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Selective G-quadruplex ligands: The significant role of side chain charge density in a series of perylene derivatives

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ABSTRACT

The human telomeric G-quadruplex structure is a promising target for the design of cancer drugs. The selectivity of G-quadruplex ligands with respect to duplex genomic DNA is of especial importance. The high selectivity of polyamine conjugated perylene derivatives appears to be regulated by side-chain charge density, as indicated by data from a FRET melting assay and induced CD spectroscopy.

Telomeres are specific nucleoprotein complexes which provide a protective cap at the end of linear eukaryotic chromosomes.[1] Telomeric DNA is characterized by short G-rich tandem repeat sequences, whose length varies in different organisms. It is guanine-rich and the extreme 3′-end extends as a single-strand overhang. This is the substrate of telomerase, a reverse transcriptase enzyme, which is involved in the maintenance of telomere length.[2] Telomerase is physiologically active in germinal, hematopoietic and epithelial cells; in addition the enzyme is reactivated in almost all human tumor cells,[3] where it is responsible for their excessive proliferation and immortality. For this reason telomerase is a suitable target for anticancer therapy and in the last few years considerable efforts have been made to design telomerase inhibitors as possible anti-cancer drugs.[4–6]

One approach to telomerase inhibition is to modify its substrate, for example by induction of G-quadruplex structures at the 3′ telomeric end.[7] Induction of a G-quadruplex conformation can be achieved by small molecules, characterized by an extended aromatic core, that favors stacking interactions with terminal G-quartets and basic side-chains (positively charged in physiological conditions) which interact with the four grooves of the G-quadruplex.[8,9] Perylene diimides,with positively-charged side chains present suitable features to interact with the G-quadruplex and have the ability to induce different G-quadruplex structures and to inhibit telomerase, depending on side-chain basicity and length.[10]

We have recently synthesized eight polyamine perylene diimide compounds, termed POL ligands (Fig. 1),[11] in an attempt to conjugate the perylene moiety (which stacks on terminal G-quartets) to stabilize G-quadruplex structures with strongly charged polyamines. We have investigated the ability of these compounds to induce inter- and intramolecular G-quadruplex structures using native polyacrylamide gel electrophoresis (PAGE), together with their ability to inhibit telomerase using a modified TRAP assay. The two properties appear to be significantly correlated, showing that the number and distances apart of positive charges in the side chains dramatically influence both intramolecular G-quadruplex induction and telomerase inhibition.[11] In common with most anticancer drugs, their ability to selectively recognize their target is of major importance. In the case of G-quadruplex ligands, this is re-

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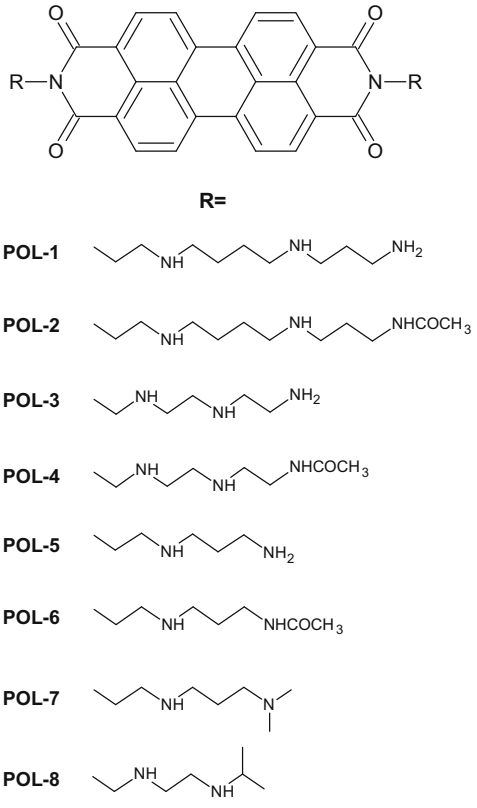

Figure 1. Structures of the eight POL perylene derivatives with different polyamine side chains.

