Interaction of Telomestatin with the Telomeric Single-strand Overhang*

Received for publication, June 2, 2004, and in revised form, July 21, 2004
Published, JBC Papers in Press, July 23, 2004, DOI 10.1074/jbc.M406123200

Dennis Gomez‡, Rajaa Paterski‡, Thibault Lemarteleur‡, Kazuo Shin-ya§, Jean-Louis Mergny¶, and Jean-François Riou‡;

From the ‡Laboratoire d’Onco-Pharmacologie, JE 2428, UFR de Pharmacie, Université de Reims Champagne Ardenne, 51 rue Cognacq-Jay, 51096 Reims, France, the §Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan, and the ¶Laboratoire de Biophysique, Museum National d’Histoire Naturelle USM 503, INSERM Unité 565, CNRS UMR 5153, 43 rue Cuvier, 75005 Paris, France

The extremities of chromosomes end in a G-rich single-stranded overhang that has been implicated in the onset of the replicative senescence. The repeated sequence forming a G-overhang is able to adopt a peculiar four-stranded DNA structure in vitro called a G-quadruplex, which is a poor substrate for telomerase. Small molecule ligands that selectively stabilize the telomeric G-quadruplex induce telomere shortening and a delayed growth arrest. Here we show that the G-quadruplex ligand telomestatin has a dramatic effect on the conformation of intracellular G-overhangs. Competition experiments indicate that telomestatin strongly binds in vitro and in vivo to the telomeric overhang and impairs its single-stranded conformation. Long-term treatment of cells with telomestatin greatly reduces the G-overhang size, as evidenced by specific hybridization or telomeric oligonucleotide ligation assay experiments, with a concomitant delayed loss of cell viability. In vivo protection experiments using dimethyl sulfate also indicate that telomestatin treatment alters the dimethyl sulfate effect on G-overhangs, a result compatible with the formation of a local quadruplex structure at telomeric overhang. Altogether these experiments strongly support the hypothesis that the telomeric G-overhang is an intracellular target for the action of telomestatin.

Telomeres are essential DNA-protein structures that cap and protect the end of the eukaryotic chromosome from illegitimate recombination, degradation, and detection as DNA damage (1). Telomeres are also of particular interest because of their role in maintaining indefinite proliferation of cells (2, 3). In humans, the telomere is composed of tandem repeats of the G-rich duplex sequence 5′-TTAGGG-3′, with a G-rich 3′ strand extending beyond its complement to form an overhang (G-overhang) averaging 130–210 bases in length (4, 5). G-overhangs are present on all chromosomal ends and are formed during S-phase through a complex mechanism that involves cleavage of the C-strand and is independent of telomerase activity (5–8). G-overhangs may be involved in different DNA conformations such as T-loops or G-quadruplexes. T-loops are created through the strand invasion of the 3′ telomeric overhang into the duplex part of the telomere and are thought to represent a strategy to protect chromosome ends from fusion by an overhang sequestration mechanism (9, 10). Because of the repetition of guanines, the G-overhang is prone to quadruplex formation in which 4 blocks of repeated guanines are engaged into 3 adjacent quartets; each quartet involves 4 guanines stabilized by Hoogsteen bonds (11–13). The G-overhang can fold into at least two different intramolecular quadruplexes that differ in the position of the adjacent loop regions (14, 15). Optimal telomerase activity requires the non-folded single-stranded telomere overhang and G-quadruplex formation has been shown to inhibit telomerase elongation in vitro (16). Hence, stabilization of telomeric G-quadruplexes by small molecule ligands has emerged as an original strategy to achieve anti-tumor therapy (12, 13, 17). Several classes of ligands are potent inhibitors of telomerase and display strong affinities for G-quadruplex structures (11). Small molecule ligands that selectively stabilize the telomeric G-quadruplex induce telomere shortening and replicative senescence (18–21). Among those, the natural product telomestatin (22) (chemical formula shown in Fig. 1) appears very promising because of its high selectivity toward quadruplexes as compared with all other nucleic acid conformations (29).1 Telomestatin induces delayed growth arrest and apoptosis in different tumor cell types and displays an interesting selectivity toward cancer cells as compared with normal progenitors (19, 24–27). Telomere shortening is also observed in cells treated with telomestatin but arises earlier than expected for a single mechanism involving telomerase inhibition (19, 26). Furthermore, the extent of telomere degradation is limited to a few kilobases and is often undetectable in cell lines bearing short telomeres. Recent results indicate that G4 ligands from the triazine series induce short-term apoptotic effects independently of the presence of telomerase activity and that resistance to these agents is associated with telomere capping alterations (28). We have examined here the effect of telomestatin on the conformation and the length of the telomeric G-overhang.

