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Perylene Diimides with Different Side Chains are Selective in Inducing Different G-Quadruplex DNA Structures and in Inhibiting Telomerase

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Abstract—Four *N,N′*-disubstituted perylene diimides, having different side chains, have been studied for their ability in inducing G-quadruplex DNA structures. We found that electrostatic interactions between ligands side chains and DNA grooves play a main role not only in the amount of G-quadruplex formed, but also in selecting its topology. Moreover, such compounds show also a different ability to inhibit telomerase. The correlation of these findings suggests the intriguing possibility that different G-quadruplex structures could differently inhibit the enzyme.

Telomerase is a ribonucleoproteic reverse trascriptase enzyme, involved in the maintenance of telomeres length of eukaryotic cells.[1] Since telomerase is not active in most somatic tissues while it is active in most human tumors (more than 85%), it has become a novel and potentially highly selective target for anti-tumor drug design.[2]

A development of potential telomerase inhibitors has been carried out by the research of small molecules able to interact with telomeric DNAs and to induce unusual DNA secondary structures, inaccessible to telomerase.[3] Telomeric DNA sequences assume mainly the canonical B-DNA structure, although organized in a peculiar chromatin structure;[4–6] nevertheless, the 3′ telomeric G-rich single strand overhangs are able to assume, in vitro and, as very recently shown,[7] in vivo, inter- or intra-molecular four-stranded structures, named 'G-quadruplex', which are stabilized by Hoogsten G–G pairing in a planar arrangement.[8] Different types of G-quadruplex are formed, depending on the number and the orientation of the DNA strands. Parallel quadruplexes arise from the association of four strands with the same orientation and the same anti-glycosidic conformation, giving rise to four grooves of equal size, all with B-DNA minor groove character. Antiparallel quadruplexes arise from the dimerization of two strands, each containing at least two G-tracts, or from the intramolecular folding of one strand containing four G-tracts. The reciprocal orientation of the strands and the positions of the loops connecting the strands lead to structures characterized by different glycosidic conformations (*syn* and *anti*), topological parameters and groove sizes.[9] These differences may provide structural diversities needed for specific G-quadruplex recognition. It is worth to note that very recently crystal structures of two G-quadruplexes formed by telomeric repeats have shown that both inter- and intra-molecular G-quadruplexes can adopt radically different structures compared to the prevailing assumptions, with all strands having a parallel orientation.[10]

Many compounds inducing and/or stabilizing G-quadruplex and inhibiting telomerase in *'cell-free'* systems, such as porphyrins,[11] disubstituted anthraquinones,[12] trisubstituted acridine,[13] dibenzophenanthroline derivatives[14] and perylene derivatives[15–17] have been studied. All these molecules are characterized by an aromatic core, that favors stacking interactions with the G-quartets of the G-quadruplex, and by positively charged side chains able to specifically interact with the DNA grooves.

