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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2292–2304
Bioorganic & Medicinal Chemistry

The number and distances of positive charges of polyamine side chains in a series of perylene diimides significantly influence their ability to induce G-quadruplex structures and inhibit human telomerase

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Received 8 October 2007; revised 16 November 2007; accepted 23 November 2007
Available online 31 December 2007

Abstract—We have synthesized eight polyamine perylene diimides to conjugate the efficiency of perylene derivatives in stabilizing G-quadruplex structures and the polyamines' biological activity, due to specific interactions with different DNA domains. Our study was carried out by investigating the ability of these derivatives to induce inter- and intramolecular G-quadruplex structures by polyacrylamide gel electrophoresis (PAGE) and to inhibit telomerase in a modified TRAP assay. The two properties appear to be satisfactorily correlated and they show that the number and distances of positive charges in the side chains dramatically influence both these features. Although our previous studies on perylene derivatives with mono-positively charged side chains indicated that self assembly in aqueous solution leads to a major efficiency, the result observed with the spermine derivative suggests that a too strong aggregation is unfavourable, because it determines a lower solubility of the compounds.

1. Introduction

Immortality and the related high proliferation of cancer cells is often due to telomerase activation.[1] Telomerase is a specific reverse transcriptase with an endogenous RNA template; it has a fundamental role in the maintenance of the eukaryotic chromosomes' terminal ends, the telomeres, which are characterized by a repeated G-rich DNA, specific proteins[2] and a single-stranded terminal region,[3] which shortens when a cellular replication occurs ('end replication problem'). DNA polymerase is unable to fully replicate the 3′-end of chromosomes and so the non-coding telomeric region of DNA acts to protect chromosomes from the risk of loosing genetic information when cells replicate. Normal somatic cells do not exhibit telomerase activity and their DNA reaches a critic length (Hayflick limit[4]), after which cellular senescence occurs. The majority of cancer cells (>85%) show telomerase activity, where the enzyme lengthens the single-stranded region of the telomere through addition of discrete repeats of bases, thereby overcoming the Hayflick limit and giving rise to the cellular immortalization. Due to the selective activation of telomerase in cancer cells compared to normal cells, the enzyme has been proposed as target for non-cytotoxic anticancer agents.[5,6] One of the strategies to inhibit the enzyme is the modification of its substrate; since telomeric DNA contains a single-stranded G-rich 3′-overhang, it is possible to induce the formation of G-quadruplex structures, in order to keep telomerase from lengthening chromosome ends.

The G-quadruplex[7] is an unusual DNA secondary structure, based on Hoogsteen G–G pairing, which gives rise

Keywords: Polyamine side chained perylene derivatives; G-quadruplex; Telomerase inhibitors.
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doi:10.1016/j.bmc.2007.11.065

to a hydrophobic planar ring of four guanines. The G-tetrad is stabilized by the co-ordination of a monovalent cation in the hole[8,9]; multiple G-tetrads can stack together by π–π aromatic interactions to form a complex quadruple helix, with four negatively charged grooves. G-quadruplex can adopt different topologies depending on the glycosidic bond conformation, on the monovalent cation[10] and on the oligonucleotide sequence.[11,12] These features give rise to many kinds of structures, different in number, orientation, polarity of the chains, loops and grooves.[13] Many studies showed that G-quadruplex structures appear to be involved in many important biological activities, such as DNA replication, gene expression and recombination,[14] and their presence in vivo has been demonstrated[15,16] in a number of biological systems.

G-quadruplex can be stabilized by the presence of small organic molecules, which consequently act as telomerase inhibitors. The types of molecules that are able to stabilize G-quadruplexes are characterized by an aromatic core, that favours stacking interactions with the G-tetrads, and basic side chains (positively charged in physiological conditions), which interact with the quadruple helix grooves, according to the model of threading intercalation.[17,18] In order to inhibit telomerase, many molecules with these characteristics have been synthesized: anthraquinones[19,20] and 3,6-disubstituted acridines[19] are examples of the first generation of these inhibitors. Subsequently, tri-substituted acridines[21] and porphyrins[22] have been synthesized to improve the efficiency in inhibiting telomerase. Natural compounds, such as berberine[23] and telomestatin,[24] have also been shown to inhibit human telomerase, the latter with high efficiency. Perylene diimides present optimal features to interact with the G-quadruplex and show a good ability to induce different G-quadruplex structures and to inhibit telomerase, depending on the side chains' basicity and length.[25] Our research group has recently synthesized new highly hydrosoluble perylene derivatives[26] and coronene derivatives[27] with various side chains.

In this work, we present a series of perylene derivatives with polyamine side chains (Fig. 1), with the aim to evaluate the role of the number and the distance of positive charges in the side chains on G-quadruplex induction and telomerase inhibition. Many polyamines are well known, both as natural and synthetic products. Natural polyamines (spermine, spermidine, putrescine) play a key role in many genetic processes, such as DNA replication and gene expression.[28] They are also involved in cellular growth, proliferation and differentiation. Acetylation of polyamines can play many biological roles, above all in the regulation of polyamine activity.[29] Natural polyamine metabolism is strongly regulated because they can interact with DNA: they are positively charged in physiological conditions and therefore able to form specific electrostatic bonds with the DNA phosphate backbone. They can interact with A, B and Z DNA,[30] and have also been demonstrated to induce structural transitions among these conformations.[31] Alterations in their metabolism are involved in cancer development, so they themselves have been identified as new anti-

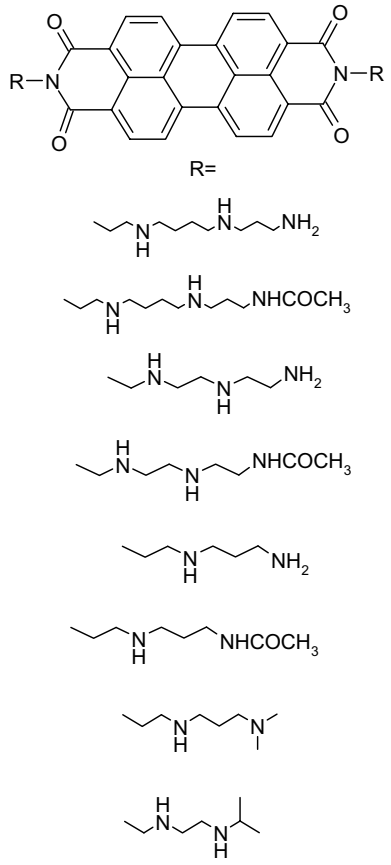


Figure 1. Structures of the eight perylene diimides with different polyamine side chains.

cancer targets.[32] In particular, spermine shows a good efficiency in interacting with G-quadruplex DNA and triethylenetetramine, a synthetic polyamine, shows the ability both to stabilize G-quadruplexes structures and to inhibit telomerase.[33] As polycations, these compounds are able to induce the same G-quadruplex structures as inorganic cations[34] and their interaction with DNA has been used for the synthesis of functionalized perylene derivatives as molecular fluorescent dyes.[35] Although these studies indicate that polyamines can interact specifically with different DNA structures, a study of the influence of the number and distances of the positive charges in polyamines or polyamine derivatives on their ability to induce G-quadruplexes and inhibit telomerase is still lacking. The eight new polyamine perylene derivatives hereby reported present different numbers and distances of positive charges in their side chains. Our results show that these two parameters are important in determining the ability of these compounds to self-assemble in water solution, to induce the G-quadruplex and to inhibit telomerase.

