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Perylene side chains modulate G-quadruplex conformation in biologically relevant DNA sequences

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ABSTRACT

The stabilisation of different G-quadruplex intra- and intermolecular structures by a number of perylene derivatives characterised by side chains ending with linear or cyclic amines was investigated by electrophoretic (EMSA) and spectroscopic (CD) techniques. The G-rich sequences included the biologically relevant human telomeric TTAGGG runs and the NHE region of the *c-myc* oncogene.

The test compounds could be subdivided into two families: derivatives carrying a cyclic amine in the side chains, which show a reduced binding to the G-quadruplex form, and linear amine congeners, exhibiting enhanced affinity. The latter efficiently induce pairing of multiple DNA chains, while the former are not able to overcome the original folding of the nucleic acid sequence which is preserved in the complex. Remarkably, addition of the perylenes to G-rich sequences paired in a double helical form results in G-quadruplex induction by weak binders only. This is likely related to the ability of strong G-quadruplex binders, but not of weak G-quadruplex binders, to efficiently intercalate into the double-stranded arrangement, which becomes stabilised and is not prone to undergo denaturation and subsequent G-quadruplex folding essentially for kinetic reasons. Hence, two apparently conflicting requirements emerge from this work. In fact, linear alkylamino terminals in the perylene side chains are capable of strong and selective G-quadruplex recognition, but only cyclic amine end groups favour duplex–quadruplex transitions that are likely crucial to produce biological and pharmacological effects in living systems.

1. Introduction

The search for small molecules able to stabilize DNA G-quadruplex structures has recently raised large attention for potential applications in anticancer chemotherapy.[1] The terminal regions of chromosomes, the telomeres, represent their main target. These regions are formed by repeated sequences maintained by a reverse transcriptase called telomerase. When this enzyme is turned off, like in somatic cells, only a limited number of cell divisions can occur before cells enter senescence and, finally, die. On turn, when telomerase is active this check point can be overcome and proliferation can continue indefinitely. Interestingly, telomerase is activated in almost 90% of cancer cells, thus rendering them immortal.[2]

The telomeric sequences, being guanine rich, can assume different G-quadruplex folded arrangements depending upon experimental conditions such as strand length and concentration, metal ions and temperature.[3] G-quadruplex structures can coexist in solution in equilibrium with the linear form. Compounds able to bind efficiently a G-quadruplex form can shift this equilibrium toward folded telomeric structures.[4] The latter arrangements prevent telomerase from binding to and processing them, hence suppressing cancer cell immortality. Additionally, these altered structural equilibria can cause telomere replication inhibition as well as telomere dysfunction leading to short-term responses.[5]

Furthermore, several other genomic sites may adopt a G-quadruplex folding.[6] These sequences are often located in close proximity to promoter regions where they may work as topological switches involved in transcription regulation. An interesting example is the NHE region of *c-myc* for which a role of the G-quadruplex form in the regulation of the expression of the oncogene emerged also in vivo.[7,8] Obviously, the pharmacological outcome resulting from the stabilization of G-quadruplex in different genomic regions will be distinct.

All G-quadruplex structures present overlapping planar arrays of four guanines in which each guanine pairs with two neighbours by Hoogsteen bonding (G-tetrads)[9]; most of the small molecules able to stabilize these nucleic acid arrangement possess an extended aromatic system that allows them to stack

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onto the G-tetrads. In addition, these ligands generally have positively charged side chain/s which are supposed to further stabilize the complex by interacting with the loops or the grooves.[10] The structure of these regions is dramatically affected by the G-quadruplex arrangement assumed by the polynucleotide chain.

Perylenes are a class of well known G-quadruplex binders.[11–13] The biophysical properties of the leading compound PIPER, as well as of a large number of its derivatives, have been extensively analyzed in terms of protonation degree, aggregation and DNA binding.[14–16] It was suggested that aggregation is a key feature in defining G-quadruplex binding efficiency although no clear cut correlations have been drawn yet.[17] In a previous work, we reported the synthesis and characterization of a group of compounds structurally related to PIPER.[18] We observed that, besides the presence of a fused aromatic ring system, the introduction of a cyclic amine in the side chains clearly favours selectivity for telomerase versus TAQ polymerase inhibition. Since this selective effect did not appear to be related to enhanced binding to telomeric G-quadruplex, we proposed that the presence of a pyrrolidine- or piperidine-containing side chain would rather reduce the affinity for other nucleic acid conformations, than improving G-quadruplex binding. This conclusion was further supported by the work of other groups.[16,19,20] Additionally, it emerges that modest changes in perylene side chains can not only alter the balance between ds-DNA versus G-quadruplex recognition but can promote selectivity among different G-quadruplex structures (dimeric vs tetrameric arrangements).[21–23] In particular, a correlation between pKa of nitrogens in the side chains and aggregation degree is not sufficient to illustrate the different possible docking processes in the G-quadruplex grooves. In fact, more information concerning the thermodynamically and kinetically driven processes occurring during selective DNA recognition is required.

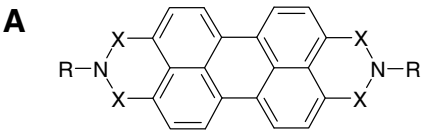
To better dissect this issue, we now performed a detailed biophysical investigation examining the binding of substituted perylene derivatives (bearing cyclic as well as linear terminal amines in the side chains) to different possible G-quadruplex arrangements (structures in Fig. 1) using different incubation conditions in terms of time and temperature. In addition, we considered an above mentioned G-rich nuclease-hypersensitive element (NHE) regulating c-*myc* transcription.[8,24] In fact, this sequence represents a pharmacologically relevant target and exhibits different folding features with reference to the telomeric structure(s). We will show that selected perylenes may favour G-quadruplex switch between distinct folding modes. Also, the perylene-induced transition from double helical to G-quadruplex arrangement can be modulated by the nature of the perylene side chains.