EXPERIMENTAL PROCEDURES

Oligonucleotides, Compounds, and Cells—All oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium). Telomestatin was prepared at 5 mM in methanol, Me2SO (50:50). Additional dilutions were made in water. A549 human lung carcinoma was from the American Type Culture Collection. These cells were grown in Dulbecco’s modified Eagle’s medium with glutamax (Invitrogen), supplemented with 10% fetal calf serum and antibiotics.

1 L. Guittat, personal communication.
Long-term Growth Assay—A549 cells were seeded at $7.5 \times 10^4$ cells/25-cm$^2$ flask in the presence of different concentrations of telomestatin. Cells were passaged every 4 days; viable cells were counted with a hemacytometer, and reseeded at the original density. Results represented the cell survival (in %), as compared with untreated cells at each passage.

Solution Hybridization Experiments—The non-denaturing hybridization assay to detect telomeric G-overhang was performed with a modification of the procedure described previously (26, 29). Aliquots of 2.5 μg of undigested genomic DNA were hybridized at 50 °C overnight with 0.5 pmol of $[^{32}P]ATP$-labeled 21C oligonucleotide (5'-CCCTAAC-CCTAAACCCTAACCC-3') in sodium/magnesium hybridization buffer containing 20 mM Tris, pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 10 mM MgCl$_2$ in a volume of 20 μl. In some experiments MgCl$_2$ was omitted (sodium buffer) and NaCl was replaced by KCl (potassium buffer) or LiCl (lithium buffer), as indicated. For competition with pu22myc (5'-GAGGTTGGGAGGTTGGGAGG-3') the reactions were performed in sodium buffer without MgCl$_2$. To detect the telomere C-overhang, genomic DNA was hybridized overnight in sodium buffer without MgCl$_2$.

Dimethyl Sulfate Treatment—After 24 to 48 h of treatment with telomestatin (1 to 5 μM), cells were washed with fresh medium and were treated with 0.05% DMS for 5 to 10 min at 4 °C. The reaction was stopped by adding 1 M β-mercaptoethanol and DNA was purified as usual.

T-OLA Analysis—T-OLA was performed as described before (29). 5 μg of DNA were added to a 20-μl reaction mixture containing 0.5 pmol of 32P-end-labeled (CCCTAA)$_4$ oligonucleotide for 12 h at 50 °C, followed by gel electrophoresis and autoradiography.

FIG. 1. Chemical formula of telomestatin and 979A.

FIG. 2. Measurement of the telomeric overhang in telomestatin-treated A549 cells. a, effect of telomestatin on the growth of human A549 lung carcinoma cells. Cells were exposed to the indicated concentrations of telomestatin and cell survival was measured 4 days after seeding (except for 5 μM telomestatin). The surviving fraction was reseeded every 4 days until the culture reached a senescence-like growth arrest. b and c, non-denaturing solution hybridization analysis of the 3' telomeric overhang in A549 cells. a, induced to delayed growth arrest by 16 days of treatment with telomestatin (2 μM) or untreated cells (0). b, treated for 24 and 48 h with telomestatin (5 μM) or untreated cells (0), as indicated. G-strand, hybridization signal of the gel with 21C probe. EtBr, ethidium bromide staining of the gel. The asterisks indicate the position of the loading well at the top of the gel.

FIG. 3. T-OLA analysis of the telomeric overhang from A549 control cells (lane 1) and cells treated with 5 μM telomestatin for 48 h (lane 2). The reaction produced DNA fragments with sizes increasing by units of 24 nucleotides as indicated. The asterisk indicates the position of the loading well at the top of the gel.