2. Results and discussion

In this work, the synthesis, the physico-chemical properties and the biological activity of eight perylene diimides are reported. These new compounds are characterized by side chains containing polyamines, which differ for

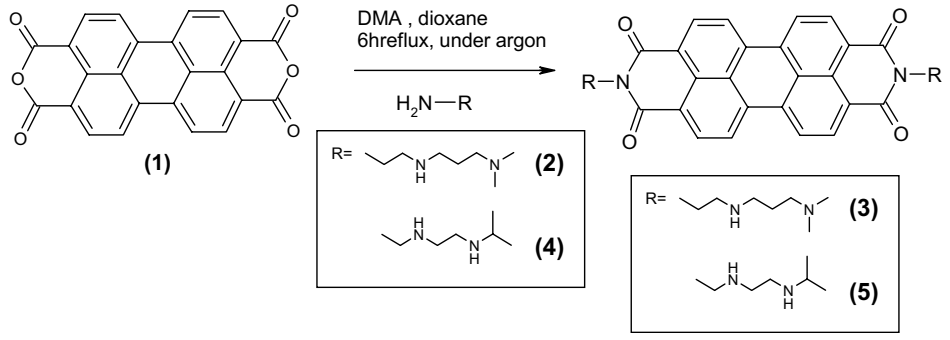
three features: the number of basic sites, the distance between them and the nature of the terminal group (primary, secondary, tertiary amine or amido-group, Fig. 1).

2.1. Chemical synthesis

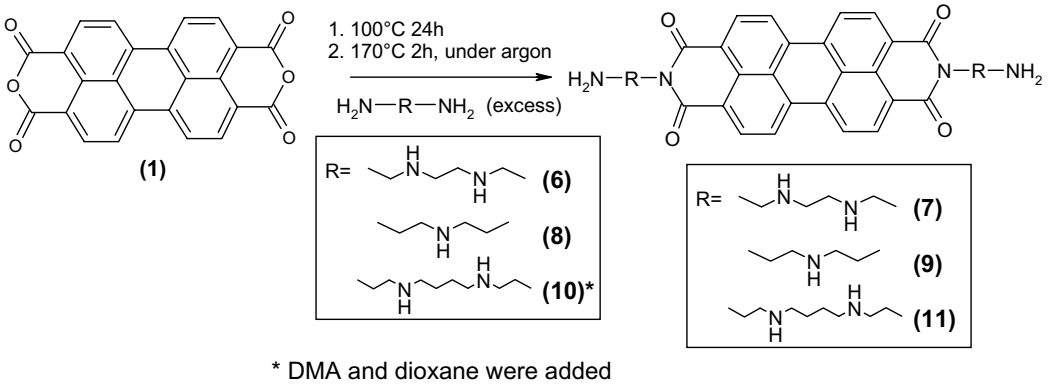
Polyamines used as reactants are characterized by the presence of one (**2** and **4**, Scheme 1) or two (**6**, **8** and **10**, Schemes 2 and 3) primary amines as terminal groups. Therefore, in order to avoid undesired side reactions, two different synthetic schemes for the synthesis of perylene diimides were carried out, depending on the nature of the amine used: when only one free primary amino group was present, Scheme 1 was followed, in the case of two primary amino groups, Scheme 2 was adopted, unless one of the two groups was previously protected, according to Scheme 3.

2.1.1. Scheme 1. The perylene diimides **3** and **5** were obtained by treatment of the perylene anhydride **1** with an almost stoichiometric amount of polyamines **2** and **4**, respectively (only 15% excess of reactant was used), in DMA/dioxane under argon. After heating for 6 h under reflux, the reaction was quenched by NaOH solution and the mixture was cooled at 4 °C overnight. The red precipitate was filtered, washed repeatedly with water and dried under vacuum to obtain the basic form of the desired product. Differently from the previously described preparations with mono-amino side chains,[25] the remaining solution was strongly coloured, suggesting the presence of a significant amount of the products. This was because the polyamine side chains are sufficiently polar to give rise to a satisfactory water solubility of the synthesized compounds, even in these conditions. In order to eliminate the polyamines that did not react, the water solution was extracted with chloroform. Finally, the hydrochlorides of compounds **3** and **5** were obtained by adding a concentrated solution of HCl to the aqueous layer, in order to guarantee the complete saturation of the basic sites, and precipitating the respective hydrochlorides with acetone.

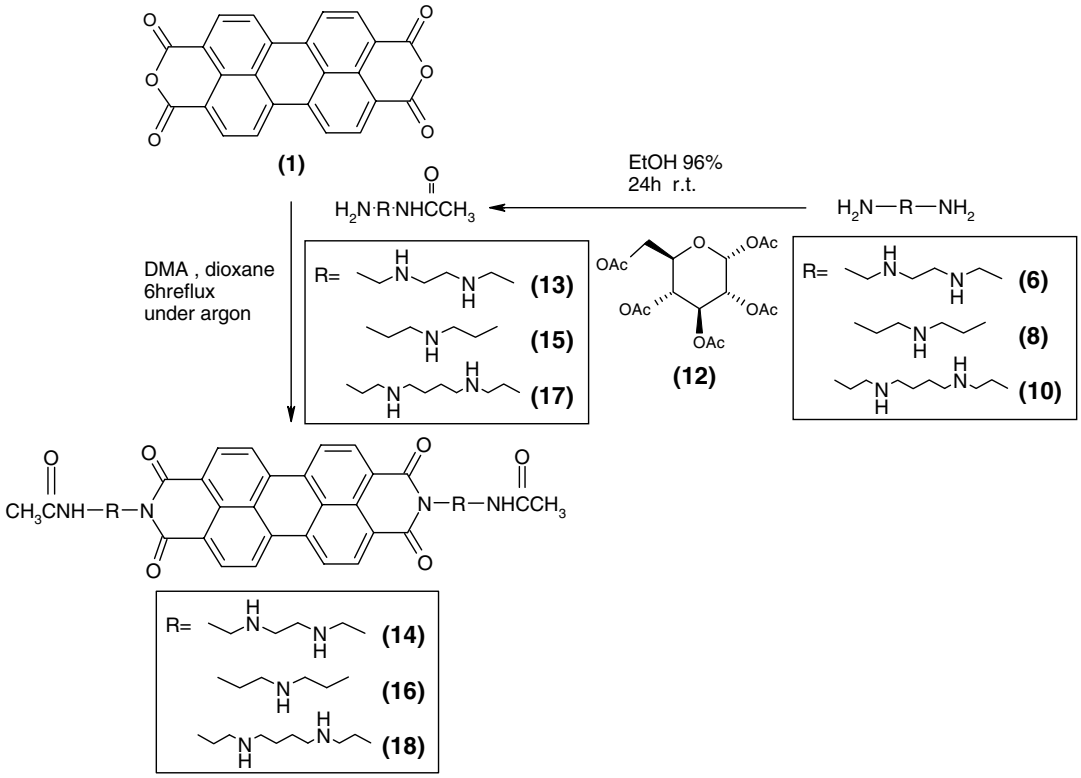
2.1.2. Scheme 2. The perylene diimides **7**, **9** and **11** were obtained by treatment of the perylene anhydride **1** with a large excess of polyamines **6**, **8** and **10**, respectively. The large excess of reactants avoided the polymerization which could occur. Compounds **6** and **8** are liquid at room temperature, so that they assumed the roles of both reactants and solvents, while, **10** being a solid, a mixture of DMA and dioxane was used as solvents in this case. The reaction was conducted under argon atmosphere, heating under stirring at 100 °C for 24 h, then at 170 °C for 2 h.[36] The purification of the products was challenging, because of the physico-chemical properties of the synthesized compounds, quite similar to those of the reacting polyamines. After quenching the crude mixture as described in Scheme 1, solvents were eliminated by filtration; the residue was then dissolved in HCl solution and precipitated as a hydrochloride, by adding acetone. The acetone solution showed a white opalescence after the complete precipitation of the salts



Scheme 1.



Scheme 2.



Scheme 3.

of **7**, **9** and **11**, due to the partial precipitation of the most soluble hydrochlorides of the residue reacting amines. Their precipitation is slower than that of the products, so the final excess of polyamines was eliminated by washing the products with acetone, until the loss of the opalescence.

