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New highly hydrosoluble and not self-aggregated perylene derivatives with three and four polar side-chains as G-quadruplex telomere targeting agents and telomerase inhibitors

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Abstract—Four new perylene derivatives with three and four basic side-chains are reported here as G-quadruplex interactive compounds. The new perylene derivatives are readily soluble in water and not self-aggregated, in contrast to what happens with the previously reported two side-chain perylene derivatives. All four compounds are able to induce the G-quadruplex and to inhibit 50% of telomerase activity at about 5 μM concentration, showing a similar efficiency with respect to each other. Molecular modelling studies are presented to try to explain these findings.

Human telomeric DNA consists of tandem arrays of the sequence TTAGGG at the 3′-end of the chromosome which can reach several kilobases in length.[1] A single-stranded overhang of about 200 nucleotides protrudes at the 3′ terminus of telomeric DNA and in the nonreplicative state this strand interacts with duplex DNA to form a loop structure that stabilizes and protects the terminal end of chromosomes.[2] The terminal single-stranded DNA of telomeres is the substrate for telomerase, a ribonucleoprotein reverse transcriptase enzyme involved in the maintenance of telomere length in eukaryotic cells.[3] Telomerase has therefore become an highly selective target for antitumour drug design since this enzyme is active in most of human tumours (more than 85%) and inactive in the somatic cells of normal tissues.[4]

In the last few years, an interesting approach for targeting telomerase has been developed with the use of molecules able to bind the G-rich strand of telomeric DNA and to force it to assume unusual secondary structures inaccessible to the enzyme, known as G-quadruplexes.[5] G-quadruplex structures are based on the association of four guanine bases in a planar stable hydrogen-bonded arrangement, called a G-tetrad or G-quartet.[6] Several tetrads can be stacked on one another and held together by intervening sequences to form G-quadruplexes.

Molecules able to induce and/or stabilize G-quadruplex structures have been intensively studied for their ability to inhibit telomerase in cell-free systems and thus act as potential antitumoural agents. Many compounds such as porphyrins,[7] trisubstituted acridines,[8] telomestatin,[9] berberine[10] and perylene[11] derivatives are well known as telomerase inhibitors and potential anticancer agents.

Recently, we have studied a library of *N*-*N*′-disubstituted perylene diimides having the same perylene core and different side chains.[12] We have shown that electrostatic interactions between ligand side-chains and DNA phosphates play a main role in the formation and stabilization of G-quadruplex structures and in selecting its topology; we also found that different side-chains attached to the perylene core led to different efficiencies in telomerase inhibition. Furthermore, we have shown that the side-chain basicity plays a significant role in drug water solubility: the tertiary amines present in the hydrophilic

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side-chains can be converted to the respective hydrochlorides, leading to a moderate water solubility of the otherwise very hydrophobic perylene core, but these compounds nevertheless undergo extensive self-aggregation in aqueous media.[12b] The self-association properties of the perylene-based ligands in water have been shown to be critical in determining the selectivity of binding to G-quadruplex DNA,[13] although the correlation between these two aspects has been suggested to be a complex matter.[14] On this basis, we decided to strongly increase the polarity of the perylene derivatives by increasing the number of basic side-chains. In fact, an increased number of

