Water Soluble Cationic Perylene Derivatives as Possible Telomerase Inhibitors: The Search for Selective G-Quadruplex Targeting

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Abstract; The search for telomerase inhibitors has been widely explored in the last few years, since telomerase activity in somatic cells can be considered as a general cancer mark. One of the possible strategies is the capping of telomere 3'-end (the enzyme subs trate) in a conformation not available to the recognition of telomerase, with particular attention to G-quadruplex structures. Small organic molecules, able to induce and/or stabilize G-quadruplexes, have been synthesized and studied in many different research groups. Here, we mean to critically analyze the class of hydrosoluble perylene diimides (HPDIs), which offers the intriguing possibility to fix the hydrophobic molecule moiety (perylene) able to bind to the terminal G-quartet of telomeric G-quadruplex, while widely varying the number and features of the hydrophilic side chains, which interact with the DNA grooves. We will show that, using this strategy, it is possible to significantly improve HPDIs efficiency in inhibiting telomerase and their selectivity for telomeric G-quadruplex with respect to duplex genomic DNA.

INTRODUCTION

The telomere is a DNA-protein structure formed at the ends of almost all eukaryotic chromosomes. It is involved in many essential functions [1], since i) it prevents telomeric DNA from being recognized as DNA breaks and allows cells to distinguish between chromosomal canonical ends and double-stranded breaks; ii) it provides a mean to faithfully replicate the whole chromosome; iii) it contributes to the spatial and functional organization of chromosomes within the nucleus; iv) it is transcribed by RNA polymerase II and the transcribed telomeric G-rich RNA appears to be involved in telomeric heterochromatin organization. This last telomere's feature has been discovered very recently [2] and has indicated that the longstanding idea that telomeres are silent genomic region should be deeply modified. Stability and length of telomeres are known as related to telomerase activity. Telomerase is a ribonucleoproteic enzyme possessing an RNA moiety, coded by the hTER gene, and a proteic moiety, coded by the hTERT gene. In human, it is physiologically active in germinal, hematopoietic and epithelial cells, but not in somatic cells; this behaviour derives from the hTERT gene silencing. As a consequence of telomerase inactivity, telomeres length decreases at each replication cycle and this feature can be considered a sort of biological clock. In principle, since cells senescence is strictly connected to telomere length decrease it is possible to consider the reactivation of telomerase as a trick to decrease aging effects. Unluckily this possibility seems to be forbidden, because of cancer-promoting activity of the enzyme. In fact, telomerase is reactivated in about 90% of transformed cells. Thus, in the last few years, telomerase has been widely considered as an attractive anti-cancer target. Moreover, differences in telomerase expressions, telomeres length and cell kinetics between normal and tumour tissues suggest that telomerase targeting could be selective for transformed cells [3].

Since terminal telomeric DNA is characterised by G-rich sequences, which have been shown to be able to fold into G-quadruplex structures, based on guanine quartets (Fig. **1**) [4], small molecules that stabilize these structures might inhibit telomerase activity according two different pathways: by locking the single-stranded telomeric substrate into a G-quadruplex structure [5] or helping the uncapping of telomere 3'-ends [6].

HYDROSOLUBLE PERYLENE DIIMIDES AS G-QUADRUPLEX INTERACTIVE TELOMERASE INHIBITORS

It is generally accepted that small organic molecules are able to bind to human telomeric G-rich DNA and induce G-quadruplex formation; on the other hand, they can bind to preformed G-quadruplex structures increasing their thermodynamic stability. These molecules are endowed of strong interest from the pharmacological point of view, representing potential anti-cancer drugs [7].

With the present review, our aim is to cover the studies, carried out in many laboratories as well as in our research group, referring to a specific class of small organic molecules, namely the hydrosoluble perylene diimides (HPDIs). The first reported compound of this series was the N,N'-bis[2-1(piperidino)-ethyl]-3,4,9,10-perylene-tetracarboxylic

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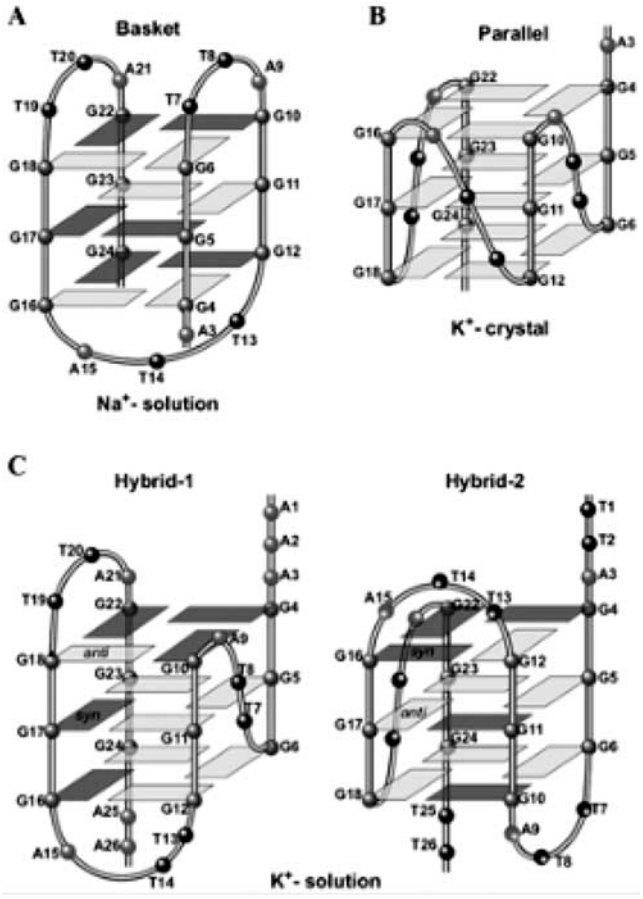

Fig. (1). A) Schematic drawing of the folding topology of the basket-type intramolecular G-quadruplex formed by wtTel22 in Na^+ solution as determined by NMR. B) Folding topology of the propeller-type parallel-stranded intramolecular G-quadruplex formed by wtTel22 in the presence of K^+ in crystalline state. C) Folding topologies of the Hybrid-1 (major conformation in Tel26) and Hybrid-2 (major conformation in wtTel26) intramolecular telomeric G-quadruplexes in K^+ solution. This figure was published in *Biochimie, 90*, Polymorphism of human telomeric quadruplex structures, Dai J., Carver M., Yang D., 1172-83, Copyright Elsevier (**2008**) [4].

diimide (PIPER) (Fig. **2A**), that was devised by use of an automated in silico procedure to find optimally sized chromophore for the interaction with G-quadruplex [8]. All the considered HPDIs (Fig. **2**) are characterized by a large flat aromatic system (the perylene moiety) able to interact with the terminal G-quartet, via π-π stacking; in addition, all of them have two up to four side chains, in most cases characterized by positive charges, able to interact with G-quadruplex grooves. Furthermore, the charged side chains assure a satisfactory water solubility necessary for a possible pharmacological application. Since the hydrophobicity of perylene moiety is balanced by side chains hydrophylic moiety, HPDIs offer a very interesting possibility to anchor the drug to the terminal G-quartet and to widely modulate the binding to the four G-quadruplex grooves by synthesizing derivatives different for the type and/or the number of side chains.

