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Research paper

Specific binding of telomeric G-quadruplexes by hydrosoluble perylene derivatives inhibits repeat addition processivity of human telomerase

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ABSTRACT

Telomerase is responsible for the immortal phenotype of cancer cells and telomerase inhibition may specifically target cancer cell proliferation. Ligands able to selectively bind to G-quadruplex telomeric DNA have been considered as telomerase inhibitors but their mechanisms of action have often been deduced from a non-quantitative telomerase activity assay (TRAP assay) that involves a PCR step and that does not provide insight on the mechanism of inhibition. Furthermore, quadruplex ligands have also been shown to exert their effects by affecting association of telomere binding proteins with telomeres. Here, we use quantitative direct telomerase activity assays to evaluate the strength and mechanism of action of hydrosoluble perylene diimides (HPDIs). HPDIs contain a perylene moiety and different numbers of positively charged side chains. Side chain features vary with regard to number and distances of the charges. IC_{50} values of HPDIs were in the low micromolar (0.5–5 μM) range depending on the number and features of the side chains. HPDIs having four side chains emerged as the best compounds of this series. Analysis of primer elongation products demonstrated that at low HPDI concentrations, telomerase inhibition involved formation of telomeric G-quadruplex structures, which inhibited further elongation by telomerase. At high HPDI concentrations, telomerase inhibition occurred independently of G-quadruplex formation of the substrate. The mechanism of action of HPDIs and their specific binding to G-quadruplex DNA was supported by PAGE analysis, CD spectroscopy and ESI-MS. Finally, competition Telospot experiments with duplex DNA indicated specific binding of HPDIs to the single-stranded telomeric substrates over double stranded DNA, a result supported by competitive ESI-MS. Altogether, our results indicate that HPDIs act by stabilizing G-quadruplex structures in single-stranded telomeric DNA, which in turn prevents repeat addition processivity of telomerase.

1. Introduction

The telomerase enzyme is repressed in most normal human somatic cells, but its reactivation has been detected in about 90% of human cancers [1]. Telomerase mediates telomere length homeostasis allowing cancer cells to avoid senescence and have an unlimited proliferative potential [2]. For these reasons, telomerase is considered an interesting target for anticancer strategies and its roles in the telomere extension process mechanisms, cellular senescence and telomere capping or stem cells maintenance are subjects of vibrant research [3–5].

The catalytic core of telomerase consists of the telomerase reverse transcriptase and a RNA subunit that provides the template for telomeric repeat synthesis (in humans hTERT and hTER, respectively) [6–8]. Telomerase is unique in that it can synthesize multiple copies of the template on the 3′ end of a primer following a single binding event, a process known as repeat addition processivity. Human telomeric DNA consists of several kilobases of double-stranded 5′-TTAGGG-3′/5′-CCCTAA-3′ repeats and a single-stranded 3′ overhang that contains 150–200 nucleotides of 5′-TTAGGG-3′ repeat sequence. The G-rich 3′ overhang can fold *in vitro* under physiological conditions into intramolecular G-quadruplexes, which are formed by three G-tetrads. G-tetrads are stabilized by the presence of monovalent cations such as K^+, Na^+

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and NH_4^+ [9]. Telomeric G-quadruplex structures have been demonstrated to exist *in vivo* in ciliated protozoan [10] but their presence at human telomeric 3′ overhangs is still uncertain. When folded in a G-quadruplex structure, the telomeric single-stranded (ss) DNA is inaccessible to extension by telomerase [11].

It has been shown, in a large number of cases in cell free systems, that small organic molecules can inhibit telomerase by inducing and stabilizing G-quadruplex structures of human telomeric ssDNA [12]. G-quadruplex promoting molecules can also induce telomere uncapping leading to senescence or apoptosis of tumor cells [13]. In most cases, typical G-quadruplex stabilizing telomerase inhibitors are characterized by the presence of an aromatic planar area and from two up to four positively charged side chains [14–18].

In the Rome research group, we have synthesized and studied during the last few years a new class of small organic molecules, the hydrosoluble perylene diimides (HPDIs). All these compounds contain a perylene moiety, a large planar aromatic system, which can bind to the terminal G-quartet of a G-quadruplex via $\pi-\pi^*$ stacking in a "threading intercalation" mode [19]. The two up to four basic side chains interact with G-quadruplex grooves. In addition, the charged side chains confer reasonable water solubility necessary for pharmacological applications. Since perylene hydrophobicity is balanced by the hydrophilic side chain moieties, we synthesized these compounds with different side chain features, in order to modulate and improve HPDIs' ability to induce and/or stabilize G-quadruplex structures. Keeping constant the central perylene area, not only the number of side chains was increased from two up to four, but we also changed single side chain features such as the number and the distances between positive charges, the chain length and the terminal groups [15–18].