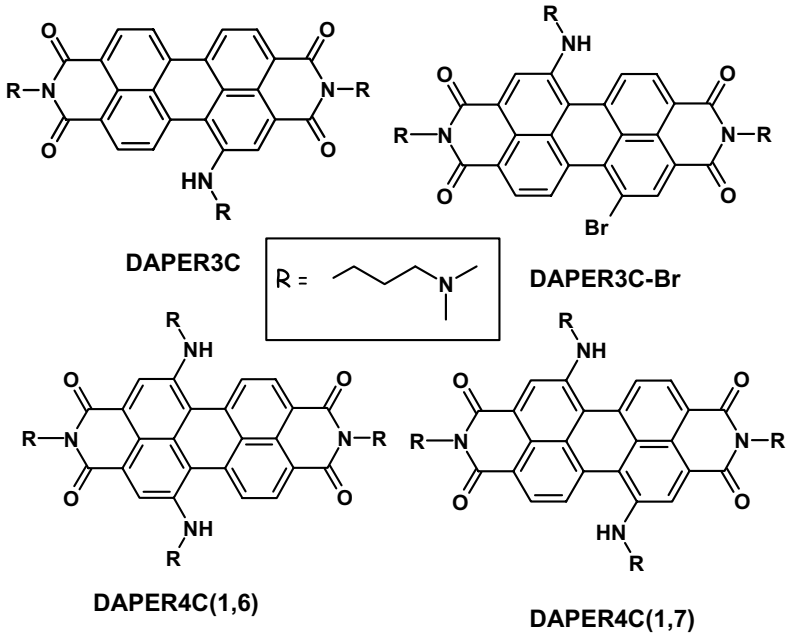


Figure 1. The new perylene derivatives with three and four polar side chains.

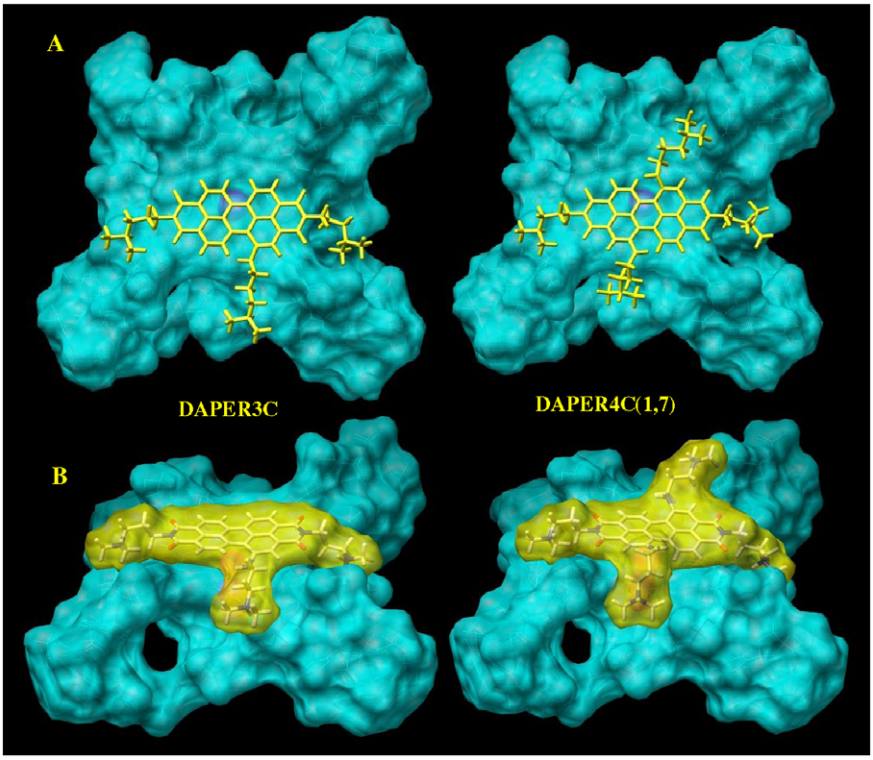
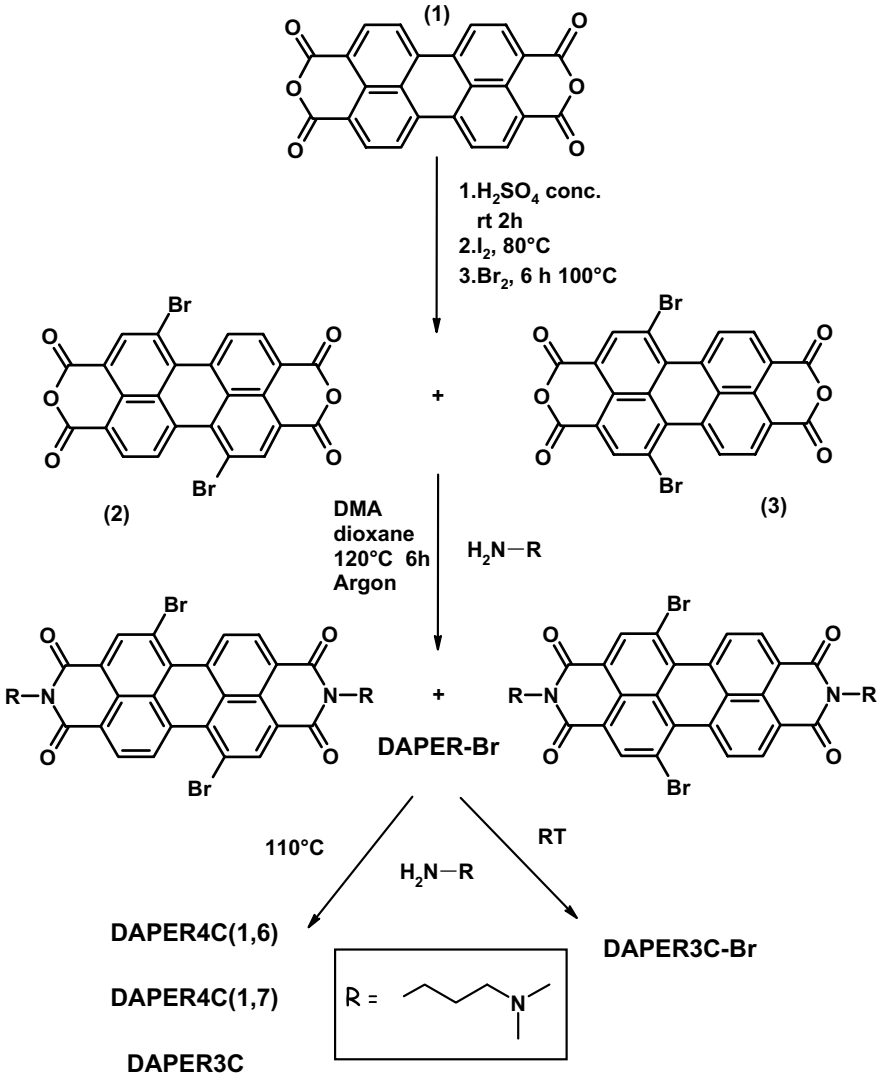