A chart of HPDIs, synthesized and studied in our as well as in many other research groups with the aim to inhibit telomerase, is reported in Fig. (**2**). The HPDIs have been divided in two main groups, according to having an unsubstituted or modified bay-area. The first group (Fig. **2A**) is characterized by two equal side chains, which can have different basicity, as in the case of PIPER derivatives [8-11], different lengths and charges number [13,14], different polarity (PEG side chains) [15] or anionic side chains [12,14]; the only HPDI able to recognize G-quadruplex and cleave it upon binding is Perylene-EDTA-Fe(II), characterized by two side chains that chelate Fe(II) [16]. In the series of first generation PIPER derivatives, a further division can be considered between HPDIs bearing cyclic or linear amino group in the side chains [17]. The second group (Fig. **2B**) is characterized by a modified bay area of the perylene moiety [18,19]. It is worth considering that this modification, which is necessary to increase HPDI side chains number, should decrease perylene planarity and thus diminish or prevent HPDI stacking on the terminal G-quartet [19].

TELOMERASE INHIBITION AND G-QUADRUPLEX INDUCTION

The Telomeric Repeat Amplification Protocol (TRAP) assay is used to evaluate the inhibitory properties of small molecules against telomerase [20]. At the beginning, it consisted of two different reactions: I) telomerase elongation of an oligonucleotide substrate, II) amplification of the products by polymerase chain reaction (PCR). After that, the samples were loaded on a nondenaturing 12% polyacrylamide gel,

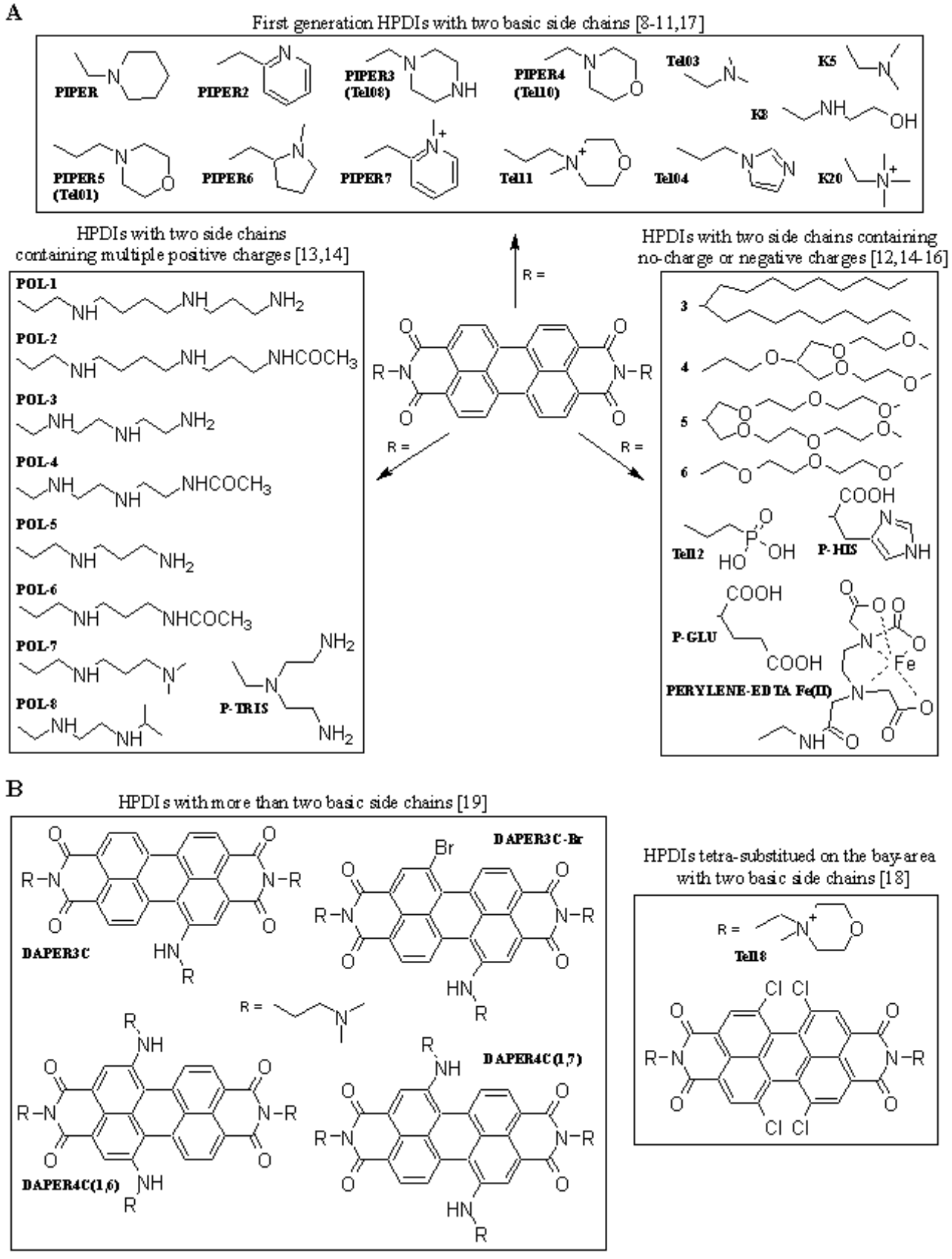

Fig. (2). Structures of hydrosoluble perylene diimides (HPDIs) with unsubstituted (A) or modified bay-area (B).

electrophoresed and finally visualized by radioactivity or fluorescence. Initially, TS oligonucleotide (5'-AATCCGT-CGAGCAGAGTT-3') was used as a substrate for the elongation by telomerase, although it does not contain the guanine repeats characteristic of the telomeric sequence. Subsequently, on the basis of studies by Mergny *et al*. [21], Rossetti *et al*. have used TSG4 (5'-GGGATTGGGATTGG-GATTGGGTT-3') as telomerase substrate [10]: it is designed to mimic the GGG tracts of the telomeric sequences, but having the sequence frame inverted. This feature decreases the thermodynamic stability of the intramolecular G-quadruplex formed by TSG4 with respect to that formed by the human telomeric sequence and makes the substrate conformation more easily controlled by drugs interactions [10,13,19]. An important topic to consider is the ability to compare telomerase inhibition, evaluated in different laboratories. In the amplification reaction the possible inhibition of Taq polymerase by the G-quadruplex ligands should be carefully considered. In fact, due to their strong interaction with G-rich sequences, G-quadruplex specific ligands could inhibit the polymerase activity on the G-rich product of telomerase elongation, even when PCR controls are not influenced. To circumvent these difficulties, an intermediate step was added to remove the inhibitors after the telomerase extension step (i.e., before PCR): this can be obtained with solvent extraction and/or precipitation [10,13,19] or a products purification step [22]. In order to definitively overcome this problem, a different strategy is also possible: a "direct assay" without the PCR amplification step [23]. This requires telomerase-enriched cell extracts [24] and a much higher quantity (≈10-fold) of radioactive material than a typical TRAP assay. For these reasons, a simple and direct comparison of results coming from different laboratories is not always correct and must be carefully considered.