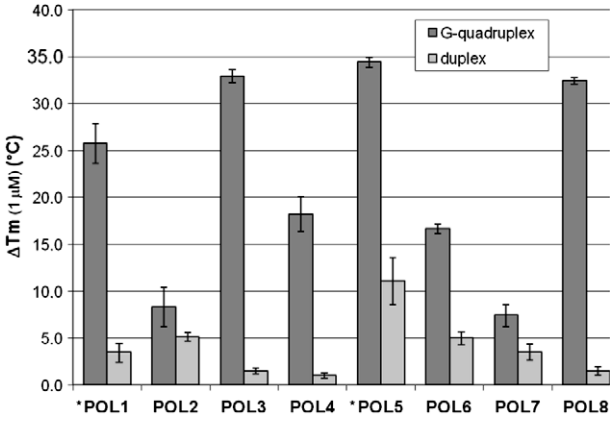

Figure 2. Histogram of the ΔT_m values obtained from the FRET melting studies at 1.0 μM drug concentration. For the compounds labeled with *(POL-1 and POL-5) the reported values correspond to 1.5 μM drug concentration. Melting temperatures are estimated as the midpoints of the melting curves for the human telomeric G-quadruplex and t-loop duplex DNA. Reported values are the means of three independent determinations.

lated to the ability of the molecule to selectively bind to G-quadruplex DNA compared to duplex DNA.

Our purpose in this study is to examine the selectivity of these new compounds for G-quadruplex DNA with respect to duplex DNA. Since their aromatic area is constant, these molecules enable one to investigate the specific role of the side chains, and if the efficiency of telomerase inhibition and induction of intramolecular G-quadruplex is coupled to selectivity between the two different DNA structural types. The study has been carried out using FRET melting assays, competition FRET melting assays, a new competition TRAP assay, absorption spectroscopy and induced circular dichroism (CD) spectroscopy with two different oligonucleotides (a human telomeric G-quadruplex and a self-complementary hairpin duplex DNA) and genomic duplex DNA. The information obtained on binding properties has enabled us to propose a model for the G-quadruplex/drug complex, which differs substantially from that for duplex DNA/drug complex.

The ability of the compounds to stabilize the two preformed DNA structures (quadruplex and duplex) was studied using a FRET melting assay.[12] ΔT_m values, reported in Figure 2, are given at a 1 μM ligand concentration for most of the drugs, since at this concentration there is a high level of G4-DNA stabilization (see Fig. S1 in Supplementary data). For two ligands (POL-1 and POL-5), however, data at 1.5 μM concentration are reported, since at 1.0 μM no relevant stabilization was observed. We also ran competition FRET experiments (Fig. 3), in which the perylene derivatives affinity for G4-DNA is evaluated in the presence of increasing concentrations of sonicated calf thymus duplex DNA (ct dsDNA).[13] In most cases, using ct dsDNA, we obtained results in agreement with those observed with hairpin duplex DNA (t-loop); surprisingly affinity for G-quadruplex DNA was not affected by the presence of the ct dsDNA also for molecules with high affinity for the t-loop (i.e., POL-2 and POL-5). This finding suggests that care must be taken in using synthetic oligonucleotides as model systems for genomic DNA, as also shown very recently by an ESI-mass spectrometry study on perylene derivatives selectivity.[14] It is evident that for all the studied ligands the number of positive charges in the side-chains has a major influence on G4-DNA thermal stabilization. Stabilization is strongly influenced by charge separation, perhaps rather more than the number of charges. For the pairs of compounds POL-1/POL-2, POL-3/POL-4 and POL-5/POL-6, ΔT_m values are significantly higher for those ligand having side-chains with three positive charges (POL-1, POL-3) or two (POL-5) compared to those with equal side-chains, except than the terminal group is acetylated. It is worth noting that selectivity is only for the interactions with G-quadruplex DNA, and is in large part absent for duplex DNA. The thermal stability of the t-loop dsDNA is only slightly increased by ligand interactions and surprisingly unaffected by the acetylation of side-chain terminal amino groups.

The two ligands POL-7 and POL-8 cannot be strictly compared since they have different terminal groups with a distinct hydrophobicity. Both have two positive charges, with the pairs POL-5 and POL-7, and POL-3 and POL-8 having the same charge separation. POL-8 shows both greater G-quadruplex thermal stabilization and greater selectivity with respect to POL-7. This result is to be well correlated with the general behavior of the other perylene derivatives. The features derived from the FRET melting assay are in good agreement with the results obtained from competitive FRET. The presence of ct dsDNA does not affect ligand stabilization of the G-quadruplex. Effective competition is only observed for the acetylated ligands POL-4 and POL-6, and for POL-7.