Figure 2. (A) Complexes between DAPER3C (left) and DAPER4C(1,7) (right) as yellow sticks and a monomeric G-quadruplex (blue surface, K^+ ions are violet spheres) obtained by simulated annealing.[16] (B) The same structures as in (A) but under a different perspective, with the perylene derivatives as atom type coloured sticks and yellow surfaces.

polar side-chains should lead to enhanced water solubility of the perylene core and to a smaller amount of self-aggregation.

In order to obtain perylene derivatives with these molecular features, in this paper we present four new perylene derivatives with three and four basic side-chains (Fig. 1). According to the model proposed by Hurley and co-workers[15] for perylene diimides interacting with G-quadruplex structures, we can suppose that four positively charged side-chains should improve the interaction between these molecules and the G-quadruplex, leading to higher binding constants and consequently to increased telomerase inhibition. In fact, molecular modelling simulations[16] of the designed compounds performed on a G-quadruplex monomeric structure[17] show a good interaction between the new perylene derivatives and the terminal G-tetrad, with the side chains correctly directed towards the DNA grooves (Fig. 2). Nevertheless, the effect of an increased amount of ligand positive charges on the electrostatic interaction with DNA phosphates could cause a decrease in selectivity for the G-quadruplex.

In order to obtain the designed perylene derivatives, we have functionalized the aromatic area of perylene by dibromination of 3,4:9,10-perylenetetracarboxylic dianhydride (**1**), as previously described.[18] We obtained a mixture of the two possible isomers **2** and **3**, which were not separable in this step of the synthesis (Scheme 1).[19,20] The mixture was then reacted with the commercially available 3-dimethylamino-1-propylamine and converted to the respective dibromo diimides (DAPER-Br).[21] In the final step of the synthesis, the mixture of the two isomeric diimides was treated with a large excess of the same amine used in the preceding step at 110 °C, obtaining a mixture of different products, which were successfully separated by column chromatography: the four-substituted perylene derivatives DAPER4C(1,7) (5% yield) and DAPER4C(1,6) (9% yield), having the additional chains in the 1,7 and 1,6 positions, respectively, and the trisubstituted derivative DAPER3C (14% yield), obtained by partial dehalogenation of the dibromo diimides.[21] Moreover, we performed the substitution of bromine atoms on the dibromoperylene diimides at room temperature and we found that only one bromine atom was readily substituted by the nitro-



Scheme 1. Synthetic route for the new perylene derivatives.

