against G-quadruplexes. Here, we consider *J19*, which is a G4 with anti-HIV activity formed by GGGT repeats, as a representative quadruplex.[19] From screening, we observed several compounds displaying significant fluorescence enhancement upon interaction with *J19*. Amongst these candidates, CyC-M716 was selected, which displayed the highest response to the G-quadruplexes. At saturation, CyC-M716 displayed an

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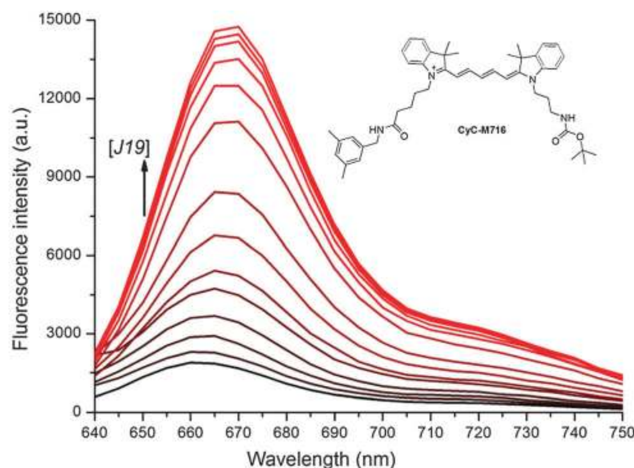
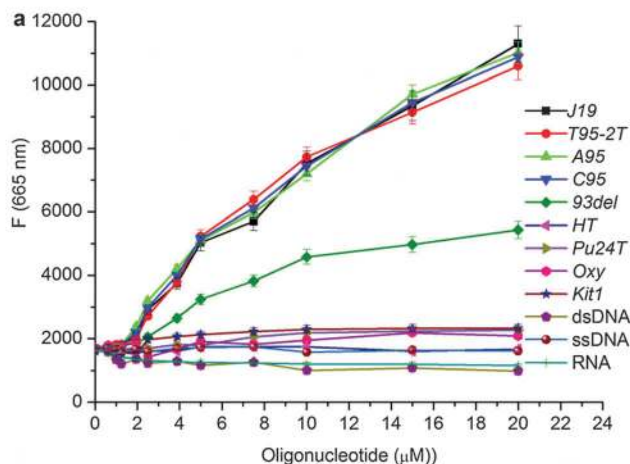


Fig. 1 Fluorescence spectra of CyC-M716 (10 μM) upon incubation with serial dilutions of *J19* (from 0–100 μM) in buffer (20 mM K_2HPO_4/KH_2PO_4, 100 mM KCl, pH 7.0). λ_{ex}: 600 nm. Φ_F (without *J19*) = 0.10 Φ_F (in 100 μM *J19*) = 0.68.



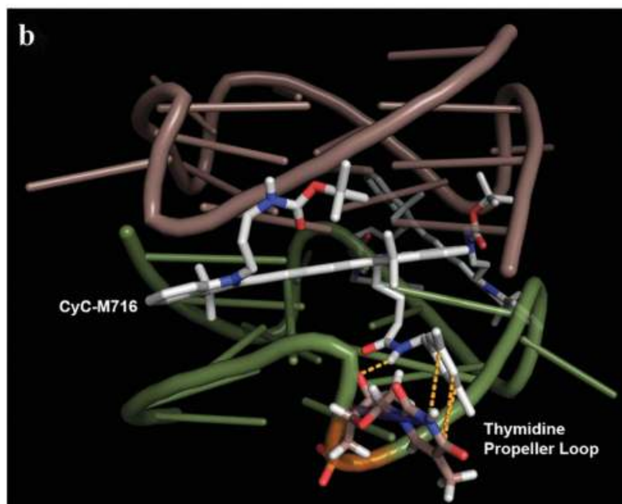


Fig. 2 (a) Fluorescence response of CyC-M716 (10 μM) toward 12 different oligonucleotides (0–20 μM) in buffer. λ_{ex}: 600 nm. (b) Molecular docking results of CyC-M716 binding to the *J19*. Pink and green for *J19* monomer; carbon atoms are colored in white for CyC-M716, oxygen and nitrogen atoms are colored in red and blue, respectively. The thymidine at 12th sequence is highlight. Carbon atoms are colored in pink. Oxygen and nitrogen atoms are colored in red and blue, respectively. Plausible hydrogen bonds between CyC-M716 and *J19* residue are shown as yellow dashed lines. Distances involved in π–π interaction display yellow dashed lines.