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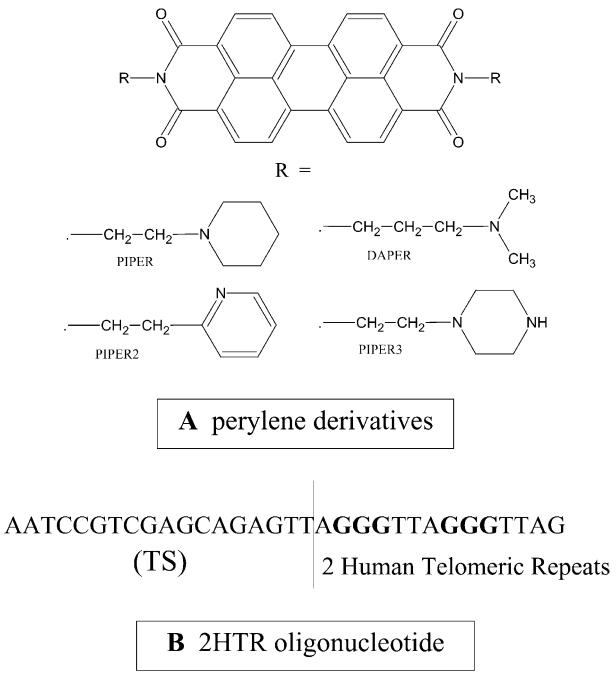
Among all these different classes of drugs, *N,N′*-bis[2-1(piperidino) - ethyl] - 3,4,9,10 - perylene - tetracarboxylic diimide (PIPER) has been well characterized for its ability in inhibiting telomerase on account of inducing and stabilizing G-quadruplex parallel four stranded DNA structures.[15,17] The molecular model of the binding, derived mainly by NMR studies, involves the stacking of PIPER aromatic moiety on the terminal G-quartet of the G-quadruplex. This model does not allow to evaluate the role of side chains. In fact the dynamics of side chains of the complex make difficult their structural characterization by NMR studies. However, the study of the influence of different side chains of the drugs in the specificity of the binding is surely of importance: with regard to this, it has been recently shown that three positional cationic porphyrins isomers exhibit different ability to induce and stabilize G-quadruplex structures.[18] Since different G-quadruplex structures may be associated with different cellular processes, it seems essential to discriminate the binding selectivity of G-quadruplex-interactive agents to achieve a therapeutic selectivity. Moreover, a very recent structure–activity relationship (SAR) study about porphyrins evidenced not only that stacking interactions and positive charges are required for telomerase inhibition, but also that a combination of hydrogen-bonds and charged substituents improves telomerase inhibition.[19] Finally, very recently, the influence of the side chains of two perylene diimides on their behavior in solution (aggregation), as well as on their binding selectivity to G-quadruplex DNA structures with respect to duplex DNA, has been reported.[16]

In this report we show that, maintaining unchanged the stacking interactions due to the aromatic core of perylene derivatives, different charged side chains are able to selectively induce and/or stabilize different G-quadruplex structures. The structural selectivity seems correlated with telomerase inhibition, suggesting the importance of taking into account the electrostatic and van der Waals interactions of the side chains with DNA grooves in planning efficient telomerase inhibitors.

We have synthesized and studied four perylene derivatives with the same aromatic core but different side chains[20] (Scheme 1A). PIPER side chains are characterized by a nitrogen atom in a piperidine ring ($pK_a \approx 11$), so that it can be considered totally charged, in the experimental conditions we adopted (pH≈6.5). In the case of *N,N′*-bis[2-(2-pyridino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER2), instead, the nitrogen atom in the aromatic pyridine ring ($pK_a \approx 5.5$) is positively charged only in a small fraction of the molecules, in the same conditions. The piperazine ring of *N,N′*-bis[2-(1-piperazino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER3) has two nitrogen atoms, whose first dissociation constant is comparable to that of PIPER, while the second one is comparable to that of PIPER2, so that a small fraction of molecules will be doubly charged in our experimental conditions, while in the molecules with a single charge, it can be positioned alternatively on one of the two nitrogen atoms; besides, the possibility exists to make different



Scheme 1.

hydrogen bonds with DNA phosphates than PIPER. The nitrogen atom in the side chains of *N,N′*-bis[3,3′-(dimethylamino)propylamine] - 3,4,9,10 - perylenetetracarboxylic diimide (DAPER),[21] even having pK_a comparable to that of PIPER, is not inside a cycle, so that its charge has a different stereochemistry; besides, its longer aliphatic side chains, with respect to the other three drugs, could give rise to different docking in the G-quadruplex grooves. On the basis of the described chemical properties, the three PIPER analogues can establish with G-quadruplex DNA structures stronger electrostatic interactions and different hydrogen bonds (PIPER3), definitely lower electrostatic interactions (PIPER2) and different docking of side chains in the G-quadruplex grooves as well as different electrostatic interactions (DAPER), with respect to PIPER.

