Combining G-quadruplex targeting motifs on a single PNA scaffold: A hybrid (3+1) PNA-DNA bimolecular quadruplex

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Abstract: We describe the first G-quadruplex targeting approach that combines an intercalation and a hybridization strategy by investigating the interaction of a G-rich PNA acridone conjugate **1** with a three repeat fragment of the human telomere **G3** to form a hybrid PNA-DNA quadruplex mimicking the biologically relevant (3+1) pure DNA dimeric telomeric

quadruplex. Using a combination of UV, CD and fluorescence spectroscopy as well as mass spectrometry, we show that PNA **1** can induce the formation of a bimolecular hybrid quadruplex even at low salt concentration upon interaction with a single-stranded three repeat fragment of telomeric DNA. However PNA **1** cannot invade a short fragment of B-DNA even if the latter

contains a CCC motif complementary to the PNA sequence. These studies could open up new possibilities for the design of a novel generation of quadruplex ligands that target not only the external features of the quadruplex but also its central core constituted by the tetrads themselves.

Introduction

It has been known for several decades that DNA sequences containing a high density of clustered guanines are able to adopt four-stranded secondary structures named guanine (G)-quadruplexes or tetraplexes in the presence of physiological cations, notably K^+ and Na^+ (Scheme 1).¹ Converging *in silico* and *in vitro* data have revealed the high prevalence of such G-rich DNA sequences throughout the human genome.² Recently there has been considerable focus on quadruplexes, their cellular functions, and their exploitation for biological intervention towards therapeutics. The most widely studied DNA quadruplexes are those derived from telomeric repeat sequences.^{3–6} Biophysical studies on the human telomeric quadruplex have provided valuable insights into its structural and dynamic properties.³ NMR and X-ray crystal structures of the intramolecular quadruplex formed from four human telomeric repeats (GGGTTA) have revealed that it is highly polymorphic.⁴ Several lines of evidence link G-quadruplexes with telomere maintenance⁵ given that telomeric DNA in its quadruplex form is not a competent substrate for telomerase.⁶ Telomerase is crucial for immortality in most human cancers and therefore ligand induced quadruplex stabilisation in telomeric DNA has potential as an anti-cancer therapeutic strategy.⁷ Thus far, strategies for targeting quadruplexes include small molecules⁸, complementary oligonucleotides⁹ or also engineered DNA binding proteins.¹⁰ An original approach was also recently reported that uses an anthracene-diethylene triamine conjugate to

induce intramolecular parallel quadruplex formation from single-stranded DNA via stacking of the anthracene moiety onto the external G-tetrad and invasion of the central ion channel by the triamine side-chain.¹¹ Peptide Nucleic Acid (PNA) is a synthetic mimic of DNA in which the negatively charged phosphate backbone is replaced by a neutral polyamide backbone, thus allowing it to hybridise in a sequence specific manner and with high affinity to either DNA or RNA. Recently, it has been shown that carefully designed G-rich PNA oligomers were also capable of forming PNA₄ quadruplexes¹², hybrid PNA₂-DNA₂¹³, PNA₂-DNA¹⁴ or (PNA-DNA chimeras)₄¹⁵ tetramolecular and trimolecular quadruplexes.

A recent NMR study reported an unprecedented structure for a DNA fragment of the human telomeric region containing three G-rich repeats, d(GGGTTAGGGTTAGGGT) in sodium solution.¹⁶ Here an asymmetric dimeric quadruplex was formed where the G-tetrad core involved all three G-tracts of one of the strands and the 3'-most G-tract of the second strand. The three-repeat sequence could also associate with a solitary G-rich unit d(GGGTTA) to form a bimolecular complex called the (3+1) assembly. We therefore reasoned that this bimolecular (3+1) structure could represent the basis of a promising new design strategy for G-quadruplex stabilising ligands. These ligands could combine on a single scaffold binding elements that target simultaneously the central core of the G-quadruplex structure as well as key external features, i.e, a G-rich PNA strand attached to a quadruplex binding platform. The G-rich PNA region could hybridize with the G-rich DNA clusters to form three hybrid G-tetrads, while the quadruplex binding platform could stack on the resultant terminal G-tetrad of the hybrid quadruplex core. We chose an acridone as our DNA binding platform as it is a well-characterised G-tetrad end-stacker¹⁷ for which high quadruplex vs duplex DNA discrimination capabilities have been demonstrated when appending appropriate side-chains (e.g. 3-pyrrolidine-propionamide). We report here the synthesis of ligand **1** (Scheme 1), a G-rich PNA-acridone conjugate and demonstrate the successful targeting of a three-repeat fragment of human telomeric DNA via the formation of a bimolecular (3+1) hybrid quadruplex.