8.5-fold increase in fluorescence and a slight 10 nm bathochromic shift in emission maximum upon interacting with *J19* (Fig. 1). A Job plot analysis revealed a binding stoichiometry of one CyC-M716 per *J19* monomer (Fig. S1, ESI† inset); a one-site binding model was thus fitted to the fluorescence titration and the dissociation constant (K_d) determined to be 20.63 ± 0.05 μM (Fig. S1, ESI†).

Next, we investigated the fluorescence response of CyC-M716 towards various model G4 oligonucleotides and canonical nucleic acids (Table S5 and Fig. S2, ESI†). Like *J19*, parallel-stranded *T95-2T, A95* and *C95* G4s elicited remarkable fluorescence enhancements upon incubating with CyC-M716 (Fig. 2). In contrast, other parallel-stranded G4s (*93del, c-kit1* and *Pu24T*) returned only modest or negligible increments in fluorescence emission. Non-parallel stranded G4s – *Oxy* and *HT* – similarly did not increase the fluorescence of the dye. Interestingly, slight quenching in the fluorescence was observed when CyC-M716 was incubated with genomic double-stranded and single stranded DNA and RNA. The binding parameters (K_d) for CyC-M716 to these G4s also indicated the selectivity to different G4s structures as well as canonical DNA and RNA (Fig. 2 and Table S6, ESI†). Corroborating the emission enhancement results, the excited-state lifetime of CyC-M716 also displayed significant changes owing to its different binding environments in the quadruplexes (Fig. S3 and Table S7, ESI†); upon incubation with the respective G4s, the longest decay times were seen with *J19* and *T95-2T* (>2.2 ns). Comparatively shorter lifetimes were observed with *93del* (≈1.8 ns), *HT* and dsDNA (≈1.6 ns); however, these fluorescence lifetimes remain higher than that of the free dye (≈0.8 ns) indicating a general association of the cyanine core with various nucleic acids. Upon careful examination of the structural features shared by *J19, T95-2T*, *A95*, and *C95* G4s recognized by CyC-M716, we observed that all of them are non-interlocked, parallel-stranded quadruplexes having propeller loops.[20,21] *93del* is an interlocked, parallel-stranded dimer and consists of propeller loops formed by adenosine and thymidine.[22] In contrast, *c-kit1* does not have propeller loops. *Pu24T* has such loops but with snapback motifs capping the G-tetrad core.[23,24]

Molecular docking of CyC-M716 to *J19* (PDB ID: 2LE6) was thus performed to understand the binding mode. As depicted in Fig. 2b the calculated binding model suggests plausible binding of the cyanine core to the grooves of *J19* with the two chains embedding further into the thymidine propeller loops of the quadruplex. Fixation of the chains is facilitated by hydrogen bonds formed between the 3′-OH of the 12th thymidine residue in *J19* and the amide hydrogen of CyC-M716 as well as π–π interactions between the aromatic planes of the thymine and the 3,5-dimethylbenzylamino motif in CyC-M716; this interaction restricts bond rotation and enhances the cyanine fluorescence accounts for the selectivity of CyC-M716 to parallel-stranded G4s with single thymidine propeller loops. Two dye molecules were found to associate with the dimeric *J19*

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quadruplex which is consistent with the binding ratio found from the Job plot (Fig. S1, ESI†).