Native PAGE assays were performed to investigate the role of the different side chains of these perylene derivatives in the assembly of G-quadruplex structures. Mobility shift assay allows to distinguish different G-quadruplex structures.[17] We have considered the DNA oligomer 2HTR (Scheme 1B), which contains a tail, corresponding to the TS primer used in the in vitro assay we have adopted to evaluate telomerase inhibition, followed by two tandem repeats of the human telomeric sequence, containing the two G-rich tracts involved in the formation of G-quadruplex. It was incubated in presence of increasing concentration of PIPER and its three analogues (Fig. 1). Sequences characterized by two guanine tracts can associate into antiparallel and parallel G-quadruplex structures that are dimers or tetramers of the oligomer, respectively. Considering previous gel-shift data obtained in similar experimental conditions[17] and adopting mobility standards obtained by G-quadruplex dimeric forms induced by potassium ions[22] and by G-quadruplex tetrameric

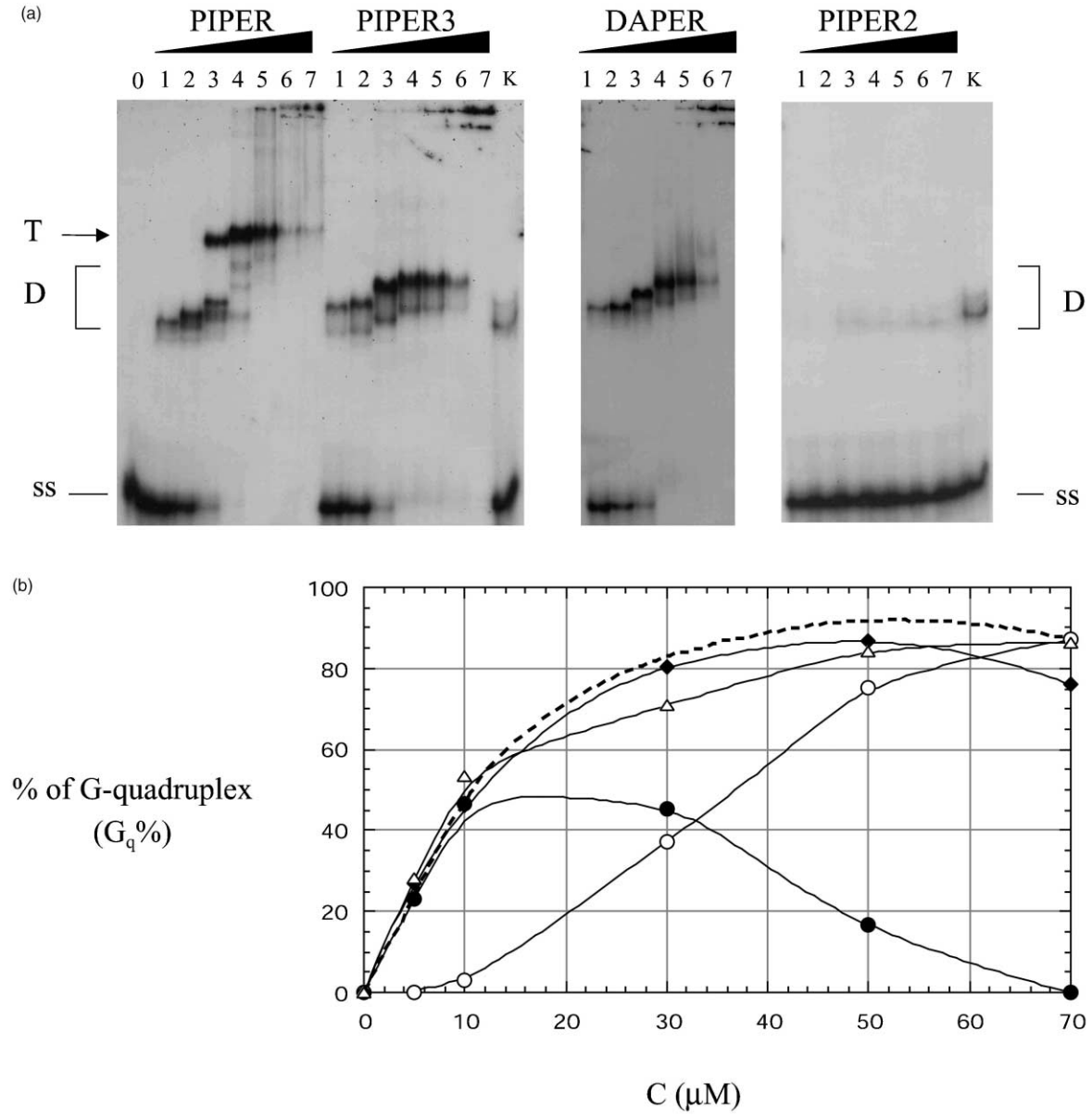


Figure 1. (a) G-quadruplex structures formation induced by PIPER, PIPER2, PIPER3 and DAPER (hydrochlorides), studied by native PAGE (15% polyacrylamide gel, TBE-KCl 0.5X buffer (KCl 20 mM), run overnight at 4 °C). 2HTR (8 μM), previously heated at 95 °C for 10 min and quickly cooled in ice, was incubated for 24 h at 37 °C in MES/KCl buffer (MES 10 mM pH 6.5, 50 mM KCl), in the presence of different drug concentrations: 5 μM (lane 1), 10 μM (2), 30 μM (3), 50 μM (4), 70 μM (5), 100 μM (6), 130 μM (7). In lane K, 2HTR was incubated with KCl 1 M. Major