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Tri-, tetra- and heptacyclic perylene analogues as new potential antineoplastic agents based on DNA telomerase inhibition

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Abstract—A recent approach in anticancer chemotherapy envisages telomerase as a potentially useful target. An attractive strategy deals with the development of compounds able to stabilize telomeric DNA in the G-quadruplex folded structure and, among them, a prominent position is found in the perylenes. With the aim to further investigate the role of drug structure, in view of possible pharmaceutical applications, we synthesized a series of compounds related to PIPER, a well-known perylene-based telomerase inhibitor. We modified the number of condensed aromatic rings and introduced different side chains to modulate drug protonation state and extent of self-aggregation. Effective telomerase inhibition was induced by heptacyclic analogues only, some showing a remarkably wide selectivity index with reference to inhibition of *Taq* polymerase. G-quadruplex stabilization was monitored by circular dichroism and melting experiments. Cell cytotoxicity measurements indicated a poor short-term cell killing ability for the best G-quartet binders. Besides the presence of a planar seven-condensed ring system, the introduction of a cyclic amine in the side chains critically affects the selectivity window.

1. Introduction

The search for novel chemotherapeutic approaches to treat cancer is still an active research field, due to the almost unavoidable onset of serious, undesirable side effects, such as resistance and toxicity, when using the presently available drugs. Recently, a new appealing target, the enzyme telomerase, has drawn the attention of medicinal chemists.

Due to the inability of DNA polymerases to replicate the end of linear DNA, a progressive shortening of the chromosomes occurs during each replication cycle.[1] However, loss of genetic information is avoided by the occurrence of non-coding repeated sequences, called telomeres, at the end of chromosomes. The length of the telomeric region has multiple functions among which the signalling to stop cell division when telomeres shorten below a critical length.[2] While this occurs in normal somatic cells, in the vast majority of cancer cells the activation of a nuclear enzyme, called telomerase, is observed.[3,4] This enzyme is a ribonucleoprotein that functions as a reverse transcriptase: using its RNA subunit as template, it adds telomeric repeats to DNA, thus preventing the shortening in telomere length. Therefore, cancer cells acquire the capability to divide indefinitely and, hence, become immortal. Impairing telomerase activity would restore the chromosome shortening process and thus produce cancer cell senescence and death. Hence, the search for compounds interfering with telomerase activity has become an attractive goal in anticancer therapy.[5,6]

Among the approaches thus far devised, blocking the protein catalytic portion (hTERT) or its RNA domain (hTR) gave successful results. In particular, a palmitoyl lipid conjugate of GRN163, an antisense thio-phosphoramidate oligonucleotide 13 bases long, which works as a 'template antagonist' for hTR, is currently undergoing phase I clinical trials.[7]

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An alternative validated approach to interfere with the telomerase-mediated DNA elongation process is to modify the structural properties of the DNA substrate. In fact, only the single-stranded linear form of the telomere is suitable for recognition by this enzyme. Interestingly, in all species, telomere sequences are rich in guanines; in human cells, the telomeric repeat is d(TTAGGG).[8] An intrinsic property of the G-rich sequences is their ability to fold into peculiar secondary structures, called G-quadruplexes, stabilized by reverse Hoogsteen hydrogen bonds connecting four guanine residues.[9,10]

Several G-quadruplex structures have been reported, differing in strand orientation, glycosidic conformation or loop geometry.[11–13] In all cases, a large planar aromatic network is formed by the four bases. A molecule that efficiently interacts with the planar quadruplex structure would substantially stabilize the folded conformation over the linear one, thus interfering with telomerase-mediated DNA elongation.[14]

A number of compounds, generally characterized by a large aromatic moiety that can stack onto a G-tetrad, were found to be active in this sense.[15] Besides G-quadruplex recognition, an ideal telomerase inhibitor, suitable for pharmacological applications, should not bind efficiently to other structures of DNA, in particular to the double helical nucleic acid, to avoid toxicity effects.