Since the most promising results in the competitive FRET assay were obtained with POL-3 and POL-8, these two molecules were selected for a further selectivity study using a competitive TRAP assay, in which telomerase inhibition is evaluated in presence of ct dsDNA.[15] This study was possible due to the high specificity of PCR primers for telomeric DNA and the internal standard DNA. The results obtained for the two drugs POL-3 and POL-8, shown in Figure 4, indicate that, for these molecules, ct dsDNA is not a competitor of human telomeric DNA up to ten times the concentration of the telomerase substrate. This issue needs further investigation, probably with an assay that does not require PCR amplification of telomerase products; however our results support the concept of selectivity of POL-3 and POL-8 for human telomeric G-quadruplex DNA.

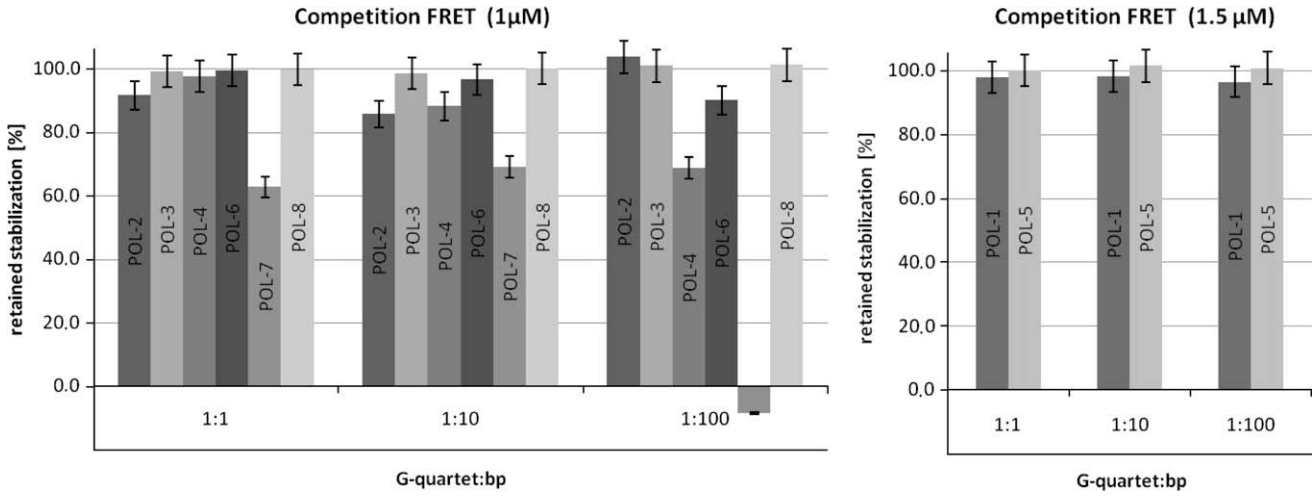

Figure 3. Competition FRET results. *Y*-axis represents the G-quadruplex stabilization ability of the compounds when in presence of a duplex DNA competitor, c.t. dsDNA (in the ratios depicted in *X*-axis, G-quadruplex:duplex) normalized using 100% for the experiment without duplex competitor.