2. Results

2.1. Perylenes mediate induction of different G-quadruplex structures

G-quadruplex formation induced by tested perylenes was evaluated by electrophoretic mobility shift assays (EMSA) using different sequences related to the human telomere and to the c-myc promoter (pu27). While pu27 and 4GGG are intramolecularly folded, 1GGG and 2GGG, which contain only 1 or 2 telomeric repeats, give rise to G-quadruplex structures involving preferentially four or two distinct DNA strands, respectively. These forms are characterized by different electrophoretic mobilities and, thus, are easily resolvable by polyacrylamide gel techniques. Examples are reported in Figure 2. As expected, in 100 mM K^+ 1GGG and 2GGG are largely present as linear monomers, whereas 4GGG and pu27 exhibit high electrophoretic mobility forms that can be safely attributed to intramolecular G-quadruplex arrangements (labelled M1).[11] In the presence of Li^+, a metal ion unable to stabilize G-quadruplex structures (Fig. S1 in supplementary material), the electrophoretic mobility of 4GGG is reduced, consistent with the presence of the linear monomer (M0), whereas pu27 still retains (at least partly) its folded conformation.

As summarized in Figure 2, the tetrameric (T) form of 1GGG is induced by addition of test ligands. In the presence of increasing concentrations of **K20**, 2GGG was initially converted into a dimeric form (D) and then transformed into the T form. It should, however, be underlined that addition of all other test perylenes to 2GGG led to formation of the T form only in the examined concentration range. The amounts of perylenes required to produce 50% folding



X = C=O	R = $(CH_2)_2N(CH_3)_2$	**K5**
	R = $(CH_2)_2$(1-piperidino)	**PIPER**
	R = $(CH_2)_2NH(CH_2)_2OH$	**K8**
	R = $(CH_2)_2N(CH_3)_3$	**K20**
X = CH_2	R = $(CH_2)_2$(1-piperidino)	**K19**

B

1GGG	5' TACAGATAGTTA**GGG**TTAGACTTA 3';
2GGG	5' TACAGATAGTTA**GGG**TTA**GGG**TTA 3'
4GGG	5' A**GGG**TTA**GGG**TTA**GGG**TTA**GGG** 3'
4GGGd	3' TCCCAATCCCAATCCCAATCCC 5'
4GGG-tail	5' TACAGATAGTTA**GGG**TTA**GGG**TTA**GGG**TTA**GGG** 3'
4GGG-tail-d	3' ATGTCTATCAATCCCAATCCC 5'
pu27	5' T**GGGG**A**GGG**T**GGGG**A**GGG**T**GGGG**AA**GG** 3'
pu27-d	3' ACCCCTCCCACCCCTCCCACCCCTTCC 5'

Figure 1. Chemical structure of tested compounds (A) and DNA sequences (B) used in this work.

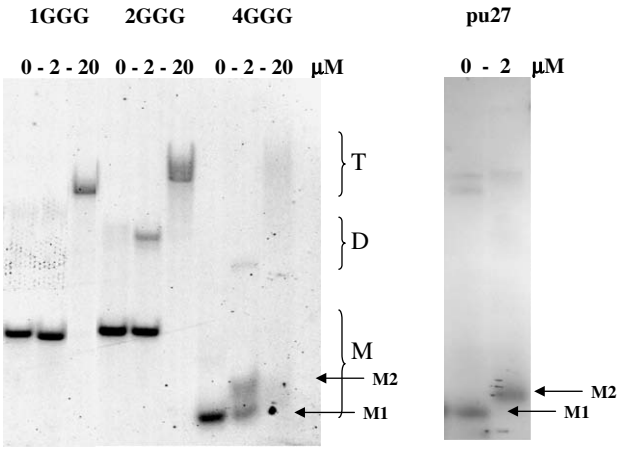


Figure 2. Effect of increasing concentrations of derivative **K20** on the assembly of G-quadruplex structures by tested oligonucleotides (1 μM, strand concentration) in 10 mM Tris, 1 mM EDTA, 100 mM KCl at pH 8.0. Drug–DNA mixtures were incubated 30 min at 25 °C and then loaded on a 16% native polyacrylamide gel in TBE 0.5× containing 20 mM KCl. M = monomeric forms: M1 refers to the intramolecularly folded G-quadruplex structure, M2 to the intramolecular G-quadruplex–perylene bound form; D = dimeric forms; T = tetrameric forms.

Table 1
Induction of G-quadruplex structures by tested perylenes in 10 mM Tris, 100 mM KCl, pH 8.0, after 30 min incubation at 25 °C

Perylene	EC_{50} (μM)	
	2GGG	1GGG
K5	4.0 ± 1.2	8.7 ± 0.9
PIPER	90 ± 10	>100
K8	3.8 ± 0.6	11.0 ± 1.3
K20	12.0 ± 2.2	10.8 ± 1.0
K19	20 ± 2.8	25.0 ± 1.5

EC_{50} values refer to the drug concentration required to induce 50% folding in 1GGG or 2GGG.

of 1GGG or 2GGG are reported in Table 1. In particular, a reduced efficiency of derivatives **PIPER** and **K19** in stimulating G-quadruplex formation emerges.

Upon addition of perylene to 4GGG or pu27 a monomeric G-quadruplex–ligand complex is observed (labelled M2 in Fig. 2). By increasing the perylene/DNA ratio, other species with electrophoretic mobility corresponding to dimeric and tetrameric forms can be detected. These complexes are difficult to monitor using pu27 due to complex precipitation events.

Experiments performed with all tested sequences in the presence of Li^+ showed no substantial differences in the amount of the drug–DNA complex although multimeric forms are largely favoured (Fig. S1 in the supplementary material). This suggests that the extent of G-quadruplex structure formed in the presence of each of the perylenes is poorly affected by the presence of K^+ or Li^+.