bands are identified as single strand DNA (ss), dimeric (D) and tetrameric (T) G-quadruplex structures. (b) Percentage of G-quadruplex structures formed (G_q%) versus drug concentration (C) of PIPER, PIPER3 (◆) and DAPER (△). In the case of PIPER, dimeric (●) and tetrameric (○) structures can be distinguished (the dashed line being their sum). In this plot, the values corresponding to concentrations higher than 70 μM have not been considered because of the increasing amount of higher-order structures. G_q% 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).

forms induced by PIPER,[17] major bands were identified as single-strand DNA (ss), dimeric (D) and tetrameric (T) G-quadruplex structures (Fig. 1a).

The obtained results show with good evidence that a strong decrease of electrostatic interactions with DNA grooves (PIPER2) does not allow the formation of G-quadruplex structures, although PIPER2 should be able to establish stacking interactions with the G-tetrads. PIPER3 and DAPER are both able to promote the formation of G-quadruplex structures, but, differently than PIPER, they induce exclusively dimeric G-quadruplex structures. We observe several bands corresponding to distinct dimeric G-quadruplex isomers,[23] but we do not observe the formation of tetrameric G-quadruplex structures either increasing the incubation time or increasing drugs concentration. On the contrary, as previously evidenced,[17] PIPER is able to promote the formation of dimeric G-quadruplex structures at low drug/oligomer ratios, while at higher ratios it promotes exclusively the formation of tetrameric G-quadruplex structures. At the highest studied