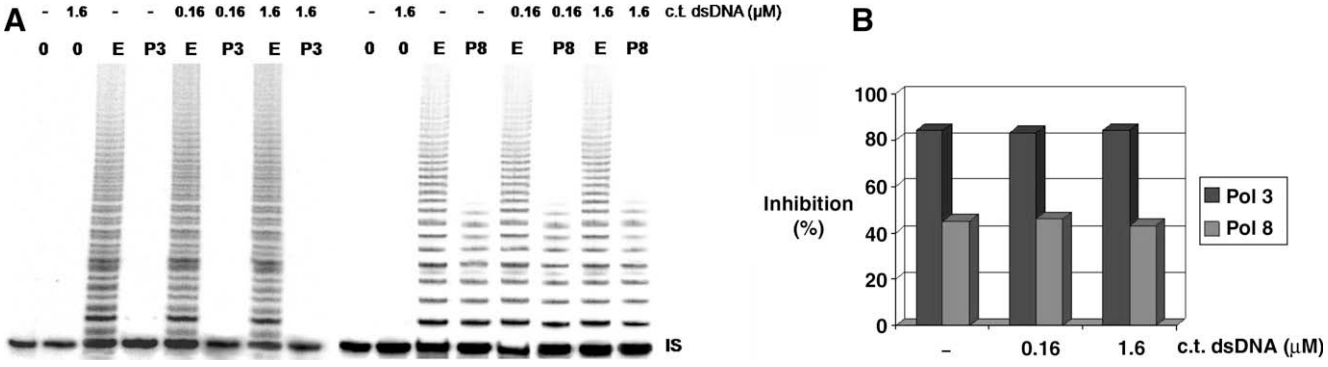

Figure 4. (A) Inhibition of human telomerase by two polyaminic perylene derivatives POL-3 (P3) and POL-8 (P8) using a modified telomeric repeat amplification protocol assay (competitive TRAP). Typical autoradiographies using TS oligonucleotide in presence of drugs (10 μM) and ct dsDNA at the indicated concentrations. In lanes '0' cell extract was not added, in lanes 'E' no drug was added. IS (internal standard) is a 36 bp oligonucleotide to control PCR amplification efficiency. (B) Percentage of telomerase inhibition by POL-3 and POL-8 (10 μM), in presence of ct dsDNA at the indicated concentrations. Errors estimated by at least three independent experiments are about ±5%.

Absorption spectroscopy and circular dichroism (CD) studies have been carried out on POL-3 and POL-8, which have the highest selectivity for the two DNA structures, as found in the FRET melting study reported here, as well as showing the highest level of telomerase inhibition in the TRAP assay.[11] Both compounds have the same charge density for their side chains. We have also studied by comparison compound POL-4, that has the same charges distance in the side chains as POL-3 and POL-8, but lacks both selectivity and biological activity. To characterize the features of their binding to G-quadruplex DNA in comparison with duplex DNA, we have carried out spectroscopic titrations of the three drugs at different DNA/drug molar ratios (*R*) and constant drug concentration, in the wavelength range 400–650 nm. The spectra of the three drugs correspond to the spectra of the perylene moiety and are not significantly perturbed by different side chains,[16] and is characterized by a broad band centered at about 497 nm, with a shoulder at 530 nm.[17] In the presence of the two DNA structures, the absorption profile of perylene changes (Fig. 5A and Fig. S4). On increasing the DNA/drug ratio R, it becomes similar to those obtained in organic solvents.[17] In all cases, the spectra are characterized by two bands at 525, 475 and a shoulder at 450 nm, except than the three maxima are red-shifted by about 25 nm each, compared to those in organic solvents. The variations in the absorption spectra of G-quadruplex-drug and duplex DNA–drug complexes as a function of *R*, are different for each drug. In all cases, presence of a DNA induces absorption spectra variations, which indicate that the solution equilibria are shifted towards the unstacked perylene monomeric form that prevails in organic solvents; nevertheless, the complexity of the equilibria involved (as suggested by the lack of an isosbestic point in titrations) prevents any attempt to derive the apparent association constants between the ligands and the two DNA structures. We report in the Supplementary data section, for the sake of comparison, the titration of POL-3 in DMSO with increasing amount of MES buffer (Fig. S3), where only one isosbestic point is evident, thus ensuring that the equilibrium is just that between the monomer in organic solvent and dimers or oligomers in aqueous solution.

Circular dichroism (CD) spectroscopy appears more suitable than absorption spectroscopy to study drug binding[18] since it can take into account the complexity of possible equilibria between free drugs and drugs bound to DNAs, in stacked or unstacked form. Perylene derivatives are symmetrical molecules and thus are not optically active. Duplex or quadruplex DNA structures have a CD spectrum only in the wavelength range 220–320 nm, so that the CD spectra of DNA–drug complexes, in the wavelength range 400–650 nm, are exclusively due to the ligand bound to DNA and thus asymmetrically perturbed. Some particular features emerge for all the studied drugs from the CD titrations (Fig. 5B and Fig. S5).[19] The CD spectra of the complexes between POL-3, POL-8 and POL-4 and G-quadruplex structure are directly correlated

