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Identification of Trinucleotide Repeat Ligands with a FRET Melting Assay

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DNA hairpin structures formed within a repeated tract might be a causative factor for triplet expansion observed in several debilitating diseases. We have designed and used a fluorescence resonance energy transfer (FRET) melting assay to screen for ligands that bind specifically to the CNG triplet repeats. Using this assay, we screened a panel of 33 chemicals that were previously designed to bind DNA or RNA secondary structures. Remarkably, we found that macrocyclic compounds, such as acridine dimers and trimers, exhibit interesting affinities and specificities for this motif.

Introduction

Recent molecular genetic studies have revealed a correlation between spontaneous expansion of DNA trinucleotide repeats and several debilitating diseases such as myotonic dystrophy. First characterized in Fragile-X syndrome[1] (for a review see ref. [2]), these neurodegenerative disorders fall into a new category of genetic diseases named TREDs, for trinucleotide repeat expansion diseases. The number of repeats often increases during parent-to-offspring gene transmission as a result of germline expansion. DNA secondary structures formed within the repeated tract could be a causative factor for triplet expansion;[3,4] these repeat regions might interfere with replication, transcription, or repair events. $(CNG)_n$ trinucleotide repeats (with N = A, T, G, or C), where *n* is less than twelve, fold into simple hairpin structures involving T–T, A–A, C–C, or G–G mismatches sandwiched between two Watson–Crick G–C base-pairs. DNA molecules with more than twelve trinucleotide repeats can fold into unusually long hairpins called broken hairpins.[5,6]

As the length of the repeated array is a crucial feature in the onset of disease and the progression of symptoms, suppression of somatic expansion could be therapeutically beneficial. Different research teams have recently tried to trigger triplet-repeat contractions by using chemotherapeutical approaches. Hashem et al. treated lymphoblastic cells from mytonic dystrophy patients with therapeutic concentrations of DNA-damaging agents.[7] After treatment, they observed significant reductions of CTG repeat length, by 150–300 CTG/CAG repeats. In an equivalent study, Gomes-Pereira et al. treated Dmt-D kidney mouse cells with another set of genotoxic agents composed of ethidium bromide, cytosine arabidoside, and 5-azacytidine. They also observed a contraction of the CTG repeated sequences of 30 to 75%, and in some cases an almost normal number of repeats was restored.[8] These studies used indirect approaches with genotoxic agents, which cause lesions all over the genome; thus, the observed contractions might have arisen from reparation events of the lesions, which affected the repeated tract or their vicinity, and could have involved different steps of the reparation process (excision or reversion). In Friedreich ataxia, another neurodegenerative disease linked to the expansion of GAA/TTC triplets, the formation of an intramolecular triplex within the expanded tract might be involved in the silencing of the frataxin gene. Hebert and colleagues have devised a strategy in which a small molecule binds preferentially to the duplex, and prevents formation of the unusual structure that restores transcription through the GAA repeat.[9] Burnett et al. also partially restored the expression of the frataxine gene in a cellular model by using a polyamidic minor groove binder.[10] These ligands recognize the GAA/TTC duplex and could, consequently, impede the formation of the triplex structure.

No therapeutic approach to prevent or revert repeat expansion is currently available, but as discussed above, in vitro studies suggest that repeat deletion can be induced by various chemotherapeutic agents. Such molecules, which cause locus-specific lesions, could reduce the number of repeats and consequently slow or stop the progression of the disorders.[11] In the literature, only a few molecules have been reported to interact specifically with CNG trinucleotide repeat sequences. The spirocycline molecules were initially synthesized to recog-

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nize DNA and RNA loops, and it was reported that these molecules stimulated expansion rather than contraction of the triplet repeats in an in vitro DNA replication assay.[12–14] A second family of molecules was designed to recognize the hydrogen-bonding motif of the bases with the aim of detecting AA, CC, and GG mismatches. Some of them—a naphthyridine azaquinolone and a naphthyridine dimer—have been shown to bind specifically to trinucleotide repeats, but these molecules have not yet been studied in any in vitro or cellular assay.[15,16]

The goal of this study was to identify molecules that would bind specifically to CNG regions of DNA by using a FRET-based screening method. To this end a panel of 33 chemicals previously designed to bind DNA or RNA secondary structures was screened. Among these, macrocyclic compounds, such as acridine dimers and trimers, exhibited remarkable affinity and specificity for hairpins formed from sequences containing seven or eight CNG repeats (N = T, A, C).