Table 1. Molar extinction coefficients of the four perylene derivatives and DAPER[12,24] at the indicated wavelengths, corresponding to a maximum in the UV/vis spectrum in DMSO

Compound	λ (nm)	$\varepsilon \times 10^{-3}$ (M^{-1} cm^{-1}) DMSO	$\varepsilon \times 10^{-3}$ (M^{-1} cm^{-1}) MES buffer, pH 6.5, T = 25 °C	$\varepsilon \times 10^{-3}$ (M^{-1} cm^{-1}) MES buffer, pH 6.5, T = 90 °C
DAPER4C(1,7)	695	24.0	17.2	19.8
DAPER4C(1,6)	681	20.0	15.8	18.0
DAPER3C	640	24.7	16.9	19.4
DAPER3C-Br	661	21.2	15.2	17.9
DAPER	528	54.1	15.2	24.6

gen of the amine, while the other one still remained on the aromatic core even after a long reaction time (12–24 h). In this way, we obtained the compound DAPER3C-Br, which presents one polar side-chain and one bromine atom on the perylene bay-area (Fig. 1). This interesting feature could allow the displacement of the two bromine atoms in two separate steps, by different reactants, carrying out the reactions at different temperatures. In this way, we could obtain asymmetric compounds with the ability to interact specifically with asymmetric G-quadruplex structures.

The study of the behaviour of the synthesized compounds in different solvents, paying particular attention to self-aggregation, was performed by UV/vis absorption spectroscopy and NMR. Previously we have shown that perylene diimides[12b] and coronene derivatives[20,22] are characterized by a strong decrease of the molar extinction coefficients in water with respect to organic solvents and by a low resolution of the NMR spectra in D_2O, because of strong self-aggregation processes. On the other hand, in the case of the new three- and four-chained perylene derivatives, the UV/vis spectra in aqueous buffer at pH 6.5 showed little differences with respect to the spectra obtained in organic solvent (Table 1).[23] Furthermore, when increasing the temperature, small changes in the spectra were observed (Fig. 3 and Supplementary data S1–S4). These data suggest that the new perylene derivatives in solution are not aggregated, in contrast to what happens for the previously reported two-side-chain perylene derivatives[12b] (DAPER[24] is reported for comparison in Table 1). NMR data confirmed this hypothesis, since the NMR spectrum in D_2O for one of these compounds [DAPER4C(1,6)] shows three distinct peaks in the aromatic region (δ 8.14, 7.96, 7.48), while the analogous spectra for the two-side-chain perylene derivatives were almost flat in the aromatic region, due to self-aggregation (Supplementary data S5). Moreover, the three aromatic peaks of DAPER4C(1,6) in D_2O remain substantially unmodified in the NMR spectra carried out at high temperature, being correctly proportional to the aliphatic signals (Supplementary data S6–S8). Furthermore, the lack of self-aggregation is confirmed by the unchanged UV/vis spectra as a function of the concentration (Fig. 3C), in contrast to the two-side-chain perylene derivatives.[12b]

It has been clearly shown that bay-substituted perylene diimides present a twisting of the two naphthalene subunits in the perylene core by about 20 deg.[25] It is