Previously, we developed a modified telomeric repeat amplification protocol (TRAP) to measure the inhibition of telomerase activity by HPDIs [20]. The assay consists of three-steps: elongation of a primer by telomerase in the presence of the inhibitor, telomerase product purification from the inhibitor and amplification of telomerase products by PCR. The extraction of the inhibitor before the PCR step is important since it has been shown that G-quadruplex ligands can also inhibit Taq polymerase during PCR amplification [14]. In addition, the widely used TRAP assay is difficult to quantify in a reliable manner, because telomerase products are detected only upon PCR amplification. Furthermore, TRAP does not provide information about the mechanisms of inhibition such as effects on telomerase processivity.

In this paper, we reexamined telomerase inhibition by several classes of hydrosoluble HPDIs using two different quantitative direct enzymatic telomerase assays, Telospot and Direct assay. Both of these assays rely on highly efficient telomerase expression and reconstitution in human embryonic kidney (HEK) 293T cell. In Telospot [21], the reaction products are detected by dotblot hybridization with radiolabeled probes in a quantitative manner whereas in a Direct telomerase assay, radioactively labeled reaction products are separated according to their length on sequencing gels and visualized upon exposure to a Phospho-imager [22]. Our analysis involved newly developed polyamine side chains derivatives (POL-HPDIs), containing chains of different length and basicity, and different terminal groups [17,18]. In addition, the most active compounds of the first generation HPDIs (PIPER, PIPER3, DAPER) [19,23] with two simple basic side chains, together with a new short side chain compound (PIPER8) were included in this study. Finally, highly hydrosoluble three and four side-chained perylene derivatives (DAPER3/4C series) were also tested [18]. IC_{50} values were determined and two modes of inhibition were uncovered. At low micromolar concentration, HPDIs inhibited telomerase by inducing G-quadruplex structures in the telomere substrate, whereas at higher concentration substrate length independent inhibition prevailed. Double stranded telomeric and genomic DNA did not affect the potency of HPDIs supporting the specificity of these compounds.

2. Materials and methods

2.1. Hydrosoluble perylene diimides (HPDIs)

Perylene derivatives (Fig. 1) have been prepared as previously described [24]. PIPER8 has been synthesized following an analogous procedure, with particular attention to the presence of two free amine functions. To avoid polymerization, ethylenediamine was used as a solvent: 100 mg of 3,4:9,10-perylenetetracarboxylic dianhydride were mixed with 6 ml of anhydrous ethylenediamine and refluxed for 6 h. Upon water addition, a dark red solid was obtained, washed repeatedly with water, separated by filtration and dried, to give 100 mg of N,N′-bis[2-aminoethyl]-perylene-3,4:9,10-tetracarboxylic diimide (PIPER8, 82% yield). ^{1}H NMR (300 MHz, CF_3CO_2D) δ 4.03 (4H), 5.01 (4H), 8.9–9.1 (8H). HPDIs stock solutions were prepared in DMSO to obtain a concentration of 400 μM and kept in the dark at 4 °C.

2.2. Oligonucleotides and DNA

DNA oligonucleotides were purchased from MWG-Biotech. The sequences of the oligonucleotides are shown in Table 1. Calf thymus double stranded DNA (c.t. dsDNA) was dissolved in double distilled water and then subjected to sonication for 6 min (Sonyprep 150 sonicator) to obtain an average length of about 200 base pairs (according to gel electrophoresis analysis with Mass Ruler DNA ladder mix). Telomeric dsDNA was obtained upon restriction digestion of a plasmid (LITMUS-telo-0.2 kb) which contains 198 bp of 5′-TTAGGG-3′ tandem repeat sequence.

2.3. Super-telomerase extract preparation

Telomerase was expressed and extracted as described [22]. Briefly, HEK293 T cells were transfected with 4 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) in 6-well plates. The optimal mass ratio of hTERT- and hTER-expressing plasmids is 1:5 (0.66 μg pVan107 and 3.33 μg pBS-U1-hTER). One day post-transfection, cells were trypsinized, transferred to a 25 cm^2 flask and grown one more day. Forty-eight hours post-transfection, cells were detached by trypsinization, washed once in PBS and lysed in 400 μl of Chaps lysis buffer (10 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 0.5% CHAPS, 10% glycerol, supplemented before use with protease inhibitor cocktail III (Calbiochem) and 5 mM β-mercaptoethanol). After incubation at 4 °C for 30 min on a rotating wheel, cell debris was removed by spinning it down at 4 °C for 10 min at 13,000×*g*. The supernatant (~4 mg/ml) was aliquoted, quick frozen in dry ice and stored without loss of activity for several months at −80 °C.

2.4. Telospot

Telospot was performed as previously described [21] with slight modifications. Briefly, telomerase reactions were carried out for 45 min at 30 °C in 20 μl containing 2 μg of super-telomerase extract, 50 mM Tris–HCl [pH 8.0], 50 mM KCl, 1 mM spermidine, 5 mM β-mercaptoethanol, 1 $MgCl_2$, 40 μM dATP, 80 μM dTTP, 40 μM dGTP and 35 nM Telo3, Telo2 or TS primer, as enzyme substrate (primers and dNTPs were used at Km concentrations). HPDIs were diluted in DMSO/H_20 in order to have always 2% final DMSO concentration in telomerase reactions. As a control for complete telomerase inhibition, 50 mM EDTA was added.