Results and Discussion

Principle of the test

The $(CTG)_7$ DNA oligonucleotide was double-labeled with FAM and TAMRA, by using a method similar to the one we had developed for labeling of quadruplex sequences.[17,18] The $(CAG)_8$ and $(CCG)_8$ oligonucleotides were labeled with identical tags. Folding into an intramolecular hairpin at moderate temperatures leads to a juxtaposition of the 5′ and 3′ ends of the molecule. The proximity between FAM and TAMRA in turn leads to FRET and quenching of donor emission (Figure 1).

Melting of the hairpin structure can thus be followed by fluorescence emission at 516 nm. The double-labeled $(CTG)_7$ DNA oligonucleotide had an apparent $T_{1/2}$ of 55 °C, similar to that of the unmodified oligonucleotide as determined by absorbance; this indicates that the attachment of the fluorophores did not greatly perturb the stability of the hairpin.[6]

Screening a panel of compounds

We chose to carry out our test of the double-labeled $(CTG)_7$ on an initial panel of 33 different chemicals. Over a hundred compounds were actually tested, but the other molecules gave few positive results. These molecules were chosen because of: 1) the presence of positive charges, 2) the planar aromatic character of some chromophores, and 3) known interaction of some of these molecules (acridine) with DNA. Figure 2A demonstrates that many of these compounds (14 out of 33) induced significant stabilization (10 °C or more) of the $(CTG)_7$ motif when tested at 1 μM. Unfortunately, for most compounds, this stabilization was lost or very significantly reduced in the presence of the unlabeled double-stranded DNA competitor ds26: only two molecules retained a stabilization potency of at least 5 °C in the presence of ds26 (Figure 2B). This loss indicated that many of these compounds exhibited little, if any, preference for the structure adopted by the trinucleotide repeats relative to classical B-DNA.

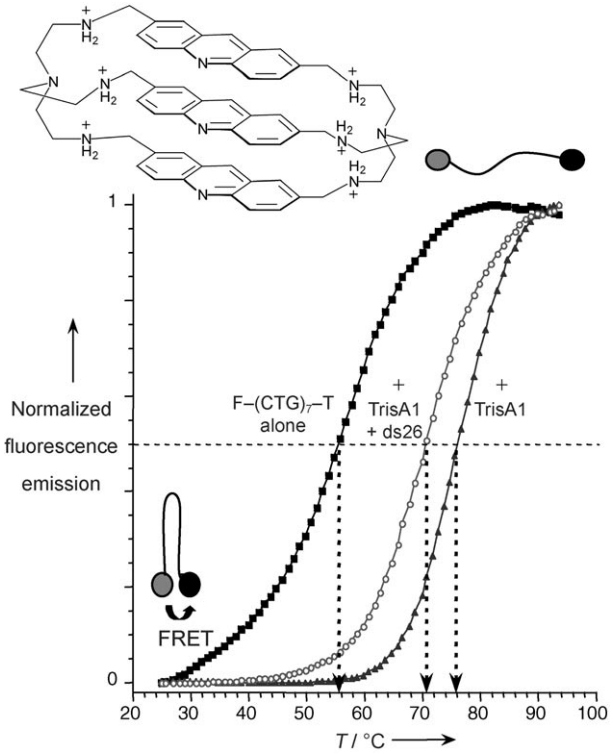

Figure 1. Fluorescence-based melting assay. The $(CTG)_7$ sequence was labeled with two chromophores (F–$(CTG)_7$–T): FAM (gray) at the 5′ end and tetramethylrhodamine (black oval) at the 3′ end. Addition of a trinucleotide ligand (TrisA1, shown at top) led to an increase in melting temperature. Further addition of a large excess of a double-stranded competitor (ds26) decreased this effect (○). In this example, the reversion is only partial. Fluorescence emission was normalized with respect to maximum emission at high temperature.