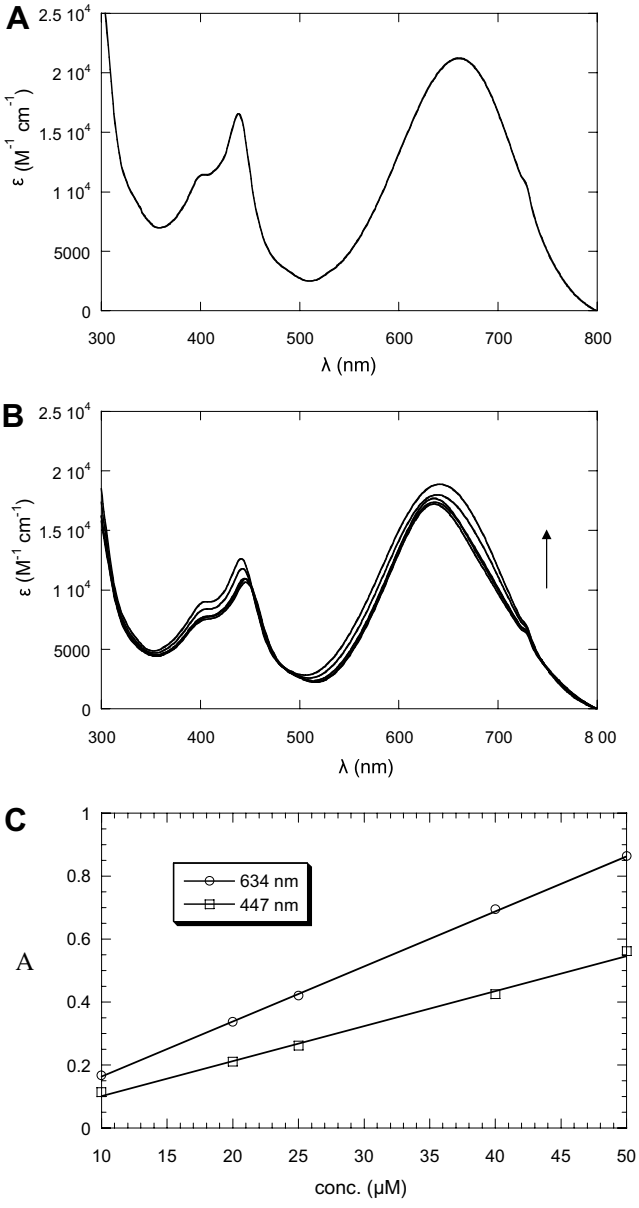


Figure 3. UV/vis absorption spectra of DAPER3C-Br performed in DMSO (A) and in MES (pH 6.5) buffer (B) at different temperatures.[23] The arrow indicates an increasing temperature from 25 to 90 °C. The analogous experiments for the other perylene derivatives are reported in the Supplementary data. (C) Absorbance of DAPER3C-Br in MES buffer as a function of the concentration.

reasonable to suppose that the self-aggregation of this class of compounds in water solution is very much reduced with respect to the two-side-chain perylene derivatives, because of the effect of the greater number

of polar side-chains introduced on the perylene moiety and of the distortion of the planar area of perylene due to the presence of one or two side-chains in the bay area. Indeed, very recently the effects of core twisting on self-assembly of perylene diimides have been exploited.[26]

The ability of the new three- and four-chained perylene derivatives to induce G-quadruplex structures was investigated by polyacrylamide gel electrophoresis (PAGE)[27], using the DNA oligomer TSG4 (5′-GGGATTGG GATTGGGATTGGGTT-3′). TSG4 is able to form an intramolecular G-quadruplex and can act as a substrate for telomerase elongation in a modified TRAP assay.[28] This oligonucleotide was used in our previous works to test the ability of berberine,[10b] perylene[12b] and coronene[22] derivatives to induce intramolecular G-quadruplex structures. The oligonucleotide was incubated in the presence of increasing concentrations of the four new perylene derivatives and the formation of G-quadruplex structures was investigated by PAGE analysis, as reported in Figure 4. Considering the electrophoretic mobility of the bands obtained for the G-quadruplex induced by 20 μM PIPER, the major electrophoresis bands were identified as single-stranded (ss) and monomeric G-quadruplex (M). The intramolecular G-quadruplex (M) corresponds to the band showing the highest mobility; its particular structure favours the faster running in the gel grid with respect to the single-stranded DNA, which has the same molecular weight. It is worth noting that the fraction of G-quadruplex structures stabilized by the new three- and four-chained perylene derivatives is approximately the same for the four compounds, if it is evaluated as the decrease of the free single-strand DNA at different drug concentrations. However, the electrophoretic band analysis shows that, while in the case of three-chained perylenes only the bands corresponding to the intramolecular G-quadruplex are present, as usually found for perylene diimides,[12b] in the case of four-chained derivatives electrophoretic bands having significantly lower mobility than ss-DNA are present. These bands, which are likely to correspond to G-quadruplex complexes characterized by a higher molecular weight with respect to ss-DNA, could be due to intermolecular G-quadruplex structures induced by these ligands or to complexes between two or more oligonucleotides held together by the four-chained perylene derivatives. At the highest drug concentration (20 μM), the electrophoretic band features suggest the presence of equivalent amounts of the two G-quadruplex structures.