A typical autoradiography of a canonical TRAP assay is reported in Fig. (**3A**), that shows telomerase strong (POL-3) or zero (POL-4) inhibition [13]. It is worth noting that in both cases, the amplification step is carried out after drug extraction with an organic solvent, and that the PCR amplification of an internal standard assures that the results, obtained for different drugs, can be safely compared. From this and many other similar experiments the telomerase inhibition activity of many HPDIs was derived. However, it is worth noting that for many others HPDIs reported in Fig. (**2**), telomerase inhibition was not studied. The lack of data could derive by the fact that the results referring to PIPER were not consistent in different research groups [9,10,17], probably due to the drug influence on the Taq polymerase [23] necessary in the amplification step. This seems to have discouraged the extensive use of TRAP assay for this class of molecules [22,23]. Thus, the results reported in Fig. (**3B**), derive mainly from the modified TRAP assay, firstly introduced by Rossetti *et al.* [10] and show that many HPDIs are better telomerase inhibitors than PIPER, the first HPDI studied by Hurley [25]. Namely the higher basicity of the side chains, as in the case of PIPER3 [9,10], or the greater number of side chains, three as in the case of DAPER3C [19], or the higher number of positive charges in the side chains (POL-3 and POL-8) [13], substantially increase HPDIs inhibition of telomerase with respect to PIPER (see histograms in Fig. **3B**). In some cases, HPDIs completely lack activity against telomerase, but these negative results are important to explain the molecular features necessary for telomerase inhibition. For example, the electrostatic interactions between side chains and G-quadruplex grooves are necessary, since side chains which are anionic [14,18] or polar but neutral [15], or terminal acetylated [13] strongly decrease the HPDI ability to inhibit telomerase activity.

To connect telomerase inhibition with drugs interactions with human telomeric G-quadruplex monomolecular structure, a suitable simplified model of telomeric 3'-end DNA is necessary. The model oligonucleotide, adopted in most biophysical studies, has the sequence of human telomeric DNA 3'-overhang and a length of 21 nucleotides. It corresponds to less than four repeats of telomeric DNA, in order to equalize sequence ends. The oligonucleotide structure has been investigated in the presence of Na^+ [26] or in the presence of K^+ [27] and give rise to two different unimolecular G-quadruplexes as illustrated in Fig. (**1A** and **B**). However, it is necessary to consider that, in different research groups, it has been recently found by NMR studies, in solution containing potassium, that human telomeric sequence forms an hybrid-type intramolecular G-quadruplex structure with mixed parallel/antiparallel strands, as illustrated in Fig. (**1C**) [4].

The kinetic aspects of G-quadruplex induction by small organic molecules have been scarcely investigated. About this topic, it is worth remembering that proteins, which interact specifically with G-quadruplex and accelerate the assembly of this structure have been identified: for example, Rap1 from *Saccharomyces cerevisiae* [28] and the β-subunit of telomere binding protein in Oxytricha [29]. The first evidence that small ligand, such as HPDIs, can act as a driver in promoting the association of the DNA oligomer $(TTAGGG)_2$ into G-quadruplex structures came out from Hurley *et al*. in 1999, who suggested that HPDIs should have a degree of self-stacking in water solution to induce G-quadruplex [25]. On the other hand, self-association of perylene derivatives has been hypothesized to favour the specific recognition of the G-quadruplex with respect to duplex DNA by Kerwin and co. [11,12,30]. On the contrary, Palumbo *et al.* have recently reported that strong HPDIs self-aggregation could lower telomerase inhibition and decrease the interactions with G-quadruplexes [17].

In many cases, the adopted assay method to evaluate G-quadruplex induction by HPDIs was PAGE (polyacrylamide gel electrophoresis assay), since the formation of intermolecular dimers or tetramers is characterized by bands with a lower mobility with respect to that of a monomer, while the induction of the more compact intramolecular G-quadruplex structure is characterized by a band of higher mobility with respect to the unstructured oligonucleotide. Typical PAGE studies are reported in Fig. (**4A** and **B**). The ability of HPDIs to induce the two G-quadruplex structures was referred to that of PIPER (previously determined by Hurley and Kerwin [8]) that allows the assignment of the bands, as an internal standard. In the case of TSG4, three different bands were observed, identified as single strand DNA (SS), intermolecular (I) and monomeric (M) G-quadruplex (Fig. **4A**). The intramolecular G-quadruplex (M) corresponds to the bands

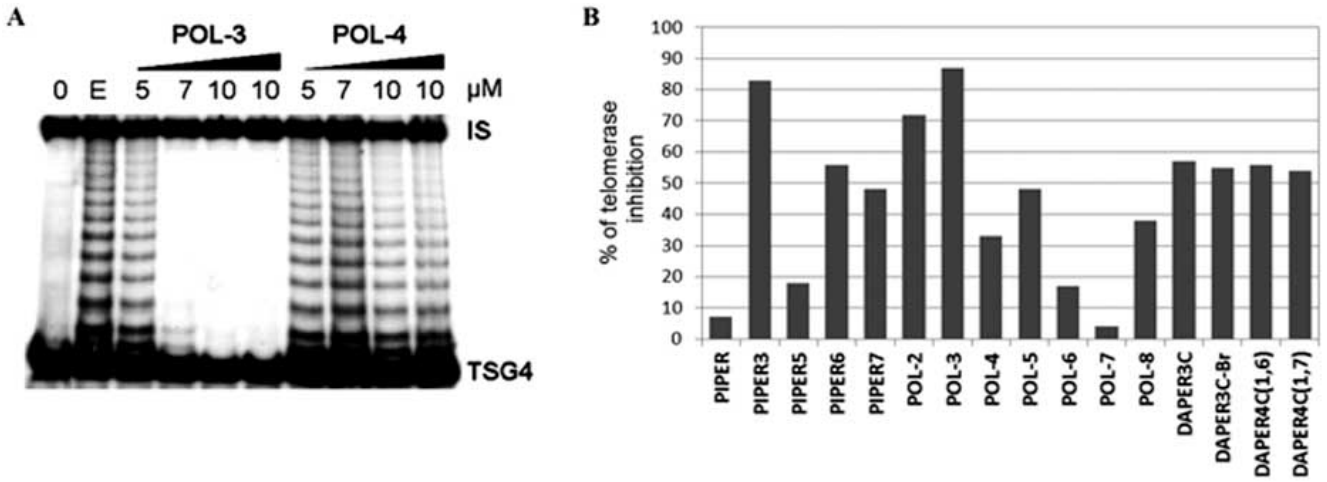

Fig. (3). A) Typical TRAP experiment showing the inhibition of human telomerase by HPDIs (POL-3 and POL-4) at different concentrations, revealed by PAGE [13]. In lane "0" cell extract was not added, in lane "E" no drug was added. IS is a 130bp "internal standard" to evaluate PCR amplification efficiency. B) Histogram of the percentage of telomerase inhibition, derived from TRAP assays, at 10 μM drug concentration. Three different series of HPDIs (PIPERs, polyamine perylene derivatives and DAPERs [10,13,19]) are reported.

showing the highest mobility; its high folded structure favours the faster running in the gel grid with respect to an unstructured oligonucleotide, model for single strand DNA. In the case of 2HTR (5'-AATCCTCGAGCAGAG-TTAGGGTTAGGGTTAG-3'), since only intermolecular G-quadruplex is possible, two bands corresponding to dimeric (D) and tetrameric (T) intermolecular G-quadruplex structures, with a lower mobility than the single strand DNA (SS) (Fig. **4B**), are evident.

The most interesting outcome from these studies indicates that the induction of intramolecular G-quadruplex structure of human telomere depends on HPDIs side chain differences. On the contrary, the induction of intermolecular G-quadruplex structures, in the same physico-chemical conditions, is less efficient and moreover the different HPDIs effect lacks selectivity.