Interesting examples are derivatives bearing a perylene or a porphyrin central core such as PIPER[16–19] or TMPyP4.[20] Other 'small' molecules based on the acridine or anthracenedione aromatic system produced selective (tetraplex vs double-stranded) DNA recognition in a fashion that is dependent upon the nature and location of charged side chains.[21,22] Among them, the trisubstituted acridine derivative BRACO 19, designed using a molecular modelling approach, exhibited a considerable efficacy in vivo.[23,24]

SAR studies on PIPER involving modifications of the piperidine ring of the side chains[25,26] evidenced that the self-aggregation properties of these molecules are largely responsible for their binding preferences to G-quadruplexes versus double-stranded DNA.[27–29] This finding is in agreement with the NMR resolved structure of a G-quadruplex bound to PIPER showing an external stacking of the drug onto the G-tetrad.[17]

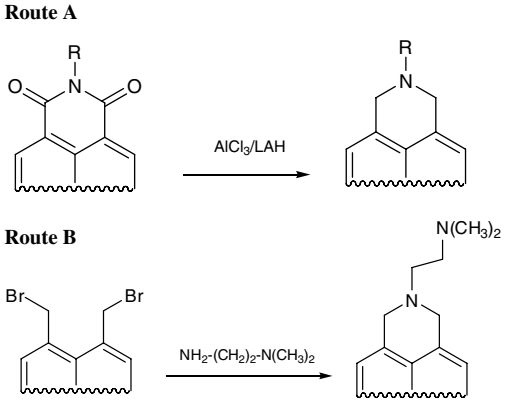
Starting from the available information, in the present paper we examined a number of perylene derivatives exhibiting different side chains to modulate their steric hindrance, protonation state and extent of self-aggregation. In addition, we progressively reduced the number of condensed aromatic rings in an attempt to understand the role of the dimension of the planar system in driving specific DNA structure recognition.

Spectroscopic studies (fluorescence, circular dichroism) as well as telomerase inhibition and cell cytotoxicity assays were performed to correlate physico-chemical and biochemical properties of the test derivatives.

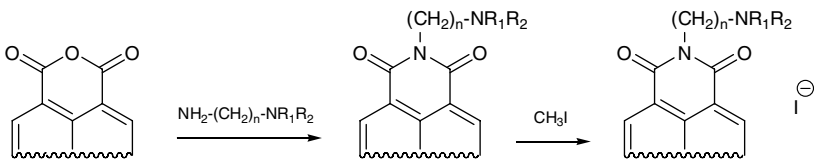
2. Chemistry

A summary of the synthetic procedures used to prepare the novel perylene analogues is reported in Schemes 1 and 2. Details are given in the Supplementary Material.

The number of condensed cycles has been progressively increased from three to four and finally to seven. Side chains exhibiting different steric hindrance as well as different pK_a were introduced by reacting the proper anhydride with the required diamine (Scheme 1). In some instances, the terminal amine groups were converted into permanently charged quaternary ammonium salts by treatment with methyl iodide. The extension of the planar system has been additionally modulated by reduction of the carbonyl groups. This was achieved either by reduction of the oxidized system with $AlCl_3$/LAH (Scheme 2, route A) or by preparation of the cyclic amine starting from the dibromo intermediate (Scheme 2, route B). The chemistry we used to obtain this class of compounds is rather flexible and allows us to introduce a vast number of substituents giving analogues of the leading compound, PIPER. The chemical structures of the novel test compounds are summarized in Figure 1.



Scheme 2.



Scheme 1.