* The abbreviations used are: DMS, dimethyl sulfate; T-OLA, telomeric oligonucleotide ligation assay; TRF$_2$, telomeric repeat factor 2.
Effects of Telomestatin against Telomeric G-overhang

Helisense 41489

Telomestatin inhibited hybridization at telomeric G-overhang. a, non-denaturing solution hybridization analysis of the telomeric G-overhang from purified A549 genomic DNA treated with different concentrations of telomestatin (0.1, 1, and 10 μM) in different salt conditions: magnesium/sodium, sodium, lithium, or potassium as described under “Experimental Procedures.” b, quantification of the telomestatin effect. G-overhang hybridization signal is normalized relative to the EtBr signal. The results are expressed relative to untreated telomestatin effect. G-overhang hybridization signal is normalized relative to untreated sample defined as 100%. 21G, complementary sequence (RevPu22 and Rev21G) used here are presented. Sequences of the oligomers used. a, sequences of the oligomers used. b, increasing concentrations of telomestatin (0.1–30 μM) were added to G-quadruplex forming oligomers and their reverse counterpart, as described under “Experimental Procedures.” c, non-denaturing solution hybridization analysis of the telomeric G-overhang from purified A549 genomic DNA treated with different concentrations of telomestatin (0.1, 1, and 10 μM) or 979A (0.1, 1, 10 μM) in sodium buffer. G-strand, hybridization signal of the gel with 21C probe. EtBr, ethidium bromide staining of the gel.

**Fluorescence Experiments**—The melting behavior of a fluorescent nucleotide F21G (5’-FAM-GGGTTAGGGTTAGGGTTAGG-DabCyl-3’) was studied alone, and in the presence of 20 μg of genomic DNA and/or 5 μM telomestatin. Assays were performed in a buffer containing 0.5 μM F21G, 10 mM cacodylate, pH 8.0, 0.1 mM LiCl, and 5 mM KCl. Excitation wavelength was 470 nm and emission of fluorescein was recorded at 530 nm using the Roche LightCycler real-time PCR apparatus as described (31).

**PCR Stop Assay**—The stabilization of G-quadruplex structures by telomestatin was investigated by a PCR-stop assay (32) using a test oligonucleotide and a complementary oligonucleotide that partially hybridizes to the last G-repeat of the test oligonucleotide. Sequences of the test oligonucleotides (Pu22myc and 21G) and the corresponding complementary sequence (RevPu22 and Rev21G) used here are presented in Fig. 4a.

Assay reactions were performed in a final volume of 25 μl in a 10 mM Tris, pH 8.3, buffer with 50 mM KCl, 1.5 mM Mg(OAc)₂, 7.5 pmol of each oligonucleotide, 1.5 units of Taq polymerase, and the indicated amount of the ligand. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Amplified products were resolved on 12% non-denaturing polyacrylamide gels in 1 × TBE and stained with SYBR Green I (Roche). Fluorescence was scanned with a phosphorimager (Typhoon 9210, Amersham). Results represent the mean ± S.D. of three independent experiments.

**RESULTS AND DISCUSSION**

**G-overhang Signal Is Strongly Decreased in the Presence of Telomestatin**—Because senescence might also result from telomere uncapping and the status of the 3’ overhang (33–35), we have examined the effects of the treatment with G-quadruplex ligands on the telomeric G-overhang by using a non-denaturing solution hybridization technique with a complementary oligonucleotide (21C) and native agarose gel electrophoresis without any digestion by restriction enzymes (see “Experimental Procedures” for details). The localization of the signal relative to undigested DNA and sensitivity to exonucleases (Ref. 29 and data not shown) supported evidence for the specificity of this assay to G-rich telomeric single-stranded DNA. Continuous treatment of A549 human lung carcinoma cells with 1 or 2 μM telomestatin induced a delayed growth arrest starting at days 16 and 8, respectively, that led to a senescent-like culture in

![Diagram](http://www.jbc.org/Downloaded_from)
which both apoptotic and SA-β-galactosidase expressing cells were detected (Ref. 26 and data not shown, see also Fig. 2a). In the presence of 5 μM telomestatin, growth arrest was observed after only 4 days of exposure (Fig. 2a, squares). DNA extracted from the A549 human lung carcinoma cell line growth arrested by 2 μM telomestatin presented a significant decrease in the G-overhang signal, as compared with untreated cells (Fig. 2b).

Treatment with 1 and 5 μM telomestatin for a shorter period (24 or 48 h), a time when the ligand has no or reduced impact on cell culture growth (Fig. 2a), also provoked a dramatic decrease in the G-overhang signal with an effect detectable within 24 h (95% at 5 μM, 70% at 1 μM) (Figs. 2c and 9b).