2.1.3. Scheme 3. This synthetic pathway was elaborated to operate in stoichiometric conditions also with polyamines **6**, **8** and **10**, to avoid the purification difficulties due to the excess of reactants. The polyamines were monoacetylated at one of the terminal amino groups, obtaining the N^1—monoacetylated compounds **13**, **15** and **17**, which can react with perylene anhydride **1** under the same conditions of Scheme 1, to obtain perylene diimides **14**, **16** and **18**. The acetyl group was chosen as a 'protecting group' because it is simple to add and because of the many interesting biological roles of N^1- and N^8-monoacetyl polyamines.[29] The risk of this reaction is to obtain the N^1—diacetylated compound as the main product, so that a strictly stoichiometric amount of the mild acetylant α-D-glucose pentaacetate**12** was used. The reaction was conducted at room temperature for 24 h, under stirring, in 96% ethanol solution.[37] Because of the presence of a small amount of diacetylated product and of a discrete amount of the reactants, which did not react completely in these mild conditions, the monoacetylated products were purified by column chromatography on silica gel ($CHCl_3$/MeOH/$CH_3CH_2CH_2OH$/NH_3 = 5:2:2:1).[37] The pure compounds **13**, **15** and **17** obtained were used as reactants in the reaction with **1**, in the same conditions of Scheme 1, obtaining compounds **14**, **16** and **18**, respectively.

2.2. Spectroscopic properties and self-aggregation of the polyamine perylene derivatives

Absorption spectroscopy can be a good tool to evaluate the self assembly of polyamine perylene derivatives in water solution. In fact, the absorption spectra between 350 and 650 nm in MES/KCl buffer solution (pH 6.5) give clear indications that all the synthesized compounds can self-interact in solution. Their extinction coefficients are characterized in all cases by a strong hypochromic effect and the bands present a large broadening with respect to the spectra in organic solvent, as shown in Figure 2 and in Supplementary data. This indicates that perylene moieties are stacked in water, as previously discussed.[25] Surprisingly, the hypochromic effect in water solution is significantly higher in the case of POL-1 and POL-5 with respect to their monoacetylated derivatives. This suggests that the assumption that the higher the number of positive charges in the side chains the greater the water solubility is surely too simple, while it is necessary to take into account the complexity of the interactions with water structure. However, as previously observed,[25] a certain degree of stacking is important in determining the ability of these compounds to induce G-quadruplex structures (see the following section), although a quantitative correlation between these two features (amount of self-stacking and ability of G-quadruplex induction) was not found so far and the underlying mechanism requires surely further investigation. In particular, it has been recently shown by Palumbo and coworkers[25d] that strong drug self-aggregation is related to a minor telomerase inhibition and weaker interactions with the G-quadruplex, suggesting

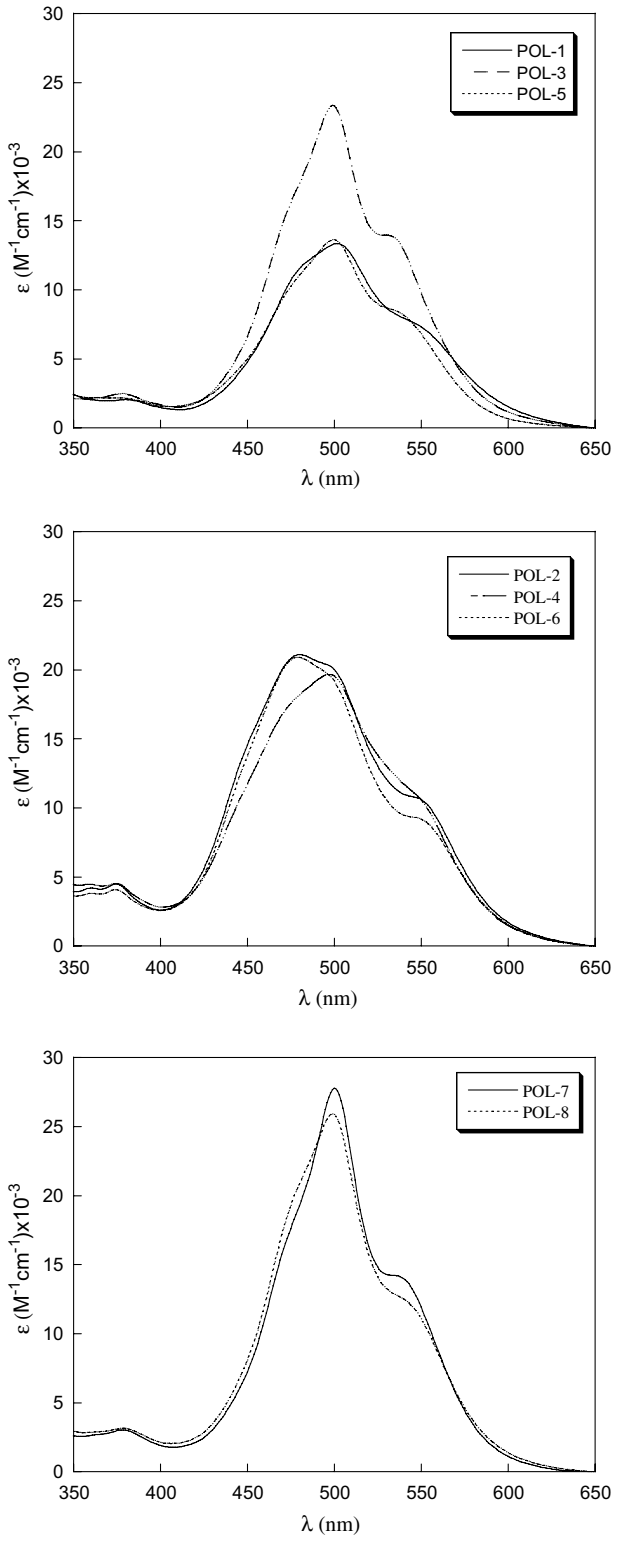


Figure 2. UV–vis absorption spectra of perylene derivatives **POL-1/8** in MES–KCl aqueous buffer (pH 6.5).

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.[27]

2.3. Inter- and intramolecular G-quadruplex DNA induction

The ability of the polyamine perylene derivatives to induce G-quadruplex structures was investigated by polyacrylamide gel electrophoresis (PAGE)[25] using the DNA oligomer TSG4 (5′-GGGATTGGGATTGGGATT GGGTT-3′). TSG4 is able to form stable intramolecular G-quadruplex and can act as a substrate for telomerase elongation in a modified TRAP assay.[38] This oligonucleotide was used, in our previous works, on berberine,[23] perylene[25,26] and coronene[27] derivatives. The oligonucleotide was incubated in the presence of increasing concentrations of polyamine perylene derivatives and the formation of quadruplex structures was investigated by PAGE analysis as reported in Figure 3. Two different bands were observed, that were identified as single strand DNA (ss) and monomeric G-quadruplex (M). The assignment of the bands was possible by comparison with the structures induced by 40 μM PIPER,[25] that was introduced in each PAGE as an internal standard. The intramolecular G-quadruplex (M) corresponds to the band showing the highest mobility; its high folded structure favours the faster running in the gel grid with respect to DNA single strand, which has the same molecular weight. It is worth noting that this increase of mobility can be less than that expected for naked DNA, since it is reasonable to suppose that one or more drug molecules could bind to the G-quadruplex structure: this explains the observed shift of this band.

The perylene derivatives, indicated as POL-1 and POL-2, which are characterized by the spermine or monoacetylated spermine as side chains, are unable to induce the intramolecular G-quadruplex (Fig. 3). In the case of POL-1, this can be attributed to its low water solubility, which also gives easily rise to aggregation. All the other perylene derivatives, except for POL-7, are able to induce the intramolecular G-quadruplex: for these six compounds we have evaluated the decrease of the free single-strand DNA at different drug concentrations (Table 1) to compare their efficiency. As in our previous papers,[25–27] we preferred this method to the evaluation of the monomeric G-quadruplex concentration, since this second approach is affected by a larger error, due to the binding of the drug. The trend appears to be more favourable in the case of POL-3 and POL-5, with respect to their monoacetylated derivatives (POL-4 and POL-6, respectively) and in the case of POL-8 with the decrease of distance between charges with respect to POL-7.