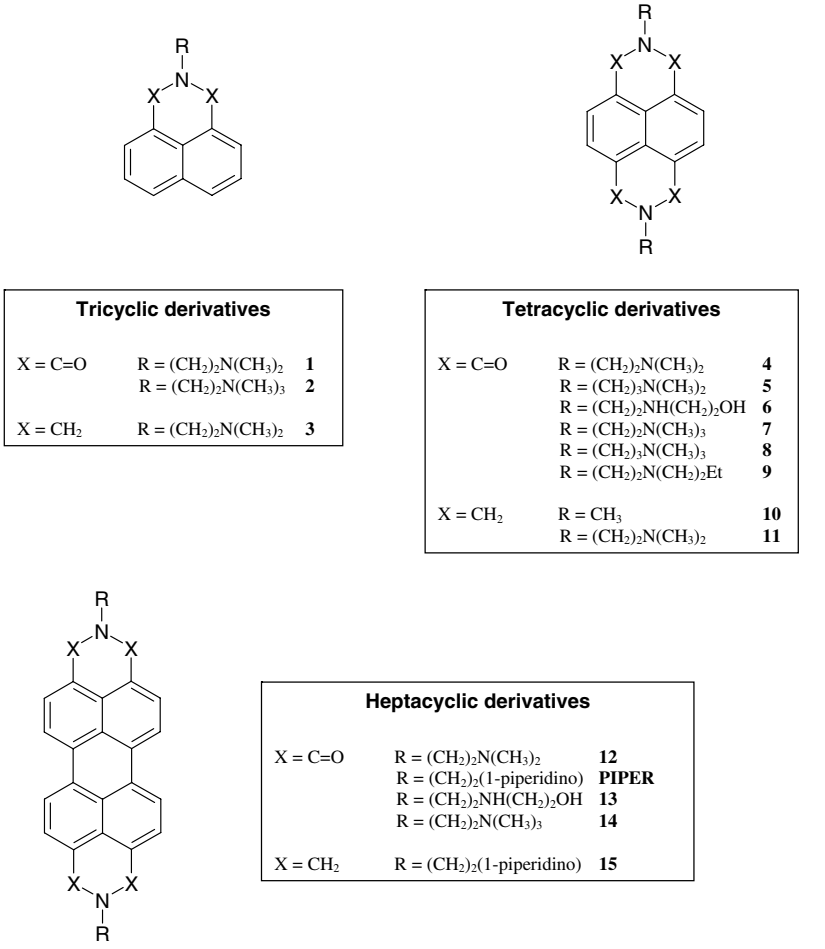


Figure 1. Molecular structure of test compounds.

3. Enzyme inhibition assays

All new test derivatives were evaluated for their telomerase inhibition properties as determined by TRAP assay (Fig. 2). To avoid false positive results due to drug interference with the amplification step, *Taq* polymerase inhibition was additionally monitored. The results are summarized in Table 1, where a selectivity index (SI, ratio between IC_{50} for *Taq* polymerase vs telomerase inhibition) is also included.

The need for a proper number of condensed rings for efficient enzyme/s inhibition clearly emerged. An analysis of the data for the analogues **1**–**3** indicated that none of these tricycles interfered efficiently either with *Taq* polymerase or telomerase.

Moving to the tetra and further to heptacyclic derivatives, *Taq* polymerase inhibition became stronger and stronger. The four-condensed ring system (tetracyclics from **4** to **9**) was sufficient to grant remarkable activity although removal of the distal protonable amine in the side chains (**10**), as well as reduction of the carbonyl groups (**11**) markedly decreased it. However, in the presence of these tetracyclic derivatives, telomerase inhibition, if any, occurred at drug concentrations comparable to those required to inhibit *Taq* polymerase.

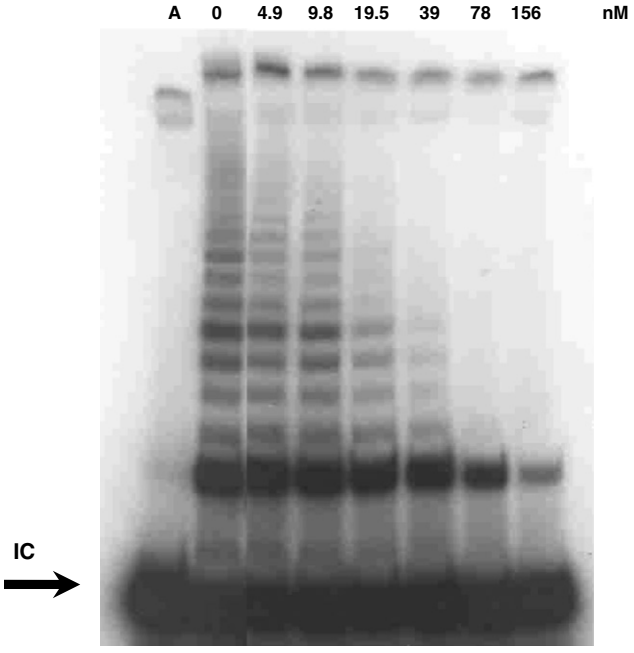


Figure 2. TRAP assay performed using a cellular extract from JR8 cells in the presence of increasing concentrations of **14** (0–160 nM). Lane A refers to the same reaction performed in the absence of cellular extract. The arrow indicates the 36 bp internal control.