To confirm these findings, we also used the T-OLA assay (29), which measures the G-overhang length (Fig. 2d). Again, telomestatin-treated A549 cells presented a marked shortening of the telomeric G-overhang to 24 nucleotides, as compared with 192 nucleotides in control untreated cells.

Both methods indicate that telomestatin is able to induce a rapid and dramatic alteration of the telomeric G-overhang. Surprisingly, the G-overhang loss was found earlier than expected and might represent a rapid degradation process triggered by telomestatin as a consequence of changes in the telomeric overhang configuration. On the other hand, because telomestatin is a potent and specific G-quadruplex ligand, we cannot exclude that the ligand modified the G-overhang in a conformation that further inhibited the hybridization reaction. This would mean that telomestatin tightly binds to the telomeric overhang during DNA extraction and purification. Several experiments were then designed to answer this point.

**Fig. 5.** Pu22myc quadruplex competes with the effect of telomestatin at the telomeric overhang. a and b, non-denaturing solution hybridization analysis of the telomeric G-overhang from A549 cells untreated or treated with 5 μM telomestatin for 24 and 48 h in sodium buffer. a, DNA from untreated cells (A549 untreated) was incubated with different concentrations of telomestatin (0.1, 1, and 10 μM) with or without the pu22myc quadruplex competitor (1 μM), as indicated. DNA from telomestatin-treated cells (A549 + Telo 5 μM) was incubated with or without pu22myc quadruplex competitor (1 or 10 μM), as indicated. b, DNA from untreated cells (A549 untreated) was incubated with 5 μM telomestatin with or without 10 μM pu22myc quadruplex competitor, as indicated. DNA from telomestatin-treated cells (A549 + Telo 5 μM) was incubated with or without pu22myc quadruplex competitor (10 μM), as indicated. c, quantification of the competition with 10 μM pu22myc for in vitro treatment with telomestatin (0.1, 1, 2, and 5 μM). d, quantification of the competition with pu22myc for 24- or 48-h treatments of A549 cells with telomestatin (5 μM). G-strand overhang signal was normalized relative to the EtBr signal and results expressed as the relative hybridization of the 21C probe from untreated A549 DNA defined as 100%.

**Fig. 6.** Telomestatin does not inhibit hybridization at the telomeric C-rich overhang. Non-denaturing solution hybridization analysis of the telomeric C-overhang from purified A549 genomic DNA treated with different concentrations of telomestatin (1 and 10 μM) in sodium buffer. C-strand, hybridization signal of the gel with 20G probe is indicated by an arrow. EtBr, ethidium bromide staining of the gel. The asterisk indicates the position of the loading well at the top of the gel.
Telomestatin Specifically Binds to the Single-stranded Conformation of G-overhang in Vitro—We first investigated whether telomestatin could stabilize the telomeric overhang in a conformation that impaired hybridization. Previous studies have shown that, under physiological conditions, formation of a G-quadruplex structure delays but does not prevent the hybridization of the complementary C-strand leading to the formation of a (TTAGGG/CCCTAA)$_n$ duplex (36). The addition of a specific ligand is expected to shift the equilibrium toward the G-quadruplex folding and therefore to inhibit hybridization. This was initially proposed for a bisacridine dye (37) and recently demonstrated for telomestatin by using short oligonucleotides mimicking four telomere repeats (23).

These results were obtained with short (21–22 bases long) synthetic oligodeoxynucleotides. Because of its size, the structure and folding of a 200-base long telomeric overhang into a quadruplex might be qualitatively and quantitatively different from that described with oligonucleotides. The potency or the nature of the ligand, as well as the ionic or protein environment, might also greatly influence this folding and the nature of the complex. We therefore studied the in vitro effect of telomestatin on the G-overhang from purified genomic DNA. Not only is the overhang length different from the model system mentioned above, but the nature of genomic DNA implies the presence of a huge molar excess of double-stranded sequences that could trap telomestatin if this ligand had an insufficient specificity toward quadruplexes. Overnight incubation of the labeled C-strand (21C) with genomic DNA in the presence of telomestatin induced a concentration-dependent inhibition of the hybridization signal in Na$^+$ and K$^+$ salt conditions (Fig. 3, a and b). The presence of Li$^+$ partially released the extent of the inhibition (Fig. 3, a and b), in agreement with the known requirements for G-quadruplex stabilization (13). Interestingly, the addition of Mg$^{2+}$ that favors duplex hybridization over G-quadruplex folding (38) did not change the extent of the inhibition (Fig. 3, a and b, compare magnesium/sodium and sodium), indicating that telomestatin created a potent and stable interaction at telomeric overhang related to its property of interacting with G-quadruplex. Consistent with these findings, a G4-inactive ligand (formula shown in Fig. 1) was unable to inhibit 21C hybridization under all conditions tested (Fig. 3c and data not shown). For in vitro treatment with telomestatin, the presence of the labeled probe was also observed in the loading well but disappeared after further pretreatment with proteinase K without any change of the telomeric overhang signal (not shown), indicating that it corresponded to aggregation with the remaining proteins from the DNA preparation.