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Scheme 1. (**Top**) Structure of first generation acridone-PNA conjugate **1**. (**Bottom**) Planar array of four guanines or G-tetrad (left) and schematic representation of a bimolecular (3+1) G-quadruplex structure determined by RMN by Patel *et al.*¹⁶ (right).

Results and Discussion

Design and synthesis: We designed PNA-acridone conjugate **1** such that (i) it incorporated a Lysine for water solubility, (ii) it contained three consecutive guanines that could each be part of a different G-tetrad, and (iii) the acridone is linked to the PNA via a flexible linker and can therefore stack favourably with the top G-tetrad of the hybrid quadruplex once formed. Compound **1** was synthesised on rink amide MBHA resin (Merck Biosciences) using standard Fmoc chemistry (supporting information). The structure of the acridone was based on the bis-aminoalkylamido acridone quadruplex ligands¹⁷ and was attached to the PNA via a glycine linker. The acridone monomer **6** was synthesized in 6 steps that required no purification on silica gel (Scheme 2).

Ullman reaction between commercially available methyl anthranilate and 2-chloro-5-nitrobenzoic acid using a modified version of a previously reported protocol¹⁸ afforded the bisarylamine which was almost quantitatively converted into the corresponding acridone **2** after treatment in polyphosphoric acid and precipitation in water. Reduction of the nitro group with tin(II) chloride under acidic conditions afforded the amino acridone **3** which reacted with 3-chloropropionyl chloride to form the corresponding acridone amide **4**. Nucleophilic addition of pyrrolidine on the aliphatic halide generated the desired 3-pyrrolidine-propionamide substituted acridone methyl ester **5** which was finally hydrolysed under basic conditions to afford the desired acridone carboxylate **6**.

Reagents and conditions: a) Cu, Cu₂O, Cu(OAc)₂, DMF, 160 °C, 1h (**50%**); b) PPA, 130 °C, 45min (**85%**); c) SnCl₂·2H₂O, AcOH, HCl, 15min r.t. then 45min 80 °C (**66%**); d) 3-CPC, 60 °C, 24h (**85%**); e) pyrrolidine, DMF, 60 °C, 30min (**92%**); f) NaOH, H₂O, DMF, 50 °C, 40min (**89%**)

Scheme 2. Synthesis of the acridone carboxylic acid building block **6**.

Characterisation of a DNA₁/PNA₁ quadruplex: Using a combination of UV, CD, fluorescence spectroscopies and mass spectrometry we have established the interactions between a three repeat fragment of human telomeric DNA d(GGGTTAGGGTTAGGG) (**G3**) and the PNA-acridone **1**. At 20 μM strand concentration, **G3** formed a stable quadruplex (*T_m* = 59.5 °C) in potassium phosphate buffer (100 mM, pH 7.4 also containing 100 mM KCl). It also exhibited a CD signature with two maxima at 260 and 295 nm characteristic of a mixture of parallel and antiparallel G-quadruplex conformations respectively. When annealed in the presence of an equimolar amount of PNA **1**, the UV thermal denaturation curve showed a melting transition at *T_m* = 60.5 °C, only one degree higher than for the DNA alone (Table 1). However the CD profile was significantly different with a main maximum at 260 nm and a smaller one at 295 nm (Figure 1), indicative of either (i) a ligand-induced conformational switch of the dimeric DNA quadruplex or (ii) the formation of an alternative hybrid PNA-DNA quadruplex structure accounting for the new CD signature.

Similar CD and UV experiments were carried out but using decreasing concentrations of salt (either in 100 mM potassium phosphate buffer with no added potassium chloride first or in neat water at controlled pH 7.4). As expected for the 20 μM **G3** solution, the *T_m* value decreased significantly when lowering the potassium concentration: from 59.5 °C to 51 °C when removing the additional 100 mM KCl of the initial potassium phosphate buffer, and down to < 10 °C when annealed in neat water (Table 1). A salt dependent *T_m* shift was also observed for the equimolar PNA/DNA mixture from 60.5 °C to 55 °C with no added KCl and down to 23 °C in neat water (Table 1). Interestingly, the difference in *T_m* values between the **G3** alone and **G3** + 1eq PNA **1** gradually increases when decreasing the salt concentration. Cation mediated stabilisation of DNA quadruplexes is mainly via (i) electrostatic screening of the negatively charged DNA strands and (ii) coordination of the eight guanine O6 functional groups between tetrads.¹⁹ In contrary to that, non-covalent PNA complexes were shown to be slightly destabilised by cations²⁰ and the previously reported PNA₄ quadruplex showed comparable stability when annealed either in neat water or in 100 mM sodium phosphate buffer.¹² Herein, we demonstrate that the PNA/DNA complex is stabilised by potassium cations although not as strongly as a pure DNA quadruplex. It is also noteworthy that in