It is known that different G-quadruplex conformations are characterized by distinct thermodynamic properties and folding/unfolding kinetics.[11] These parameters can be modulated by drug binding to different extents. Thus, we monitored whether incubation conditions in the presence/absence of perylenes can induce stabilization of otherwise unfavoured folded structures.

In the presence of 1GGG, the time or the temperature of incubation did not affect the structural properties of perylene–DNA complexes (Fig. S2 in the supplementary material). This is in line with the fact that, using this sequence, G-quadruplex can be generated only by assembling four individual strands.

Also, the perylene–2GGG complexes, which are mainly tetrameric, did not show detectable changes upon increasing incubation time when temperature was kept at 25 °C. The only exception is represented by derivative **K20**, for which a long incubation time at room temperature is sufficient to allow formation of dimeric G-quadruplex arrangements at low drug/DNA ratio. However, if the perylenes are added during the DNA annealing step (95 °C for 5 min), dimeric forms appear to be preferentially stabilised. An example is reported in Figure 3. Notably, the total amount of folded structure (dimers plus tetramers) is not affected by incubation conditions. This agrees with previous results suggesting that binding of PIPER to the tetrameric form of a two human repeats sequence is kinetically driven, whereas binding to the dimeric form is thermodynamically favoured.[11]

When the test sequence was 4GGG, the intramolecular G-quadruplex was efficiently converted into a tetrameric DNA complex when **K5** or **K8** were present in the annealing step. Using comparable conditions no such a change was observed with **PIPER** or **K19** (Fig. 4).

The pu27–perylene complexes were not modified by incubation at high temperature (Fig. S3 in the supplementary material).

2.2. Conversion of ds-DNA into G-quadruplex does not parallel G-quadruplex affinity

At physiological conditions, both the telomeric region and the *c-myc* promoter are (at least partly) coupled to their complementary strands. Thus, the ligand-induced conversion of preformed double-stranded pu27 and 4GGG sequences into G-quadruplexes was evaluated by electrophoretic mobility gel shift assays. In agreement with literature data, we observed that PIPER was very efficient in inducing G-quadruplex folding from the duplex form of pu27.[25] Unexpectedly, derivative **K5** was completely ineffective (Fig. 5). Only by increasing incubation temperature up to 95 °C it was able to promote intramolecular folding of the G-rich strand.

Using a double-stranded 4GGG telomeric sequence, all tested perylenes failed to promote G-quadruplex formation. To render the G-quadruplex nucleation step easier, we prepared a construct containing the 4GGG sequence only 50% (from the 5′-end) paired to its complementary sequence. Indeed, using this substrate, **PIPER** was able to induce formation of the intramolecular G-quadruplex (Fig. 5). Interestingly, **K5** that proved to be a very strong G-quadruplex binder to the telomeric sequences, was again much less efficient in inducing the displacement of the C-rich strand and favoured preferentially multimeric structures (data not shown).

2.3. Perylene binding to G-quadruplex is modulated by DNA architecture

To analyze the DNA folding of the G-quadruplex complexes in deeper detail, we used circular dichroism spectroscopy.[26] Addition of the optically inactive perylenes to DNA can lead to generation of induced CD signal deriving from the drug when bound to DNA. However, we monitored the optical properties of DNA in the range 250–320 nm where (free and bound) perylene absorption is almost negligible. In addition, all test compounds showed superimposable UV absorption properties. Hence, we do not expect appreciable induced CD contribution in this region. In any case, the dichroic data were not used for a quantitative analysis of the DNA–perylene binding process.

The spectral features of the tested G-rich DNA sequences are summarized in Figure 6.

1GGG and 2GGG in 100 mM KCl showed low optical activity. In agreement with the PAGE results, this confirms that in these experimental conditions the oligos are essentially unfolded.