Since the more effective molecules appear also to be able to induce small amounts of intermolecular G-quadruplex, we have carried out similar studies, using an oligonucleotide able to form only intermolecular structures, 2HTR (5′-AATCCGTCGAGCAGAGTTAGGGTTA GGGTTAG-3′).[25] All the eight polyamine perylene derivatives are able to induce intermolecular G-quadruplex structures (Fig. 4). The obtained results reported in Figure 4 show that all the studied molecules are less efficient in inducing inter- with respect to intramolecular G-quadruplex, since, in the former case, also at the highest drug concentration, a substantial amount of

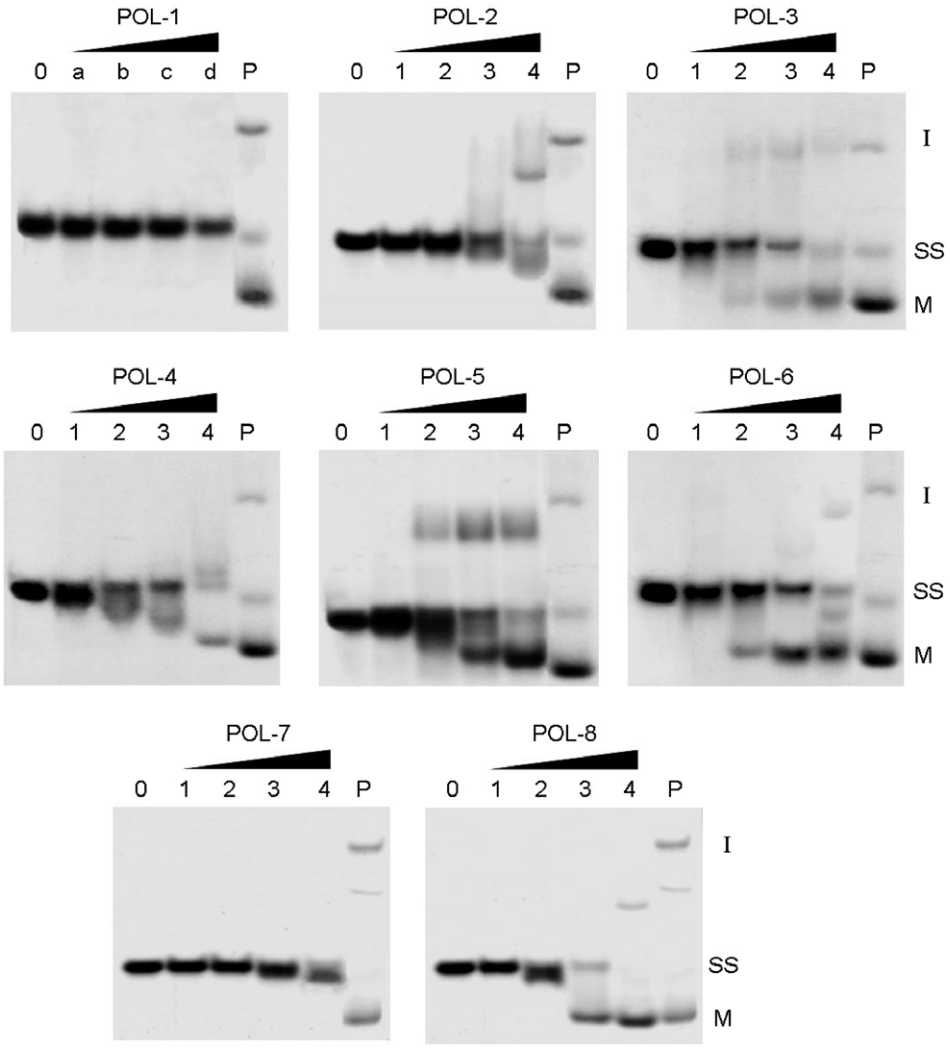


Figure 3. G-quadruplex structure formation induced by the eight polyamine side-chain perylene derivatives, studied by native PAGE. (A) Typical autoradiographies obtained using TSG_4 oligonucleotide (12 μM) in the presence of different drug concentrations: 1 μM (lane a), 2 μM (lane b), 5 μM (lane c and lane 1), 10 μM (lane d and lane 2), 20 μM (lane 3), 40 μM (lane 4) and with no drug (lane 0). As standard in lane P the structures induced by PIPER at concentration 40 μM were reported. Major bands were identified as single-stranded DNA (SS), monomeric (M) and intermolecular (I) G-quadruplex.

Table 1. Percentage of single strand DNA decrease as evaluated by PAGE with TSG_4 oligonucleotide (Fig. 3) at the indicated concentrations

Compound	10 μM	20 μM
POL-3	55%	30%
POL-4	56%	55%
POL-5	32%	25%
POL-6	70%	26%
POL-7	86%	68%
POL-8	47%	17%

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

single strand DNA is present, and the behaviour of the eight perylene derivatives appears practically equal in the limit of experimental errors (Supplementary data).

2.4. Biological activity (TRAP assay)

The ability of the eight polyamine perylene derivatives to inhibit human telomerase was investigated in a cell-free system by means of a modified Telomerase Repeat Amplification Protocol (TRAP).[38] Taking into account that the influence of six of eight molecules in inducing the intramolecular G-quadruplex appears significantly larger than in inducing intermolecular G-quadruplex structures, we have carried out the TRAP assay using TSG4 as a telomerase substrate.[26,27] This oligonucleotide is able to fold into the intramolecular G-quadruplex at the KCl concentration used in the TRAP assay (68 mM). Nevertheless, a similar structure may be efficiently unfolded and extended by telomerase, unless a suitable concentration of G-quadruplex stabilizing molecule is present. A typical experiment for each molecule is reported in Figure 5a and the results obtained are reported as an histogram in Figure 5b.

The results obtained by performing the TRAP assay, in the absence and in the presence of the polyamine perylene derivatives in the 2–20 μM concentration range, indicate a large difference in their ability to inhibit telomerase. Namely POL-3 and POL-5 are effective