c-Myc Quadruplex Competes with the Telomestatin Effect at Telomeric Overhang—Further evidence of the involvement of G-quadruplexes in hybridization inhibition was obtained by using competition experiments with another G-quadruplex forming oligonucleotide (pu22myc) derived from the NHE IIII element of the c-myc promoter (39). This quadruplex was found to be efficiently stabilized by telomestatin in a PCR-stop assay with an IC$_{50}$ equal to that of the telomeric sequence (Fig. 4b) and presented several mismatches with the 21C oligonucleotide that prevent duplex formation. As shown in Fig. 5a, the addition of 1 $\mu$M pu22myc did not interfere with 21C hybridization to the telomeric overhang and abolished the inhibitory effect of telomestatin (up to 1 $\mu$M). At a higher telomestatin concentration (10 $\mu$M), pu22myc was unable to prevent the loss of the hybridization signal, in agreement with competition between the telomeric overhang and pu22myc G-quadruplexes for telomestatin binding. In the presence of 10 $\mu$M pu22myc, the reversion of the telomestatin-induced inhibition was further improved and was complete for a telomestatin concentration up to 2 $\mu$M and partial (40%) with 5 $\mu$M (Fig. 5, a and c).

As a control, hybridization experiments were also performed with the 20G oligonucleotide that probed the transient telomeric C-rich overhang described during the S phase of proliferating cells (40). A very weak hybridization band corresponding to the C-overhang was detected in the absence of treatment. This signal was not modified by the treatment with telomestatin up to a concentration of 10 $\mu$M (Fig. 6). These in vitro experiments allow us to conclude that telomestatin specifically targets the telomeric G-overhang structure from purified genomic DNA through the formation of stable G-quadruplex structures, as previously reported for oligonucleotides (23, 25).

Telomestatin Remains Attached to the G-overhang and Prevents Hybridization of a Complementary Probe—We then investigated whether telomestatin might alter the conformation of the telomeric G-overhang and remains tightly bound to the telomeric overhang after cellular treatment. DNA from A549-treated cells (5 $\mu$M, 24 h) was incubated in the presence of the quadruplex competitor pu22myc. A partial reversion of the hybridization signal loss was observed in the presence pu22myc (Fig. 5, a and b). The recovery was up to 41% of the G-overhang signal as a function of the pu22myc competitor concentration and slightly decreased to 35% when telomestatin
treatment was prolonged to 48 h (Fig. 5d). This suggests that telomestatin was strongly bound to the telomeric overhang in a conformation that impaired 21C hybridization. We conclude that short-term treatment (up to 48 h) with telomestatin did not completely degrade the G-overhang. Such tight binding of telomestatin to the G-overhang implies a very strong selectivity of this ligand for telomeric ends over duplex DNA, in agreement with previous selectivity studies with different oligonucleotides (23, 25). This was previously demonstrated by using short oligonucleotides by MS-MSI (23) and fluorescence resonance energy transfer (FRET) experiments using a 26-nucleotide long duplex oligonucleotide as a competitor with the 21G oligonucleotide (the minimal telomeric sequence able to form a quadruplex). In the presence of a 650-base molar excess of the ds26 oligonucleotide, the melting temperature induced by telomestatin was not modified (not shown). Under the same experimental conditions, the stabilization induced by other ligands such as ethidium derivatives was lost (41), demonstrating that telomestatin is more selective toward quadruplexes than these derivatives. To investigate the selectivity toward human double-stranded genomic DNA, fluorescence experiments using P21G-Dabcyl in the presence of telomestatin were performed with purified A549 DNA. The melting temperature of the oligonucleotide (74 °C in the presence of 5 μM telomestatin) was not modified up to a 3 × 10^3 base molar excess of DNA, indicating a very potent selectivity for telomestatin between telomeric G-quadruplex and duplex genomic DNA (Fig. 7). Such tight and specific binding of telomestatin to the G-overhang is also in agreement with the results of the in vitro competition experiment with the pu22myc quadruplex.