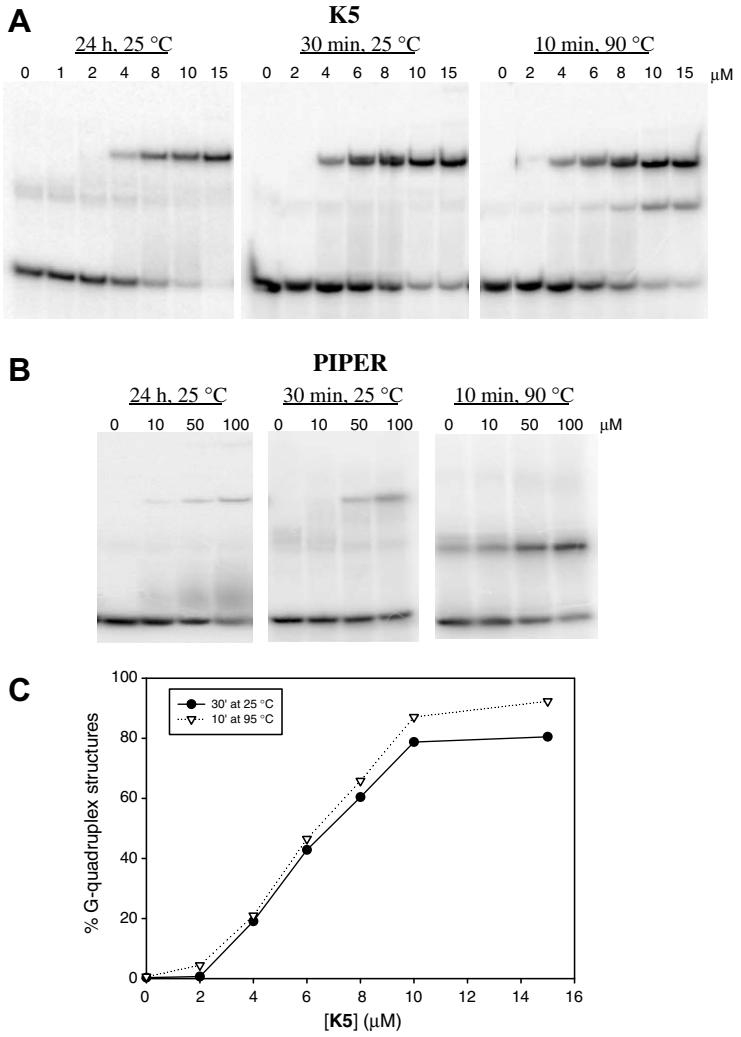


Figure 3. Effect of increasing perylenes concentrations on the assembly of 2GGG (1 μM, strand concentration) into G-quadruplex structures in 10 mM Tris, 1 mM EDTA, 100 mM KCl at pH 8.0, at the reported incubation conditions. Reaction products were resolved on a 16% native polyacrylamide gel in TBE 0.5× containing 20 mM KCl. (A) refers to **K5**, (B) to **PIPER**, in (C) the total amount of G-quadruplex structures (dimer + tetramer) induced by increasing concentrations of **K5** using two different incubation conditions is reported.

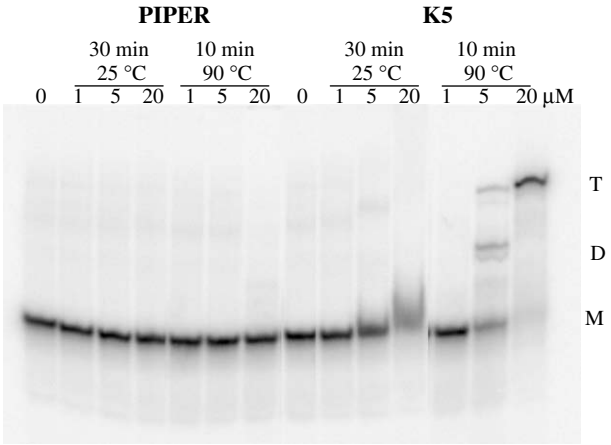


Figure 4. Electrophoretic gel shift assay showing the effect of increasing concentration of **PIPER** and **K5** on the assembly of 4GGG (1 μM, strand concentration) into G-quadruplex structures in 10 mM Tris, 1 mM EDTA, 100 mM KCl at pH 8.0, at the given incubation conditions. Reaction products were resolved on a 16% native polyacrylamide gel in TBE 0.5× containing 20 mM KCl.

4GGG is known to form a mixture of G-quadruplex structures in the presence of K^+, among which a hybrid-type intramolecular (mixed parallel/antiparallel) is most relevant.[27] This arrangement is characterised by a strong positive dichroic peak at around 290 nm with a shoulder at 265 nm and by a weak negative peak at 235 nm.

Addition of tested perylenes to these sequences induces an increase in the optical activity depending upon the nature and concentration of test perylenes. The relative increments in molar ellipticity recorded at the wavelength of maximal response are reported in Table 2. In particular with 1GGG and 2GGG intense peaks located near 265 and 245 nm were observed, which suggests a preferential G-quadruplex parallel arrangement of the bound DNA (Fig. 6).[28] Using 4GGG an increase in the optical activity was recorded at 290–295 nm (Table 2) supporting a preferential antiparallel folding.

Monitoring CD intensity variations, the test compounds appear to fall into two distinct classes. In fact, derivatives having a piperidine residue in the side chain (**PIPER** and **K19**), produce modest variations in the dichroic signal, whereas **K5, K8** and **K20** cause remarkable increments in molar ellipticity.

The *c-myc* promoter sequence in 100 mM K^+ showed a very intense dichroic signal corresponding to a parallel folding (Fig. 6).