Similar experiments performed with $(CAG)_8$ and $(CCG)_8$ trinucleotide motifs led to qualitatively similar results (Figure 3). Stabilization values ($\Delta T_{1/2}$s) obtained for the three oligonucleotides were relatively similar, although $\Delta T_{1/2}$ values were systematically slightly higher for F–$(CCG)_8$–T (Figure 3A). The stabilization was then measured in the presence of a moderate amount of double-stranded DNA (3 μM ds26; Figure 3B, right panel). The preference for CCG repeats is also illustrated by the higher T_ms observed for this repeat in the presence of duplex DNA.

Identification of a lead molecule

Remarkably, one compound, the acridine trimer TrisA1, displayed interesting affinity and specificity for $(CTG)_7$, as shown by stabilizations of +17.5 °C, +13.8 °C, and +12.8 °C in the absence, or in the presence of 3 or 10 μM, respectively, of a double-stranded competitor. In order to confirm that the observed stabilization was not the result of an artifactual interaction with FAM or TAMRA, we performed thermal denaturation experiments followed by UV-absorbance spectroscopy with unlabelled strands. These experiments confirmed that TrisA1 and two other acridine derivatives (BisA1, BisA2) stabilized the hairpin structure adopted by unmodified $(CTG)_8$ (see Figure S2 in the Supporting Information). Unfortunately, aggregation of the complexes prevented us from performing quantitative titration