experiments carried out with stoichiometric mixtures of **G3** and acridone **6** lacking the PNA strand, no thermal stabilisation or structural changes of the quadruplex were observed (data not shown). This rules out the possibility of a conformational change of the **G3** dimeric quadruplex induced by the acridone only and reinforces the initial hypothesis of an active participation of the PNA in PNA-DNA hybrid quadruplex formation.

Table 1. Melting temperatures of DNA (20 μ M **G3**) and DNA/PNA (20 μ M **G3** + 20 μ M PNA **1**) in buffers containing different potassium concentrations.

Buffer composition	complex	T_m ($^{\circ}$ C)	ΔT_m ($^{\circ}$ C) [a]
100 mM KH_2PO_4 pH 7.4 + 100 mM KCl	G3	59.5	+ 1
	G3 + PNA 1	60.5	
100 mM KH_2PO_4 pH 7.4	G3	51.0	+ 4
	G3 + PNA 1	55.0	
H_2O pH 7.4	G3	< 10	> + 13
	G3 + PNA 1	23.0	

[a] Difference in T_m values obtained under identical conditions for the solution of (20 μ M **G3** + 20 μ M PNA **1**) and for (20 μ M **G3**).

CD experiments were also carried out on **G3** and **G3/PNA 1** complexes annealed either in potassium phosphate buffer (with or without 100 mM KCl) or in neat water at pH 7.4. The results are summarised in Figure 2. **G3** formed a mixed parallel/antiparallel quadruplex in potassium phosphate buffer but remained unstructured when annealed in neat water, which is consistent with the data existing in the literature.³ When annealed in the presence of an equimolar amount of PNA **1**, **G3** formed a mixed parallel/antiparallel quadruplex in potassium phosphate buffer but also in neat water, which is consistent with the UV melting experiments demonstrating that PNA **1** could induce the formation of a quadruplex even under conditions where the dimeric DNA quadruplex cannot form. Interestingly, (i) the absolute amount of quadruplex formed decreased when decreasing the salt concentration, (ii) the 260/295 CD ratio remains almost constant for either the **G3** or for the **G3/PNA 1** complex whatever the buffer and (iii) the 260/295 ratio remains significantly higher for the PNA/DNA quadruplex than for the pure DNA quadruplex. Taken all together, these CD and UV data confirm that **G3** forms a stable mixed parallel/antiparallel quadruplex structure, the stability of which is highly dependent on salt concentration. When annealed with a stoichiometric amount of PNA **1**, **G3** also folds into a quadruplex but of different conformation (more parallel-like) and the stability of this new quadruplex is significantly less influenced by the salt concentration. This is consistent with the formation of a hybrid PNA/DNA quadruplex with a participation of the PNA guanines into mixed tetrads with the guanines of the **G3** DNA strand. The difference in CD spectra between the pure DNA and hybrid PNA/DNA quadruplex could be explained by an acridone-induced orientation of the PNA strand. Indeed, while in the dimeric DNA quadruplex, the lagging strand that participates in only $\frac{1}{4}$ of each G-tetrad can freely and equally orientate in either way (all three guanines in a syn or anti conformation), it is anticipated that the acridone will preferentially bind to the most polar face of a

quadruplex and by this mean will direct the positioning of the PNA with respect to the DNA strand. Indeed, it was previously reported that as a consequence of the position of the loops, quadruplexes often have one face rich in negative charge potential while the other is more hydrophobic.²¹ Given that the acridone possesses a positively charged side chain it is likely that it will preferentially interact with the most polar face.

Figure 1. CD spectra of (20 μ M **G3**) and (20 μ M **G3** + 20 μ M PNA **1**) solution annealed in either potassium phosphate buffer 100 mM, pH 7.4 containing 100 mM KCl (turquoise and purple), potassium phosphate buffer 100 mM, pH 7.4 (black and red) or neat water pH 7.4 (blue and green).

Figure 2. Nano ESI mass spectrum of an equimolar solution of **G3** DNA and PNA **1** annealed in ammonium acetate buffer.