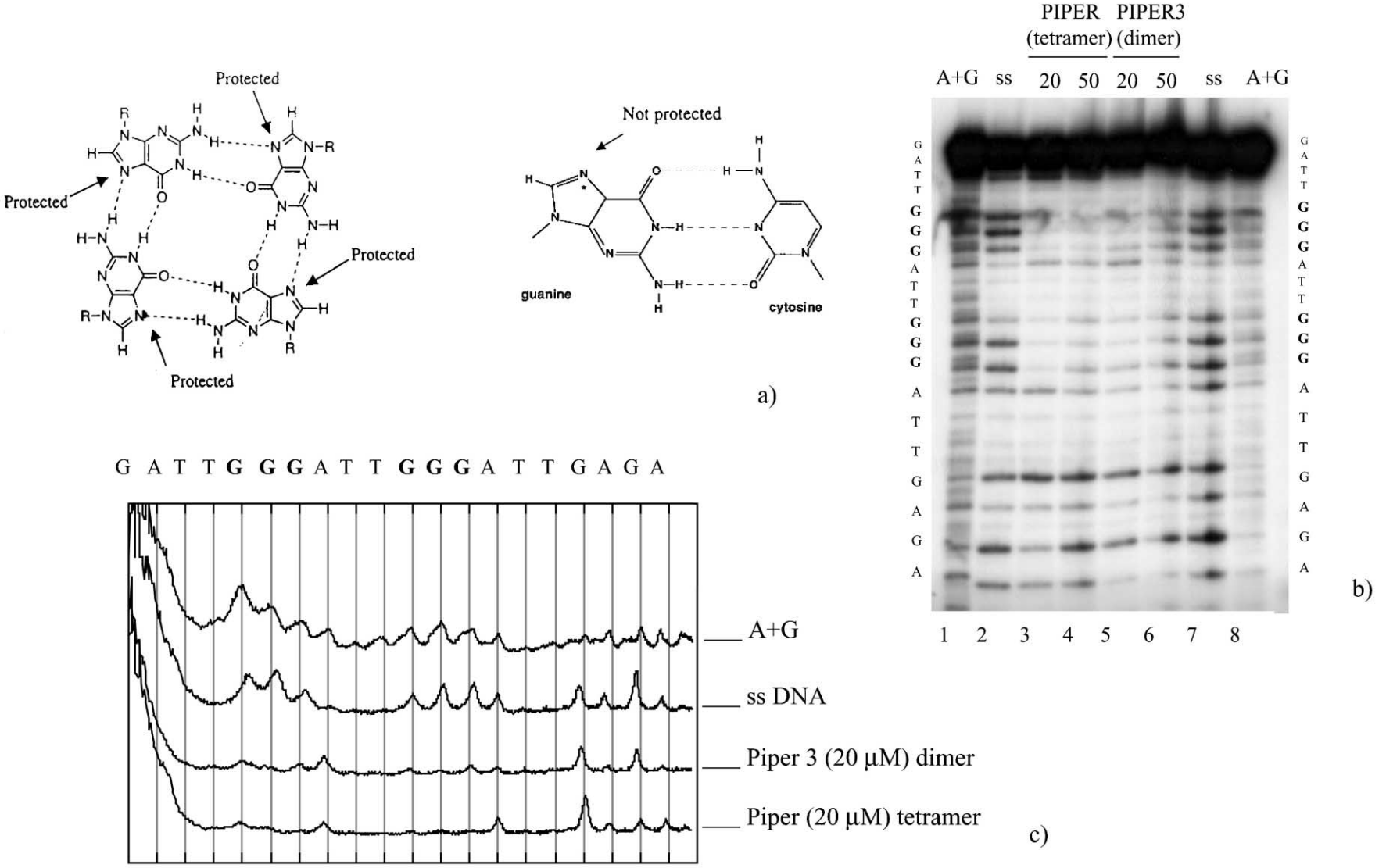


Figure 2. Dimethyl sulfate (DMS) footprinting. (a) Comparison between Hoogsteen G–G base pair and Watson–Crick G–C base pair. (b) 2HTR (free and incubated with PIPER and PIPER3 at 20 and 50 μM) was treated with DMS at a final concentration of 1% (v/v), for 10 min at room temperature. Immediately thereafter, the products (tetramer, dimer and free oligomer) were gel-purified by native 16% PAGE: successively they were treated with piperidine to induce strand cleavages at methylated bases. Finally, the reaction products were loaded on a denaturing gel 20% polyacrylamide gel. ss: free single strand DNA. 20 and 50: PIPER and PIPER3 at 20 and 50 μM respectively. A + G: Maxam and Gilbert reaction for purins. (c) Densitometric profiles of the corresponding lanes.