Evidence of CyC-M716's groove binding mode is established from competitive experiments utilizing our previously reported dye – GQR – which was proven to bind at the grooves of several parallel-stranded G4s.[9] As illustrated in Fig. S4a (ESI†), systematic addition of GQR to a fixed solution of *J19* and CyC-M716 induced a significant decrease in the fluorescence response of CyC-M716. The reverse was observed, whereby the increasing concentration of CyC-M716 led to a displacement of GQR from *J19* (Fig. S4b, ESI†). These observations clearly indicate that CyC-M716 binds at the grooves of the G4s as suggested by the docking model.

A closer analysis of the CyC library's response to *J19* revealed interesting structure activity relationships that further corroborate the calculated model. In most cases, variations in the electron density of the benzyl ring by the incorporation of electron-donating or electron-withdrawing substituents led to annihilation of the turn-on response (Fig. S5, ESI†). The emission is however less sensitive to changes in the steric bulk; methyl substituents at the *meta* positions of the benzyl ring seem to provide some stability to the ring conformation for optimal π-stacking although in general all derivatives displayed a similar fluorescence increase when bound to *J19*. The lack of response from CyC-O374 further supports the importance of having the amide hydrogen bond donor for chain stabilization and turn-on response as suggested by the docking model. Together, the evidence supports the selective recognition of CyC-M716 for propeller loops found in G4s. Selectivity is achieved by the cooperative binding of the dye to both the groove and loop; interlocking of the monomers blocks the grooves closest to the loop thereby prohibiting a favourable fluorescence response.[25] Based on these results, CyC-M716 is the cyanine dye that is able to discern a subset of structures with propeller loops in parallel-stranded quadruplexes.

The planar G-tetrad is a hallmark for G4s. Consequently, the earliest G4 probes were based on planar aromatic systems that interact with G-tetrads through π–π stacking interactions. However, sole recognition of this shared structural feature is unlikely to confer selectivity amongst the wide diversity of G4 structures.[7] In addition to G-tetrads, G4s are also made up of loop elements that connect the tetrad planes. Unlike the tetrads, loops are highly polymorphic varying in terms of the type (edge-wise, diagonal, propeller or V-shaped), length and sequence; therefore, several selective G4-binding ligands have been developed to recognize these loop structural elements.[26] In the same vein, a selective G4 binding probe can be designed to recognize the various loop features of G4s. From our survey, ETC is the only cyanine probe that interacts with the loops in hybrid and parallel-stranded G4s.[27] Nevertheless, ETC lacks selectivity for the loop types. In contrast, CyC-M716 displays additional selectivity for propeller loops. The employment of selective fluorescent probes such as CyC-M716 in displacement assays enables rapid access to new specific ligands recognizing similar structural elements, some of which may eventually be useful drugs with important applications.

Despite the tremendous value of fluorescent probes targeting specific structural features of G4s, discovery of such new sensors remains sluggish. This is due in large to our limited understanding of the highly polymorphic G4 structures which in turn restricts rational design of new fluorescent ligands. Although tedious and resource intensive, the alternative approach of screening chemical libraries has been very effective in revealing novel scaffolds and ligands capable of distinguishing additional G4 features.[28,29] Using the same high-throughput screening strategy and the combinatorial chemistry to build large fluorescent libraries, we have demonstrated the systematic discovery of CyC-M716 with only limited knowledge about binding characteristics. This represents a general and effective strategy for discovering highly selective probes for G4s.

In summary, we have described the systematic discovery of a novel cyanine-based fluorescent probe – CyC-M716 – through the high-throughput screening of a combinatorial cyanine library. CyC-M716 exhibits an 8.5-fold fluorescence increase when bound to parallel-stranded G4s with propeller loops. Subsequent experiments and molecular docking revealed that plausible π–π interactions and hydrogen bonding to the loop account for the specific turn-on response to G4s with such structural elements. CyC-M716 represents a convenient tool for the sensitive differentiation of G4 structures and discovery of new ligands recognizing similar loop characteristics.

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