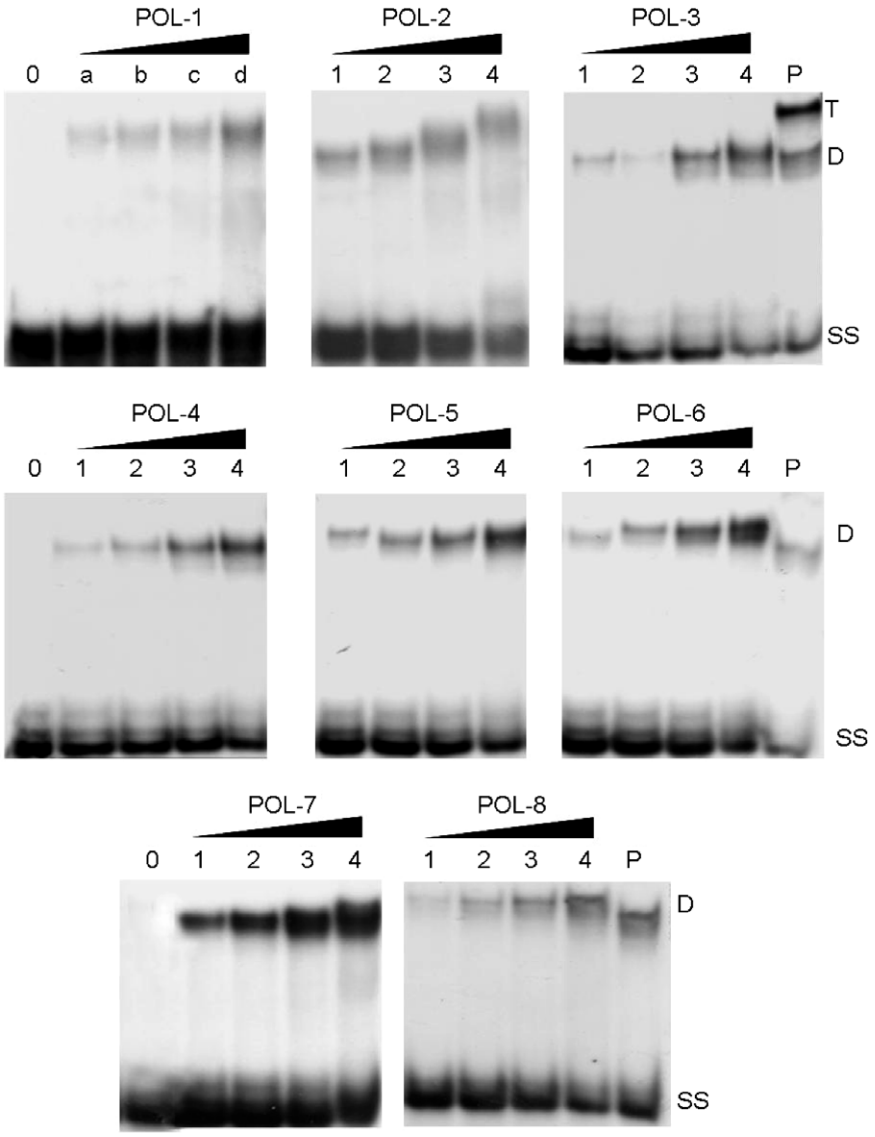


Figure 4. G-quadruplex structure formation induced by the eight polyamine side-chain perylene derivatives, studied by native PAGE. (A) Typical autoradiographies obtained using 2HTR oligonucleotide (12 μM) in the presence of different drug concentrations, as indicated in the legend of Figure 3. Major bands were identified as single-stranded DNA (SS), dimeric (D) and tetrameric (T) G-quadruplex.

telomerase inhibitors with respect to their acetylated counterparts POL-4 and POL-6, while POL-8 is a better inhibitor than POL-7. In the case of POL-1 (and analogously POL-2 for comparison) the drug concentration values were decreased to 2, 5 and 7 μM, for solubility reasons. POL-2 has surprisingly shown an activity comparable to that of POL-3, despite it is acetylated at the end of the side chain. POL-3 clearly emerges as the most efficient molecule in inhibiting the enzyme, while POL-5 and POL-8 are both efficient, but at higher drug concentration with respect to POL-3.

3. Conclusions

The reported results show a strong selectivity of polyamine perylene derivatives in inducing G-quadruplex structures and in inhibiting telomerase in a cell-free system. The structural features of the side chains which seem to be significant in determining the behaviour of the different compounds are the distances between positive charges, the charges number and the length of the side chain. As for the first aspect, the distance corresponding to $(CH_2)_2$ appears to be the optimum size: in fact, POL-3 shows the best activity, which decreases if the distance between N atoms is increased to $(CH_2)_3$, as in POL-5. This is also shown by the higher activity of POL-8 with respect to POL-7. However, distance variation should not be considered a critical parameter,[39] due to the high flexibility of polyamines. On the other hand, POL-5 has a basic nitrogen atom less than POL-3, reducing the number of positive charges in physiological conditions. The importance of positive charge number also emerges clearly from the strong decrease of activity if the end of the side chains is acetylated and thus not able to establish electrostatic interactions with the DNA phosphates. This is shown by the behaviour of POL-4 and POL-6 with respect to POL-3 and

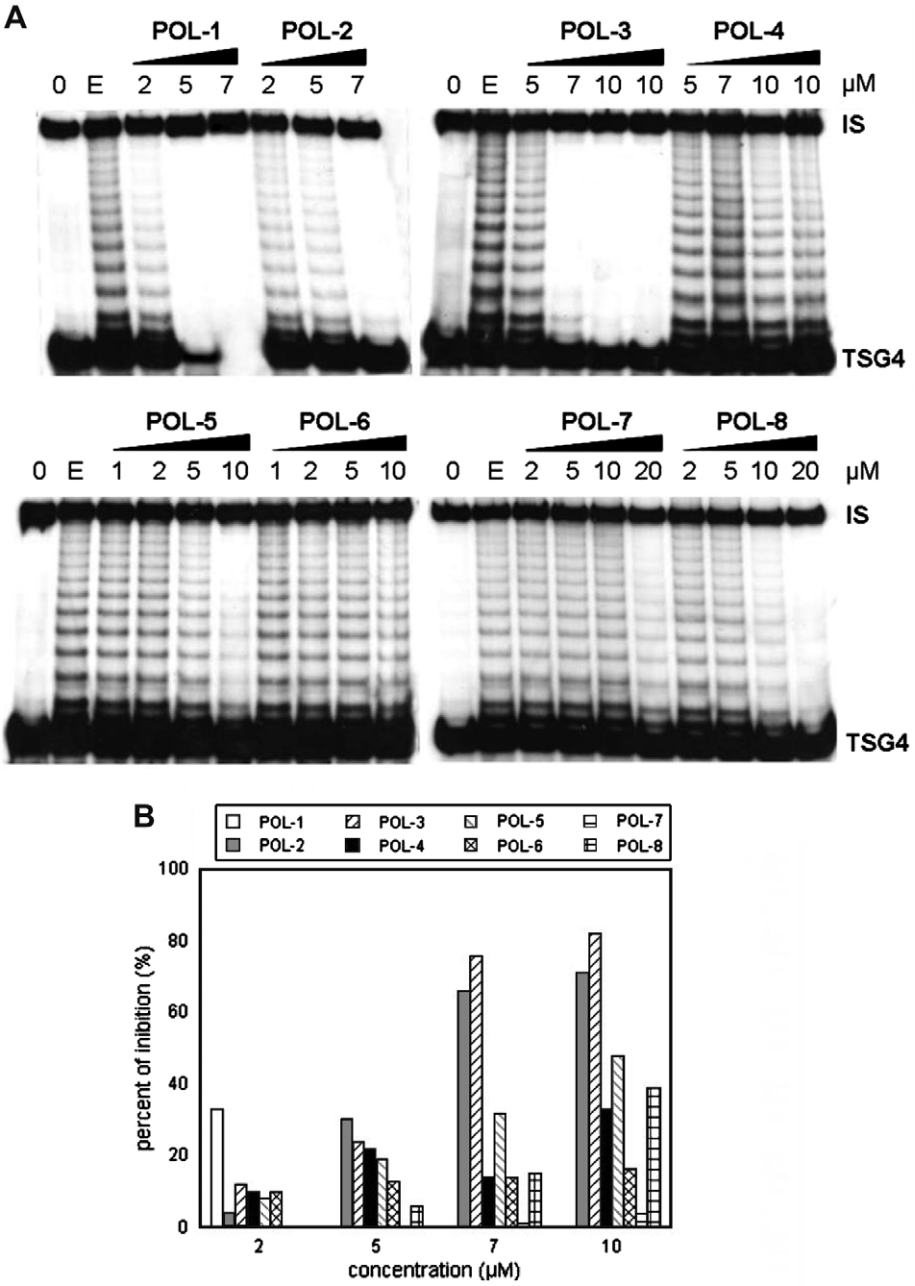


Figure 5. (A) Inhibition of human telomerase by the eight perylene derivatives by telomerase repeat amplification protocol assay (TRAP). Typical autoradiographies obtained using TSG4 oligonucleotide in the presence of the indicated drug concentrations. In lane 0 cell extract was not added, in lane E no drug was added. IS is a 130-bp 'internal standard' to control PCR amplification efficiency. (B) Percentage of telomerase inhibition for the polyamine perylene derivatives at the indicated concentrations. The results of POL-1 are not reported because at concentrations higher than 2 μM precipitation occurs. Errors estimated on at least three independent experiments are about ±5%.