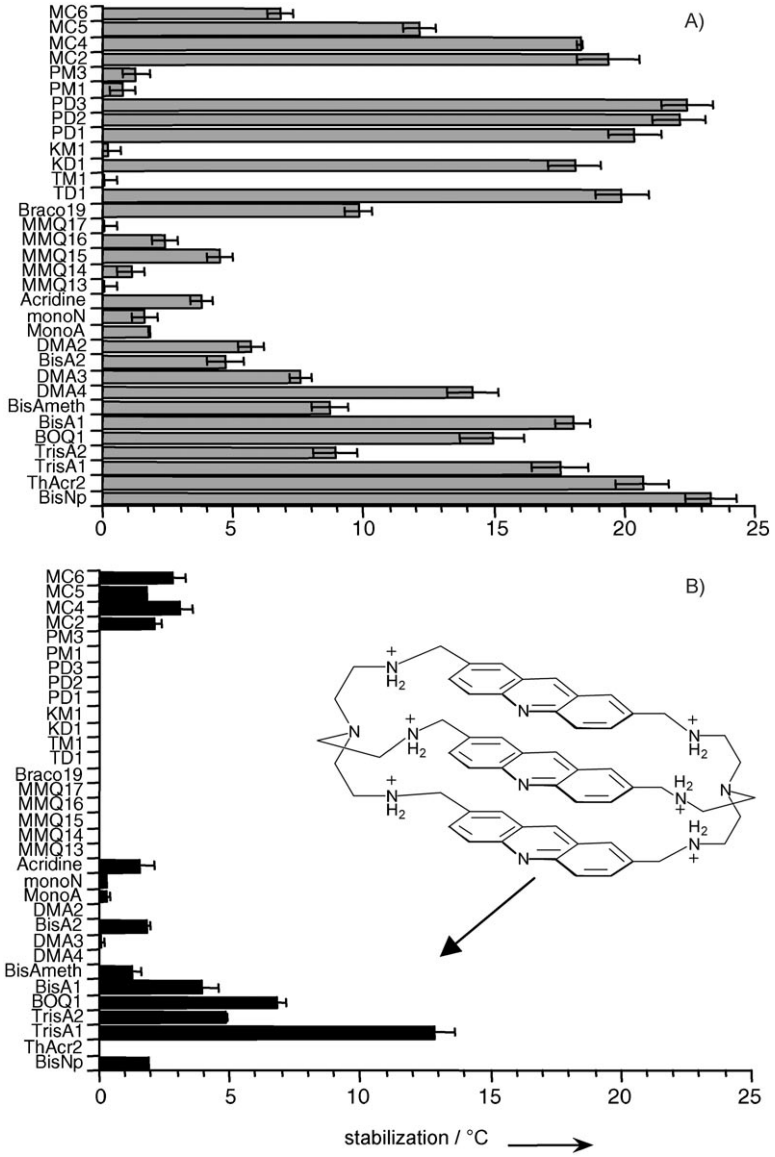

Figure 2. Results obtained with different compounds. Each molecule was tested at 1 μM. Full names, formulas, and/or references are provided in the Supporting Information. Stabilization of the hairpin conformation of the $(CTG)_7$ oligonucleotide (0.2 μM) is indicated in °C either A) in the absence or B) in the presence of the double-stranded competitor ds26 (10 μM); insert: chemical structure of TrisA1.

experiments by UV spectroscopy. Likewise, significant adsorption on the dialysis membranes also hampered the use of competitive dialysis experiments.[19] However, electrospray ionization mass spectrometry (ESI-MS) confirmed that BisA1 and BisA2 interacted with $(CAG)_8$, $(CTG)_8$, $(CCG)_8$, and $(CGG)_8$ repeats in a predominantly 1:1 binding mode (Figure S3).

To understand the structure–activity relationships of the selective interaction of TrisA1 with the CTG trinucleotide repeat motif, we compared the binding characteristics (stabilization and selectivity) of other acridine derivatives with the same motif (Figure 4).

In the acyclic series, on comparing the monomeric acridine (MonoA) with its dimeric (BisA2) and trimeric (TrisA2) counterparts, we detected improvements in both stabilization and specificity when the number of acridine units was increased from one to three. Similarly, in the macrocyclic series the specificity was drastically increased on changing from two units (BisA1) to three (TrisA1). Finally, on comparing macrocyclic dimers that possessed different aromatic units linked by the same spacer, such as BisNP, BisA1, and BOQ1, we observed a significant improvement in the specificity when the number of cycles that made up each aromatic moiety was increased from two to five. Surprisingly, this trend is associated with an 8 °C decrease in stabilization. From these observations, we may conclude that specificity is significantly improved when the number and the surface of the polycyclic aromatic units are increased. This gain in specificity, however, involves a loss in stabilization, which in the case of TrisA1 is compensated for by the unspecific electrostatic interactions between the positive charges harbored by the linkers and the negatively charged phosphate backbone. TrisA1 contains three acridine moieties linked by diethylenetriamine polycationic linkers; the lengths of these linkers space out each acridine unit by a distance of 0.7 nm. TrisA1 belongs to a class of macrocycles that were initially synthesized to trap free nucleotides in solution[20] and were subsequently shown to insert at thermodynamically weakened sites, such as abasic sites and mismatches, in duplex DNA.[21,22] We calculated one of the conformations that TrisA1 might adopt (Figure S4B).