The new perylene derivatives were evaluated for their telomerase inhibition properties as determined by a TRAP assay[29] (Fig. 5). A modified TRAP protocol,[28] using the DNA oligomer TSG4 as a substrate for telomerase elongation, was performed. Previously we have shown that the results obtained with this assay are the same as in the standard TS-based TRAP assay.[12b] We have found a similar efficiency by the four different per-

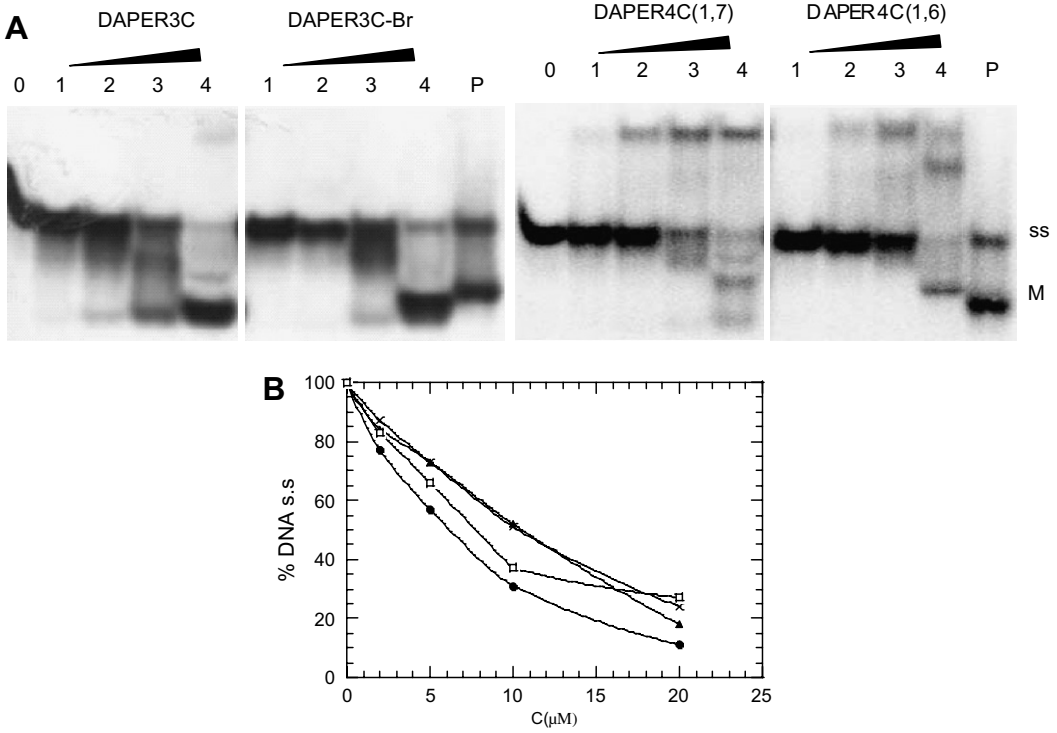


Figure 4. G-quadruplex structure formation induced by the four perylene derivatives, studied by native PAGE.[27] (A) Typical autoradiographies obtained using TSG4 oligonucleotide (12 μM) in the presence of different drug concentrations: 2 μM (lane 1), 5 μM (lane 2), 10 μM (lane 3), 20 μM (lane 4) and with no drug (lane 0). As standard in lane P the structures induced by PIPER at concentration 20 μM were reported. Major bands were identified as single-stranded DNA (ss) and monomeric G-quadruplex (M). (B) Percentage of single-strand DNA (% ss-DNA) versus drug concentrations (C(μM)). DAPER3C (●), DAPER3C-Br (▲), DAPER4C(1,6) (**x**), DAPER4C(1,7) (□).