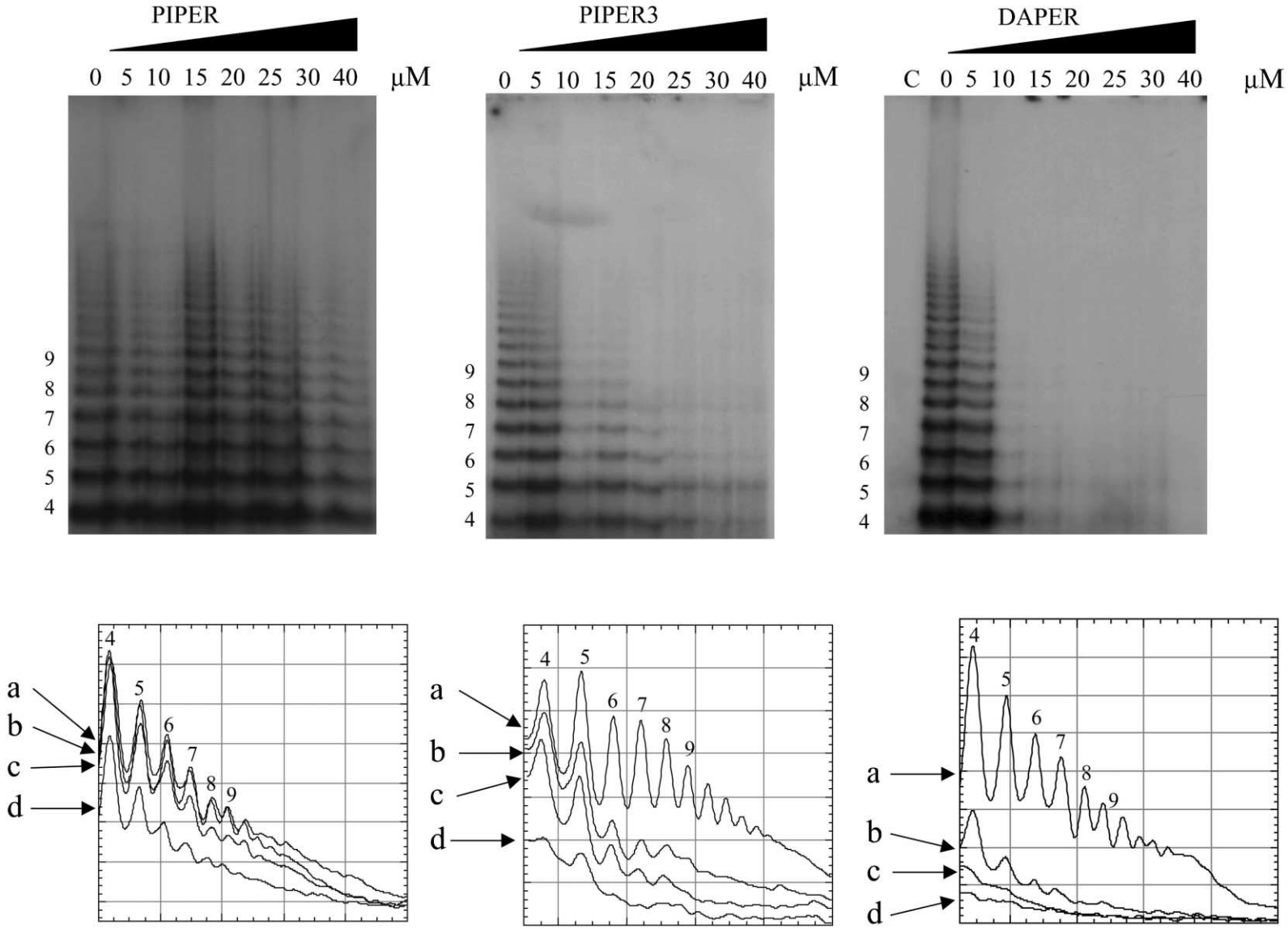


Figure 3. Inhibition of human telomerase by different perylene derivatives, by telomerase repeat amplification protocol (TRAP) assay. The reaction mixture (50 μL) contains 200 mM Tris–HCl (pH 8.3), 680 mM KCl, 15 mM $MgCl_2$, 10 mM EDTA, 0.5% Tween20, 50 μM dNTPs, 0.5 μM TS primer, 1 μL of cell extract (prepared from 10^9 cultured HeLa cells, as previously described).[26] In each sample, perylene derivatives were added at the final shown concentration, before the addition of the cell extract. In lane 0 no drug was added, in lane C cell extract was not added. Also heat inactivation was performed by heating 10 μL extract at 75 °C for 10 min prior to assaying 1 μL by TRAP analysis, giving rise to the same result. After 30 min of incubation at 30 °C, the samples were purified from drugs by phenol/chloroform extraction. ^{32}P radiolabeled TS, 0.5 μM ACT primer[27] and 2U Taq DNA polymerase (Promega) were added and 27 PCR cycles were performed (94 °C 30″, 50 °C 30″, 72 °C 1′30″). Finally, the samples were loaded on non-denaturing 12% polyacrilamide gel. The numbers to the left of the gels and on the peaks of corresponding densitometric profiles refer to the telomerase extensions in telomeric repeats. (a) No drug added, (b) 10 μM, (c) 20 μM, (d) 40 μM drug concentration.

concentrations of PIPER, PIPER3 and DAPER, additional slow migrating species are observed (lanes 6 and 7), probably corresponding to higher-order structures.

To better compare the different behavior of the different perylene derivatives, we have reported the percentage of the induced G-quadruplex structures as a function of drugs concentration (Fig. 1b). Considering the formation of all the G-quadruplex structures, the three molecules have a similar efficiency in inducing such structures. Considering the different G-quadruplex forms, PIPER efficiency in inducing dimers is lower than PIPER3 and DAPER, while PIPER is the only one able to induce tetrameric G-quadruplex structures.

To verify the attribution of electrophoretic bands to G-quadruplex structures, dimethyl sulfate (DMS) methylation protection experiments were performed. The N7 of guanine is directly involved in the Hoogsteen hydrogen bond of G–G base pair, so the formation of G-quadruplex structures should protect guanine residues from methylation by DMS, while guanines not involved in quadruplex structures remain susceptible[17,24] (Fig. 2a).