This conformation is rather open and shows the central acridine protruding from the cavity; it might thus be able to sandwich two base pairs by intercalating within a double helix as hypothesized with the model represented in Figure S4A. Although intercalation at contiguous sites in DNA is forbidden by the nearest-neighbor exclusion principle,[23] this mode of interaction is obviously sensitive to the quality of base-pair stacking. Hence, the specificity of TrisA1 for the CNG motif is likely to be linked to the lower stability of the NN mismatch, which allows an easier insertion than the classical base-pair

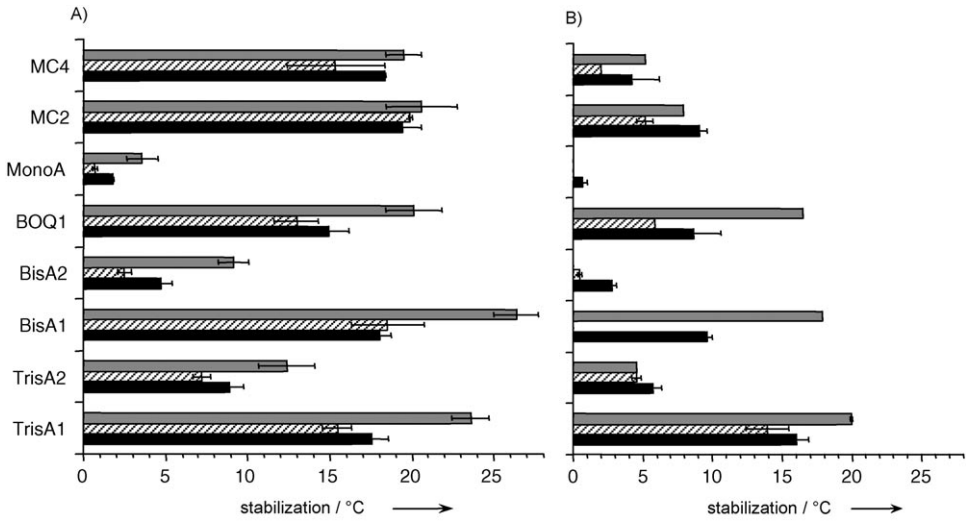

Figure 3. Stabilization of F–$(CCG)_8$–T (black), F–$(CAG)_8$–T (hatched), and F–$(CTG)_7$–T (gray) repeats by different compounds either A) in the absence, or B) in the presence of ds26 (3 μM double-stranded competitor).

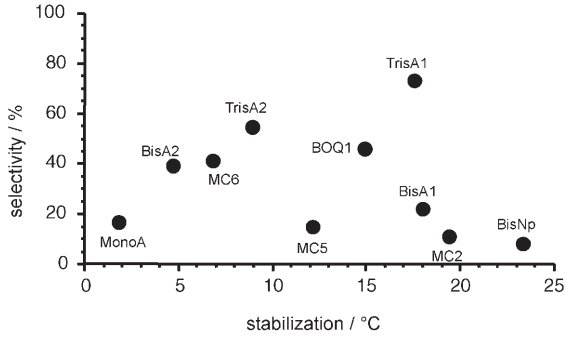

Figure 4. Illustration of the stabilization of the F–$(CTG)_7$–T oligonucleotide vs. selectivity for a selection of compounds. The stabilization induced by each ligand (1 μM) is plotted on the *x* axis, and the *y* axis shows the percentage of stabilization maintained in the presence of ds26 (10 μM). The most desirable compounds are those in the upper right-hand corner (high stabilization and high selectivity).

stacking involved in Watson–Crick B-DNA. Finally TrisA1 is a highly cationic compound (4–5^+ at pH 7.4), and its binding obviously involves strong electrostatic interactions. Although interactions between the positively charged amine groups and the negatively charged backbone of the DNA structure are known to induce unspecific binding and are likely to be responsible for the observed aggregation/precipitation of the complex, the molecule, nevertheless, retains a high specificity for the folded TNR-containing structure.