G-Quadruplex Binding and Selectivity by FRET Melting and ESI-MS

The possible pharmacological use of HPDIs, as in the case of most drugs, requires a high selectivity for the target, in this case telomeric G-quadruplex DNA with respect to duplex DNA, which is present in the genoma, in dramatically prevailing amount. Fluorescence resonance energy transfer (FRET) and surface plasmon resonance (SPR) methods have been most commonly used in studying the selectivity of G-quadruplex ligands. However, in the studies involving HPDIs, SPR has not been adopted so far, probably on account of aggregation troubles. Thus, in this review, only studies based on FRET melting assay will be reported

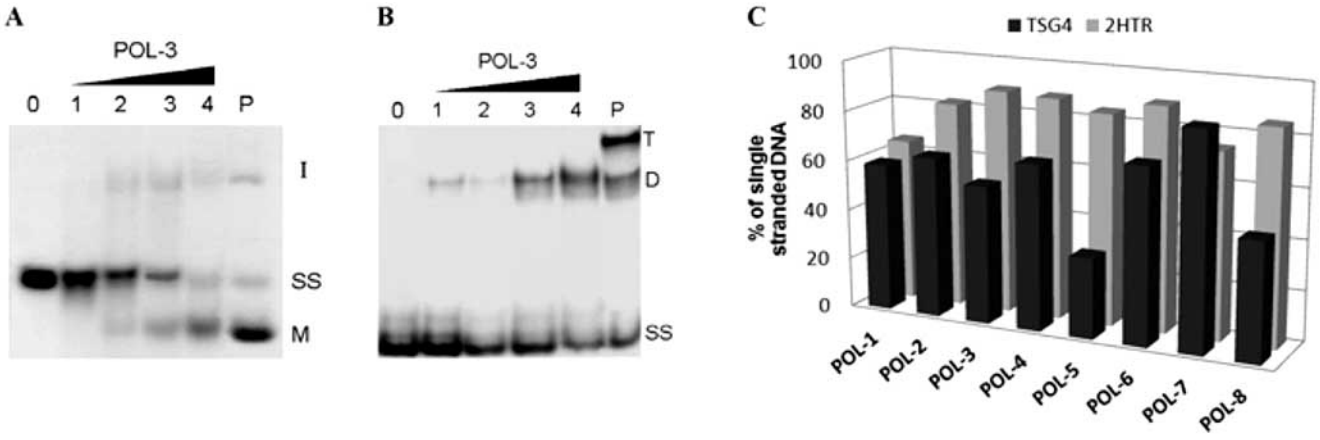

Fig. (4). Typical PAGE experiments of (A) intramolecular (TSG4) and (B) intermolecular (2HTR) G-quadruplex induction by POL-3, studied by native PAGE [13]. The oligonucleotides (12 μM) are in the presence of different drug concentrations: 5 μM (lane 1), 10 μM (lane 2), 20 μM (lane 3), 40 μM (lane 4) and with no drug (lane 0). As standard, in lane P, the structures induced by PIPER (40 μM) were reported. Major bands were identified as single-stranded DNA (SS), monomeric (M) and intermolecular (I) G-quadruplex. C) Histogram of percentage of single stranded DNA decrease due to intramolecular and intermolecular G-quadruplex induction, studied by PAGE, at 10 μM drug concentration.

[31], regarding two series of HPDIs (Fig. **2A**). FRET is a spectroscopic method, based on the energy transfer between two fluorophores (a donor and an acceptor) bound to two residues of a molecule, having a distance lower than 50 Å. It is very useful to probe the structure of guanine rich sequences in which a donor and an acceptor are attached to each end of a G-rich oligonucleotide [32]. FRET melting assay is based on the use of a quadruplex-forming oligonucleotide double-labeled with a pair of FRET donor and acceptor (usually FAM, 6-carboxyfluorescein, and TAMRA, 6-carboxy-tetramethylrhodamine) that allows to monitor the melting of the quadruplex via FRET variations [32,33]. Semi-quantitative evaluation of ligand binding affinity is obtained by measuring the increase in melting temperature induced by the ligand (ΔT_m), on this oligonucleotides, as well as, similarly, on an autocomplementary duplex forming oligonucleotide. The quadruplex versus duplex DNA selectivity is further assayed by competitive FRET melting assay, which is carried out in the presence of duplex DNA as competitor. This rapid and convenient method has been adapted for high-throughput screening and used for an overwhelming majority of recently reported quadruplex ligands.

The ability in stabilizing monomeric G-quadruplex of HPDIs with different side chains basicity or with polyamine side chains with positive charges different in number and in distance was studied in our research group [31]. We used two different DNA sequences able to fold into the two desired DNA structures: a human telomeric G-quadruplex and a self-complementary hairpin duplex DNA (t-loop). ΔT_m values, reported in Fig. (**5**), are given at a ligand concentration of 1.5 μM, since at this concentration they give rise to a significant G4-DNA stabilization. The reported results can be summarized as follows: first, in most cases HPDIs are able to greatly increase intramolecular G-quadruplex thermal stability, while, on the contrary, their influence on thermal stability of duplex DNA is negligible; second, side chains basicity (in the case of PIPERs) and number of charges and distance between them (POL derivatives) influence the effect on G-quadruplex thermal stability. Another drugs feature that seems to strongly influence the different molecules activity is the nature of the side chains terminal group. In fact, considering the couples POL-1/POL-2, POL-3/POL-4 and POL-5/POL-6, the ΔT_m values appear significantly higher in the case of the side chains with three positive charges (POL-1, POL-3) or two (POL-5) in comparison with the molecules which have equal side chains, except that the terminal amino group is acetylated. It is worth noting that this effect is present only for the interactions with G-quadruplex DNA, while it is lacking in the case of dsDNA: in fact, the thermal stability of the t-loop dsDNA is only slightly increased by drug interactions and surprisingly not influenced by the acetylation of the side chains terminal amino group. The results from FRET melting assay are in good agreement with those obtained from competitive FRET. In fact the presence of sonicated calf thymus duplex DNA (c.t. dsDNA) does not generally influence the drug stabilization of the G-quadruplex.

Competitive FRET melting allows us to extend the comparison of drugs interactions between the two DNA structures to genomic DNA, since c.t. dsDNA surely represents a better biological model than t-loop DNA. Moreover, the competition at increasing concentration of c.t. dsDNA permits a semiquantitative evaluation of the drugs association constants with G-quadruplex and duplex DNA.

On these basis, it is worth considering that competitive FRET melting should be carried out on most of HPDIs reported in Fig. (**2**). In this case, a more sound basis to compare the influence of HPDIs with different features on G-quadruplex binding would be achieved.

Electrospray Ionization Mass Spectrometry (ESI-MS) allowed us to obtain binding constants of HPDIs with telomeric G-quadruplex structures in comparison with those with duplex DNA. A recent paper reported an extensive ESI-MS study of the non-covalent interactions between different inter- and intramolecular G-quadruplex structures and several perylene and coronene ligands [34]. The formation of stable complexes between the studied perylene derivatives and the G-quadruplex-forming oligonucleotides is clearly demonstrated by the presence in the mass spectra of the peaks corresponding to drug-quadruplex complexes (Fig. **6A**). A quantitative analysis of binding affinities towards quadruplex DNA structures has been possible, since the as-