In order to determine the stoichiometry of this quadruplex, we performed nano-electrospray ionization mass spectrometry (Nano ESI-MS) on a mixture of **G3** DNA and PNA **1**. The nano ESI mass spectrum showed multiple equidistant peaks in the regime m/z 1540-1560 (Figure 2). The peak centred at m/z 1546.2 showed fine structure (Inset) with a constant separation of 0.25 ± 0.01 indicating that the peaks in this regime correspond to a quadruply charged species. The associated molecular weight of this species (6181.5 ± 2.5 Da) corresponds to a bimolecular complex that is composed of one DNA strand and one PNA strand (calc. molecular weight 6181.6 Da). Moreover, the family of peaks in this regime centred at m/z 1550.59 and 1559.59 corresponded to the same complex associated with one NH_4^+ and one K^+ cation respectively. These results confirm the formation of a (3+1) hybrid PNA-DNA quadruplex.

In order to delineate the ability of acridone-bearing PNA **1** to efficiently target such three G repeat sequences, its binding with the former was further investigated. Quadruplex ligands such as quinacridines²² or carbazoles²³ exhibit changes in their fluorescent properties upon quadruplex binding that efficiently report on their DNA binding affinity and/or binding mode. Fluorescently labeled nucleic acid probes that are capable of detecting DNA single-base mismatches were recently reported that exploit the fact that the fluorescence of the intercalator is directly responsive to local perturbations arising upon hybridisation of the probe to the DNA target.²⁴ In these systems, interaction of the fluorophore with G bases or G-C base pairs often result in a significant quenching of fluorescence.

The emission of fluorescence of a solution of PNA **1** was recorded under different conditions to ascertain whether indeed the acridone moiety behaved as a quadruplex binding ligand (Figure 3). When 3 μ M PNA **1** was annealed with 3 μ M **G3** DNA under quadruplex forming conditions, a palpable increase of the fluorescence intensity (c.a. 40%) was observed. This fluorescence enhancement was also accompanied by a moderate red-shift from 431 nm to 436 nm. However, when 3 μ M PNA **1** was incubated with 3 μ M ssDNA (of sequence similar to that of **G3** but carrying three G $\rightarrow$ T mutations to prevent quadruplex formation), it did not lead to any significant changes in the fluorescence properties of the acridone moiety. This result is consistent with a specific interaction of PNA **1** with **G3** that additionally incorporates a stacking mode of the acridone moiety on a quadruplex core. Indeed, while in PNA **1** the fluorescence of the acridone is likely to be quenched by free neighbouring G bases, a structuration of PNA **1** within a quadruplex conformation, and of the three free guanines in G-tetrad formation could prevent such quenching and account for the observed fluorescence exaltation.

Figure 3. (Left) Fluorescence emission spectra ($\lambda_{\text{exc}} = 410$ nm) of 3 μ M PNA (green), 3 μ M PNA + 3 μ M ssDNA (black) and 3 μ M PNA + 3 μ M **G3** DNA (red) in 100 mM potassium phosphate buffer pH 7.4 containing 100 mM KCl. (Right) Plots of F/F_0 vs **G3** DNA concentration for the titration of a 3 μ M solution of PNA **1** in potassium phosphate buffer 100 mM, pH 7.4 containing 100 mM KCl with a solution of annealed **G3** (black squares) or unstructured **G3** (red triangles). Excitation was at 410 nm and emission was recorded at 436 nm.

To further investigate the nature of the binding interaction between PNA **1** and the DNA fragment **G3** we first titrated in a solution of **1** (3 μ M) with increasing amounts of **G3** pre-folded into its quadruplex form as confirmed by CD. This resulted in a gradual decrease in the fluorescence intensity of the acridone moiety. Interestingly, when the same experiment was performed with increasing amounts of **G3** in its unfolded single stranded state it led to a significant increase in the acridone fluorescence (Figure 3). Additionally, when a fixed amount of PNA **1** was annealed with increasing amounts of **G3** DNA an increase in fluorescence intensity was observed (data not shown) similar to that seen for unfolded **G3**

DNA (Figure 3). These results reflect two different binding modes for **1** when interacting with either a preformed quadruplex or a quadruplex forming sequence in its single stranded state. Unlike non-functionalised G-rich PNAs, PNA **1** is not capable of invading the dimeric quadruplex of **G3** once formed within the timescales investigated. However, it is capable of inducing G-rich single-stranded DNA folding into a quadruplex conformation even at room temperature and in neat water, leading to a thermodynamically favoured hybrid structure (Scheme 3).

Scheme 3. Proposed modes of interaction of PNA **1** with **G3** DNA.