Conclusions

In conclusion, we have demonstrated that a FRET-based melting assay is applicable for the identification of specific trinucleotide repeat-binding ligands. While it was relatively easy to find ligands that bound to trinucleotide repeats with high affinities, most compounds exhibited limited specificity for the CNG repeat structure relative to duplex DNA. Many compounds were derived from well known DNA binder scaffolds, such as the acridine system. These intercalators generally have relatively strong affinities for duplex DNA, which are well documented in the literature (see, for example, refs. [24–26]). This might explain the relatively disappointing selectivity found for most compounds. The acridine derivative TrisA1 was an interesting exception. It should be noted that most of the selected derivatives have macrocyclic or macrobicyclic scaffolds, which might be key features for limiting bisintercalation into regular B-DNA, and thus for favoring trinucleotide hairpin binding. Our preliminary results suggest that the acridine derivatives might also recognize RNA trinucleotide repeats[27] (Figure S5), and could offer a potential strategy for blocking the translation of these mRNAs into toxic polypeptides. It will be interesting in the near future to compare the effect of this class of ligands with molecules that prefer the duplex B-DNA form over alternate structural forms. This strategy is of special interest in the case of the triplex-prone GAA/CTT repeats found in Friedreich ataxia (reviewed in ref. [28]).

Experimental Section

Oligonucleotides and compounds: All oligonucleotides and their fluorescent conjugates were purchased from Eurogentec (Belgium) and were dissolved in double distilled water (at a concentration of about 300 μM), stored at −20 °C, and used without further purification. Concentrations were determined by UV absorption from extinction coefficients determined according to the nearest-neighbor model.[29]

The synthesis of neomycin-capped macrocycles (MC2, MC4, MC5, MC6),[30] aminoglycoside monomers and dimers (PD1–3, PM1,3, KD1, KM1, TM1, TD1),[31] metaquinacridines (MMQ13–MMQ17),[32,33] and trisacridine (TrisA1)[34] were described previously. Compounds TrisA2, BisA2, and DMA2–4 were synthesized by reductive amination of acridine-7-carbaldehyde with the corresponding diamine by using standard procedures.[32] The resulting neutral aminated dimers were then dissolved in HCl (1 N), followed by subsequent precipitation with ethanol and filtration to yield the final salts.

TrisA2: Brown crystals; ^{1}H NMR (D_2O, DCl): $\delta = 2.95$ (t, $J = 6$ Hz), 3.34 (t, $J = 6$ Hz), 4.35 (s, 6H), 7.52 (t, $J = 7.5$ Hz, 3H), 7.67 (d, $J = 9$ Hz, 3H), 7.83 (t, $J = 7.5$ Hz, 3H), 7.86 (d, $J = 7.5$ Hz, 3H), 7.87 (d, $J = 7.5$ Hz, 3H), 7.95 (d, $J = 9$ Hz, 3H), 8.05 (s, 3H), 8.97 ppm (s, 3H); ^{13}C NMR (D_2O, DCl): $\delta = 45.5$ (CH_2), 49.9 (CH_2), 50.8 (CH_2), 119.0 (CH), 120.4 (CH), 124.9 (C_q), 125.5 (C_q), 128.9 (CH), 129.6 (CH), 131.0 (C_q), 131.8 (CH), 138.7 (2C, CH + C_q), 138.9 (CH), 139.1 (C_q), 148.6 ppm (CH); elemental analysis calcd (%) for $C_{48}H_{51}N_7Cl_6 \cdot 8.3\,H_2O$: C 52.97, H 6.22, N 9.01; found: C 52.92, H 5.63, N 8.90.