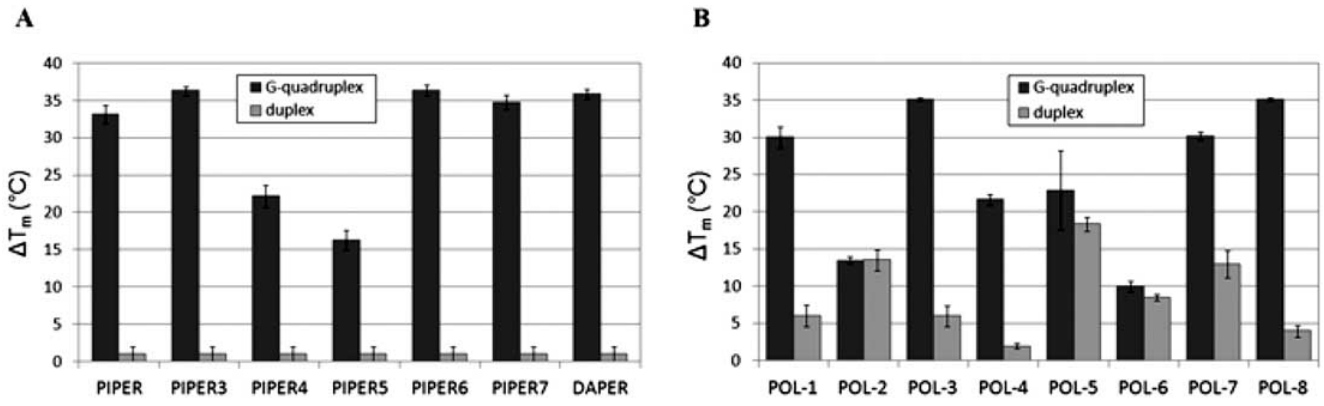

Fig. (5). Histograms of the ΔT_m values obtained from FRET melting at 1.5 μM HPDI concentration ([31]). Melting temperatures are estimated as the midpoints of the melting curves for the human telomeric G-quadruplex and t-loop duplex DNA. Reported values are the means of three independent experiments.

sociation constants can be calculated directly from the relative intensities of the corresponding peaks found in the mass spectra, with the assumption that the relative intensities in the spectrum are proportional to the relative concentrations in the injected solution [34]. A summary of the results obtained for two-, three- and four-chained perylene derivatives is reported in Table **1**, in terms of binding constants for different G-quadruplex-forming oligonucleotides. In particular two intramolecular quadruplexes have been considered: the human sequence F21TTT (5'-GGGTTAGGGTTAGGGTT-AGGGTT-3') and the inverted sequence TSG4, used in PAGE and TRAP assays. All the tested compounds show a good ability to bind to the G-quadruplex, with an order of magnitude of the binding constants ranging from 10^5 to 10^7 M^{-1} for K_1 and from 10^4 to 10^6 M^{-1} for K_2. The highest values have been obtained with three- and four-chained perylene derivatives.

As in the case of other techniques, the selectivity between quadruplex and duplex DNA, a highly relevant topic related to the specificity of the biological activity, can be investigated also by ESI-MS, choosing an appropriate model for duplex DNA. Dickerson-like dodecamers represent duplex models [35] and one of them has been used for perylene derivatives (DK66, 5'-CGTAAATTTACG-3', Fig. **6B**), leading to the evaluation of the relative binding constants to be compared to those for quadruplex DNA (Table **1**). Since the possibility of performing experiments in the simultaneous presence of G-quadruplex structures and a c.t. dsDNA is of particular interest for the biological relevance of this system, a competition ESI-MS experiment in the presence of genomic DNA fragments was reported for the first time (Fig. **6C**) [34]. Provided that c.t. dsDNA cannot be detected in these conditions due to its high molecular weight, any decrease in intensity of the peaks relative to the complexes between the tested drug and the G-quadruplex structures must be caused by the binding of the drug to genomic duplex DNA. The effect of the presence of c.t. dsDNA upon G-quadruplex binding can be evaluated in terms of percentage of bound quadruplex DNA [36]. A general decrease in the percentage values of bound G-quadruplex when dsDNA was added to the samples has been observed, but to a different extent for the various compounds: three- and four-chained perylene derivatives appear to be less selective than PIPER, despite their high efficiency of binding [34].

It would be very useful extending this quantitative analysis, both on quadruplex binding and quadruplex/duplex selectivity, to all the HPDIs, since so far only qualitative assays have been performed by this powerful technique in other labs [36].

Both these methods (FRET melting assays and ESI-MS) are suitable to derive important features (association constants and their variation with the temperature) of drugs binding to the two DNA structures (duplex and quadruplex). Taking also in consideration that while FRET assays are carried out in the presence of the physiological cation K^+, while ESI-MS requires the presence of NH_4^+ ions, it is possible to consider the two methods as complementary.

SPECTROSCOPICAL STUDY OF HPDIS-DNA BINDING

Binding studies of ligands to DNA structures, using spectroscopic methods, are a well known resource for deriving association constants [37]. It is important to underline that the method is easy to apply if DNA binding sites are independent and a unique type of binding mechanism is involved. In the case of cooperative binding and when more than one type of binding sites is involved, the evaluation of the association constant as well as of the number of binding sites can be rather complicated. However, in many cases, it is still possible to compare, at least qualitatively, the binding of the same ligand to different DNA structures or of different ligands to the same DNA structure. In the case of HPDIs, multiple equilibria are involved, on account of their aggregation processes in water, which can be significantly different for different derivatives, depending on the length and number of charges in the side chains. The following scheme illustrates, tentatively, a probably simplified mechanism for these equilibria in solution:

1) HPDI (monomer) <=> HPDIs (oligomer)

2) HPDI (monomer) + DNA <=> [HPDI (monomer)/DNA]

3) HPDIs (oligomer) + DNA <=> [HPDIs (oligomer)/DNA]

The process can vary on account of HPDI side chain features and of DNA structure. We consider in this review only intramolecular G-quadruplex of human telomeric DNA and canonical duplex DNA.

The first UV-visible absorption spectroscopy analyses on HPDIs were carried out by Kerwin *et al.* [11,12]. The perylene moiety absorption spectra modifications in water with respect to organic solvents (wavelength range between 450 and 600 nm) indicate that HPDIs are strongly aggregated, forming π–π stacked supramolecular organization: in fact, the spectra are characterized by a strong hypochromism. The presence of DNA in solution significantly decreases the hypochromism and in most cases spectra similar to those in organic solvents are obtained [31]. From these results HPDI monomer seems prevalent with respect to oligomer, if a suitable amount of DNA is present in solution. G-quadruplex DNA seems more efficient than duplex DNA in favouring HPDIs unstacking, indicating a different mechanism of binding and probably higher association constants than those for duplex DNA [31]. However, in both cases the absence of well defined isosbestic points prevents to derive association constants and allows only a mere comparison between the two trends.