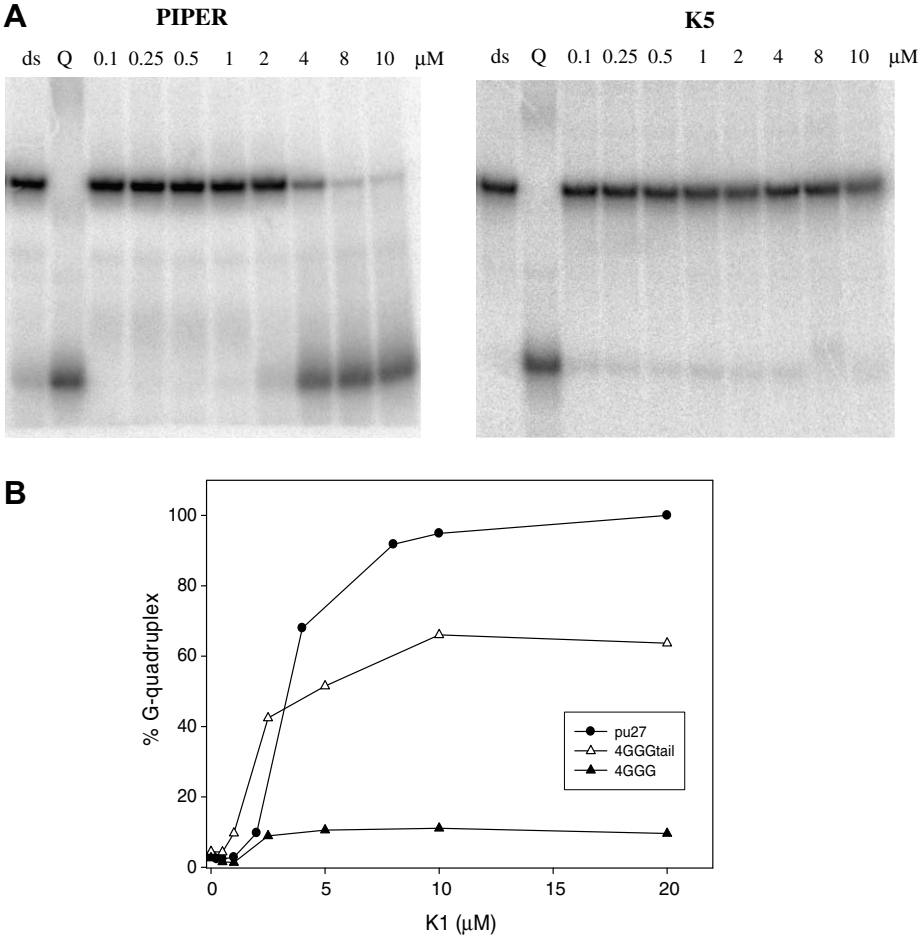


Figure 5. (A) Perylene-mediated induction of G-quadruplex in the G-rich strand of the *c-myc* promoter region (pu27). The DNA fragment (1 μM, strand concentration) was annealed with its complementary strand and then incubated with increasing concentration of perylene in 10 mM Tris, 1 mM EDTA, 100 mM KCl at pH 8.0 for 24 h at 37 °C. The double stranded (ds) and the G-quadruplex (Q) folds are included for comparison. Reaction products were resolved on a 16% native polyacrylamide gel in TBE 0.5× containing 20 mM KCl. (B) Extent of G-quadruplex formation when double-stranded DNA obtained by annealing of the corresponding complementary strands of sequences related to telomeric (4GGG and 4GGGtail) or *c-myc* promoter (pu27) region (1 μM, double helix concentration) are incubated for 24 h at 37 °C with increasing concentration of **PIPER** in 10 mM Tris, 1 mM EDTA, 100 mM KCl at pH 8.0. Data refer to quantification of EMSA experiments (corresponding to the example reported in (A)).

Its melting temperature was 83 °C, about 20 °C higher than the one found for 4GGG (64 °C). These data indicate that under our experimental conditions the promoter sequence is stably folded and, as a consequence, no major CD change can be expected to occur upon perylene addition (data not shown). According to literature data, in the absence of K^+, a remarkable reduction in the CD signal is observed although still suggesting an overall parallel structure.[29] In these experimental conditions, addition of test perylenes induces a significant increment in the optical activity with a modest red shift of the maximal absorption (corresponding to K^+ effect). Again, derivatives containing a cyclic amine in the side chains are less efficient in affecting DNA structure/stability in comparison to their congeners (Fig. 6).

The above described effects were obtained by addition of perylenes to an already folded nucleic acid sequence. Monitoring the melting/cooling processes of 1GGG or 2GGG bound to tested perylenes, we observed complete reversibility. This was true also for 4GGG bound to **PIPER** or **K19** (Fig. 7). However, when thermal cycling was performed using 4GGG bound to **K5** or **K8**, the prevalence of a different DNA complex was indeed observed upon cooling to room temperature. In fact, the final CD spectra are characterized by a positive and a negative peak at 260 and 240 nm, respectively, suggesting a conformational change to a predominant parallel arrangement of the G-quadruplex structure. Derivative **K20** showed an intermediate behaviour (data not shown). Taking into account EMSA results, this process corresponds to the formation of a tetrameric G-quadruplex, not induced by **PIPER** and **K19**.

Using the pu27 DNA sequence, tightly organized in a parallel folding, no major G-quadruplex structural changes were observed as a result of thermal cycling (Fig. 7).

As different perylenes appear to give rise to different preferential G-quadruplex arrangements only when present in the folding step of telomeric DNA, we investigated drug binding to the unfolded form of 4GGG. In 100 mM Li^+ the sequence 4GGG is characterized by a weak optical signal (Fig. 8), which supports lack of G-quadruplex folding in agreement with PAGE data. Upon addition of perylenes, the remarkable modification in the dichroic signal can be safely attributed to a preferential parallel DNA arrangement. Interestingly, in these experimental conditions **K19** and **PIPER** do stabilize a folded G-quadruplex structure but, again, they are largely less efficient than **K20**, **K5** and **K8**.

2.4. Perylene side chains affect intercalation efficiency into ds-DNA

As previously reported, perylenes can interact with double-stranded DNA by an intercalative process. To clarify this issue with the test perylenes, we performed DNA unwinding assays. When