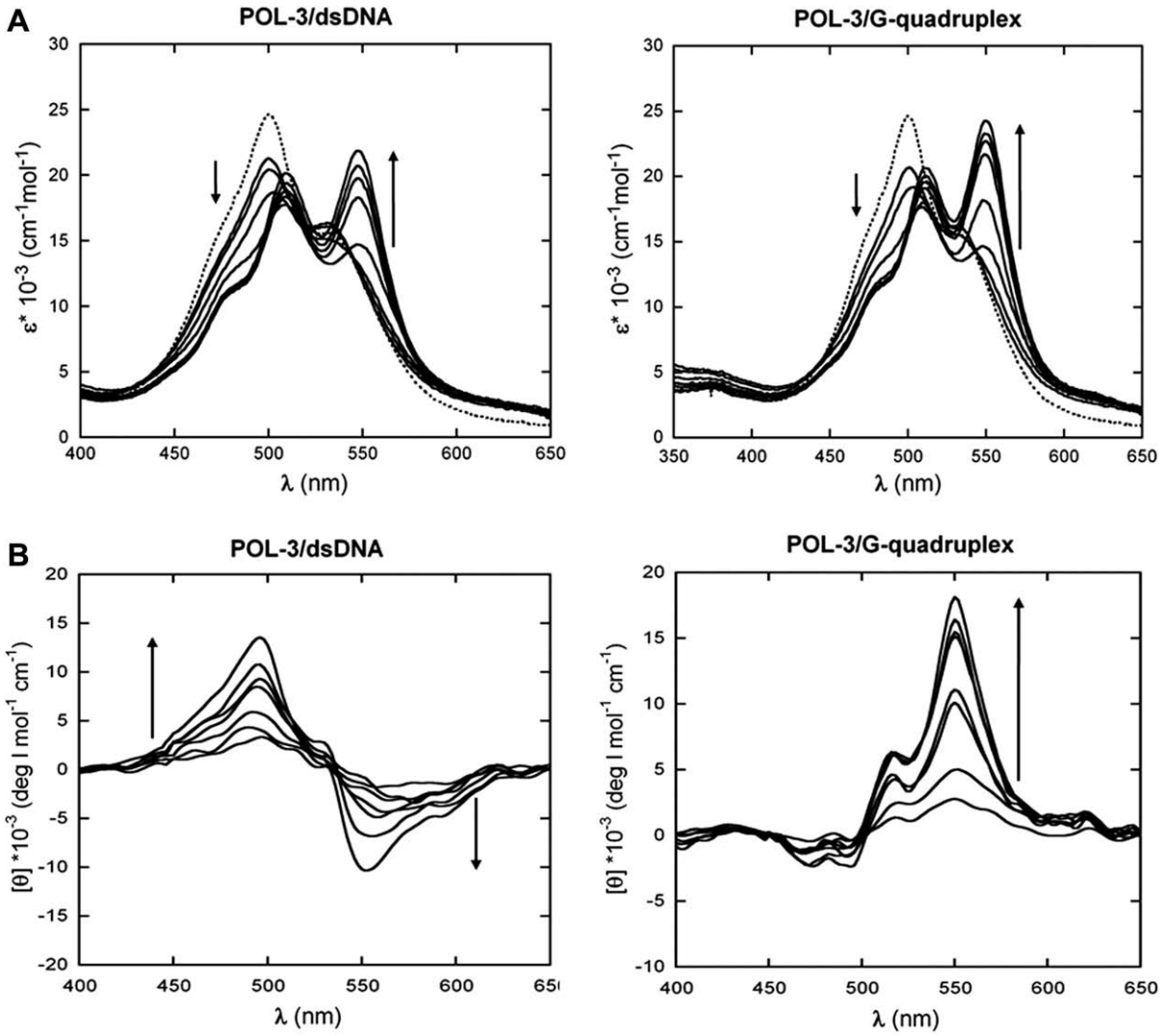

Figure 5. Absorption (A) and CD (B) spectra of POL-3 at constant concentration (20 μM) in the presence of duplex or G-quadruplex DNA at increasing DNA/drug molar ratio (*R*). The arrows indicate increasing R and the spectra correspond to the following values of *R*: 0.5, 1, 2, 4, 8, 12, 15, 20. In the panel (A) the dotted lines represent the spectra of POL-3 alone in MES/KCl buffer.