With the aim to obtain a more significant comparison between HPDI binding to the two DNA structures, circular dichroism (CD) spectroscopy appears more suitable than absorption spectroscopy. In fact, HPDIs are symmetrical molecules and thus are not optically active. Duplex or quadruplex DNA structures show a CD spectrum only in the wavelength range from 220 to 320 nm, so that the CD spectra of HPDI/DNA complexes, in the wavelength range from 350 to 700 nm, are exclusively due to the ligand bound to DNA and thus asymmetrically perturbed. Two typical CD

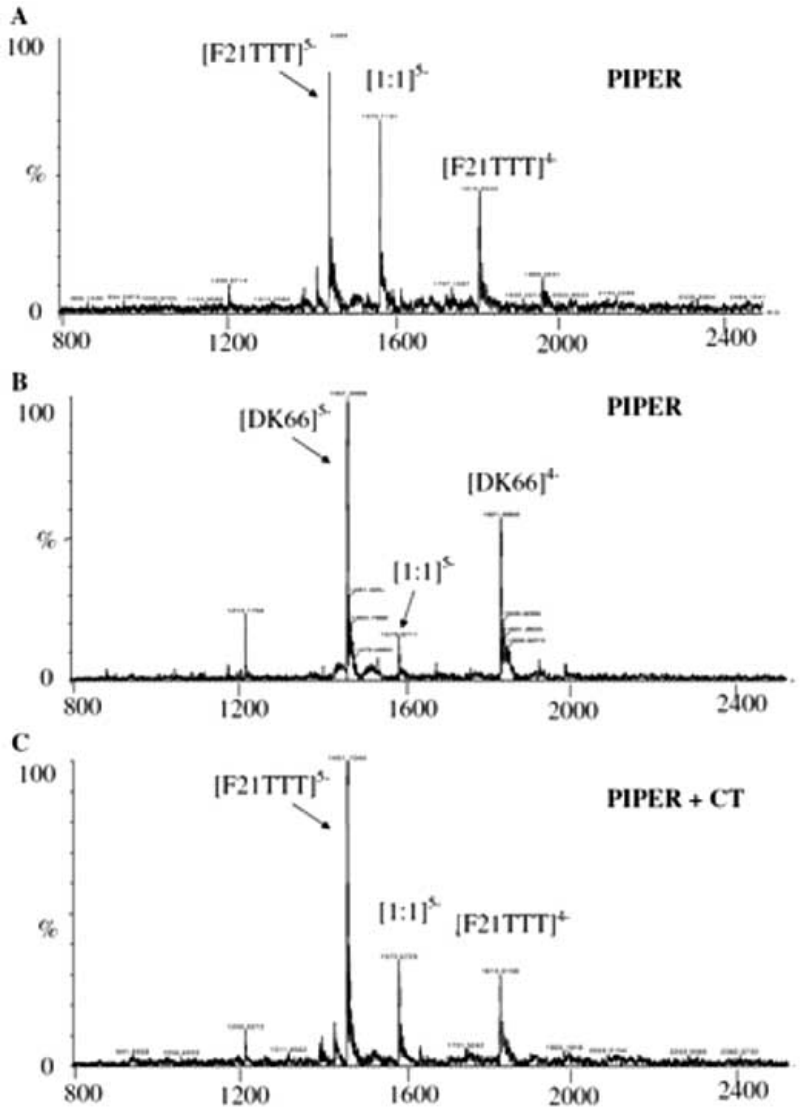

Fig. (6). ESI mass spectra of 1:1 equilibrium solutions of PIPER and G-quadruplex (A) or duplex DNA (B). At the same quadruplex-forming oligonucleotide (F21TTT) as in A, calf thymus DNA at 1:1 quadruplex/duplex ratio (in phosphate ions) has been added, together with PIPER (1:1 molar ratio) (C). Labels and arrows indicate the main ions identified, corresponding to DNA alone or drug/DNA complexes at the indicated stoichiometry. Thanks are due to Antonello Alvino and Valentina Casagrande for these spectra.

Table 1. K_1 and K_2 Values for the Indicated Oligonucleotides and Compounds Derived by ESI-MS, on a Logarithmic Scale, with Standard Deviations Reported Over at Least Three Independent Experiments [34]

Compound	TSG4		F21TTT		DK66	
	Log K_1	Log K_2	Log K_1	Log K_2	Log K_1	Log K_2
PIPER	5.6 ± 0.1	4.5 ± 0.1	5.2 ± 0.2	4.4 ± 0.1	4.7 ± 0.1	4.8 ± 0.2
DAPER4C(1,7)	6.3 ± 0.2	5.5 ± 0.3	5.8 ± 0.1	4.9 ± 0.3	5.3 ± 0.3	4.8 ± 0.1
DAPER4C(1,6)	6.4 ± 0.2	5.2 ± 0.2	6.6 ± 0.1	5.0 ± 0.2	5.9 ± 0.2	4.9 ± 0.1
DAPER3C	6.5 ± 0.3	5.4 ± 0.2	6.0 ± 0.1	4.8 ± 0.2	4.8 ± 0.1	6.2 ± 0.1

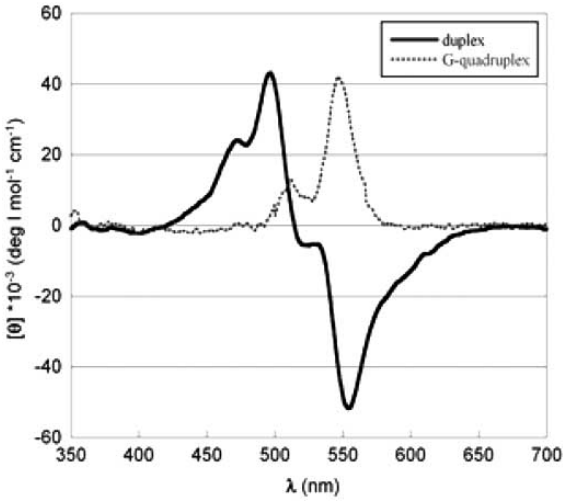

Fig. (7). Circular dichroism spectra of PIPER3 in the presence of G-quadruplex or duplex DNA (DNA/drug molar ratio equal to 10), in 100 mM KCl MES buffer (10 mM, pH 6.5).

spectra of HPDI (referring to PIPER3) with telomeric G-quadruplex and genomic duplex DNA are reported in Fig. (**7**). As in the case of all the other studied HPDIs [31], the CD spectra are dramatically different in the two cases. In the case of the HPDI complex with duplex DNA, a conservative CD spectrum is observed, characterized by the presence of two bands (having approximately the same area) with opposite signs, where the maximum of the absorption spectrum at 500 nm, nearly corresponds to zero CD intensity. This feature suggests that two or more perylene chromophores are stacked upon each other, giving rise to spectra exciton splitting and to a Cotton effect [38]. Differently, the CD spectra due to the interactions of HPDIs with telomeric G- quadruplex structure are similar to the absorption spectra and indicate that there is not coupling between chromophores, well in agreement with the threading intercalation model, previously proposed by Hurley and al. on the basis of NMR spectra [25]. Taking into account the reported spectra and those of a number of other HPDIs/DNA complexes investigated with the same methodology, we have recently proposed a schematic model of interaction between HPDIs and the two DNA structures as shown in Fig. (**8**) [31].

It would be of interest if CD spectroscopic studies on the induced asymmetry of HPDIs chromophores could be extended also to the other perylene derivatives, synthesized and studied in other research groups.