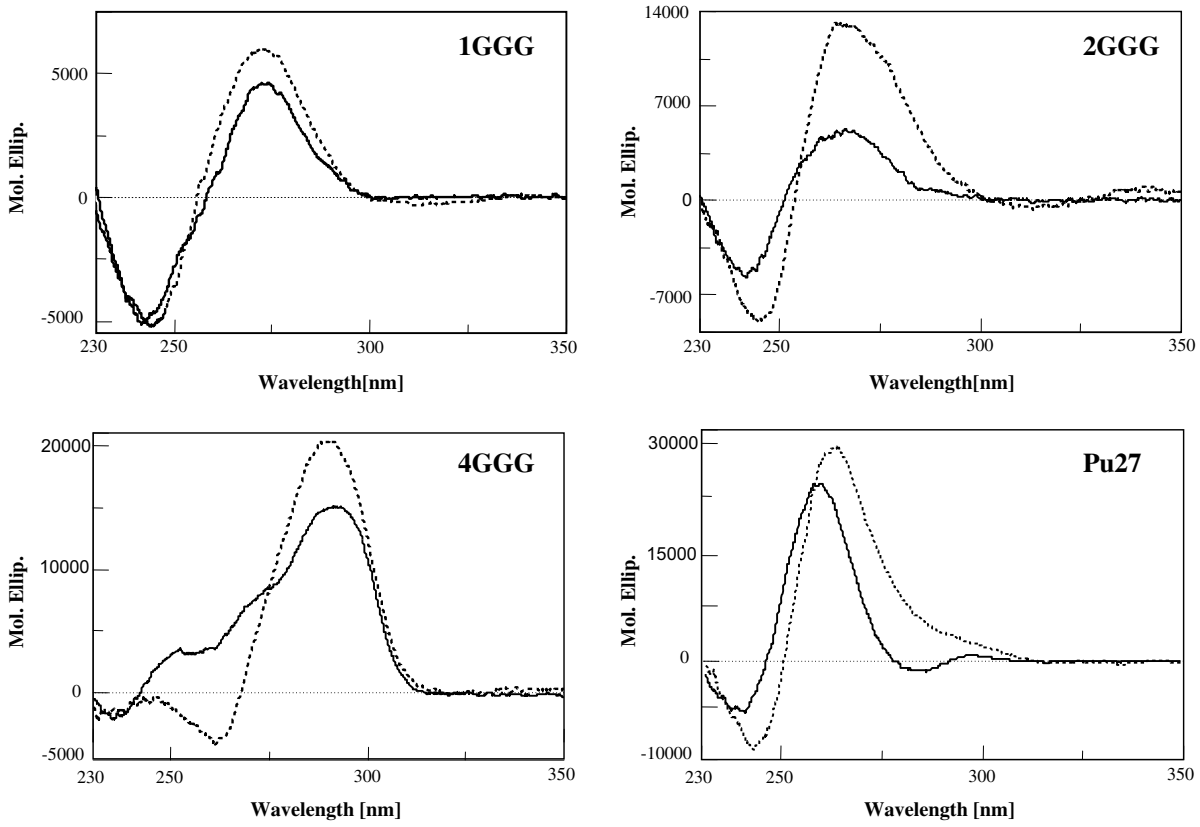


Figure 6. CD spectra of the tested oligonucleotides (1GGG, 2GGG, 4GGG and pu27 are 4 μM, strand concentration) in 10 mM Tris, 1 mM EDTA, KCl 100 mM, pH 8.0, in the absence (solid line) and in the presence (dotted line) of **K20** (14 μM) recorded at 25 °C. Perylene was added to the previously folded oligonucleotide and the spectrum recorded after 30 min incubation at 25 °C.

Table 2
Molar ellipticity increase (%) for selected oligonucleotides (4 μM, strand concentration) produced by addition of the tested perylenes (10 μM) in 10 mM Tris, 100 mM KCl, pH 8.0, at 25 °C (wavelength in brackets)

Perylene	2GGG (265 nm)	4GGG (295 nm)
K5	136 ± 5	16 ± 2
PIPER	14 ± 1	11 ± 1
K8	139 ± 7	16 ± 1
K20	176 ± 6	46 ± 3
K19	40 ± 2	5 ± 1

drug–DNA incubation was performed at pH 8.0, **K20**, **K5** and **K8** completely unwound negatively supercoiled DNA at concentrations between 10 and 20 μM (Fig. S4 in the supplementary material). By further increasing drug concentrations plasmid DNA was positively supercoiled, thus confirming an efficient intercalation process. In the presence of **PIPER** or **K19** (up to 100 μM), no appreciable variation in the plasmid electrophoretic mobility was observed. Interestingly, these two compounds undergo self-aggregation under the test unwinding conditions. In order to reduce aggregation, the same experiments were performed at pH 6.0, at which intercalation was confirmed to occur with all test compounds at comparable concentrations (20–50 μM). Comparable results were obtained using relaxed DNA as a substrate, thus ruling out a role for DNA topology in driving binding to the double helix (Fig. S5 in the supplementary material).

3. Discussion

The stabilization of G-quadruplex is an attractive target to interfere with the unrestrained replication of cancer cells, possibly by more than one mechanism.[5] Besides telomeres, other genomic regions have the potential to form G-quadruplex DNA, with important biological consequences. Considering the plasticity of the quadruplex arrangement, which can be formed intra- and intermolecularly in parallel, antiparallel and mixed arrangements, it would be very useful to devise molecular tools to efficiently discriminate among distinct G-quadruplex architectures. To date, only very few examples are known stemming from the porphyrin family, in which selectivity for *c-myc* promoter has been incremented upon extension of the cyclic core, or from telomestatin and diseleno sapphyrin for which preferential binding to different telomeric folding (basket type and hybrid structure, respectively) has been suggested.[30,31]

It has been previously reported that among perylene derivatives, it is possible to modulate the selective binding of double stranded versus G-quadruplex DNA by affecting the aggregation properties through side chains modifications.[14,16,22] In this work, we show that also selectivity within G-quadruplex structures can be modulated by the perylene family, since test compounds are able to bind to different G-quadruplex arrangements in a side-chain dependent fashion.

Indeed, the perylene congeners we tested can be divided into two distinct groups according to their affinity for the G-quadruplex structures: derivatives carrying a cyclic amine in the side chains, which show a reduced binding to the folded form, and linear amine congeners, exhibiting enhanced binding. Worth noting is the similar behaviour of PIPER and K19. Although the two compounds do not share the same planar system, they showed comparable DNA affinity and recognition selectivity in all our assays. This suggests that the effects produced by an increase in nitrogen basicity as a result of amide reduction (likely increasing DNA affinity) are