Table 1. Biological properties of test compounds

Compound	IC_{50} (μM) *Taq* polymerase	IC_{50} (μM) telomerase	Selectivity index[a] (SI)	Cell cytotoxicity IC_{50} (μM)[b]	
				H460	HT29
1	>40	no	—	3.42 ± 0.04	5.05 ± 0.43
2	22 ± 2.4	no	—	⩾100	nd
3	>40	no	—	⩾100	⩾100
4	1.5 ± 2.0	no	—	0.16 ± 0.03	nd
5	6.4 ± 2	3.5 ± 2	1.8	0.42 ± 0.03	0.13 ± 0.01
6	5.8 ± 2	no	—	9.3 ± 1.6	11.7 ± 0.25
7	5.0 ± 2	2.5 ± 2	2.0	2.21 ± 0.28	3.24 ± 0.30
8	5.0 ± 2	2.5 ± 2	2.0	1.14 ± 0.21	8.89 ± 0.57
9	14 ± 2	6.8 ± 2	2.0	4.40 ± 0.62	8.82 ± 0.75
10	>40	no	—	⩾100	⩾100
11	25 ± 2	no	—	⩾100	15.2 ± 0.99
12	0.9 ± 2	0.16 ± 0.02	5.6	2.30 ± 0.20	0.23 ± 0.03
PIPER	5 ± 2	0.20 ± 0.02	25	⩾100	9.02 ± 1.03
13	0.2 ± 2	0.06 ± 0.01	2.0	2.62 ± 0.90	1.17 ± 0.09
14	0.15 ± 2	0.03 ± 0.01	4.2	⩾100	⩾100
15	10 ± 2	0.40 ± 0.03	25	28.1 ± 5.31	5.40 ± 0.63

no, not observed in the tested concentration range 0–40 μM and, in any case, lower than *Taq* polymerase inhibition nd, not determined.
[a] Ratio between the drug concentrations at which 50% *Taq* polymerase/telomerase inhibition was observed.
[b] Drug concentration producing 50% cancer cell death.

On the contrary, all tested heptacyclic derivatives (from **12** to **15**) showed a concentration window for selective interference with telomerase activity, with the notable exception of derivative **13**. Among heptacyclics, it is interesting to note that the presence of a cyclic amine in the side chains as in PIPER increased this concentration window irrespective of the reduction of the carbonyl groups of the planar ring system (**15**).

The whole set of substitutions tested here showed that the activity against telomerase and *Taq* is similarly affected by the nature of the perylene side chains although to a larger extent in the case of the DNA amplification reaction. In particular, the most efficient inhibition of both enzymes was observed with derivatives **13** and **14**. For compound **14**, this can reasonably rest in the presence of positively charged quaternary nitrogens that facilitate interaction of the drug with the negatively charged DNA, independently of the polynucleotide conformation. For **13**, in addition to the basic nature of the amine, the presence of a hydroxyl moiety that can form hydrogen bonding with DNA bases may account for more efficient DNA complexation.

4. Self-aggregation properties

Perylene derivatives are known to self-aggregate producing a substantial quenching of their fluorescence emission.[27,28] Additionally, self-aggregation is reported to favourably increase the selective recognition of G-quadruplex versus dsDNA. Thus, we monitored self-aggregation properties of our heptacyclic derivatives from **12** to **15** fluorometrically as a function of pH (Fig. 3).