BisA2: Yellow amorphous powder; ^{1}H NMR (CD_3OD): $\delta = 3.54–3.66$ (m, 8H), 4.66 (s, 4H), 7.89–7.93 (m, 1H), 8.28–8.36 (m, 4H), 8.42 (d, $J = 8.6$ Hz, 1H), 8.64 (s, 1H), 9.81 ppm (s, 1H); ^{13}C NMR (CD_3OD): $\delta = 45.6$ (CH_2), 45.9 (CH_2), 52.3 (CH_2), 121.8 (CH), 123.1 (CH), 127.3 (C_q), 128.2 (C_q), 130.0 (CH), 131.6 (CH), 132.3 (C_q), 133.3 (CH), 139.2 (CH), 139.7 (CH), 142.1 (C_q), 142.8 (C_q), 150.1 ppm (CH); UV/Vis (buffer, pH 6): λ_{max} (ε, $cm^{-1}M^{-1}$) = 252 (204200), 357 nm (15900); MS (ESI^+) *m/z* 486 $[M+H]^+$; elemental analysis calcd (%) for $C_{32}H_{31}N_5 \cdot 5HCl \cdot 3.3H_2O$: C 52.84, H 5.90, N 9.63; found: C 52.88, H 5.68, N 9.81.

DMA-2: ^{1}H NMR (D_2O, DCl): $\delta = 1.45$ (s, 6H), 1.98–2.05 (d, 4H), 2.95–3.08 (t, 4H), 3.50–3.62 (m, 14H), 4.52 (s, 4H), 7.80–7.88 (m, 2H), 8.12–8.40 (m, 10H), 8.47 (s, 2H), 9.72 ppm (s, 2H); ^{13}C NMR (D_2O, DCl): $\delta = 26.6$ (CH_2), 37.0 (CH), 41.9 (CH_2), 51.3 (CH_2), 52.1 (CH_2), 54.1 (CH_2), 119.5 (CH), 121.0 (CH), 125.9 (C_q), 126.9 (C_q), 128.9 (CH), 130.4 (2 C, CH + C_q), 132.2 (CH), 138.2 (CH), 139.1 (CH), 139.6 (C_q), 140.4 (C_q), 150.2 ppm (CH); UV/Vis (buffer, pH 6): λ_{max} (ε, $cm^{-1}M^{-1}$) = 252 (204400), 357 nm (18000); MS (ESI^+): *m/z* 637.9 $[M+H]^+$; elemental analysis calcd (%) for $C_{42}H_{48}N_6 \cdot 6HCl \cdot 2.9H_2O$: C 55.56, H 6.64, N 9.26; found: C 55.53, H 6.53, N 9.34.

DMA-3: ^{1}H NMR (D_2O, DCl): $\delta = 2.05–2.10$ (m, 4H), 2.94–3.02 (t, 4H), 3.25 (s, 14H), 4.50 (s, 4H), 7.82–7.90 (m, 2H), 8.18–8.42 (m, 10H), 8.50 (s, 2H), 9.80 ppm (s, 2H); ^{13}C NMR: $\delta = 21.2$ (CH_2), 44.7 (CH_2), 49.3 (CH_2), 50.9 (CH_2), 53.9 (CH_2), 119.5 (CH), 120.9 (CH), 125.8 (C_q), 126.8 (C_q), 128.9 (CH), 130.4 (CH), 130.7 (C_q), 132.1 (CH), 138.3 (CH), 139.1 (CH), 139.6 (C_q), 140.3 (C_q), 150.1 ppm (CH); UV/Vis (buffer, pH 6): λ_{max} (ε, $cm^{-1}M^{-1}$) = 251 (222100), 357 nm (17200); MS (ESI^+): *m/z* 583.8 $[M+H]^+$; elemental analysis calcd (%) for $C_{38}H_{42}N_6 \cdot 6HCl \cdot 5.6H_2O$: C 50.58, H 6.61, N 9.31; found: C 50.34, H 6.29, N 9.49.

DMA-4: This compound was not isolated in a pure state but only as a mixture with a monoacridine derivative. MS (ESI^+): *m/z* 665.7 $[M+H]^+$

All compounds (1–10 mM) were dissolved in DMSO or double distilled water. Stock solutions were stored at −20 °C. Further dilutions were made in double distilled water. The formulas of all the studied compounds are shown in Table S1.