Telomestatin Induces an Effective G-overhang Degradation Associated with the Delayed A549 Growth Arrest—At this point, because of its tight binding to the telomeric overhang, we were unable to conclude whether a short-term exposure of A549 cells to 5 μM telomestatin resulted, at least partially, in a rapid degradation of the telomeric overhang, because the pu22myc competition induced a partial reversion of the hybridization that was nearly equal for in vitro experiments (40%) and cell treatment (35–41%). This ligand concentration also provoked a rapid growth arrest after 4 days of exposure of the cells (Fig. 1a). To determine whether the onset of the delayed growth arrest induced by telomestatin corresponded to an effective degradation of the telomeric overhang, we studied the long-term effect of telomestatin exposure at a lower concentration.

After 16 days of treatment with 2 μM telomestatin, the remaining A549 cells were washed in fresh culture medium without telomestatin and maintained for a further 9 days in the absence of the drug to achieve a density suitable for DNA extraction (Fig. 8a, open triangle). During that period, cell population doubling was equal to 3.2, corresponding to a 67-h doubling time, as compared with 22 h for control cells, indicating that these cells have not fully recovered from the effects of the drug. Interestingly, the telomeric G-overhang signal was still found to be decreased by 70% (Fig. 8b), a value suggesting that the G-overhang was substantially degraded.

After 4 to 12 days of treatment with 2 μM telomestatin, the telomeric G-overhang signal decreased from 33 to 10%, respectively (Fig. 8c). In vitro, the effect of this telomestatin concentration is fully reversed by the competition with 10 μM pu22myc (Figs. 5c and 8c). DNA from A549-treated cells (4 to 12 days) were incubated in the presence of the pu22myc competitor (10 μM). The recovery was up to 71% of the hybridization at 4 days, but markedly decreased to 48% when telomestatin treatment was prolonged to 12 days (Fig. 8c). Importantly, and contrary to the previous experiments at higher telomestatin concentrations, the addition of pu22myc allowed to determine the effective G-overhang signal. This observation supports the conclusion that telomestatin induced a bona fide and persistent reduction in G-overhang length. In other words, the reduction of the radioactive signal resulting from the stabilization of a quadruplex during hybridization that prevents formation of a duplex with the radiolabeled probe cannot fully account for the reduction observed in Fig. 8. G-overhang degradation correlated with the telomestatin-induced delayed loss of viability of A549 cells.

DMS Treatment Alters Telomestatin Effects on G-overhangs—To confirm that the telomeric overhang signal decrease was not the sole result of a tenacious interaction of the ligand with the telomeric overhang during hybridization, we used in vivo protection experiments with DMS. Treatment of A549 cells with DMS before DNA extraction modifies the electrophoretic migration of genomic DNA and creates discrete double-stranded DNA breaks (Fig. 9a, EtBr), as previously described (30). The radioactive band intensity was also reduced to about 70% of the control (Fig. 9, a and b), indicating a rapid decrease...
in G-overhang size that corresponded to the onset of a rapid endonucleolytic process of the telomeric G-overhang. When telomestatin treatment (24 h, 5 μM) was followed by DMS treatment before DNA extraction, a 2-fold increase in the G-overhang hybridization was observed, as compared with telomestatin treatment alone (Fig. 9, a and b). Similar results were obtained in the presence of 1 μM telomestatin for 24 h (Fig. 9, b and c), suggesting that DMS is able to decrease the effect of telomestatin on the G-overhang.