At pH 8.0, PIPER and **15** showed essentially no fluorescence emission, compounds **12** and **13** presented an intermediate behaviour, whereas compound **14** was the most fluorescent derivative. Lowering pH to 3.0 produced an increment in fluorescence intensity for all test compounds. In acidic solution, full protonation of the side chains occurs, hindering self-aggregation and, in particular, the face-to-face interactions that mainly contribute to the quenching process.[30] Thus, the ratio between the emission intensities at pH 3.0 and 8.0 can be used as a parameter related to the extent of self-association at physiologically relevant conditions. In agreement with literature data according to which PIPER aggregation starts at pH 7.5,[27] our results (Table 2) indicate

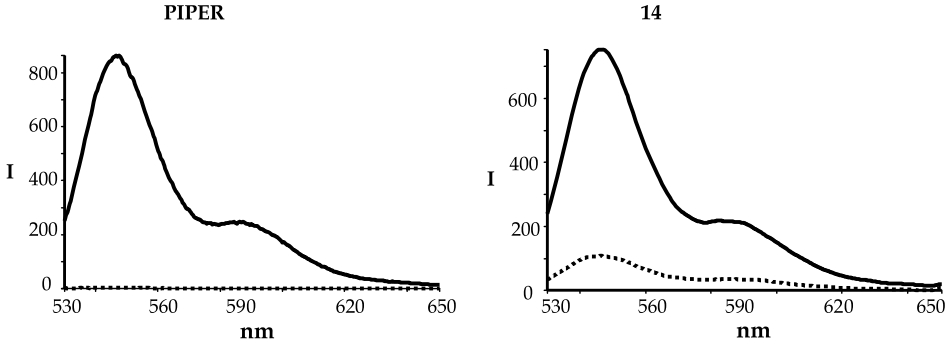


Figure 3. Emission spectra (λ_{ecc} = 520 nm) of 0.5 μM PIPER or **14** in 10 mM Tris, 1 mM EDTA at pH 8.0 (dotted line) or 3.0 (solid line).

Table 2. Biophysical properties of test heptacyclic compounds

Heptacyclics	Side chains	Fluorescence quenching[a]	% CD increment (295 nm)	T_m[b] (°C)
18a	$(CH_2)_2N(CH_3)_2$	140	16.0	73.4
PIPER	$(CH_2)_2$Nc[c]	580	11.3	68.8
20a	$(CH_2)_2NH(CH_2)_2OH$	10	16.3	73.4
21	$(CH_2)_2N(CH_3)_3$	7	46.3	69.0
22a	$(CH_2)_2$Nc[c]	520	5.3	62.6

[a] Ratio between the fluorescence quantum yields measured at pH 3.0 and pH 8.0, respectively.
[b] The melting temperature for 4GGG in the absence of drugs is 62.0 °C.
[c] Nc, 1-piperidino.

that, at pH 8.0, PIPER and **15** are largely aggregated, **12** and **13** exhibit an intermediate extent of self-association, whereas compound **14** is essentially not aggregated. This latter finding is explained by the permanent charge exhibited by the quaternary nitrogens of **14**, which substantially reduce aggregation at any pH.

5. Circular dichroism studies

The effects of the test derivatives on G-quadruplex folding/stability were further assayed by CD spectroscopy (Fig. 4). These studies were performed using a synthetic DNA sequence containing four human telomeric repeats, 4GGG. In 100 mM KCl, this sequence assumes an intramolecular G-quadruplex structure producing a CD spectrum characterized by a positive and a negative peak at 290 and 235 nm, respectively.[13] All tested perylenes were able to modify the oligonucleotide spectrum, in particular inducing an increment in the positive band alone with a shift to 295 nm. The extent of such an increment is largely modulated by the nature of perylene side chains (Table 2). Among tested compounds, derivatives **14** and **15** emerged as the most and the least active. In the presence of a 4GGG mutated sequence, unable to form G-quadruplexes (MUT), no major changes in DNA optical activity were monitored in the presence of perylene analogues. A negligible effect was observed also in the presence of a double helical nucleic acid.

The perylene interaction with the folded form of 4GGG was further investigated by monitoring the oligonucleotide melting profile. In our experimental conditions, the G-quadruplex structure assumed by the oligonucleotide showed a thermal transition at 62.0 °C. In the presence of all tested perylenes, this temperature was incremented. As reported in Table 2, **12** and **13** proved to be the most effective G-quadruplex stabilizers, followed by PIPER and **14**, and finally by **15**. Interestingly, the increment in T_m generally parallels that in the CD band at 295 nm, only **14** being an outlier. Indeed, this latter derivative is the only one presenting permanently charged side chains, whereas all other derivatives display acid–base equilibria which are surely affected by temperature changes.