with the absorption spectra. The shapes of the two spectra are similar, since the absorption spectrum is characterized by two bands with maxima at 550 and 500 nm and a broad shoulder at 475 nm and the CD spectrum, in a similar way, is characterized by two positive bands at about 550 and 510 nm and a low shoulder at 475 nm. The similarity of absorption and CD spectra suggests that the perylene chromophores are strongly coupled with the terminal G-quartet of G-quadruplex DNA. The ellipticities of the CD spectra are significantly lower in the case of POL-4 with respect to POL-3 and POL-8 (Fig. S5), suggesting a lower association constant for POL-4 compared to the other two compounds. The features of the CD spectra are dramatically different for the complexes with ct ds DNA. In all cases, a conservative CD spectrum is observed, characterized by the presence of two bands with opposite signs, where the maximum of the absorption spectrum at 500 nm nearly corresponds to zero CD intensity. This feature suggests that two or more perylene chromophores are stacked upon each other, giving rise to spectra exciton splitting.[20] It is worth noting that the presence of the two maxima in the absorption spectrum makes the relative CD spectrum more complex and not strictly conservative, especially in the case of G-quadruplex/POL-4 complexes. A perfectly conservative exciton CD spectrum (equal intensity of the negative and positive bands) occurs only if the coupled chromophores have only one allowed transition.[21] In the case of the POL compounds, the strong coupling between two molecules could give rise to CD spectra characterized by split positive and negative bands for the S_0–S_1 transition of the perylene chromophore at 525 nm. Also, the vibronic structure that can be ascribed to the vibration of the perylene skeleton, which is strongly coupled with the S_0–S_1 transition, should be characterized by the same splitting at about 550 and 475 nm.

The values of induced ellipticities, in the presence of G-quadruplex, are significantly different, comparing POL-3 and POL-8 with POL-4. The perylene derivatives which are the most efficient in the FRET melting assay (POL-3 and POL-8), give CD spectra characterized by larger ellipticities than POL-4. On the other hand, the induced dichroism of the three drugs bound to duplex DNA appears similar. These results indicate that the differences in the side chains determine selective drug interactions with the G-quadruplex, while they are not significantly different in the behavior towards duplex DNA.

Although a direct correlation between thermal stability, derived from FRET melting assays, and differences in induced ellipticity is not appropriate, both properties are ultimately dependent on the

molecular features of binding. It is tempting to propose two outline structural models, taking into account the dramatic differences in the induced CD spectra of the G-quadruplex-drug and duplex-drug complexes. Two different models in the case of POL-3 are shown in Figure 6.

The drug complex with the monomeric G-quadruplex (Fig. 6C) has a 1:1 stoichiometry, consistent with the perylene moiety end stacking on the terminal G-quartet (a 2:1 stoichiometry is possible, with a second ligand molecule stacking on the other terminal G-quartet, but in any case for each binding site the ligand interacts in the monomeric form). The two side chains interact in the DNA grooves, possibly with phosphates groups, either directly or mediated through water molecules.[22] This model can explain the importance of physico-chemical properties (mainly the charge density and the terminal acetylation) of the polyamine side chains in their ability to induce and stabilize a G-quadruplex structure. Since the POL compounds show a range of ability to bind to the G-quadruplex (as shown by FRET melting studies and CD spectra), as well as in inducing the intramolecular G quadruplex structure,[11] we suggest for POL-3 and POL-4, that the number of charges on the side chains is a significant factor, provided that the overall charge density of each side chain remains constant.

The hydrophobic ends of the side-chains appear to have a surprisingly prominent role in interacting with G-quadruplex grooves. Ligand POL-8, which has the same charge separation as POL-4, but with a different terminal group ($-CH(CH_3)_2$), has high selectivity toward G-quadruplex structure, unlike POL-4. This is likely to be a consequence of the POL-4 terminal acetyl group having more extensive interactions with water molecules compared to methyl groups in POL-8.

It is worth noting that recently new G-quadruplex structures have been found in K^+ solution by NMR, confirming the well known polymorphism of human telomeric quadruplex structures.[23] It is evident the importance of different loops orientation and conformation in defining the binding sites and thus the relative mode of interactions between ligands and G-quadruplex,[22] nevertheless the experimental data reported in this study cannot lead to distinguish among different hypotheses, but confirm unambiguously that perylene ligands can interact with quadruplex DNA only in the monomeric form.