Further in vitro treatment with telomestatin (0.1 to 10 μM, Fig. 9c) was applied to DNA from DMS- and/or telomestatin-treated cells in lithium buffer conditions. In that case, telomestatin-induced inhibition of G-overhang was increased in DNA from DMS-treated cells (IC50 = 0.8 μM), as compared with control cells (IC50 = 1.8 μM), suggesting that DMS-induced overhang shortening facilitated the effect of the ligand in vitro. Similar results were obtained in sodium buffer conditions (Fig. 9d). DNA from cells treated with telomestatin (1 μM, 24 h) provided a stronger inhibition with further in vitro treatment with the ligand (IC50 = 0.5 μM) (Fig. 9c). In contrast, DNA from cells treated with both DMS and telomestatin had a reduced effect on G-overhangs (IC50 = 2 μM) in agreement with the formation of a stable G-quadruplex was found, and prolonged treatment of the cells with telomestatin resulted in a marked decrease in the G-overhang signal that correlated with the onset of the delayed growth arrest. Recent reports indicate that TRF2 is essential for protection of the telomeric overhang and prevents the action of the nucleotide excision repair nuclease ERCC1/XPF that participates in the overhang removal (34). We propose that the alteration of the G-overhang conformation by telomestatin might alter its capping by essential factors such as TRF2, therefore leading to its degradation.

Because DMS mostly induces N7-alkG modifications in vivo (30), the protection is compatible with the formation by telomestatin of quadruplex Hoogsteen bonds at the telomeric overhang in vivo.

CONCLUSIONS

Our data indicate that telomestatin is able to impair the telomeric overhang structure in human cells. A tight and specific interaction of the ligand with telomeric overhang compatible with the formation of a stable G-quadruplex was found, and prolonged treatment of the cells with telomestatin resulted in a marked decrease in the G-overhang signal that correlated with the onset of the delayed growth arrest. Recent reports indicate that TRF2 is essential for protection of the telomeric overhang and prevents the action of the nucleotide excision repair nuclease ERCC1/XPF that participates in the overhang removal (34). We propose that the alteration of the G-overhang conformation by telomestatin might alter its capping by essential factors such as TRF2, therefore leading to its degradation. Our results are also in agreement with the finding that another ligand from the 2,6-pyridocarboxamide series is preferentially associated with mitotic telomeres during metaphase. Our results represent the first evidence that telomeric overhangs are an important target for the biological effect of these classes of agents.

Acknowledgments—We thank L. Lacroix (MNHN, Paris) for helpful discussions, F. Boussin (CEA, Saclay, France) and L. Guittat (MNHN, CEA, Saclay, France) and L. Guittat (MNHN, Paris) for helpful discussions.
Effects of Telomestatin against Telomeric G-overhang

Paris for sharing unpublished results, and S. Bacchetti (Istituto Regina Elena, Roma) and S. Stewart (Washington University, St Louis) for critical reading of the manuscript.