In order to demonstrate the influence of the acridone moiety on both the stability and conformation of the complex formed between PNA **1** and **G3**, an analogue of **1** but lacking the acridone platform, PNA **1b** (of general sequence Lys-GGG-NH₂) was synthesised (Figure 4). The ability of compound **1b** to form a stable bimolecular quadruplex upon binding to **G3** DNA was investigated using both UV and CD spectroscopy in either 100 mM potassium phosphate buffer pH 7.4 with no added KCl or in neat water pH 7.4. In 100 mM potassium phosphate buffer a stoichiometric mixture of PNA **1b** and DNA **G3** formed a stable quadruplex (T_m value of 52.5 $^{\circ}$ C), slightly more stable than the **G3** dimeric quadruplex (+ 1.5 $^{\circ}$ C) but less stable than the bimolecular hybrid quadruplex formed from PNA **1** and **G3** (- 2.5 $^{\circ}$ C) under similar conditions. The CD spectrum of this new hybrid **1b**:**G3** quadruplex revealed a mixed parallel/antiparallel conformation with a main maximum at 260 nm (Figure 4) as previously observed with PNA **1**. More striking is the observation that PNA **1b** (lacking the acridone platform) proved unable to form a hybrid PNA:DNA quadruplex in the absence of any salt, unlike what was observed with PNA acridone conjugate **1** (Figure 4). This observation along with the differences in T_m values measured in phosphate buffer demonstrates the influence of the acridone moiety on the stability of the hybrid quadruplex formed. The acridone appears necessary to form a hybrid quadruplex in neat water although it has little effect on the conformation of the hybrid PNA:DNA quadruplex formed.

Figure 4. Structure of PNA **1b** and CD spectra of a stoichiometric mixture of (20 μ M **G3** + 20 μ M PNA **1b**) annealed in either in potassium phosphate buffer 100 mM, pH 7.4 (black) or in water pH 7.4 (red)

Interaction of PNA **1 with duplex DNA:** The ability of the PNA acridone conjugate to stabilise double stranded DNA was also examined using UV and fluorescence spectroscopy. PNA binding to double stranded DNA can happen via different binding modes. For instance, one PNA strand can interact with two complementary DNA strands to form a PNA-DNA triplex or can also lead to duplex invasion with the formation of a PNA-DNA heteroduplex. Such invasion has been demonstrated using a homopurine PNA decamer which forms only a duplex with the complementary oligonucleotides.²⁵ Herein, we investigated the capacity of our PNA **1** to either invade or associate with two B-DNA undecamers, containing either a CCC motif complementary to the GGG PNA sequence (duplex B) or no sequence complementarity with PNA **1** (duplex A).

First, UV thermal denaturation experiments were carried out on duplexes A and B alone and with a stoichiometric amount of PNA **1**. Melting temperatures (T_m) of 53 °C were obtained for both duplexes A and B. Interestingly, no T_m shift was observed when incubating either duplex A or B with PNA **1** suggesting that our GGG PNA acridone conjugate cannot invade and stabilise B-DNA even if the latter contains a CCC complementary sequence (Figure 5). In order to further investigate the specificity of PNA **1** for quadruplex versus double-stranded DNA, additional UV thermal denaturation studies were carried out under low salt conditions (i.e. 10 mM potassium phosphate buffer pH 7.4, no added salt). Low salt conditions were previously reported to favour PNA binding to double helix DNA.^[26] Under those conditions duplex B proved moderately stable (T_m value of 36 °C). When incubated with a stoichiometric amount of PNA **1**, it showed a similar melting temperature of 36 °C indicative again of an absence of PNA induced B-DNA stabilisation. It is also noteworthy (i) that most PNAs commonly designed to invade duplex DNA and therefore interfere with DNA transcription are significantly longer than PNA **1** and (ii) that hybridisation of **1** with a single strand of DNA containing a complementary CCC motif (i.e. ACGCCCTAAGC) did not lead to the formation of stable hybrid PNA:DNA duplexes or triplexes.

When titrating in a solution of **1** (3 μ M) with increasing amounts of B-DNA, a decrease in the fluorescence intensity of the acridone was observed which is comparable to that observed for the titration with a solution of folded **G3** quadruplex (Figure 5).

This is consistent with our previous model that non specific interaction of PNA **1** with either pre-folded quadruplex or duplex DNA results in a fluorescence quenching while only the specific involvement of the PNA guanines in G-tetrad formation leads to a significant fluorescence enhancement.