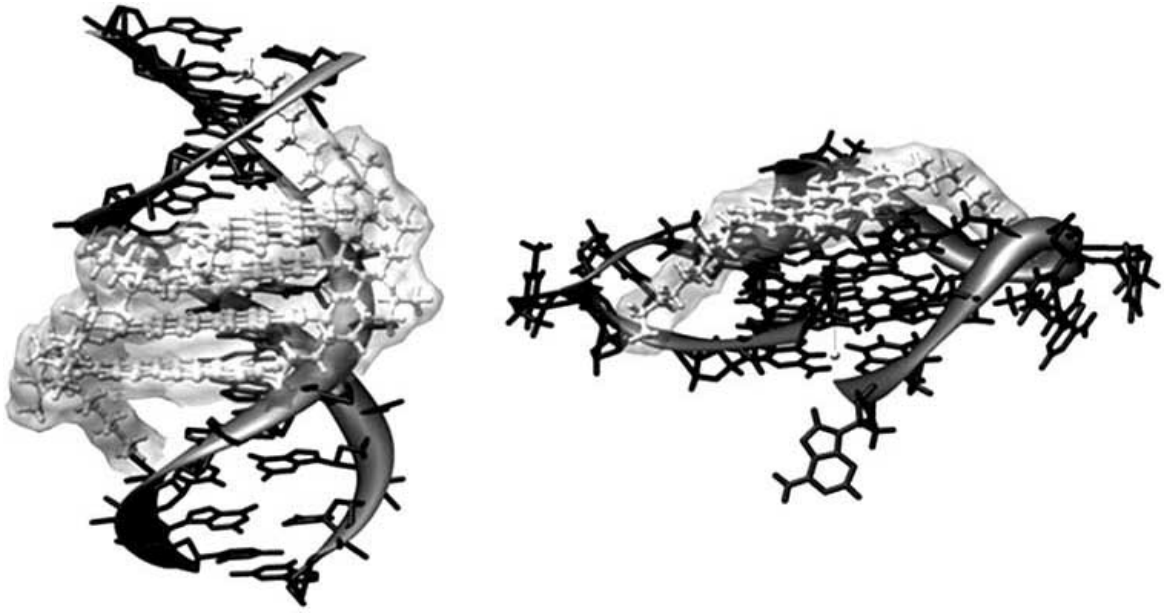

Fig. (8). Representative models of a HPDI with duplex (left) and G-quadruplex (right) DNA. DNA backbone is in black with dark gray ribbons following strands conformation, while ligand molecules are ball-stick with light gray surfaces. The complex with the monomeric G-quadruplex has been obtained by simulated annealing [13], on the basis of the molecular model proposed by Hurley [8], while the model with duplex DNA is a schematic representation, compatible with CD data [31].

CONCLUSIONS AND PERSPECTIVES

According to the proposed models, perylene self-stacking should favour the binding to duplex DNA and be unfavourable to G-quadruplex binding, since in this case it is stacked on the terminal G-quartet as a monomer. On the other hand, the correlation between G-quadruplex induction and self-stacking, previously studied by Hurley [25], suggests that HPDIs should have a suitable degree of self-stacking in water solution to induce G-quadruplex, although we propose that large self-stacking could be costly for binding selectivity. As it often happens in biological systems, the same molecular feature gives rise to a dual behaviour. The right balancing between side chains polarity and perylene hydrophobicity to obtain a convenient G-quadruplex stacking and induction requires HPDI side chains to be very carefully chosen. It seems worth concluding that the versatility of side chains chemical features of HPDIs represents an interesting and useful tool to obtain ligands that can optimize telomeric intramolecular G-quadruplex induction and stabilization, coupled with a satisfactory selectivity respect to duplex DNA. These HPDIs features could be useful in the design of new telomerase inhibitors.

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REFERENCES

[1] Krauskopf, A.; Blackburn, E.H. Telomeres and their control. *Ann. Rev. Genetics*, **2000**, *34*, 331-358: Cech, T.R. Life at the end of the chromosome: telomeres and telomerase. *Angew. Chem. Int. Ed.*, **2000**, *39*, 34-43: Blackburn, E.H. Switching and signaling at the telomere. *Cell*, **2001**, *106*, 661-673.

[2] Azzalin, C.M.; Reichenbach, P.; Khoriauli, L.; Giulotto, E.; Lingner, J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science*, **2007**, *318*, 798-801: Schoeftner, S.; Blasco, M.A. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat. Cell. Biol.*, **2008**, *10*, 228-236: Azzalin, C.M.; Lingner, J. Telomeres: the silence is broken. *Cell Cycle*, **2008**, *7*, 1161-1165.

[3] White, L.K.; Wright, W.E.; Shay, J.W. Telomerase inhibitors. *Trends Biotechnol.*, **2001**, *19*, 114-120; Incles, C.M.; Schultes, C.M.; Neidle, S. Telomerase inhibitors in cancer therapy: current status and future directions. *Curr. Opin. Investig. Drugs*, **2003**, *4*, 675-685.

[4] Dai, J.; Carver, M.; Yang, D. Polymorphism of human telomeric quadruplex structures. *Biochimie*, **2008**, *90*, 1172-1183.

[5] Zahler, A.M.; Williamson, J.R.; Cech, T.R.; Prescott, D.M. Inhibition of telomerase by G-quartet DNA structures. *Nature*, **1991**, *350*, 718-720: Sun, D.; Thompson, B.; Cathers, B.E.; Salazar, M.; Kerwin, S.M.; Trent, J.O.; Jenkins, T.C.; Neidle, S.; Hurley, L.H. Inhibition of human telomerase by a G-quadruplex-interactive compound. *J. Med. Chem.*, **1997**, *40*, 2113-2116.

[6] Gunaratnam, M.; Greciano, O.; Martins, C.; Reszka, A.P.; Schultes, C.M.; Morjani, H.; Riou, J.F.; Neidle, S. Mechanism of acridine-based telomerase inhibition and telomere shortening. *Biochem. Pharmacol.*, **2007**, *74*, 679-689: Brassart, B.; Gomez, D.; De Cian, A.; Paterski, R.; Montagnac, A.; Qui, K.H.; Temime-Smaali, N.; Trentesaux, C.; Mergny, J.L.; Gueritte, F.; Riou, J.F. A new steroid derivative stabilizes g-quadruplexes and induces telomere uncapping in human tumor cells. *Mol. Pharmacol.*, **2007**, *72*, 631-640.

[7] Cuesta, J.; Read, M.A.; Neidle, S. The design of G-quadruplex ligands as telomerase inhibitors. *Mini Rev. Med. Chem.*, **2003**, *3*, 11-21: Arola, A.; Vilar, R. Stabilisation of G-quadruplex DNA by small molecules. *Curr. Top. Med. Chem.*, **2008**, *8*, 1405-1415: Franceschin, M. G-quadruplex DNA structures and organic chemistry: more than one connection. *Eur. J. Org. Chem.* **2009**, 2225-2238.

[8] Fedoroff, O.Y.; Salazar, M.; Han, H.; Chemeris, V.V.; Kerwin, S.M.; Hurley, L.H. NMR-Based model of a telomerase-inhibiting compound bound to G-quadruplex DNA. *Biochemistry*, **1998**, *37*, 12367-12374.

[9] Rossetti, L.; Franceschin, M.; Bianco, A.; Ortaggi, G.; Savino, M. Perylene diimides with different side chains are selective in inducing different G-quadruplex DNA structures and in inhibiting telomerase. *Bioorg. Med. Chem. Lett.*, **2002**, *12*, 2527-2533.

[10] Rossetti, L.; Franceschin, M.; Schirripa, S.; Bianco, A.; Ortaggi, G.; Savino, M. Selective interactions of perylene derivatives having different side chains with inter- and intramolecular G-quadruplex structures. A correlation with telomerase inhibition. *Bioorg. Med. Chem. Lett.*, **2005**, *15*, 413-420.

[11] Kern, J.T.; Thomas, P.W.; Kerwin, S.M. The relationship between ligand aggregation and G-quadruplex DNA selectivity in a series of 3,4,9,10-perylenetetracarboxylic acid diimides. *Biochemistry*, **2002**, *41*, 11379-11389.

[12] Kern, J.T.; Kerwin, S.M. The aggregation and G-quadruplex DNA selectivity of charged 3,4,9,10-perylenetetracarboxylic acid diimides. *Bioorg. Med. Chem. Lett.*, **2002**, *12*, 3395-3398.