POL-5. POL-2 shows a surprising behaviour, being almost as efficient as POL-3 in terms of activity in the TRAP assay, despite its terminal acetylation. In principle, POL-1, carrying the same side chain as POL-2 without the terminal acetyl group, should be even more active than POL-2. The activity of POL-1 in the TRAP assay looks very good indeed, since it shows about 30% of inhibition at 2μM (Fig. 5b). Unfortunately, its poor solubility and the great tendency to self-aggregate in water (Fig. 2) cause precipitation of drug and DNA at higher concentrations (Fig. 5a). It is interesting to note that POL-1 and POL-2 are not good inducers of the G-quadruplex, as derived by PAGE studies, but their ability to inhibit telomerase could be related to their stabilization of the G-quadruplex. In fact, in the modified TRAP assay a preformed G-quadruplex structure by TSG4 oligonucleotide is expected to be present.

In the case of G-quadruplex/spermine interactions, recent studies carried out with NMR[40] showed that this polyamine interacts strongly with the G-quadruplex and suggest that its binding could derive from two different mechanisms: the spanning of the quadruplex groove or the interaction with the fold of quadruplex loops.

Table 2. Medium number of H-bonds per structure as derived by the 25 lowest energy complexes between the indicated ligands and the monomeric G-quadruplex (Fig. 6) obtained by simulated annealing[27]

Compound	POL-3	POL-5	POL-7	POL-8
Hbonds/structure	5.0	2.9	1.8	2.2

However, it is reasonable to assume that both these interactions are disfavoured by the conjugation of the polyamines with the perylene moiety. We suggest that this conjugation leads to the formation of micellar aggregates, which are too large in the case of POL-1 to allow the induction of the G-quadruplex, causing also the poor water solubility of this compound, although further investigations are necessary to safely assess this topic.

Moreover, hydrogen bonding is surely another very important feature in determining the different ability of the studied compounds to interact with the G-quadruplex structure. In fact, performing simulated annealing experiments of the analyzed compounds on a human intramolecular G-quadruplex[41] as previously described,[27] we estimated the medium number of H-bonds per structure as derived by the 25 lowest energy complexes (Table 2). We excluded from this analysis the acetylated compounds, because of their poor activity in the PAGE study, and the spermine containing derivative POL-1, because of its poor solubility. The values obtained for the analyzed compounds show a good correlation with their efficiency in inhibiting telomerase. In particular, POL-3, which is the best efficient compound of this series, shows a very strong tendency to make hydrogen bonding with the G-quadruplex structure. In Figure 6, the ability of POL-3 to make a multiple pattern of H-bonds is clearly shown, differently from the other compounds.

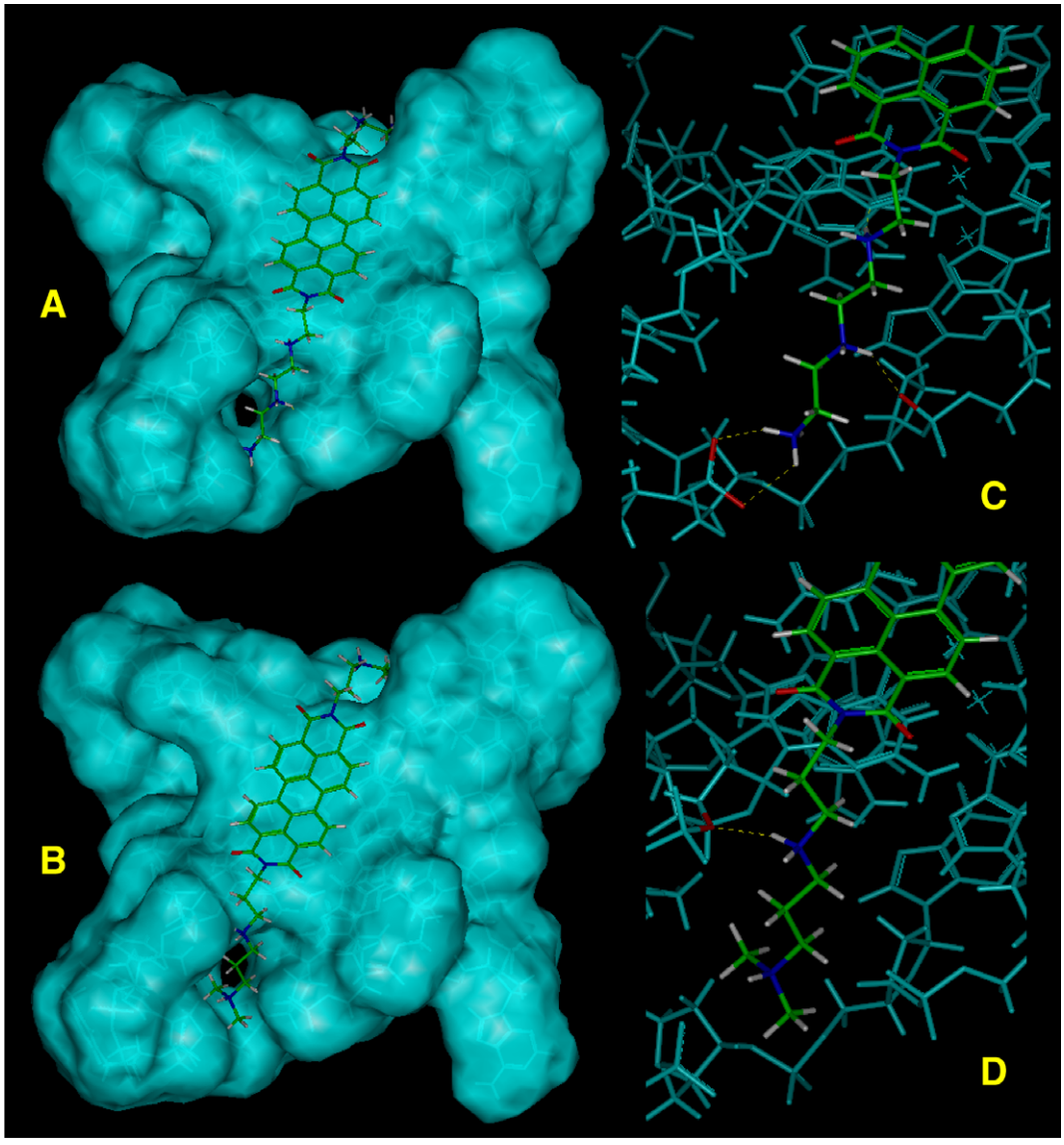


Figure 6. Models obtained by molecular modelling[27] for the complexes between a monomeric G-quadruplex[41] (blue) and the perylene ligands **POL-3** (A–C) and **POL-7** (B–D). Semi-transparent surfaces are in blue for the DNA (A,B), while ligands are atom-type coloured. H-bonds are evidenced as dotted yellow lines (C,D), with red coloured oxygen atoms on DNA phosphates when involved in hydrogen bonding.

From this research, POL-3 combines a good water solubility, an efficient ability to induce G-quadruplex structures and an activity of telomerase inhibition under 5 μM, improving all these aspects with respect to the previously synthesized perylene diimides,[25] as in the case of three and four side-chained perylene derivatives.[26] For these reasons, POL-3 clearly emerges to be considered as the best polyamine perylene diimide, which surely deserves further testing in biological systems. These studies have been currently carried out in our laboratories.

4. Experimental

4.1. Chemistry

All the commercial reagents and solvents were purchased from Aldrich. TLC plates (silica gel 60 F_{254}) and silica gel 60 (0.063–0.200 mm) for column chromatography were purchased from Merck. NMR spectra were obtained with Varian Gemini 200 and Varian Mercury 300 instruments. High resolution ESI-MS spectra were recorded on Micromass Q-TOF MICRO spectrometer. Elemental analyses (C, H, N) were carried out on EA1110 CHNS-O (CE instruments).