Figure 5. **(Left)** UV thermal denaturation curve (heating curve) of a 4 μ M duplex A (black), 4 μ M duplex A + 4 μ M PNA **1** (red), 4 μ M duplex B (green) and 4 μ M duplex B + 4 μ M PNA **1** (blue). Buffer was potassium phosphate buffer 100 mM pH 7.4 also containing 100 mM KCl. **(Right)** Plots of F/F_0 vs B-DNA concentration for the titration of a 3 μ M solution of PNA **1** in potassium phosphate buffer 100 mM, pH 7.4 containing 100 mM KCl with a solution of either non complementary duplex A (black squares) or complementary duplex B (red triangles). Excitation was at 410 nm and emission was recorded at 436 nm.

Conclusion

We report a novel approach to targeting the human telomeric DNA sequence that combines within a single scaffold a hybridisation and an intercalation motif. We have shown that this strategy is successful and have characterised the resultant (3+1) hybrid PNA₁:DNA₁ quadruplex, which is the first of a new genre of hybrid quadruplexes that also mimics one of the biologically relevant structures of the human telomeric quadruplex. The hybrid PNA-DNA bimolecular quadruplex showed increased stability at low salt concentration compared to the corresponding DNA₂ dimeric quadruplex. These studies open up new possibilities for the next generation of quadruplex ligands that target not only the external features of the quadruplex but also its central core constituted by the tetrads themselves. The absence of PNA-induced B-DNA stabilisation also reinforces the potential of such conjugates as quadruplex specific ligands. However, because of the inability for PNA **1** to invade dimeric quadruplex via displacement of one DNA strand, PNAs with increased affinity will be necessary before such quadruplex targeting strategy can be used *in vivo*. The design of second generation ligands containing two quadruplex binding platforms at both ends of the PNA are currently being developed in our group that could significantly improve the stability of the hybrid quadruplex. Considering the increasing interest for DNA quadruplexes as either therapeutic targets in biology or supramolecular objects in nanosciences, ligands that can target DNA sequences containing only three clusters of guanines, even under conditions where DNA quadruplexes cannot easily form (low salt concentration), could be highly valuable.

Experimental Section

¹H and ¹³C NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer at 400 and 100.6 MHz, respectively. Chemical shifts are reported as δ values (ppm) with reference to the residual solvent peaks. All reagents and solvents were obtained from commercial sources and used without further purification. Purification of PNA acridone

conjugate **1** was performed using a Gilson HPLC using a reverse-phase C₁₈ column with gradients combining buffer A and B. Buffer A = (H₂O + 0.1 % TFA); Buffer B = (MeCN + 0.1 % TFA).

2-[[2-(methoxycarbonyl)phenyl]amino]-para-nitrobenzoic acid¹⁷: A mixture of 2-chloro-para-nitrobenzoic acid (5 g, 25 mmol), methyl anthranilate (5 g, 33 mmol), Cu powder (190mg, 3 mmol), Cu₂O (140mg, 1 mmol), Cu(OAc)₂ (360mg, 2 mmol) is stirred at 160 °C in DMF (120 mL) for 3 hours and then allowed to slowly reach room temperature. After filtration through a thin layer of silica between two layers of celite, addition of aqueous 0.1M HCl (120 mL) and vigorous stirring leads to the formation of an orange precipitate. The desired product is obtained as an orange solid (4g) by filtration and is used for the subsequent step without further purification. Yield = 50%.

¹H-NMR (DMSO, 400 MHz): 8.15 (2H, br m), 7.98 (1H, d, J = 7.6 Hz), 7.67 (1H, d, J = 7.8 Hz), 7.66 (1H, s), 7.60 (1H, t, J = 7.8 Hz), 7.17 (1H, t, J = 7.6 Hz), 3.88 (3H, s).

Methyl-3-nitro-acridone-5-carboxylate (**2**)¹⁷: A mixture of 2-[[2-(methoxycarbonyl)phenyl]amino]-p-nitrobenzoic acid (1g, 3.2 mmol) in polyphosphoric acid (10 g) is heated at 130 °C for 45 min. After cooling to room temperature, water is added that leads to the formation of a bright yellow solid. The solid is finally filtered off, washed with water and dried under vacuum. The acridone is obtained pure as a yellow solid (0.8g). Yield = 85%.

¹H-NMR (DMSO, 400 MHz): 9.02 (1H, s), 8.55 (1H, d, J = 7.2 Hz), 8.47 (1H, d, J = 7.2 Hz), 8.41 (1H, d, J = 8.4 Hz), 8.02 (1H, d, J = 8.4 Hz), 7.44 (1H, t, J = 7.2 Hz), 4.03 (3H, s).