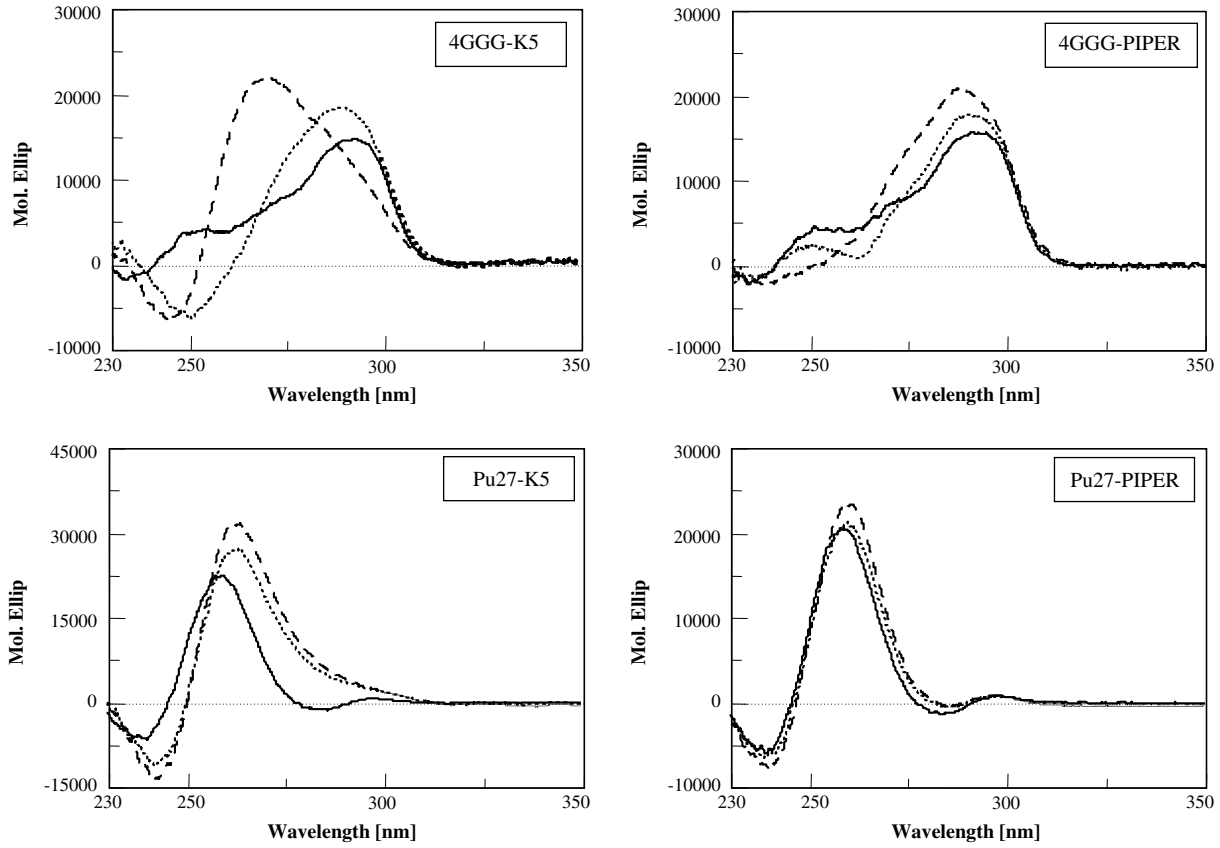


Figure 7. CD spectra of oligonucleotide 4GGG or pu27 (4 μM, strand concentration) in 10 mM Tris, 1 mM EDTA, KCl 100 mM, pH 8.0, recorded at 25 °C. Solid line refers to the spectrum of the DNA folded alone, dotted line corresponds to the same oligonucleotide after addition of perylene (14 μM) followed by an incubation of 30 min at 25 °C, dashed line is the spectrum of the oligonucleotide folded in the presence of perylene (14 μM).

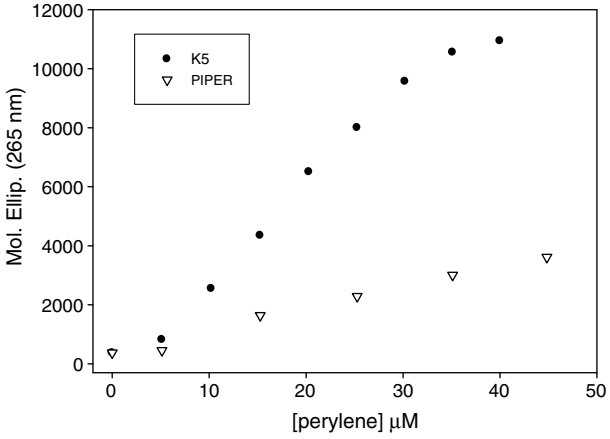


Figure 8. CD titration of 4GGG (4 μM, strand concentration) with perylenes in 10 mM Tris,1 mM EDTA, 100 mM LiCl, pH 8.0, 25 °C. The molar ellipticity recorded at 260 nm is reported as a function of **PIPER** or **K5** concentration.

possibly counterbalanced by partial loss of perylene planarity due to the same process (decreasing drug/base stacking efficiency and, hence, DNA affinity). In addition, it can be inferred that the carboxyl groups, missing in K19, are not actively involved nucleic acid binding.

The different binding efficiency of the two identified groups of derivatives was confirmed with different experimental approaches both using telomere- and *c-myc* promoter-related sequences. Since in our experimental conditions these two types of sequence assume distinct conformations, at first glance our results could be interpreted in terms of inability to bind G-quadruplex DNA in a structure-specific fashion.

However, in the case of the telomeric sequence, the presence of the test high-affinity perylenes **K5**, **K8**, and **K20** forces G-quadruplex into a distinct (possibly parallel) arrangement essentially through the pairing of multiple DNA chains. An interesting finding refers to the rearrangement of 2GGG from a tetramer to a dimer G-quadruplex upon increasing the incubation temperature. This behaviour could be simply explained by the mass action law, considering the higher number of complex units (terminal stacking) that can be formed with the perylene structure when a tetramer is split into two dimers. However, this is not the case using 4GGG, the monomeric form of which is converted into the tetrameric one upon annealing in the presence of perylenes **K5** or **K8** (and not of **PIPER** or **K19**). Interestingly enough, this process is associated with a dramatic change in the folding of the G-quadruplex structure that evolves from an intramolecular mixed type to a multimeric (likely parallel) form.

The annealing process is required to promote perylene-driven stabilisation of the latter. Hence, the complex exhibiting the mixed form appears to be kinetically favoured, whereas the tetrameric complex is thermodynamically favoured.