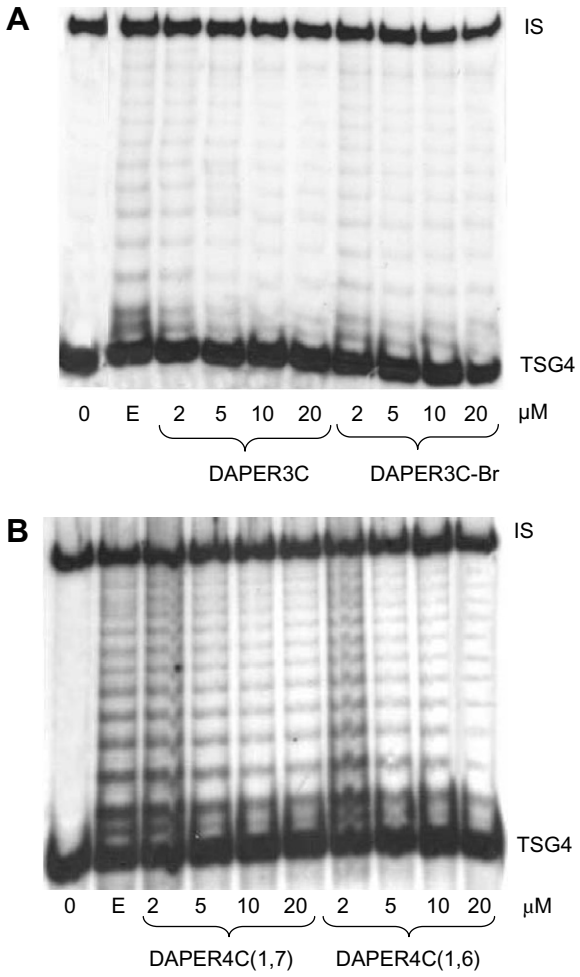


Figure 5. Inhibition of human telomerase by the two three-chained perylene derivatives (A) and by the two four-chained perylene derivatives (B) by telomerase repeat amplification protocol assay (TRAP).[29] Typical autoradiographies obtained using TSG4 oligonucleotide in the presence of the indicated drug concentrations. In lane 0 cell extract was not added, in lane E no drug was added. IS is a 130 bp 'internal standard' to control PCR amplification efficiency.

Table 2. Percentage of telomerase inhibition for the four perylene derivatives at the indicated concentrations

	2 μM (%)	5 μM (%)	10 μM (%)	20 μM (%)
DAPER3C	24	39	57	59
DAPER3C-Br	18	49	55	59
DAPER4C(1,7)	8	41	54	54
DAPER4C(1,6)	12	47	56	59

Errors estimated on at least three independent experiments are about ±5%.

ylene derivatives (Table 2): all of them are able to inhibit 50% of the enzyme activity at about 5 μM concentration. This value is lower than those found in the case of the two-side-chained perylene derivatives, which we previously showed to be in the range of 10–20 μM.[12] The inhibition trend of the new perylene derivatives is characterized by an asymptotic behaviour around 60% of inhibition starting from 10 μM drug concentration, which is quite unexpected and requires further investigations.

It is interesting to note that perylene derivatives with three and four side-chains show a similar efficiency with respect to each other, both in the PAGE and in the TRAP assay. This can be explained by looking more closely at the molecular models (Fig. 2): the perylene area is superimposed on approximately half the G-tetrad area. In fact, as in the case of trisubstituted acridines,[8b] the third side-chain on the minor axis of the perylene anchors the ligand to one orthogonal groove with respect to the two grooves where the two side chains on the major axis of the perylene lie. On the contrary, the fourth side-chain (when present) cannot reach the opposite groove when the ligand is in this position, so that its presence is not useful for improving the binding to the G-quadruplex. The presence of this highly mobile side-chain could explain the unique ability of these compounds to hold together two different TSG4 oligonucleotide chains (Fig. 4). This behaviour of the four-chained perylene derivatives surely deserves further investigation, since it seems to characterize only these molecules, in contrast to the perylene derivatives with two and three side-chains.