Methyl-3-amino-acridone-5-carboxylate (**3**)¹⁷: A mixture of compound **2** (1g, 3.4 mmol), tin(II) chloride (3g) and concentrated HCl (5 mL) is stirred vigorously for 20 min in 20 mL glacial AcOH. The mixture is heated at 80°C for 40 min then cooled to room temperature before water (80 mL) is added to allow the amino acridone to precipitate. After filtration and subsequent washes with AcOEt and diethyl ether, the desired product is obtained pure as a yellow powder (0.6 g). Yield = 66%.

¹H-NMR (DMSO, 400 MHz): 8.46 (1H, dd, 2J = 8 Hz, 3J = 1.8 Hz), 8.33 (1H, dd, 2J = 8 Hz, 3J = 1.8 Hz), 7.93 (1H, d, J = 8 Hz), 7.28 (1H, t, J = 8 Hz), 6.62 (1H, dd, 2J = 8 Hz, 3J = 1.8 Hz), 6.54 (1H, d, J = 1.8 Hz), 3.98 (3H, s); HRMS: *m/z*: calcd for C₁₅H₁₃N₂O₃⁺: 269.0921; found: 269.0914.

Methyl-3-(3-chloropropionamide)-acridone-5-carboxylate (**4**): A suspension of **3** (0.5g, 1.9 mmol) in 3-chloropropionyl chloride (20 mL) is heated at 60°C for 24h. Product precipitates at room temperature by addition of diethylether and is then filtered off. The resulting dark yellow powder is then washed with AcOEt, water and diethyl ether, thus affording the desired product pure as a pale brown solid (0.6 g). Yield = 85%.

¹H-NMR (DMSO, 400 MHz): 7.61 (1H, d, J = 8.4 Hz), 7.47 (1H, d, J = 8 Hz), 7.32 (3H, s), 7.26 (1H, d, J = 8.4 Hz), 6.44 (1H, t, J = 8 Hz), 6.40 (1H, d, J = 8 Hz), 3.08 (3H, s), 3.02 (2H, t, J = 6 Hz), 2.03 (2H, t, J = 6 Hz); ¹³C-NMR (DMSO, 100 MHz): 176, 169.5, 168, 144.5, 141.4, 141.3, 136.5, 133, 127.5, 122, 120.5, 117, 115.2, 114.8, 106.4, 53.2, 41, 24; HRMS: *m/z*: calcd for C₁₈H₁₅ClLiN₂O₄⁺: 365.0875; found: 365.0861.

Methyl-3-(3-(pyrrolidin-1-yl)propanamido)-acridone-5-carboxylate (**5**): To a solution of **4** (0.5g, 1.4 mmol) in 2.5 mL DMF is added pyrrolidine (500μL, 5.6 mmol). The solution is then stirred at 60°C for 30 min until the reaction is complete. After cooling to room temperature, diethyl ether is added resulting in the precipitation of a yellow solid. After recrystallisation in diethyl ether, the desired product is obtained pure as a yellow solid (0.5 g). Yield = 92%.

¹H-NMR (DMSO, 400 MHz): 8.54 (1H, d, J = 7.8 Hz), 8.42 (1H, d, J = 7.8 Hz), 8.20 (1H, s), 8.20 (1H, d, J = 7.8Hz), 7.39 (1H, t, J = 7.8 Hz), 7.38 (1H, d, J = 7.8 Hz), 4.00 (3H, s), 3.47 (2H, t, J = 6 Hz), 1.85 (2H, t, J = 6 Hz), 3.10 (4H, br m), 1.97 (4H, br m); ¹³C-NMR (DMSO, 100 MHz): 176, 169.5, 168, 144.5, 141.4, 141.3, 136.5, 133, 127.5, 122, 120.5, 117, 115.2, 114.8, 106, (53.5, 2C), 53.2, 50, 33, (23, 2C); HRMS: *m/z*: calcd for C₂₂H₂₄N₃O₄⁺: 394.1761; found: 394.1789.

Sodium-3-(3-(pyrrolidin-1-yl)propanamido)-acridone-5-carboxylate (**6**): To a solution of **5** (0.25g, 0.64 mmol) in DMF (10 mL) is added a 1M NaOH aqueous solution (0.96 mL, 1eq) and the reaction mixture is stirred at 50°C for 40 min. After evaporation of water, diethyl ether is added and the resulting precipitate filtrated off and triturated with diethyl ether until a yellow solid is obtained. The solid is finally washed with AcOEt and diethyl ether, thus leading to the desired carboxylate acridone pure as a light yellow solid (0.23g). Yield = 89%.