4.1.1. Synthesis of compounds 3 and 5: General procedure (Scheme 1). Perylene anhydride (**1**) was treated with the appropriate polyamine (**2** or **4**), in a refluxing mixture of *N*,*N*-dimethylacetamide (DMA, 15 ml) and 1,4-dioxane (15 ml) for 6 h.[25] The basic forms of the products (**3** and **5**, respectively) were precipitated by adding a diluted solution of aqueous NaOH, separated by filtration and dried under vacuum. The solution was extracted with chloroform and the aqueous layer was acidified with concentrated HCl. Hydrochlorides of the products were precipitated with acetone.

4.1.1.1. Synthesis of *N*,*N*′-bis(7-dimethylamino-4-azaheptyl)-3,4,9,10-perylenetetracarboxylic diimide (3). Compound **1** (1.02 g) was treated with 1 ml of *N*,*N*-dimethyldipropylenetriamine (**2**), according to the general procedure described, so that both the basic form (462 mg) and the hydrochloride (1.38 g) of compound **3** were obtained. The overall yield was 92%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.20 (4H, d, J = 8 Hz, aromatic H), 9.15 (4H, d, J = 8 Hz, aromatic H), 4.88 (4H, m, N_{imidic}—CH_2), 3.81 (12H, m, N_{aminic}—CH_2), 3.44 (12H, s, N—CH_3), 3.0–2.8 (8H, br, CH_2—CH_2—N_{aminic}) ppm. ^{13}C NMR (CF_3COOD): δ 162.3 (C=O), 132.4 (ar), 129.2 (ar), 125.4 (ar), 122.5 (ar), 120.5 (ar), 117.8 (ar), 51.4, 42.8, 41.6, 39.4, 33.7, 20.6, 18.0. MS (ESI) *m*/*z*: 675.3663 [(M+H)$^+$] (calcd for $C_{40}H_{47}N_6O_4$: 675.3659).

4.1.1.2. Synthesis of *N*,*N*′-bis(5-isopropylamino-3-azapentyl)-3,4,9,10-perylenetetracarboxylic diimide (5). Compound **1** (0.99 g) was treated with 1.1 ml of N^1—isopropyldiethylenetriamine (**4**), according to the general procedure described, so that both the basic form (0.97 g) and the hydrochloride (260 mg) of compound **5** were obtained. The overall yield was 73%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.24 (4H, d, J = 8 Hz, aromatic H), 9.14 (4H, d, J = 8 Hz, aromatic H), 5.18 (4H, m, N_{imidic}—CH_2), 4.3–4.1 (12H, m, N_{aminic}—CH_2), 3.96 (2H, m, J = 6 Hz, N_{aminic}—CH $(CH_3)_2$), 1.78 (12H, d, J = 6 Hz, —CH_3) ppm. ^{13}C NMR (CF_3COOD): δ 162.7 (C=O), 132.9 (ar), 129.2 (ar), 125.5 (ar), 122.5 (ar), 120.5 (ar), 117.7 (ar), 50.3, 45.1, 41.7, 38.2, 33.4, 13.4. MS (ESI) *m*/*z*: 647.3345 [(M+H)$^+$] (calcd for $C_{38}H_{43}N_6O_4$: 647.3346).

4.1.2. Synthesis of compounds 7, 9 and 11: General procedure (Scheme 2). Perylene anhydride (**1**) was treated with a large excess of the appropriate polyamine (**6**, **8** or **10**): when it is liquid (**6** and **8**), the amine could be used as a reactant and a solvent, when solid (**10**), DMA and dioxane were added. The mixture was heated at 100 °C for 24 h, then at 170 °C for 2 h. The crude product was treated with a mixture of diethyl ether/1-propanol = 4:1, so that a colloidal precipitate was obtained, which was filtered under vacuum.[36] It was dissolved in HCl solution, then hydrochlorides of the products were precipitated by adding acetone and they were purified by washing many times with acetone, to remove the hydrochloride of the reactant polyamine.

4.1.2.1. Synthesis of *N*,*N*′-bis(8-amino-3,6-diazaoctyl)-3,4,9,10-perylenetetracarboxylic diimide (7). Compound **1** (98 mg) was treated with 2.0 ml of triethylenetetramine (**6**), according to the general procedure described, so that 96 mg of the hydrochloride of compound **7** were obtained, with a yield of 44%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.19 (4H, d, J = 8 Hz, aromatic H), 9.09 (4H, d, J = 8 Hz, aromatic H), 5.12 (4H, m, N_{imidic}—CH_2), 4.3–4.0 (20H, br, N_{aminic}—CH_2) ppm. ^{13}C NMR (CF_3COOD): δ 162.8 (C=O), 132.7 (ar), 129.4 (ar), 125.6 (ar), 122.7 (ar), 120.5 (ar), 117.8 (ar), 45.1, 41.9, 41.5, 41.3, 33.9, 33.3. MS (ESI) *m*/*z*: 649.3273 [(M+H)$^+$] (calcd for $C_{36}H_{41}N_8O_4$: 649.3251).

4.1.2.2. Synthesis of *N*,*N*′-bis(7-amino-4-azaheptyl)-3,4,9,10-perylenetetracarboxylic diimide (9). Compound **1** (107 mg) was treated with 2.0 ml of bis(3-aminopropyl)amine (**8**), according to the general procedure described, so that 102 mg of the hydrochloride of compound **9**were obtained, with a yield of 48%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.00 (8H, m, aromatic H), 4.71 (4H, m, N_{imidic}—CH_2), 3.68 (12H, m, N_{aminic}—CH_2), 2.9–2.5 (8H, br, CH_2—CH_2—N_{aminic}) ppm. ^{13}C NMR (CF_3COOD): δ 161.7 (C=O), 131.7 (ar), 128.5 (ar), 124.8 (ar), 121.8 (ar), 119.7 (ar), 117.1 (ar), 41.9, 41.0, 33.3, 32.8, 19.9, 19.2. MS (ESI) *m*/*z*: 619.3054 [(M+H)$^+$] (calcd for $C_{36}H_{39}N_6O_4$: 619.3033).

4.1.2.3. Synthesis of *N*,*N*′-bis(12-amino-4,9-diazadodecyl)-3,4,9,10-perylenetetracarboxylic diimide (11). Compound **1** (72 mg) was treated with 0.82 g of spermine (**10**), according to the general procedure described, in

2 ml of anhydrous dioxane and 2 ml of DMA, so that 111 mg of the hydrochloride of compound **11** was obtained, with a yield of 62%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.13 (8H, m, aromatic H), 4.97 (4H, m, N_{imidic}—CH_2), 3.86 (20H, m, N_{aminic}—CH_2), 2.9–2.5 (16H, br, CH_2—CH_2—N_{aminic}) ppm. ^{13}C NMR (CF_3COOD): δ 161.4 (C=O), 131.5 (ar), 128.5 (ar), 124.6 (ar), 121.7 (ar), 119.7 (ar), 117.2 (ar), 43.8, 41.7, 41.2, 35.8, 33.4, 20.1, 19.3, 18.5. Elemental analysis: $C_{44}H_{56}N_8O_4$(6HCl): calcd C 53.9%, N 11.4%, H 6.3%; found C 52.8%, N 10.8%, H 6.2%.

4.1.3. Synthesis of reactants 13, 15 and 17: General procedure. α-D-Glucose pentaacetate (**12**) was dissolved in 40 ml of 96% ethanol, under stirring in conditions of mild heating, and then were gradually added to the opportune polyamine (**6**, **8** or **10**). The crude product is a light yellow dense liquid, which was purified by column chromatography on silica gel ($CHCl_3$/MeOH/$CH_3CH_2CH_2OH$/NH_3 = 5:2:2:1),[37] obtaining the monoacetylated products (**13**, **15** and **17**).