A major improvement in the biological activity of the new perylene derivatives hereby reported, due to the greater number of positively charged side-chains with respect to the two-side-chained perylene derivatives, could have been expected. However, the distortion of the planar area of the perylene core due to the presence of the new side chains[25,26] (Fig. 2) should be taken into account to explain this result. Nevertheless, the balance between hydrophobic and hydrophilic interactions is a complex topic, which surely requires further study.

Further investigation will be carried out to fully exploit the experimental data here reported, but the distortion of the planarity of the perylene core (well known for the bay-substituted perylene diimides) and the self-aggregation of the synthesized compounds must be carefully considered in the model of the interaction between the different ligands and the G-quadruplex DNA. In fact, self-association is reported to favour the specific recognition of the G-quadruplex with respect to duplex DNA.[13] Nevertheless, it has been recently shown by Palumbo and coworkers[14] that strong drug self-aggregation is related to a minor telomerase inhibition and weaker interactions with the G-quadruplex, suggesting that the higher selectivity for G-quadruplex arrangements upon aggregation is due to a reduced binding efficiency to duplex and single-stranded DNA rather than a greater affinity for G-quartets. We have recently derived analogous conclusions in a series of coronene derivatives.[22] Moreover, Hurley and co-workers[15,30] proposed also that the self-stacking properties of perylene diimides are important in determining their ability to induce the formation of G-quadruplex structures, acting in the manner of a chaperone protein. Since the perylene derivatives hereby reported do not show significant self-stacking in water (Table 1), further investigation will be devoted by our group to clarify the effects of the lack of self-association on the kinetic aspects of the induction of G-quadruplex structures by

these new compounds and on their selectivity for the G-quadruplex with respect to duplex DNA.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.021.

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27. Radio-labelled TSG4 oligonucleotide (5′-GGGATTGGGATTGGGATTGGGTT-3′) was heated at 95 °C for 10 min and quickly cooled in ice, at a concentration of 12 μM. It was then incubated for 2 h at 30 °C in MES–KCl buffer (10 mM MES, pH 6.5, and 5 mM KCl) in the presence of different drug concentrations and with no drug. Complexes and structures formed after incubation were studied by native PAGE (15% polyacrylamide gel, TBE 0.5×, KCl 20 mM, run overnight at room temperature). In all electrophoresis runs, the G-quadruplex induction by PIPER (20 μM) was also reported as a useful standard to assign the electrophoresis bands to different DNA conformations. Percentage of ss-DNA represents the ratio between the intensity of the relative band on the electrophoresis gel and the total amount of DNA, obtained by Instant Imager (Packard).
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29. The reaction mixture for assaying inhibition of human telomerase (50 μl) contained 50 μM dNTPs, 0.4 μM TSG4 primer, in TRAP buffer (20 μM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween 20 and 1 mM EGTA). The mixture was heated at 90 °C for 10 min, and then kept in ice for 10 min. In each sample, perylene derivatives were added at different concentrations and incubated for 2 h at 30 °C. After that, 1 μl of cell extract (prepared from 10^7 cultured human SKN-Be Neuroblastoma cells) was added. After 30 min of incubation at 30 °C, the samples were purified by phenol/chloroform extraction to eliminate Taq polymerase inhibition by the investigated drugs[31]. ^{32}P radiolabelled TSG4 (0.14 μM), 0.4 μM CXext primer and 2 U Taq DNA polymerase (Eppendorf) were added and 30 PCR cycles were performed (94 °C 10′, 92 °C 30″, 54 °C 30″, 72 °C 5′30″). Finally, the samples were loaded on a nondenaturing 12% polyacrylamide gel and electrophoresis was run overnight at room temperature. Samples with no drug and with no cell extract were references. A 130 bp 'internal standard' (IS) was used to evaluate PCR amplification efficiency.[31]
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