¹H-NMR (D₂O, 400 MHz): 7.88 (1H, d, J = 7.2 Hz), 7.72 (1H, d, J = 7.2 Hz), 7.18 (1H, d, J = 8.8 Hz), 6.85 (1H, t, J = 7.2 Hz), 6.51 (3H, s), 6.17 (1H, d, J = 8.8 Hz), 2.64 (2H, t, J = 7.2 Hz), 2.55 (4H, br m), 2.20 (2H, t, J = 7.2 Hz), 1.73 (4H, br m); ¹³C-NMR (D₂O,

100 MHz): 177, 173, 171, 141.5, 140, 139, 136, 129, 126, 120.5, 119.9, 119.8, 115, 113.5, 104, (53.5, 2C), 50, 35, (23, 2C); HRMS: *m/z*: calcd for C₂₁H₂₂N₃O₄⁺: 380.1605; found: 380.1616.

MALDI-TOF Spectra of PNA 1, 1b and 1c

PNAs **1** and **1b** were synthesised on solid support using the Fmoc chemistry. The PNA was cleaved from the resin with a 95% TFA solution and purified by HPLC. The final compounds were characterised by HR-MS (MALDI-TOF).

PNA **1** [M+H]⁺ = 1437.675 (Calc mass for C₆₂H₇₇N₂₈O₁₄: 1437.617)

PNA **1b** [M+H]⁺ = 1019.542 (Calc mass for C₃₉H₅₅N₂₄O₁₀: 1019.446)

DNA oligonucleotide preparation. All oligonucleotides were purchased from Sigma Genosis. All concentrations were expressed in strand molarity with a nearest-neighbour approximation for the absorption concentrations of the unfolded species. The DNA and hybrid PNA: DNA quadruplexes were prepared in buffer 100 mM potassium phosphate buffer at pH 7.4 also containing 100 mM KCl. After heating to 95 °C for 5 min the solution was slowly cooled for 6 h to room temperature and the oligonucleotide solutions were finally stored at 4 °C.

Oligonucleotide sequences: **G3** d(GGGTTAGGGTTAGGG); **ssDNA** d(GTGTAGTGTAGTG); **duplex A** d(GCATAGTGGCT) hybridised with its complementary sequence; **duplex B** d(GCTTAGGGCGT) hybridised with its complementary sequence.

Fluorescence Spectroscopy. Fluorescence emission spectra were recorded in quartz cells at 20 °C on a Jobin Yvon Fluorolog 3.22 instrument. The excitation and emission bandwidths were fixed to 5 and 5 nm respectively. Fluorescence titration experiments were carried out using a 500 μL quartz cuvette containing a solution of 3 μM PNA-acridone conjugate **1** in 100 mM potassium phosphate buffer pH 7.4 containing 100 mM KCl. Concentrated DNA aliquots (from a 150 μM stock solution) were directly added to the PNA solution. The spectra were recorded between 420 and 520 nm while exciting at 410 nm.

UV Spectroscopy. Absorption spectra were recorded on a Uvicon XL spectrophotometer. Quadruplex and duplex DNA melting curves were recorded at a 295-nm and 260-nm wavelength respectively between 15°C and 90°C at 0.5°C min⁻¹. For quadruplex melting, the DNA (and PNA when appropriate) concentration was 20 μM. For duplex melting, the DNA (and PNA when appropriate) concentration was 4 μM.

Circular Dichroism. Circular dichroism spectra were recorded on a Jobin-Yvon spectrophotometer in a 2 mm pathlength cuvette (300 μL). Spectra were recorded at 25°C from 320 to 220 nm and are presented at an average of three successive scans. Finally, all the spectra were subtracted from a baseline corresponding to the buffer alone (100 mM potassium phosphate buffer containing 100 mM KCl) when appropriate.

Nano-electrospray ionization mass spectrometry (Nano ESI-MS). All mass spectrometry measurements were performed on a Micromass ESI-MS Q-TOF Ultima Mass Spectrometer (Manchester, UK) with micro-channel plate detector. Non-covalent nano ESI-MS spectra was collected in positive ion mode for the PNA-DNA complex and in the negative ion mode for the sample containing DNA alone. A 1:1 PNA/DNA solution at 1 mM strand concentration, prepared in 150 mM ammonium acetate buffer (pH 7) was heated to 95°C, annealed to room temperature over 3h and equilibrated at 4°C overnight. A 500 μM solution containing DNA was also prepared similarly. Just prior to injection the equilibrated DNA/ PNA complex was diluted 5-fold with 1:4 isopropanol/water mixture. The DNA solution was similarly diluted 5-fold with 1:4 methanol/water mixture. Source temperature of 20°C, capillary voltage of 2 kV for the PNA/DNA complex, -2.23 kV for the DNA complex and a cone voltage of 70 V were the source parameters employed during acquisition of mass spectra.

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