Finally, when comparing PIPER to its analogue with reduced carbonyls, it emerges that partial loss of planarity in the heptacyclic system reduces G-quadruplex recognition and this well correlated with its reduced telomerase inhibition.

6. Cell cytotoxicity

The cytotoxic effects of test derivatives, summarized in Table 1, were studied using human lung and colon cancer cell lines (H460 and HT29, respectively) following 168 h of continuous drug exposure. Almost negligible effects were observed for PIPER, **14** and **15**. As far as **14**, this could be reasonably ascribed to the permanently charged quaternary amine side chains, which likely impair cellular uptake. In the other cases, the presence of the cyclic amine groups, irrespective of the presence of

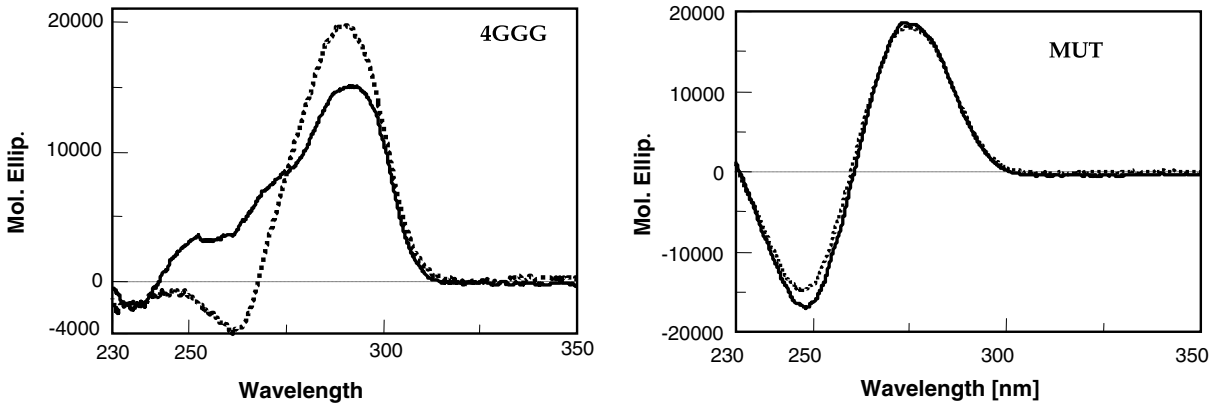


Figure 4. CD spectra of oligonucleotides 4GGG and MUT (4 μM) in 10 mM Tris, 1 mM EDTA and 100 mM KCl, pH 8.0, in the absence (solid line) and in the presence (dotted line) of **14** (14 μM).

carbonyl groups in the planar portion, appears to be responsible for poor short-term cell killing ability. In fact, the congeners **12** and **13**, exhibiting non-cyclized secondary and tertiary amine substituents, are at least one order of magnitude more potent than PIPER and **15** as cytotoxic agents. It is to note that increasing the selectivity index produces a decrease in cytotoxicity. This is in line with the fact that interaction with telomeric DNA is expected to produce cell death only after several population doublings.

Interestingly, derivatives with reduced extension of the planar ring system were shown to be lethal to cancer cells to a notable extent. For tetracyclic derivatives, this is in agreement with their efficient binding to dsDNA. This is at odds with the tricyclic derivative **1**, a poor DNA-binder but exhibiting a cytotoxic IC_{50} in the low micromolar range. Considering its strong structural similarity to the well-known topoisomerase poison amonafide,[31] this compound is likely to produce its cytotoxic effects by interfering with enzyme(s) other than telomerase or polymerase, considered here.

7. Conclusions

Our results indicate that the dimensions of the planar portion of DNA-directed drugs subtly modulate their ability to recognize different DNA structural arrangements. In particular, we showed that among PIPER-related ligands, a conjugated system consisting of more than four rings is required to efficiently target G-quartets. This finding is at odds with data reported on G-quadruplex binders structurally related to anthracene or acridine, for which good tetraplex selectivity can be reached in the presence of just three-condensed rings, the number and location of the side chains being crucial for selective targeting of DNA structures.

A key role of the side chains clearly emerged also among perylenes as they differentially modulated a large number of physico-chemical properties.