The free oligomer 2HTR (ss) was uniformly methylated by DMS at every guanine residue (Fig. 2b and c; lanes 2 and 7). On the contrary, as regards the tetrameric (lanes 3 and 4) and dimeric (lanes 5 and 6) G-quadruplex complexes, induced, respectively, by PIPER and PIPER3, the guanines that were within the two human telomeric repeats resulted protected, while the guanine residues in the tail remained always sensitive. All these data confirm that the shifted bands induced by PIPER, PIPER3 and DAPER correspond to G-quadruplex structures stabilized by the association of four guanines in a cyclic Hoogsteen hydrogen-bonding arrangement.

On the basis of the obtained results as well as of the data previously obtained by Han et al.,[18,25] suggesting that different interactions with G-quadruplex DNA of two cationic porphyrins give rise to differential effects on telomerase inhibition, it appears of interest to compare the ability of PIPER, PIPER3 and DAPER in inhibiting telomerase. To detect the telomerase activity, we have used a highly sensitive PCR-based telomerase activity assay, known as Telomeric Repeat Amplification Protocol (TRAP assay).[26] The technique is based on the ability of telomerase to recognize and elongate in vitro a specific oligonucleotide substrate (TS primer); the resulting products were then amplified via PCR, using as primers the TS oligonucleotide and a 24 mer oligonucleotide partially complementary to the telomeric repeats (ACT primer).[27] The assay we used was modified with respect to the standard TRAP method, since perylene derivatives inhibit Taq polymerase. To overcome this problem, after telomerase extension step, the reaction mixtures were purified from the drugs by phenol/chlorophorm extraction.

It was known[15] that PIPER is able to inhibit telomerase at higher concentrations than 20 μM, so we have considered drugs concentration in the range between 5 and 40 μM. As expected, PIPER has no effect at concentrations lower than 20 μM. The behavior of PIPER3 and DAPER is remarkably different since they are able to inhibit telomerase activity also in the range between 10 and 20 μM (Fig. 3), acting on both its catalytic activity and processivity. In fact, at those concentrations, we observe the decreasing of the intensity and of the number of bands, corresponding to the telomerase extension products, with respect to the control (no drug added). It is also interesting to note that while at 40 μM drug concentration telomerase activity is still remarkable in the case of PIPER, at the same concentration PIPER3 and DAPER are able to almost completely inhibit the enzyme.