4.1.3.1. Synthesis of the reactant N^1—acetyltriethylenetetramine (13). Compound **12** (0.78 g) was mixed with 1.5 ml of **6**, according to the general procedure described, so that 0.82 g of reactant **13** were obtained, with a yield of 44%.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 3.35 (2H, m, N_{amidic}—CH_2), 2.9–2.6 (10H, br, N_{aminic}—CH_2), 1.95 (3H, s, CH_3—C=O) ppm.

4.1.3.2. Synthesis of the reactant N^1—acetyldipropylenetriamine (15). Compound **12** (0.78 g) was mixed with 1.4 ml of **8**, according to the general procedure described. The crude product was purified as described before, so that 0.51 g of reactant **15** were obtained, with a yield of 28%.

^{1}H NMR (200 MHz, $CDCl_3$) : δ 3.45 (2H, m, N_{amidic}—CH_2), 2.9–2.6 (6H, br, N_{aminic}—CH_2), 1.90 (3H, s, CH_3—C=O), 1.8–1.5 (4H, br, CH_2—CH_2—N_{aminic}) ppm.

4.1.3.3. Synthesis of the reactant N^1—acetylspermine (17). Compound **12** (0.76 g) was mixed with 2.01 g of **10**, according to the general procedure described. The crude product was purified as described before, so that 0.80 g of reactant **17** were obtained, with a yield of 33%.

^{1}H NMR (200 MHz, $CDCl_3$): δ 3.35 (2H, m, N_{amidic}—CH_2), 2.9–2.6 (10H, br, N_{aminic}—CH_2), 1.95 (3H, s, CH_3—C=O), 1.8–1.5 (8H, br, CH_2—CH_2—N_{aminic}) ppm.

4.1.4. Synthesis of compounds 14, 16 and 18: General procedure (Scheme 3). Perylene anhydride **1** was treated with the opportune monoacetyl-polyamine (**13**, **15** or **17**), following the same procedure of Scheme 1.

4.1.4.1. Synthesis of *N,N′*-bis(8-acetamido-3,6-diazaoctyl)-3,4,9,10-perylenetetracarboxylic diimide (14). Compound **1** (312 mg) was treated with 331 mg of **13**, according to the general procedure described, so that both the basic form (124 mg) and the hydrochloride (161 mg) of compound **14** were obtained. The overall yield was 43%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.17 (8H, m, aromatic H), 5.15 (4H, m, N_{imidic}—CH_2), 4.9–4.0 (20H, br, N_{aminic}—CH_2, N_{amidic}—CH_2), 2.67 (6H, s, CH_3—C=O) ppm. ^{13}C NMR (CF_3COOD): δ 174.4 (C=O), 162.5 (C=O), 132.5 (ar), 129.3 (ar), 125.5 (ar), 122.5 (ar), 120.4 (ar), 117.7 (ar), 46.1, 41.7, 41.0, 33.2, 33.0, 16.1 (CH_3—C=O). MS (ESI) *m/z*: 367.1751 [(M+2H)$^{++}$] (calcd for $C_{20}H_{23}N_4O_3$: 367.1770).

4.1.4.2. Synthesis of *N,N′*-bis(7-acetamido-4-azaheptyl)-3,4,9,10-perylenetetracarboxylic diimide (16). Compound **1** (200 mg) was treated with 190 mg of **15**, according to the general procedure described, so that both the basic form (189 mg) and the hydrochloride (90 mg) of compound **16** was obtained. The overall yield was 38%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.05 (4H, d, J = 8 Hz, aromatic H), 8.99 (4H, d, J = 8 Hz, aromatic H), 4.68 (4H, m, N_{imidic}—CH_2), 3.83 (4H, m, N_{amidic}—CH_2), 3.53 (8H, m, N_{aminic}—CH_2), 2.7–2.3 (14H, br, CH_2—CH_2—N_{aminic}, CH_3—C=O) ppm. ^{13}C NMR (CF_3COOD): δ 173.4 (C=O), 162.2 (C=O), 132.3 (ar), 129.0 (ar), 125.3 (ar), 122.3 (ar), 120.2 (ar), 117.6 (ar), 42.2, 41.7, 33.2, 33.1, 21.0, 20.4, 15.5 (CH_3—C=O). MS (ESI) *m/z*: 703.3244 [(M+H)$^+$] (calcd for $C_{40}H_{43}N_6O_6$: 703.3244).

4.1.4.3. Synthesis of *N,N′*-bis(12-acetamido-4,9-diazadodecyl)-3,4,9,10-perylenetetracarboxylic diimide (18). Compound **1** (100 mg) were treated with 136 mg of **17**, according to the general procedure described, so that 121 mg of the hydrochloride of compound **18** were obtained, with a yield of 48%.

^{1}H NMR (300 MHz, CF_3COOD): δ 9.13 (8H, m, aromatic H), 4.76 (4H, m, N_{imidic}—CH_2), 3.85 (4H, m, N_{amidic}—CH_2), 3.56 (16H, m, N_{aminic}—CH_2), 2.8–2.2 (22H, br, CH_2—CH_2—N_{aminic}, CH_3—C=O) ppm. ^{13}C NMR (CF_3COOD): δ 173.8 (C=O), 162.2 (C=O), 132.3 (ar), 129.1 (ar), 125.5 (ar), 122.4 (ar), 120.3 (ar), 117.7 (ar), 44.0, 42.3, 41.7, 33.4, 33.1, 21.0, 20.4, 18.9, 15.5 (CH_3—C=O). MS (ESI) *m/z*: 423.2380 [(M+2H)$^{++}$] (calcd for $C_{24}H_{31}N_4O_3$: 423.2396).

4.2. UV–vis absorption spectroscopy

UV–vis absorption spectra were obtained using a JASCO V-530 spectrophotometer. They were registered between 350 and 650 nm in quartz cuvettes. Four hundred micromolar drug stocks, prepared by dissolving the hydrochlorides in DMSO, were diluted to observe spectra in aqueous solution (10 mM MES–50 mM KCl buffer, pH 6.5).

4.3. Polyacrylamide gel electrophoresis (PAGE)

Previously 32P radio-labelled 2HTR and TSG4 oligonucleotides were heated at 95 °C for 10 min and quickly

cooled in ice, at a concentration of 12 μM. Then they were incubated for 2 h at 30 °C in MES–KCl buffer (10 mM MES, pH 6,5, 50 mM KCl for 2HTR and 5 mM KCl for TSG4) 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 (40 μM) was also reported as a useful standard to assign the electrophoresis bands to different DNA conformations. Major bands were so identified as single-stranded DNA (ss), dimeric (D), tetrameric (T) and monomeric (M) G-quadruplex structures.[25]

4.4. Telomerase repeat amplification protocol (TRAP) assay

The reaction mixture for assaying inhibition of human telomerase (50 μl) contains 50 μM dNTPs, 0.4 μM TSG4 primer and 1 μl of cell extract (prepared from 10^7 cultured HeLa cells) in TRAP buffer (20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween 20 and 1 mM EGTA). In each sample, polyamine perylene derivatives were added at different concentrations and incubated for 2 h at 30 °C, before addition of the cell extract. After 30 min of incubation at 30 °C, the samples were purified by phenol/chloroform extraction. ^{32}P radiolabelled TSG4 (0.14 μM), 0,4 μM CXext primer and 2U *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. Samples with no drug and with no cell extract were used as references. A 130-bp 'internal standard' (IS) was used to evaluate PCR amplification efficiency.[38]

Acknowledgments

Thanks are due to Antonello Alvino for the MS characterization of the synthesized compounds, to F. Piccioni for the NMR spectra, to S. Bacchetti for the protocol of telomerase extraction and to Julie E. Reed for help in the final revision of the manuscript. The research has been financially supported by MIUR FIRB 2001, MIUR COFIN 2005 and by Istituto Pasteur Fondazione Cenci Bolognetti.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.11.065.

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