For ligands complexed with duplex DNA (Fig. 6A and B), the proposed model, which is compatible with CD data, considers several perylene diimide molecules stacked upon each other following the DNA helix, thus determining spectral exciton splitting (in the

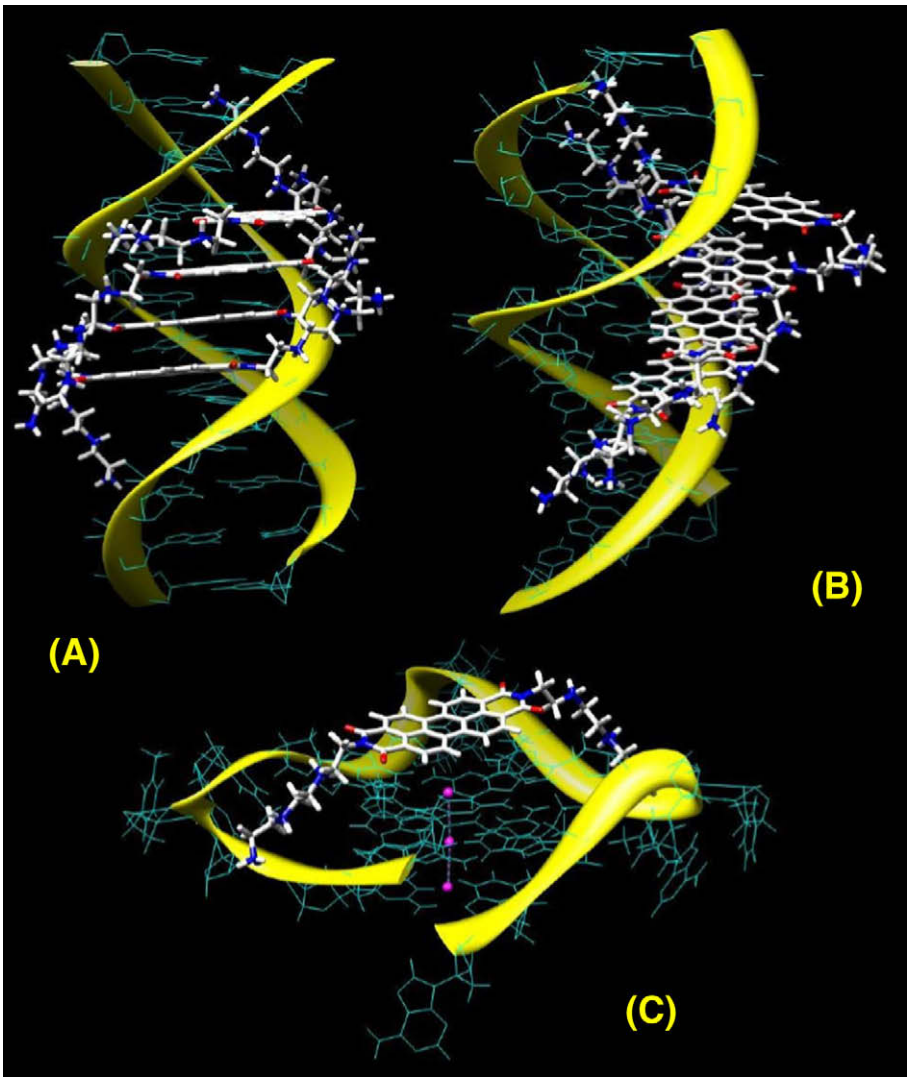

Figure 6. Representative models of POL-3 with duplex (A and B) and quadruplex (C) DNA. POL-3 molecules are atom-type colored, DNA is in blue with yellow ribbons showing the backbones. The complex with the monomeric G-quadruplex (C) has been obtained by a simulated annealing protocol, considering only one binding site (another independent binding site is possible at the opposite end of the structure),[11] while the model with duplex DNA is a schematic representation compatible with CD data, showing an arbitrary number of ligand molecules, which in any case must be higher than two.

figure four molecules are shown, but the number can be equal to or higher than two). Since the perylene moiety is inserted into the DNA major groove, the side-chains do not fully interact with DNA phosphates, distinct from what may occur in the G-quadruplex complex. This may explain why duplex thermal stability is only slightly increased by drug binding at high ionic strength (50 mM KCl) and there are only negligible differences between compounds bearing acetylated compared to non-acetylated side-chains.