First of all, we observed that strong interactions with G-quadruplex arrangements favour telomerase inhibition. For the oligonucleotide 4GGG, increments in optical activity at 295 nm and in melting temperature produced by the test compounds are safely reflecting increased stability of the G-quadruplex conformation. As clearly shown in Figure 5 a good linear correlation occurs between either of these two data sets and the telomerase inhibition in vitro. Based on these results and on the structural similarities of these active compounds to PIPER, it can be reasonably inferred that enzyme inhibition is linked to stabilization of G-quadruplex folded forms. In connection to this, derivative **14** is the most efficient analogue. We argue that the permanent positive charges present in its side chains strongly stabilize the DNA-drug complex by ionic contacts.

Additionally, the substituents tested here show different steric hindrance and different p*K*a values, which leads to different degrees of drug self-association in experimental conditions relevant to physiological processes. Indeed, it has been suggested that the extent of aggregation can be critical for improving the selective inhibition of telomerase.[13,27] In fact, we observed that the selectivity index obtained from enzyme inhibition data parallels the extent of self-aggregation in the perylene family.

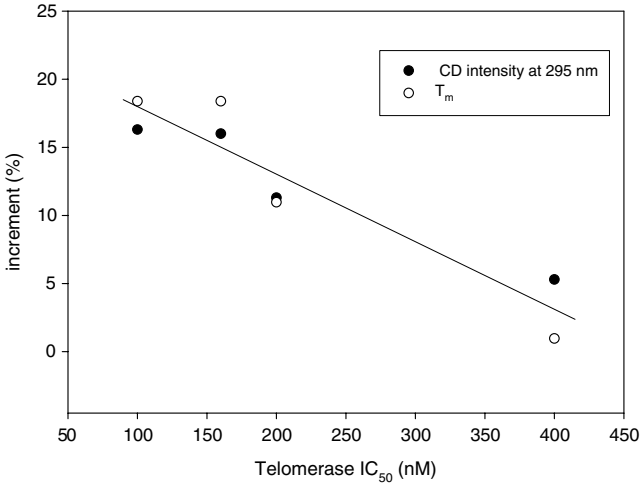


Figure 5. Plot of % increment in 4GGG CD intensity at 295 nm (full symbols) or T_m (empty symbols) as a function of telomerase IC_{50} determined for the test perylene derivatives.

If we assume that this parameter is preferentially linked to G-quadruplex recognition, we would expect also an increment in telomerase inhibition upon aggregation. However, this is not the case. In fact the more aggregated the compounds, the lower the activity on the enzyme and additionally, the lower the interaction with G-quadruplex (assuming the increment in CD intensity at 295 nm and T_m as indicative of binding efficiency).

Thus, it follows that drug aggregation increases selectivity for G-quadruplex arrangements with respect to double helical and single-stranded structures not by enhancing affinity for G-quartets but by reducing binding efficiency to other nucleic acid conformations.

Finally, the modulation of the cell killing ability observed among our heptacyclic derivatives appears to be promising for the development of effective anticancer compounds based upon recognition of telomeric DNA and exhibiting less severe short-term toxic effects.

8. Experimental

Test derivatives were synthesized and characterized accordingly to procedures reported in Supplementary Material. Synthetic oligonucleotides were purchased from Eurogentec and used without further purification. The sequences used in this study were **4GGG** 5′-AGG GTT AGG GTT AGG GTT AGG G-3′ and **MUT** 5′-TACAGATAGTTAGACTTAACGTTA-3′. Solutions of the test derivatives were prepared freshly just before use.

8.1. Fluorescence spectroscopy

Fluorescence spectra were recorded on a FL-20 Perkin-Elmer spectrofluorimeter. Sample solutions at 0.5 μM were prepared in 10 mM Tris and 1 mM EDTA and were incubated 1 h at room temperature before scan acquisition. Each measurement was averaged over 3 scans and recorded at 25 °C using an excitation wavelength of 500 nm.