If we remind that in our experimental conditions all test telomeric sequences likely assume preferentially parallel arrangements while interacting efficiently with **K5**, **K8** and **K20**, we conclude that the strong (linear amine) perylene binders efficiently recognise this G-quadruplex structure. This is true also in the case of pu27 to which the above perylenes bind very strongly. The same conclusions do not apply to our derivatives with cyclic amine side

chains (**PIPER** and **K19**) which are not able to overcome the preferential folding of the nucleic acid sequence which then 'rules' in the complex.

Given the prominent affinity of **K5** for the parallel G-quadruplex structure assumed by pu27, one would expect addition of the perylene to the double helical form of the *c-myc* promoter to favour the displacement of the G-rich strand from its complementary sequence and folding into the G-quadruplex conformation. Interestingly enough, this behaviour can be observed with the weak binders only. Hurley and co-workers previously suggested that the ability of **PIPER** to produce such a displacement rests in the stacking of perylene molecules aligned with duplex DNA, hence promoting denaturation of the duplex and subsequent folding into the G-quadruplex.[25] On the other hand, compounds that can efficiently intercalate into ds-DNA do not promote denaturation, but stabilization of the double helix and render the strand-scission process less likely. As we previously suggested and herein confirmed, cyclization of the terminal amine in the perylene side chains dramatically reduces the drug's intercalative potential, possibly due to self-aggregation processes.[18] On the other hand, **K5** behaves as a classical intercalator at physiological conditions as shown by our unwinding experiments. Hence, its inability to produce complementary strand separation. Probably, even if G-quadruplex might exhibit higher affinity for **K5** than its double helical form, the activation energy of the process is possibly too high to allow ds melting and G-quadruplex formation at 37 °C. Again, its appearance at higher temperatures indicates that the perylene-G-quadruplex complex is the thermodynamically favoured form. The importance of a less organized double helix to allow perylene-promoted G-quadruplex fold is emphasized by the finding that, using a DNA construct with the target telomeric sequence annealed to a shorter complementary strand, the duplex–G-quadruplex transition occurs.

In conclusion, our results highlight how minor variations in the amine substitution at the side chains ends critically modulate the recognition of G-quadruplex structures by perylene derivatives, both in terms of binding affinity and of selectivity among different G-quadruplex arrangements. Two apparently conflicting requirements have emerged: (i) to generate strong and selective G-quadruplex binders linear alkylamino residues must be connected to the planar ring system and (ii) to favour duplex–quadruplex transitions, as reasonably required to produce biological and pharmacological effects in living systems, cyclic amines are preferred. The reconciliation of these two features into a single pharmacophore is the challenging goal for the rational design of novel effective G-quadruplex directed drugs based on the perylene template.

4. Experimental

4.1. General Procedures

Tested perylenes were synthesized accordingly to previously reported procedures.[18] Solutions were prepared freshly just before use.

Synthetic oligonucleotides were purchased from Biosense (Belgium) and were used with no further purifications.

4.2. Circular dichroism measurements

Oligonucleotides circular dichroism spectra from 230 to 350 nm were recorded using 10 mm path length cells on a Jasco J 810 spectropolarimeter equipped with a NESLAB temperature controller and interfaced to a PC 100 in 10 mM Tris–HCl, 1 mM EDTA, 100 mM KCl, pH 8.0. Before data acquisition, DNA solutions were heated at 95 °C for 5 min and left to cool at room temperature over night. The reported spectrum of each sample represents the average of three scans recorded with 1-nm step resolution. Observed ellipticities were converted to mean residue ellipticity $[\theta] = \text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ (Mol. Ellip.).

Thermal denaturation experiments were performed by recording the DNA optical signal as a function of the temperature. Oligonucleotide solutions (4 μM) were equilibrated at 25 °C, then the signal was recorded while increasing the temperature at 0.8 °C/min and stirring the solution to allow equilibration. Experiments were performed in the presence or absence of tested derivatives (4 μM final concentrations). Two to four scans were repeated for each experimental condition. When required, the melted solution was cooled down at the same temperature change rate.

4.3. Electrophoretic mobility shift assay

Single stranded oligomers were 5′-labeled with ^{32}P and T4 polynucleotide kinase, by incubating the reaction mixture at 37 °C for 30 min. Kinase was inactivated by heating the reaction mixture at 85 °C for 5 min, followed by two phenol extractions.

A mixture of purified labelled and unlabelled oligonucleotides (total final concentration 1 μM) was heated to 95 °C for 10 min in Tris–HCl 10 mM, EDTA 1 mM, KCl 100 mM, pH 8.0 buffer and let to cool at room temperature over night. When annealing with the complementary strand was required an equimolar solution of the two strands was heated to 95 °C for 10 min and let to cool at room temperature. The folding of the starting material was monitored by native 16% polyacrylamide gel electrophoresis. After the DNA folding step, 5 μL of compound was dispensed to each sample to produce the required concentrations in a total volume of 20 μL. Reaction mixtures were incubated at 25 °C, 37 °C or 95 °C for the reported time. After incubation, 4 μL of gel loading solution (50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) were added and the samples were subsequently analyzed by native 16% polyacrylamide gels. Electrophoresis proceeded for 6 h in TBE 0.5× containing 20 mM KCl running buffer. Resolved bands on dried gels were visualized and quantified on a PhosphorImager (Amersham).

4.4. Unwinding assay

Supercoiled or relaxed pBR322 DNA (0.15 μg) was incubated in the presence or absence of increasing concentrations of tested ligands in 10 mM Tris·HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, pH 8.0 or 6.0. After 30′ incubation at 37 °C, the reaction mixtures were loaded on 1% agarose gel, and run in TAE (40 mM TRIS, 18 mM acetic acid, 1 mM EDTA). The reaction products were visualized by ethidium bromide staining.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.08.068.

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