We conclude that on the basis of the results reported in the present paper, as well as those obtained previously,[11] compounds POL-3 and POL-8 possess features which suggest that future biological and pharmacological level studies on them should be undertaken.

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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.2009.03.106.

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12. The FRET DNA melting assay was performed as described previously (Schultes, C. M.; Guyen, B.; Cuesta, J.; Neidle, S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4347). Tagged DNA sequences were used, 5′-FAM-d(GGG[TTAGGG]$_3$)-TAMRA-3′ for the G-quadruplex and 5′-FAM-dTATAGCTATA-HEG-TATAGCTATA-TAMRA-3′ (HEG linker: [(–CH_2–CH_2–O–)$_6$]) for the duplex experiment (t-loop). The assay employs 96-well plates and used a DNA Engine Opticon instrument (MJ Research).
13. Competition FRET experiments were performed as described above for the G-quadruplex experiment but with addition of varying concentrations of double strand DNA from calf thymus, c.t. dsDNA (Sigma–Aldrich, UK) (Moorhouse, A. D.; Santos, A. M.; Gunaratnam, M.; Moore, M.; Neidle, S.; Moses, J. E. *J. Am. Chem. Soc.* **2006**, *128*, 15972). The ct dsDNA has a length of about 200 bp and was obtained by sonication.
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15. The ability of two polyamine perylene derivatives, POL-3 and POL-8, to inhibit human telomerase, in the presence of a dsDNA in competition with enzyme substrate, was investigated in a cell-free system by means of a modified Telomeric Repeat Amplification Protocol (TRAP), using the TRAPeze® Kit. The TS primer was the oligonucleotide (18nt) used as telomerase substrate; it does not contain human telomeric repeats, so it can fold in a intramolecular G-quadruplex structure only if it is elongated by telomerase with at least four human telomeric TTAGGG repeats. The reaction mixture (50 μl), used for assaying inhibition of human telomerase, contains 50 μM dNTPs, 0.16 μM TS primer, 0.2 μg of cell-extract (prepared from HeLa E1 cells, over-expressing the telomerase subunits, hTER and hTERT, kindly provided from Professor J. Lingner) in TRAP buffer (20 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween 20, 1 mM EGTA). Where required, 10 μM polyamine perylene derivative (POL-3 or POL-8), 0.16 μM (the same concentration of TS) or 1.6 μM (a ten times higher concentration than TS primer) calf thymus 200 bp dsDNA were added. After 30 min of incubation at 30 °C, the samples were purified by phenol/chloroform extraction. ^{32}P-radiolabelled TS (0.16 μM final concentration) and 1 μl/sample of Primer Mix, 2U Taq DNA polymerase (Taq DNA polymerase/HotMaster™) were added in each sample. 30 PCR cycles were performed (94°/10′, 92°/30″, 54°/30″, 72°/5′30″). Finally, the samples were loaded on a native 12% polyacrylamide gel. Samples without the compound and without the cell-extract were used as references. A 36 bp oligonucleotide (IS) was used as internal standard to evaluate PCR amplification efficiency.
16. Absorption spectra were obtained by recording the spectra of the solution of one of the three ligands (POL-3, POL-4 and POL-8) kept at constant concentration, equal to 20 μM in MES/KCl buffer (pH 6.5), in the presence either of G-quadruplex DNA or of c.t. dsDNA: G-quadruplex oligonucleotide was a 21mer with the same sequence used in the FRET assay,[12] annealed in 50 mM KCl; ct dsDNA was sonicated as previously reported.[13] The concentrations of DNAs started at 400 μM so to have a DNA/drug molar ratio *R* equal to 20 and then were decreased up to 0.5 μM, by substituting a volume of drug/DNA solutions with an equal volume of 20 μM drug solution. Absorption spectra were performed with a Varian Cary 50 spectrophotometer, using a cell with a 1 cm path length. Each spectrum was acquired after the sample has been equilibrated for at least 4 min.
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