REFERENCES

1. Blackburn, E. H. (2001) Cell 106, 661–673
2. Shay, J. W. (1997) J. Cell. Physiol. 171, 266–270
3. Hahn, W. C., Stewart, S. A., Brooks, M. W., York, S. G., Eaton, E., Kurschis, A., Beijersbergen, R. L., Knoll, J. H., Meyerson, M., and Weinberg, R. A. (1999) Nat. Med. 5, 1164–1170
4. Wright, W. E., Tesmer, V. M., Huffman, K. E., Levene, S. D., and Shay, J. W. (2004) Genes Dev. 11, 2801–2806
5. Makarov, V. L., Hirose, Y., and Langmore, J. P. (1997) Cell 88, 657–666
6. Hemann, M. T., and Greider, C. W. (1999) Nucleic Acids Res. 27, 3964–3969
7. Wright, W. E., Tesmer, V. M., Liao, M. L., and Shay, J. W. (1999) Exp. Cell Res. 251, 482–499
8. Jacob, N. K., Kirk, K. E., and Price, C. M. (2003) Mol. Cell 11, 1021–1032
9. Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) Cell 97, 503–514
10. De Lange, T. (2004) Cell 116, 899–909
11. Mergny, J. L., Riou, J. F., Mailliet, P., Teulade-Fichou, M. P., and Gilson, E. (2002) Nucleic Acids Res. 30, 839–845
12. Neidle, S., and Parkinson, G. (2002) Nat. Rev. Drug Discov. 1, 503–513
13. Davies, J. T. (2004) Angew. Chem. Int. Ed. 43, 668–698
14. Parkinson, G. N., Lee, M. P., and Neidle, S. (2002) Nature 417, 876–880
15. Wang, Y., and Patel, D. J. (1993) Structure 1, 285–289
16. Zahler, A. M., Williamson, J. R., Cech, T. R., and Prescott, D. M. (1991) Nature 350, 718–720
17. Mergny, J. L., and Hélène, C. (1998) Nat. Med. 4, 1366–1367
18. Riou, J. F., Guittaud, L., Mailliet, P., Laoui, A., Renou, E., Petitgenet, O., Megnin-Chanet, F., Hélène, C., and Mergny, J. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2673–2677
19. Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J. H., and Ohyashiki, K. (2003) Oncogene 22, 5338–5347
20. Gowan, S. M., Harrison, J. R., Patterson, L., Valenti, M., Read, M. A., Neidle, S., and Kelland, L. R. (2002) Mol. Pharmacol. 61, 1154–1162
21. Riou, J. F., Mailliet, P., Teulade-Fichou, M. P., and Gilson, E. (2002) Mol. Pharmacol. 60, 981–988
22. Shin-ya, K., Wierzba, K., Matsuo, K., Ohtani, T., Yamada, Y., Furuta, H., Hayakawa, Y., and Seto, H. (2001) J. Am. Chem. Soc. 123, 1262–1263
23. Rosu, F., Gabelica, V., Shin-ya, K., and De Pauw, E. (2003) Chem. Commun. 2702–2703
24. Shammas, M. A., Reis, R. J., Li, C., Koley, H., Hurley, L. H., Anderson, K. C., and Munshi, N. C. (2004) Clin. Cancer Res. 10, 770–776
25. Kim, M. Y., Gleason-Guzman, M., Ishizaka, E., Nishioka, D., and Hurley, L. H. (2003) Cancer Res. 63, 3247–3256
26. Gowan, S., Aouali, N., Renaud, A., Douarre, C., Shin-Ya, K., Xuri, J., Martinez, S., Trentesaux, C., Morjani, H., and Riou, J. F. (2003) Cancer Res. 63, 6149–6153
27. Kim, M. Y., Vankayalapati, H., Shin-ya, K., Wierzba, K., and Hurley, L. H. (2002) J. Am. Chem. Soc. 124, 2098–2099
28. Gowan, S., Aouali, N., Londono-Vallejo, A., Lacroix, L., Megnin-Chanet, F., Leventelet, T., Douarre, C., Shin-ya, K., Mailliet, P., Trentesaux, C., Morjani, H., Mergny, J. L., and Riou, J. F. (2003) J. Biol. Chem. 278, 50554–50562
29. Cimino-Reale, G., Pascale, E., Battiloro, E., Starace, G., Vernas, R., and D’Ambrosio, E. (2001) Nucleic Acids Res. 29, E35
30. Cloutier, J. F., Castonguay, A., O’Connor, T. R., and Drouin, R. (2001) J. Mol. Biol. 306, 169–188
31. Darby, R. A., Sologoub, M., McKeen, C., Brown, L., Risitano, A., Brown, N., Barton, C., Brown, T., and Fox, K. R. (2002) Nucleic Acids Res. 30, e39
32. Gomez, D., Lemarteleur, T., Lacroix, L., Mailliet, P., Mergny, J. L., and Riou, J. F. (2004) Nucleic Acids Res. 32, 371–379
33. Stewart, S. A., Ben-Porath, I., Carey, V. J., O’Connor, B. F., Hahn, W. C., and Weinberg, R. A. (2003) Nat. Genet. 33, 492–496
34. Zhu, X. D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J. H., and de Lange, T. (2003) Mol. Cell 12, 1489–1488
35. Masutomi, K., Xu, E., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Morjani, H., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., and Hahn, W. C. (2003) Cell 114, 241–253
36. Phan, A. T., and Mergny, J. L. (2002) Nucleic Acids Res. 30, 4618–4625
37. Alberti, P., Riou, J. F., Teulade-Fichou, M. P., Guittaud, L., Riou, J. F., Chaires, J., Hélène, C., Vigneron, J. P., Lehn, J. M., and Mergny, J. L. (2001) J. Biomol. Struct. Dyn. 19, 505–513
38. Alberti, P., and Mergny, J. L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1549–1553
39. Siddiqui-Jain, A., Grand, C. L., Bearsar, D. J., and Hurley, L. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11593–11598
40. Cimino-Reale, G., Pascale, E., Alvino, E., Starace, G., and D’Ambrosio, E. (2003) J. Biol. Chem. 278, 2139–2146
41. Rosu, F., De Pauw, E., Guittaud, L., Alberti, P., Lacroix, L., Mailliet, P., Riou, J. F., and Mergny, J. L. (2003) Biochemistry 42, 10361–10371

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