In this study, we show that perylene diimides with different side chains are able to inhibit telomerase to a different extent. The compounds we have studied show also a high selectivity towards different topological G-quadruplex structures. Although further studies are surely necessary to develop molecular models to connect telomerase inhibition with selective induction of G-quadruplex structures, the obtained results suggest the intriguing possibility that different G-quadruplex structures could be selective in telomerase inhibition, as previously suggested in the case of porphyrins.[25] This can derive either by different kinetics in inducing tetrameric and dimeric G-quadruplex structures or by different thermodynamic stabilization of the distinct structures by perylene derivatives having side chains able to establish different interactions with G-quadruplex grooves.

The synthesis and the study of a number of perylene derivatives with different side chains, which has at the moment being carried out in our laboratory, as well as the evaluation of their association constants with tetrameric and dimeric G-quadruplex structures will allow to clarify the role of charged side chains in inducing and stabilizing different G-quadruplex structures. On the basis of the results here reported, this appears a relevant topic in the finding of new perylene derivatives that could inhibit telomerase at nanomolar concentrations, as required for a possible therapeutic use.

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20. Perylene derivatives (PIPER, PIPER2, PIPER3) were prepared from 3,4,9,10-perylenetetracarboxylic dianhydride and 1-(2-aminoethyl)piperidine, 2-(2-aminoethyl)pyridine and 1-(2-aminoethyl)piperazine, respectively, in a refluxing mixture of *N,N*-dimethylacetamide and 1,4-dioxane. The reaction products were precipitated adding water and separated by filtration. The resulting products were purified dissolving in HCl solution and precipitating the respective hydrochlorides with acetone.

PIPER: ^{1}H NMR (CF_3CO_2D) δ 1.8–2.0 (2H), 2.1–2.5 (10H), 3.37 (4H), 3.95 (4H), 4.19 (4H), 5.06 (4H), 9.0–9.2 (8H); ^{13}C NMR (CF_3CO_2D) δ 16.7, 19.0, 31.8, 51.2, 52.5, 117.5, 120.5, 122.5, 125.3, 129.3, 132.5, 162.2; elemental analysis calculated ($C_{38}H_{36}N_4O_4$): %C = 74.5%H = 5.9%N = 9.2, found: %C = 73.9%H = 6.1%N = 9.0.

PIPER2: (hydrochloride) ^{1}H NMR (CF_3CO_2D) δ 4.00 (4H), 5.09 (4H), 8.26 (2H), 8.45 (2H), 8.86 (2H), 9.00 (2H), 9.0–9.2 (8H); ^{13}C NMR (CF_3CO_2D) δ 26.0, 33.0, 115.0, 117.9, 119.2, 119.9, 121.9, 122.8, 126.6, 129.8, 134.5, 141.3, 147.5, 159.1; elemental analysis (base) calculated ($C_{38}H_{24}N_4O_4$): %C = 76.0%H = 4.0%N = 9.3, found: %C = 75.3%H = 4.2%N = 9.25

PIPER3: ^{1}H NMR (CF_3CO_2D) δ 3.9–4.4 (16H), 4.67 (4H), 5.03 (4H), 8.9–9.1 (8H); ^{13}C NMR (CF_3CO_2D) δ 31.2, 37.8, 45.5, 52.9, 117.4, 120.5, 122.5, 125.4, 129.3, 132.6, 162.2; elemental analysis calculated ($C_{36}H_{34}N_6O_4$): %C = 70.4%H = 5.5%N = 13.7, found: %C = 69.1%H = 5.9%N = 13.6

DAPER[19] was purchased from Pierce, as a solution 1 mM in MES buffer (10 mM, pH 6.5).
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