[13] Franceschin, M.; Lombardo, C.M.; Pascucci, E.; D'Ambrosio, D.; Micheli, E.; Bianco, A.; Ortaggi, G.; Savino, M. 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. *Bioorg Med Chem.*, **2008**, *16*, 2292-2304.

[14] Tuntiwechapikul, W.; Taka, T.; Béthencourt, M.; Makonkawkeyoon, L.; Randall Lee, T. The influence of pH on the G-quadruplex binding selectivity of perylene derivatives. *Bioorg. Med. Chem. Lett.*, **2006**, *16*, 4120-4126.

[15] Samudrala, R.; Zhang, X.; Wadkins, R.M.; Mattern, D.L. Synthesis of a non-cationic, water-soluble perylenetetracarboxylic diimide and its interactions with G-quadruplex-forming DNA. *Bioorg. Med. Chem.*, **2007**, *15*, 186-193.

[16] Tuntiwechapikul, W.; Salazar, M. Cleavage of telomeric G-quadruplex DNA with perylene-EDTA*Fe(II). *Biochemistry*, **2001**, *40*, 13652-13658.

[17] Sissi, C.; Lucatello, L.; Krapcho, A.P.; Maloney, D.J.; Boxer, M.B.; Camarasa, M.V.; Pezzoni, G.; Menta, E.; Palumbo, M. Tri-, tetra- and heptacyclic perylene analogues as new potential antineoplastic agents based on DNA telomerase inhibition. *Bioorg. Med. Chem.*, **2007**, *15*, 555-562: Pivetta, C.; Lucatello, L.; Krapcho, A.P.; Gatto, B.; Palumbo, M.; Sissi, C. Perylene side chains modulate G-quadruplex conformation in biologically relevant DNA sequences. *Bioorg. Med. Chem.*, **2008**, *16*, 9331-9339.

[18] Tuesuwan, B.; Kern, J.T.; Thomas, P.W.; Rodriguez, M.; Li, J.; David, W.M.; Kerwin, S.M. Simian virus 40 large T-antigen G-quadruplex DNA helicase inhibition by G-quadruplex DNA-interactive agents. *Biochemistry*, **2008**, *47*, 1896-1909.

[19] Franceschin, M.; Pascucci, E.; Alvino, A.; D'Ambrosio, D.; Bianco, A.; Ortaggi, G.; Savino, M. New highly hydrosoluble and not self-aggregated perylene derivatives with three and four polar side chains as G-quadruplex telomere targeting agents and telomerase inhibitors. *Bioorg. Med. Chem. Lett.*, **2007**, *17*, 2515-2522.

[20] Cathers, B.E.; Sun, D.; Hurley, L.H. Accurate determination of quadruplex binding affinity and potency of G-quadruplex-interactive telomerase inhibitors by use of a telomerase extension assay requires varying the primer concentration. *Anticancer Drug Des.*, **1999**, *14*, 367-372.

[21] Gomez, D.; Mergny, J.L.; Riou, J.F. Detection of telomerase inhibitors based on g-quadruplex ligands by a modified telomeric repeat amplification protocol assay. *Cancer Res.*, **2002**, *62*, 3365-3368.

[22] Reed, J.; Gunaratnam, M.; Beltran, M.; Reszka, A.P.; Vilar, R.; Neidle, S. TRAP-LIG, a modified telomere repeat amplification protocol assay to quantitate telomerase inhibition by small molecules. *Anal Biochem.*, **2008**, *380*, 99-105.

[23] De Cian, A.; Cristofari, G.; Reichenbach, P.; De Lemos, E.; Monchaud, D.; Teulade-Fichou, M.P.; Shin-Ya, K.; Lacroix, L.; Lingner, J.; Mergny, J.L. Reevaluation of telomerase inhibition by quadruplex ligands and their mechanisms of action. *Proc. Natl. Acad. Sci. USA*, **2007**, *104*, 17347-17352.

[24] Cristofari, G.; Lingner, J. Telomere length homeostasis requires that telomerase levels are limiting. *EMBO J.*, **2006**, *25*, 565-574.

[25] Han, H.; Cliff, C.L.; Hurley, L.H. Accelerated assembly of G-quadruplex structures by a small molecule. *Biochemistry*, **1999**, *38*, 6981-6986.

[26] Wang, Y.; Patel, D.J. Solution structure of the human telomeric repeat d[AG3(T2AG3)3] G-tetraplex. *Structure*, **1993**, *1*, 263-282.

[27] Parkinson, G.N.; Lee, M.P.; Neidle, S. Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature*, **2002**, *417*, 876-880.

[28] Giraldo, R.; Suzuki, M.; Chapman, L.; Rhodes, D. Promotion of parallel DNA quadruplexes by a yeast telomere binding protein: a circular dichroism study. *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 7658-7662.

[29] Fang, G.; Cech, T.R. Characterization of a G-quartet formation reaction promoted by the beta-subunit of the Oxytricha telomere-binding protein. *Biochemistry*, **1993**, *32*, 11646-11657.

[30] Kerwin, S.M.; Chen, G.; Kern, J.T.; Thomas, P.W. Perylene diimide G-quadruplex DNA binding selectivity is mediated by ligand aggregation. *Bioorg. Med. Chem. Lett.*, **2002**, *12*, 447–450.

[31] Micheli, E.; Lombardo, C.M.; D'Ambrosio, D.; Franceschin, M.; Neidle, S.; Savino, M. Selective G-quadruplex ligands: the significant role of side chain charge density in a series of perylene derivatives. *Bioorg. Med. Chem. Lett.*, **2009**, *19*, 3903-3908: Franceschin, M. Polycyclic aromatic compounds able to induce and stabilize G-quadruplex DNA structures as new telomerase inhibitors: synthesis, physicochemical properties and biochemical studies. *PhD Thesis*, University of Rome "La Sapienza". **2004**.

[32] De Cian, A.; Guittat, L.; Kaiser, M.; Saccà, B.; Amrane, S.; Bourdoncle, A.; Alberti, P.; Teulade-Fichou, M.P.; Lacroix, L.; Mergny, J.L. Fluorescence-based melting assays for studying quadruplex ligands. *Methods*, **2007**, *42*, 183-195.

[33] Mergny, J.L.; Maurizot, J.C. Fluorescence resonance energy transfer as a probe for G-quartet formation by a telomeric repeat. *ChemBioChem*, **2001**, *2*, 124-132.

[34] Casagrande, V.; Alvino, A.; Bianco, A.; Ortaggi, G.; Franceschin, M. Study of binding affinity and selectivity of perylene and coronene derivatives towards duplex and quadruplex DNA by ESI-MS. *J. Mass Spectrom.*, **2009**, *44*, 530-540.

[35] Gornall, K.C.; Samosorn, S.; Talib, J.; Bremner, J.B.; Beck, J.L. Selectivity of an indolyl berberine derivative for tetrameric G-quadruplex DNA. *Rapid Commun. Mass Spectrom.*, **2007**, *21*, 1759-1766.

[36] Mazzitelli, C.L.; Brodbelt, J.S.; Kern, J.T.; Rodriguez, M.; Kerwin, S.M. Evaluation of binding of perylene diimide and benzannulated perylene diimide ligands to DNA by electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.*, **2006**, *17*, 593-604.

[37] Cantor, C.R.; Schimmel, S. *Biophysical Chemistry*, W. H. Freeman and Company: New York, **1980**, Vol. *3*.

[38] Cantor, C.R.; Schimmel, S. *Biophysical Chemistry*, W. H. Freeman and Company: New York, **1980**, Vol. *2*.

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