8.2. Circular dichroism measurements

Oligonucleotide circular dichroism spectra from 230 to 350 nm were recorded using 10 mm path length cells on a Jasco J 810 spectropolarimeter equipped with a NESLAB temperature controller and interfaced to a PC 100 in 10 mM Tris–HCl, 1 mM EDTA and 100 mM KCl, pH 8.0. Before data acquisition, DNA solutions were heated at 95 °C for 5 min and left to cool at room temperature on. The reported spectrum of each sample represents the average of 3 scans recorded with 1-nm step resolution. Observed ellipticities were converted to mean residue ellipticity $[\theta] = \mathrm{deg} \times \mathrm{cm}^2 \times \mathrm{dmol}^{-1}$ (Molar Ell.).

Thermal denaturation experiments were performed by recording the DNA optical activity at 295 nm as a function of the temperature. Oligonucleotide solutions (4 μM) were equilibrated at 25 °C, then the signal was recorded while increasing the temperature at 0.8 °C/min and stirring the solution to allow equilibration. Experiments were performed in the presence or absence of tested derivatives (4 μM final concentrations). T_m was determined by locating the maxima/minima of the first derivative of the curve describing the melting profile (CD vs T). Two to four scans were repeated for each experimental condition. When required, the melted solution was cooled down at the same temperature change rate.

8.3. *Taq* polymerase assay

To meet proper working conditions, compounds were assayed with *Taq* polymerase reaction by using pBR322 (2.5 ng) as a DNA template and appropriate primer sequences (0.5 μM) to amplify the 906–1064 sequence of plasmid by PCR. The reaction was carried out in a Perkin-Elmer thermocycler performing 25 cycles of: 30 s at 94 °C, 30 s at 65 °C and 30 s at 72 °C. The reaction products were resolved on a 2% agarose gel in TBE 1X (89 mM Tris base, 89 mM boric acid and 2 mM Na_2EDTA) and stained by ethidium bromide.

8.4. Telomerase activity assay

An aliquot of 5×10^6 JR8 cells in exponential phase of growth was pelleted and lysed for 30 min on ice using 100 μl of 0.5% Chaps, 1 mM EGTA, 25% 2-mercaptoethanol, 1.74% PMSF and 10% w/v glycerol. The lysate was centrifuged at 13,000 rpm for 30 min at 4 °C and the supernatant collected, stored at −80 °C and used as the telomerase source.

Telomerase activity was assayed using a modified telomere repeat amplification protocol (TRAP) assay.[32] Briefly, a proper primer (TS) has been 5′-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After enzyme inactivation (85 °C for 5 min), a 50 μl TRAP reaction mix (50 μM dNTPs, 0.2 μg of labelled TS, 0.1 μg of return primer ACX, 500 ng of proteic extract and 2 U *Taq* polymerase) was prepared in the presence/absence of increasing drug concentration. According to (41), an internal control template (0.01 amol TSNT) with its return primer (1 ng NT) was added to the reaction mixture. Then, telomerase elongation step has been performed (30 min at 30 °C) followed by a PCR amplification step (30 cycles of: 30 s at 37 °C and 30 s at 58 °C). The reaction products were loaded onto a 10% polyacrylamide gel (19:1) in TBE. Gels were transferred to Whatman 3MM paper, dried under vacuum at 80 °C and read using a phosphorimager apparatus (Amersham).

Values are expressed as percent of telomerase inhibition relative to control (no drug) lanes.

8.5. Cell cultures and toxicity assays

H460 human lung cell line was maintained in defined RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2 mM of L-glutamine 50 U/ml of penicillin G and 50 μg/ml of streptomycin, at 37 °C in humidified atmosphere and 5% of CO_2. HT-29 colon cancer cell line was grown in McCoy's defined medium supplemented with 10% heat-inactivated foetal calf serum, 2 mM of L-glutamine and 10 mM Hepes.

To evaluate toxic profiles of the potential antitelomerasic compounds, MTT assays were performed as described: cells were plated in 96-well plates at 10,000 cells/well and cultured overnight. Afterwards, compounds were added in octuplicate and plates were incubated in presence of the drug for 168 h. At the end of this period, MTT was added to a final concentration of 50 μg/ml, and two additional hours of incubation were performed. After that, medium was aspirated carefully and 100 μl of DMSO was added per well. Soluble formazan salts were homogenated by manual pipetting and absorbance at 570 nm was read. Curves consisted in 10 serial dilutions in octuplicate in each case, and results were analysed as sigmoidal dose–response curves using GraphPad Software®.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.09.029.

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