Absorbance (UV) melting experiments: The thermal stability of the unlabeled oligonucleotide either alone or in complexation with a ligand was estimated by heating/cooling experiments. UV absorbance was recorded as a function of temperature in a quartz cuvette (1 cm pathlength) at several wavelengths by using Kontron Uvikon 940 spectrophotometer thermostated with an external ThermoNeslab RTE111. The temperature of the bath was typically increased or decreased at a rate of 0.2 °C min^{-1}. Evaporation at high temperatures was reduced by use of a layer of mineral oil, and condensation at low temperatures was minimized with the aid of a dry air flow in the sample compartment. All experiments were carried out in sodium cacodylate buffer (10 mM, pH 7.0) containing KCl (30 mM). UV absorbance denaturation profiles of $(CTG)_8$ (3 μM) alone or in the presence of ligands (15 μM) were recorded at 230 or 275 nm. These wavelengths, at which the variation of the absorbance of the ligands as a function of temperature is negligible, were carefully chosen by using the thermal differential spectrum approach.[35]

Electrospray mass spectrometry (ESI-MS) experiments: The experiments were performed with a QTOF Ultima Global electrospray mass spectrometer (Micromass, now Waters, Manchester, UK). The electrospray capillary voltage was set to −2.2 kV and the cone voltage to 35 V. The RF lens 1 voltage was set to 33 V. Argon pressure inside the collision hexapole (3.0×10^{-5} mbar) and the source pressure (2.70 mbar) were kept constant (±5%). Source block and desolvation temperatures were set to 70 and 100 °C, respectively. Unlabeled $(CNG)_8$ sequences (5 μM) were incubated in the presence of ligand (10 μM) in ammonium acetate (60 mM, pH 7) over 15 min at room temperature. Methanol (15%) was added just before injection. The ESI-MS experiment with TrisA1 could not be performed, due to low solubility and/or high aggregation of the drug-DNA complex.

Fluorescence: FRET melting experiments were carried out as described previously[17,18] with double-labeled DNA trinucleotide repeats of the general formula ^{5}FAM-$(CNG)_{7-8}$-TAMRA^3, where N = A, C, or T. The melting temperatures of $(CGG)_7$ and $(CGG)_8$ oligonucleotides were too high for monitoring ligand-induced stabilization.[5] Seven repeats of the CTG motif were tested, in comparison with eight for CAG, CUG, and CCG. However, no significant difference in melting temperature was found between $(CTG)_7$ and $(CTG)_8$;[6] this explains why we were able to compare the results obtained with a slightly different length. The FAM emission at 516 nm (excitation at 492 nm) of double-labeled sequences (FAM and TAMRA at 5′ and 3′ ends, respectively) in the absence and presence of a single compound was recorded as a function of temperature with a Stratagene Mx3000P thermal block (La Jolla, CA, USA) in 96-well microplates. To investigate the trinucleotide vs. duplex selectivity of each compound, a 26-base self-complementary competitor was added (ds26: ^{5}dCAATCGGATCGAATTCGATCCGATTG^3).[18] The temperature was varied from 25 °C to 95 °C at a rate of 1 °C min^{-1} (the temperature range of the Mx3000P thermal block). The FAM emission vs. temperature plots were normalized with respect to maximum emission at high temperature between 0 and 1, and the temperature of half-dissociation ($T_{1/2}$), which corresponds to an emission value of 0.5, was determined.[18] The (quasi)complete folding of the labeled sequences at 25 °C, under our experimental conditions, was verified by performing FRET melting experiments between 0 °C and 80 °C by using a SPEX Fluorolog instrument (data not shown). These melting experiments were performed in Na cacodylate (10 mM, pH 7), KCl (30 mM). The fluorescent oligonucleotide and the competitor were added at the same time, at concentrations of 0.2 μM and 10 μM, respectively, just before addition of the ligand (1 μM). The mixture was then incubated for 10 min at room temperature before the melting experiment was started.

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Keywords: DNA ligands · DNA recognition · DNA · FRET · nucleic acids

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